Proceedings of

New Paradigms in Laboratory Animal Science

A Joint FELASA/Scand-LAS Symposium
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Proceedings

of the Eleventh FELASA Symposium and the 40th Scand-LAS symposium

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Introduction to the Proceedings of the 11th FELASA Symposium. FELASA Past, Present and Future.

Introduction

The eleventh FELASA symposium 'New Paradigms in Laboratory Animal Science' was held on the 14th-17th June 2010 in Helsinki, Finland. The symposium was organised as a joint meeting with Scand-LAS, celebrating its 40th anniversary year. The triennial FELASA symposium has grown to be the largest laboratory animal scientific conference in Europe, also facilitating other associations to organise some of their activities during the meeting days.

We had a pleasure to host 1200 participants and exhibitors from altogether 42 countries. Almost 150 invited and free oral presentations contributed to the high quality scientific programme covering a wide range of topics in laboratory animal science and related disciplines. In nine workshops, the participants were able to interactively discuss the most current items in their field. The scientific output was completed in a poster session with 170 posters.

This proceedings book continues the series of the FELASA symposium proceedings. The proceedings book aims to be both a take-home-message to the participants, but also an opportunity for the speakers to present their work to wider scientific community. With the four themes, the book gives a representative overview of the scientific contents of the meeting, highlighted with the paper by FELASA award-winning professor Michael Festing.

The funding by Laboratory Animals Limited has been substantial in realising this proceedings book. The editors Eila Kaliste and Johanneke van der Harst; designer Timo Päivärinta for the lay-out and Penny Alborough for checking the language are all cordially thanked for their excellent and hard work in finalising the manuscripts into a form of the book.

Hanna-Marja Voipio
Chair of the Organising Committee
President of Scand-LAS

FELASA Past and Present

Being the FELASA President at the time of the eleventh FELASA Symposium was one of the most extraordinary experiences I have ever had. Sometimes in the public appearances, sometimes hidden among the hundreds of delegates, I could observe the force of laboratory animal science professionals from all around the world. A force transformed in over 300 pieces of scientific work in the Symposium. Part of them will be available for ever in this Proceedings Book.

After many years of experience in FELASA, I still cannot believe that events like this Symposium can be produced by an organisation such as FELASA that relies almost entirely on the voluntary work of the people appointed by its constituent associations. But this Proceedings Book is proof that Helsinki 2010 was not a dream and that laboratory animal science is a driving force that has no frontiers. FELASA is itself an international organisation that was created in 1978 by 3 national associations and is currently composed of 18 associations that represent several thousands professionals across Europe. The scientific work presented in Helsinki and published now in the Proceedings shows that the same principles and research interests are shared not only within Europe but also by the rest of the world.

Special thanks to Prof. Timo Nevalainen, Chair of the Scientific Committee and also a former FELASA President who, as always, made everything work perfectly to produce a remarkable scientific programme for this Symposium. The FELASA Board of Management recently decided to replace the name of Symposium with Congress in order to better reflect the importance and size of these scientific events. I wish that the spirit of Helsinki 2010 is kept and enhanced in next FELASA Congresses, but this is already refers to the future…

Javier Guillen
FELASA President 2009-2010

FELASA Present and Future

I think that Hanna-Marja and Javier have already put into words many of my comments and recollections of the Symposium in Helsinki. This was probably my fourth attendance at a FELASA Symposium and each has surpassed the excellence of the previous one. Whether measured in terms of the quality of the scientific presentations, posters, trade exhibition or general organisation of the event, each meeting has added a further improvement which seemed impossible at the outset. This challenge is now thrown open to the scientific and local organising committees of the Barcelona Congress being planned for 2013. Our expectations of SECAL and FELASA are even higher but I am confident we will be rewarded with an excellent scientific, trade and social programme.

This triennial FELASA Congress is firmly established in the calendar of scientists involved in laboratory animal research both in Europe and beyond. Although by the time of our Barcelona Congress I will be your Immediate Past-President, I look forward to another successful meeting and the opportunity to renew old friendships and foster new ones.

David Smith
FELASA President 2011-2012

Education and training in Laboratory Animal Science and Welfare

Laboratory Animals Ltd

Laboratory Animals Ltd. is a limited company with charitable status. Its main aim is to promote education and training in laboratory animal science, technology and welfare. This is achieved primarily through publication of the journal Laboratory Animals, but a variety of other activities are supported, such as sponsoring speakers at scientific meetings, providing training grants for individuals wishing to develop their expertise and supporting the organisation of training courses in laboratory animal science and welfare. Funding has been provided to pprojects such as a course in statistics and experimental design by FRAME, working groups of FELASA, and translations of FELASA working group reports into other languages including Spanish and Russian. Bursaries have been given to individuals from a variety of countries e.g. Bangladesh, Sri Lanka, Kenya, India, Kazakhstan, Thailand, Cuba, and Mexico for participation in university courses in Laboratory Animal Science in Utrecht, Copenhagen and Barcelona.

Laboratory Animals Ltd. has sponsored the publication of these FELASA proceedings plus a number of speakers at the FELASA symposium June 2010. Our mission statement is as follows: "Laboratory Animals Limited is dedicated to the advancement of all aspects of laboratory animal science, technology and welfare, and we achieve this through supporting education and training in the field. Animal experimentation must only be performed when considered absolutely necessary and all work must conform to the highest ethical standards. By supporting education and training in laboratory animal scienc, technology and welfare, where Reduction, Refinement and Replacement (the 3Rs) are the central focus, the 3Rs will become a key part of research, leading to reduced numbers of animals being used and improvements in animal welfare, as well as better scientific results."

Laboratory Animals journal

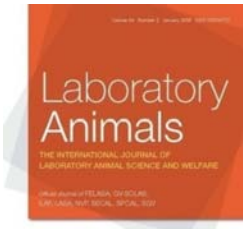
Laboratory Animals is the official journal of the following organisations: FELASA, GV-Solas (Germany), ILAF (Israel), LASA (UK), NVP (Netherlands), SECAL (Spain), SGV (Switzerland), SPCAL (Portugal), ESLAV (European Society for Laboratory Animal Veterinarians), LAVA (Laboratory Animal Veterinary Association UK) and AFSTAL (France). A reciprocal membership arrangement for membership exists with AALAS (US).

The Editorial Board of Laboratory Animals wishes to give especial encouragement to new scientific data in the field of 3R alternatives. There is full on-line access available at no extra charge to subscribers. The journal is published by the Royal Society of Medicine (UK) and indexed/abstracted in the following: Index Medicus, ISI/BIOMED, Excerpta medica (EMBASE), Current contents, CABS (Current Awareness in Biological Sciences) and Chemical Abstracts. Open access publication is possible, which makes full text articles immediately available to everyone, thereby supporting the idea of the 3Rs.

All individuals contributing to the Executive Committee, Council of Management and Editorial board are greatly acknowledged for their tremendous contributions to making our organisation work so succesfully throughout Europe and accross the World.

Merel Ritskes-Hoitinga
Chair

laboratory animals limited



FELASA Members

Members of FELASA are laboratory animal science associations of nations and regions of Europe, members of the Council of Europe

AFSTAL
l'Association Française des Sciences et Techniques de l'Animal de Laboratoire

AISAL
Associazione Italiana per le Scienze degli Animali da Laboratorio

ARSAL
Asociatia Romana pentru Stiinta Animalelor de Laborator

Balt-LASA
Baltic Laboratory Animal Science Association

BCLAS
Belgian Council for Laboratory Animal Science

CLASA
Czech Laboratory Animal Science Association

CroLASA
Croatian Laboratory Animal Science Association

GV-SOLAS
Die Gesellschaft für Versuchstierkunde

HLASA
Hungarian Laboratory Animal Science Association

HSBLAS
Hellenic Society of Biomedical and Laboratory Animal Science

ILAF
The Israeli laboratory Animal Forum (affiliate member)

LASA
Laboratory Animal Science Association

NVP
Nederlandse Vereniging voor Proefdierkunde

LASA Turkey
Laboratory Animal Science Association - Turkey (observer)

Scand-LAS
Scandinavian Society for Laboratory Animal Science

SECAL
Sociedad Española para las Ciencias del Animal de Laboratorio

SGV
Schweizerische Gesellschaft für Versuchstierkunde

SLASA
Serbian Laboratory Animal Science Association

SPCAL
Sociedade Portuguesa de Ciências em Animais de Laboratório

felasa
Federation of European Laboratory Animal Science Associations

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FELASA 2010 Award & Plenary lecture:

Inbred strains and toxicity testing

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Abstract
Methods of toxicity testing have hardly changed in the last 50 years and far too many investigative new drugs which have passed the animal tests are subsequently discarded following expensive clinical trials, due to toxicity. Most toxicity testing is done using outbred Sprague-Dawley or Wistar rats and outbred CD-1 or other mouse stocks. There are two reasons why this is a bad strategy. First, stocks and strains differ in susceptibility to toxic chemicals. Should a resistant stock be used, it could lead to a false negative result. Second, outbred stocks are phenotypically variable due to inter-individual genetic variation. This creates "noise" which could obscure the effects of a toxic agent. Both these problems can be overcome by using small number of animals of several inbred strains in a factorial experimental design, without increasing the total number of animals which are used. Such a design would be more powerful and better able to detect toxicity with the added advantage that it would highlight genetic variation in response. Although these suggestions for improving methods of toxicity testing have been stated repeatedly over the past 50 years, neither toxicologists nor the regulatory authorities have ever attempted to justify their use of outbred animals. The use of outbred stocks in poorly designed experiments is no longer ethically justifiable.

Keywords: inbred strains, toxicity

According to the US Food and Drug Administration (FDA) in their "critical path" white paper "The traditional tools used to assess product safety -- animal toxicology and outcomes from human studies -- have changed little over many decades and have largely not benefited from recent gains in scientific knowledge. The inability to better assess and predict product safety leads to failures during clinical development and, occasionally, after marketing."[1]. The attrition rate of investigative new drugs (INDs), i.e. new chemical entities which have passed all the animal tests is, based on 1099 investigative new drugs, as high as 96%, with 46% being rejected due to lack of efficacy, 27% due to safety, 23% due to economics and 5% for other causes.[2] If this percentage could be reduced even by quite a small amount it could have a significant impact on reducing the cost of drug development.

One way of improving screening methods was suggested by Russell and Burch more than fifty years ago. According to them "Toxicity testing, as usual.., is the scene of some confused thought, which may be delaying the exploitation of statistical methods. We have not infrequently heard the opinion expressed that…. in toxicity tests you need a thoroughly heterogeneous mass of animals, and plenty of them. The physician, it is argued, is going to deal with patients with a very wide range of sensitivities to a given toxic action. There is a vague feeling that since this variation is quite uncontrolled, that of the test animals ought to be uncontrolled too. On this subject Hume has written "The fallacy consists in supposing that in order to obtain a wide inductive base a heterogeneous stock should be used… The proper procedure is, of course to use several different homogeneous samples, by using a plurality of pure lines (or preferably F1 crossbreds)… for otherwise the experimenter deprives himself of the possibility of making a relatively precise estimate of the error (Fisher 1942)."[3]

The aim of this paper is to show how toxicity testing in laboratory animals could be improved without using any more animals by choosing more appropriate, genetically defined inbred or F1 hybrid animals and a better experimental design almost exactly as suggested by Russell and Burch half a century ago.

Inbred strains and their value in research

The first inbred strain of mice was developed by C.C. Little, when he was a graduate student at Harvard in 1909. At the same time Dr. Helen King bred the first inbred rat strain designated PA or WKA.[4] Geneticists soon realised that these strains were of enormous importance in many areas of research. As early as 1936 Dr. Little stated that "…. the purity of the mouse stock can assure a research scientist of a true and sure experiment ..... In experimental medicine today .... the use of in-bred genetic material...is just as necessary as the use of aseptic and anti-septic precautions in surgery".[5] Other geneticists echoed these views with comments such as ".. within the near future all research on mice should be carried out on inbred animals or on hybrid mice of known (genetically controlled) origin where the degree of biological variability has been carefully controlled." [6], "...the development of inbred strains has constituted probably the greatest advance in all cancer research." [7] and "The introduction of inbred strains into biology is probably comparable in importance with that of the analytical balance into chemistry." [8]

Inbred strains are like immortal clones of genetically identical individuals. They are stable, only changing relatively slowly as a result of the fixation of new mutations.[9] Within a strain all individuals are virtually genetically identical. The genetic uniformity leads to phenotypic uniformity. This in turn means that either experiments can be done using fewer animals, or when using the same number of animals the experiments will be more powerful, giving fewer false negative results (Type 2 errors).

There are several hundred inbred strains of both mice and rats, each of which has its own unique characteristics. So, strains can be chosen to suit any research project. For example, a cancer research worker might select a strain with a high incidence of a particular type of tumour or, in contrast, a toxicologist could select one or more strains which are relatively free of early pathology.

Each strain is defined by a unique set of polymorphic genetic markers so genetic quality control is relatively easy. This contrasts with an outbred stock. There is, for example, no set of markers which will distinguish between Sprague-Dawley (SD) and Wistar rats, so it is impossible to determine whether any given rat is a "genuine" Sprague-Dawley. Thus, samples of SD rats from different breeders will be genetically different and may respond differently to toxic agents.[10, 11] In one study the immunological response to a synthetic polypeptide in SD rats in serial samples of about 30 rats, ranged from about 8% to 100% over period of two years although the response in seven inbred strains remained constant.[12]

Why don't toxicologists use inbred strains in toxicity screening?

Given the many advantages of inbred strains as research animals, why would any research scientist choose not to use inbred strains when working with mice or rats? Why is nearly all toxicity testing done using outbred stocks? Are scientists being irrational when they choose to work with outbred stocks rather than inbred strains?

As there have been no scientific papers attempting to justify the use of outbred stocks, we are reliant on quotes from individual toxicologists. For example, one toxicologists wrote that: "The variability of toxicity obtained in less well defined animals is a strength in itself, not a problem, when trying to predict safety margin in the non-isogenic human population." (anonymous industrial toxicologist 2005). Similarly another claimed that "It is notable, at least from an industry standpoint, that outbred stocks may in fact be more relevant to the human condition, as we are all outbred to various extents!..." (anonymous toxicologist with industrial & academic experience, 2005). However, "Good experiments minimize random variation, so that any variation due the factors of interest can be detected more easily."[13] So the choice of an outbred stock violates this fundamental principle of experimental design.

An example showing the consequences of using genetically heterogeneous animals is given in Table 1. Toxicologists sometimes study the effect of a compound on sleeping time under anesthetic in order to see whether it affects drug metabolizing enzymes. Table 1 shows the mean sleeping time under barbiturate anesthetic in five inbred strains and two outbred stocks of mice and also the standard deviation, based on reasonably large sample sizes. Note sleeping time was much more variable in the outbred stocks than in the inbred strains, as shown by the standard deviations. Using a power analysis it is possible to estimate the number of mice of each of these strains or stocks which would be needed to detect a 4-minute change in sleeping time between a treated and control group of mice, assuming the mean sleeping times are compared using a t-test with some other assumptions shown in the table. Such a study would need 7-23 mice per group using one of the inbred strains compared with 191 to 297 mice per group using one of the outbred stocks. Alternatively, if a fixed sample size of 20 mice per group were to be used there would be a better than 86% chance of detecting a four minute change if using inbred strains, but only a 13-17% chance of detecting such a difference when using an outbred stock. In other words there would be about a 4/5 chance of a false negative result using one of the outbred stocks. So

Table 1. *Sleeping time under barbiturate anaesthetic in mice of five inbred strains and two outbred stocks.*
Columns 2-4 give the sample size, the mean sleeping time and the standard deviation, respectively. Column 5 shows the number that would be needed in an experiment to compare a treated and control mean, if the aim were to be able to detect a 4 minute change in mean, with the assumptions shown. The last column shows the estimated power of an experiment to detect a 4 minute change if the sample size is fixed at 20 mice per group.

Strain	Sample size	Mean	Standard deviation	Number needed*	Power**
A/N	25	48	4	23	86
BALB/c	63	41	2	7	>99
C57BL/HeN	29	33	3	13	98
C3HB/He	30	22	3	13	98
SWR/HeN	38	18	4	23	86
CFW (outbred)	47	48	12	191	17
Swiss (outbred)	47	43	15	297	13

* Number needed in a two-sample t-test to detect a 4 min. change in the mean (2-sided) with α =0.05 and a power of 90%
** power of an experiment to detect a 4 min. change in the mean if the sample size is fixed at 20 mice/group
Data from ref. 26.

just supposing it was easier to "extrapolate" to man using outbred stocks (which is highly debatable), the message that would be extrapolated would involve a high frequency of false negative results. This is exactly what the FDA is complaining about in their 2004 "critical path" white paper.

The most common justification for choosing an outbred stock seems to be simply that humans are outbred, so outbred animals should be used to model them. However, this is faulty logic. Humans weigh about 70 kg. That doesn't mean that to model them we should use 70 kg animals. Models do not need to be similar to their target in every respect. Indeed they must *differ* from the target in many ways, otherwise they would not be models.

According to Russell and Burch, there are two important dimensions when considering models in research. The first is what they call the "fidelity" of the model: how like the thing being modeled it is in every respect. So a non-human primate will be a high fidelity model, whereas a bacterium would be a very low fidelity model of humans. The other important dimension is the ability to discriminate between our treated and control groups for outcomes which are of interest, such as toxicity. The "high fidelity fallacy" is the assumption that we are better off using high fidelity models. On the contrary, what is needed is a good ability to discriminate, in this case between toxic and non-toxic compounds (at some specified dose, of course). For this reason we don't all use non-human primates. Toxicologists frequently use bacteria, such as in the Ames test, or they use mammalian cell cultures. These have very low fidelity, but are often good at discriminating between toxic and non-toxic substances. Indeed a high powered committee of the US National Academy of Sciences has suggested that in future most toxicity testing should be done using *in-vitro* methods.[14] But this will require the investment of many billions of dollars, it will take decades, and it is by no means certain that it will be successful.[15] Changing to the use of inbred strains would be trivial in comparison, with the added advantage that it also makes it possible to explore genetic variation in toxicological responses as the difference between strains.

The geneticist Walter Heston summed up the situation nicely in 1968: "Yet, the question is sometimes asked, why not use genetically heterogeneous stock mice so the results will be more applicable to the genetically heterogeneous human population? The answer is that we are not trying to set up a model with mice exactly comparable to human beings. This would be impossible because mice and men are different animals. What we are trying to do is to establish certain facts with experimental animals and this can be done, or done more easily, when the genetic factors are controlled. Once the facts are established we then, with much common sense, see how the facts can be applied to man. When genetic variability is desired this can be obtained in the highest degree by using animals of a number of inbred strains. This variation between strains is usually much greater than is found in animals of a non-inbred stock which actually may be rather uniform although more variable than in an inbred strain."[27]

Heston's last paragraph introduces the other important modification that is needed in order to improve the power of current testing methods, namely

the need to use more than one strain or stock. Some strains and stocks can be relatively resistant to test chemicals. If such a stock or strain should be chosen then that will result in a false negative result. For example, outbred Sprague-Dawley rats were found to be totally resistant to the carcinogenic effects of diethylstilbestrol (a known human carcinogen) at doses which cause more than 70% of tumours in the ACI strain of rats.[16] Many other important strain and stock differences have been found which have important implications for toxicity testing.[17,18]

Unfortunately, it is not possible to predict which strains are likely to be sensitive. But it is usually possible to use more than one strain without having to increase the total number of animals by using a factorial experimental design. Such designs are described in most statistical textbooks.[19-21] They are very efficient. According to RA Fisher "If the investigator ... confines his attention to any single factor we may infer either that he is the unfortunate victim of a doctrinaire theory as to how experimentation should proceed, or that the time, material or equipment at his disposal is too limited to allow him to give attention to more than one aspect of his problem..... Indeed in a wide class of cases (by using factorial designs) an experimental investigation, at the same time as it is made more comprehensive, may also be made more efficient if by more efficient we mean that more knowledge and a higher degree of precision are obtainable by the same number of observations."[22]

The use of several strains has been studied extensively for binary outcomes such as the presence of a tumour or histological lesion, using simulations, and it was found that in a situation where there are no strain differences (a rare situation), little is lost by using such designs. However, in the much more common situation where there are strain differences, then the multi-strain study is more powerful, with maximum power being found when as many strains as possible are used. The main exception being if the strain which would have been chosen happened to be the most sensitive (a rare situation).[23]

One important advantage of using more than one strain is that strain differences can be attributed to genetic factors, and in some cases it is possible to isolate the genes involved. For example, strain differences in the nephro-toxicity of doxorubicin in mice have recently been shown to be due to a single Mendelian locus.[24] Such findings have important implication for "personalised medicine" in the future. Most genes in humans have their equivalent in the mouse and the rat, and it is much easier to identify the genes involved in these species than in humans, particularly when a toxic agent is being investigated.

Multi-strain toxicity studies

Unfortunately, toxicologists have not done any research into the use of a multi-strain study. However, a small biotech company in the USA called Physiogenix has tested a number of compounds in this way in rats. Unfortunately they have not published their results. Currently they have a number of genetically determined disease models in rats, and are providing services to the pharmaceutical industry based on these models. However, in their study of the toxicity of gentamicin they had three parallel studies all using exactly the same number of rats. The first one used Sprague-Dawleys, the second a single inbred strain F344 and the third one used seven isogenic strains. They found that of the 34 outcomes which they studied (mostly clinical biochemistry and organ weights) there were five (15%) statistically significant ($p<0.05$) differences between treated and control group in the outbred stocks, fourteen significant differences (41%) when using the F344 and nineteen (56%) significant differences with the multi-strain study. Thus the experiments using the genetically defined animals were clearly more sensitive than the one using the outbred stock. In the multi-strain study about half the significant differences also showed strain differences. (Howard Jacob, unpublished personal communication)

A study of the effects of chloramphenicol on haematology in mice of four inbred strains and one outbred stock has, however, been used to demonstrate the value of the multi-strain assay.[25] The study involved six dose levels of chloramphenicol with approximately eight mice of each strain/stock at each dose level. A random sample from the resulting data was used to make up two parallel studies. One had eight CD-1 mice at each dose level (48 mice, although on mouse was missing) while the other had two mice of each of the four strains at each level (a 6x4 factorial design) with a total of 48 mice. The results (Table 2) show clearly that the multi-strain study using inbred strains was more sensitive than the one using a single outbred stock because there were more significant differences and these occurred at a lower dose than in the outbred stock.

Why no debate?

It is remarkable that none of the papers published during the last half century explaining the potential benefits of inbred strains in toxicity testing has provoked any response from toxicologists. A survey using PubMed of more than 48 thousand papers involving the rat (the species favoured by toxicologists, but not widely used by geneticists), published between 2001 and 2003 showed that 47% of the papers used SD rats, 38% used Wistar rats, 14% used inbred strains and

Table 2. Dose of chloramphenicol (mg/kg) at which significant differences first detected

Character	CD-1	Multi-strain
Hematocrit	2500	1500
Hemoglobin	-	1500
Lymphocytes	-	2500
Neutrophils	-	-
Platelets	2500	-
Red blood cells	-	1500
Reticulocytes	2000	1500
White blood cells	-	2500

For more details see ref. 25.

the remaining 1% involved mutant rats. So the problem is not confined to toxicologists. Why do most scientists fail to understand the value of controlling the genetic variation in their research. After-all it is an important variable?

Maybe it is because the belief outbred stocks should be used because humans are outbred is not based on science, but is simply part of the culture in that branch of science. Imagine a young toxicologist just starting in a new job with a CRO or pharmaceutical company being shown round by his (or her) new boss. He is shown the animal rooms, and the rat cages, and is told "we use Sprague-Dawley rats". He is hardly likely to query this, but if he does do so he may be told that this is what the client specifies, or that this is what the FDA wants, or even that humans are outbred etc. It is highly unlikely that he/she will have sufficient knowledge of genetics and statistics to take this much further. Eventually, he finds that everyone he knows uses SD rats, and that toxicolgists have used SD rats for more than 50 years. Nobody queries it. That is the way that toxicology is done.

Cultural beliefs of this sort are usually defended with great tenacity. Toxicologists will claim that a multi-strain study is not practical. They will say that they are reliant on many years of historical data. They will claim that outbred stocks are more representative of human populations, although how a couple of hundred rats are supposed to represent several billion humans of many races is never explained. They may claim that a multi-strain study needs to be validated, forgetting that the use of a single outbred stock is far from validated, according to the FDA Critical Path white paper. What they seem unable to do is look critically at what they are doing, and whether they are using the most appropriate animals.

Conclusions

Inbred strains have many properties that make them the animals of choice in most research involving rodents, including toxicity testing. No scientific case has ever been made for the use of outbred stocks in toxicity testing. The *intuitive belief* that we should use outbred stocks because humans are outbred is non-scientific and increases the chance of false negative results. Several strains should be used as one may be resistant to a toxic agent leading to a false negative result.[3] Genetic variation is then detected as strain differences.

This collective lunacy is not confined to toxicologists. Thousands of scientists in many disciplines are allowing belief based on intuition and tradition rather than scientific principles to dictate the animals which they use. This is leading to a gross waste of scientific resources and poorer quality research. In toxicity testing it is contributing to the high attrition rate of new drugs and is increasing the cost of drug development.

For more than 50 years geneticists have been urging scientists to use inbred strains. Are we going to have to wait another 50 years for sense to prevail?

A fantasy in 2020

As you know, the new edition of the New Universal Dictionary was published in 2019. In it toxicology is defined as:

Toxicology *n*. Branch of genetics concerned with inter-individual variation in response to poisons, toxic chemical and drugs (see also Personalized medicine).

In 2020 we know toxicology as a dynamic, fast-moving discipline which is making a major contribution to the development of personalised medicine. The first Nobel prize in Toxicology was awarded in 2015 jointly to scientists working in Finland, the USA and Singapore. But it was not always like this. Up until 2010 toxicology was a scientific backwater. Methods of toxicity screening had not changed in over 50 years. However, in 2010 an international meeting of laboratory animal scientists was held in Helsinki. They were outraged when they heard that the outbred rats and mice which they were breeding and holding in their animal houses for toxicological screening were being used in badly designed experiments which were giving the wrong results. The outcome was this document:

The Helsinki Rat-ification 2010

1. *A basic rule of good experimental design is that all variables should be controlled except that due to the treatment.*
2. *The use of genetically undefined rats and mice violates this rule*
3. *The result is excessive numbers of false negative results*
4. *The use of such animals is therefore unethical and uneconomical*
5. *We hereby pledge that we will no longer breed, maintain or allow genetically-undefined mice or rats to be used in our animal houses, and when serving on an ethical committee, we will not agree to their use in research*

Signed _____

This was circulated rapidly by email throughout the world and signed by laboratory animal scientists in their thousands. Within six months Sprague-Dawley rats had become extinct, and Wistar rats soon followed.

Of course it created an enormous row. The first to accept the Rat-ification were the commercial breeders. They soon realised that although they might sell a few less animals, they could charge more for them, so it made little financial difference to them. The laboratory animal scientists were delighted because they were no longer required to run unethical experiments. Research scientists soon found that by using only inbred animals their result were more repeatable and they could use fewer animals.

The pharmaceutical industry was delighted because they found that the toxicological screening was much more effective so fewer toxic compounds were having to be discarded following clinical trials. Some of the cost saving were passed on to the public with lower prices for drugs. Toxicologists took the longest time to come around to the new ways. Some of them had been collecting historical data on Sprague-Dawley rats for 25 years, and suddenly these were extinct. Many of these scientists took early retirement. But a new, more dynamic group of toxicologists replaced them. The real turning point came in 2016 when it was realised that toxicology is simply the branch of genetics concerned with inter-individual variation in response to toxic agents. Suddenly all the tools which geneticists had developed over the years became available to them and there was an enormous pent-up demand for scientists to work in the new field of personalised medicine. In 2016 a Nobel prize was awarded jointly to three scientists for their work in identifying over 500 genes associated with response ot toxic chemicals. It is because of this work that now in 2020 we can predict with considerable accuracy the drugs to which each individual patient will respond most effectively. The rest is history.

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Scientists and Communities

Enhancing effective Three Rs searches – the CCAC Three Rs search guide

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Abstract
In Canada, investigators are required to submit their animal-use protocols to the institutional animal care committee (ACC) for ethical review before beginning their work. The submission should indicate that they have considered how they might implement the Three Rs in their study, including whether replacement alternatives could be used. Searching for Three Rs-alternatives is not an easy task; investigators may know their field of study, but are less well versed in other fields which might offer potential Three Rs methods. There are many useful resources available on the internet, but these can be confusing to navigate. For these reasons, the CCAC's Three Rs Program has developed a Three Rs microsite, including a Search Guide for investigators. The Three Rs Search Guide provides detailed instructions on how to conduct a Three Rs information search in a Step-by-Step Three Rs Search Strategy. The resources themselves are not new, but have been selected as the best available, and presented in a manner which we believe is easiest for Canadian investigators. In addition, the Search Guide provides a list of questions that investigators can ask themselves, or ACC members can use, to determine whether a Three Rs search is complete. As a further tool, a worksheet has been developed, that investigators can complete as they proceed through the search, to capture the relevant information, prior to completion and submission of an animal use protocol. Case studies are used to demonstrate the functioning and outcomes of the Three Rs Search Guide for research and teching.

Keywords: alternatives, Three Rs, search strategy

Effective Three Rs searches, that is, a search for Replacement, Reduction and Refinement-alternatives to using animals, have long been a requirement for the use of animals in science in Canada and other jurisdictions. The Three Rs provides a set of guiding ethical principles that help to minimize adverse impacts to animals when used for research, teaching or testing purposes. In Canada, investigators wishing to use animals for a scientific purpose must prepare an 'animal use protocol'. The protocol is then subject to review and approval by an animal care committee (ACC), usually located at the investigator's home institution. As part of the protocol, investigators must indicate that the use of alternative methods has been considered and that a thorough, structured, Three Rs information search has been done.[1]

The concept of the Three Rs was first described approximately 50 years ago in Russell & Burch's book, The Principles of Humane Experimental Technique.[2] The Canadian Council on Animal Care (CCAC; the national organization with the responsibility for overseeing the ethical use and care of animals in science) incorporated adherence to the Three Rs tenet as the fundamental basis for its work. This is described in the *CCAC policy statement on: the ethics of animal investigation*, which defines the Three Rs for the CCAC and provides the ethical framework for the conduct of animal-based science in Canada.[3] Adherence to these principles is directly in line with other international and national bodies responsible for overseeing animal use in science. For a number of years, the CCAC has been viewed as the Canadian centre for the Three Rs by other like-minded organizations (e.g. UK, National Centre for Three Rs (NC3Rs); US, Johns Hopkins Center for Alternatives to Animal Testing (CAAT); Netherlands Centre for Alternatives (NCA) etc.). The recently launched CCAC Three Rs Program aims to consolidate CCAC's role as Canada's national centre for the Three Rs (e.g. through hosting the 8th World Congress on Alternatives and Animal Use in the Life Sciences in Montreal in 2011). Similar to other Three Rs centres the CCAC Three Rs Program focuses on promoting the Three Rs through communication with investigators, laboratory animal veterinarians, ACC

members and other stakeholders, and supporting the implementation of the Three Rs in all areas of animal use covered by the CCAC Program.

Difficulties in Three Rs-alternatives searches

One way in which the CCAC is supporting implementation of the Three Rs in research is by providing assistance for searches for Three Rs-alternative methods prior to the preparation of animal use protocols. Searching for Three Rs-alternatives is not a straightforward task. It typically requires a multi-database literature search, a review of relevant guidelines and polices on animal use, a search of Three Rs internet resources, and could also include consultations with appropriate experts (such as laboratory animal veterinarians, animal welfare specialists and statisticians). Currently large amounts of information about the Three Rs are available on the internet; however, an investigator with limited familiarity of these resources may have difficulty in deciding what is the most useful and relevant. The peer-reviewed literature is highly credible and most journals are online, but finding information specifically related to Three Rs principles within the primary literature of diverse research areas and extrapolating to a different project can be time consuming and difficult.

Specific problems have been documented in the literature. For example, a workshop to discuss accessing Three Rs information identified problems with large search databases returning "a considerable amount of irrelevant material retrieved… (that) must be discarded before the desired information can be identified— if at all".[4 (p242)] In addition, much of the information that might be directly relevant to Three Rs is not usually published in typical research papers. Another study found that not all relevant information is indexed as "alternatives methods" and, if the text of an article does not specifically indicate that its content relates to an alternative method, it cannot be indexed to terms covering the Three Rs-alternatives to animal experiments.[5 (p125)] More recently, Leenaars et al.[6] conducted a survey of scientists to ask specifically about their experiences with Three Rs literature searches and found that obstacles to Three Rs searching included lack of time and money, inadequate search results, and lack of access to relevant sources of information. To summarize, investigators may know their own field of study, but are less well versed in other fields which might offer potential Three Rs methods.

CCAC Three Rs Microsite and Search Guide

As discussed, people working to implement the Three Rs face difficulties finding relevant and reliable information to guide Three Rs efforts in a timely way. In recognition of this barrier, the CCAC produced a new internet resource: the CCAC Three Rs Microsite[7] and included a Three Rs Search Guide[8]. The Microsite is a compilation of science-based information with resource lists that are carefully selected and curated. Information is organized by topic, and provides a summary of key principles related to the topic followed by a selection of links to other credible Three Rs-related resources and/or peer-reviewed publications. It can be useful for those seeking to access information quickly, or it can be used as the gateway to begin a more comprehensive and thorough search for information.

Development of the Three Rs Search Guide was informed by a number of factors. Firstly, we aimed to encourage investigators to integrate consideration of the Three Rs into experimental planning and the overall scientific process (instead of a stand-alone task to complete). Secondly, we assumed that investigators or other researchers would conduct the searches themselves and not use information specialists. Third, we were guided by the specific Three Rs-related requirements of Canadian animal use protocols.[1, 9]

The main component of the Three Rs Search Guide is a detailed step-by-step search strategy that includes descriptions of how to complete specific tasks (such as "collecting pre-search information") (see Figure 1). Each step embeds links to relevant resources, such as databases. The step-by-step strategy is targeted to novice investigators and those who are unfamiliar with Canadian requirements. For those who prefer to bypass the strategy, relevant information is collated separately, and includes a list of searchable databases and a list of "questions to assess if your Three Rs search is complete". Another component is an "animal use protocol worksheet", a downloadable, printable template that investigators may use to compile Three Rs-related information when they prepare protocols (see Figure 2).[8]

Each research question is unique and detailed examples of any one Three Rs strategy cannot cover the possibilities that will arise. Moreover, each investigator will have a unique way of approaching Three Rs information gathering that cannot be converted into a formula. In the following case studies, the CCAC Three Rs Search Guide and worksheet were used to promote careful consideration of incorporation of the Three Rs into animal use protocols for funded research and teaching.

Case study: integrating a Three Rs search in the research planning process

An established investigator was funded to study changes in gene expression in Huntington's disease, which is a neurodegenerative disorder. The

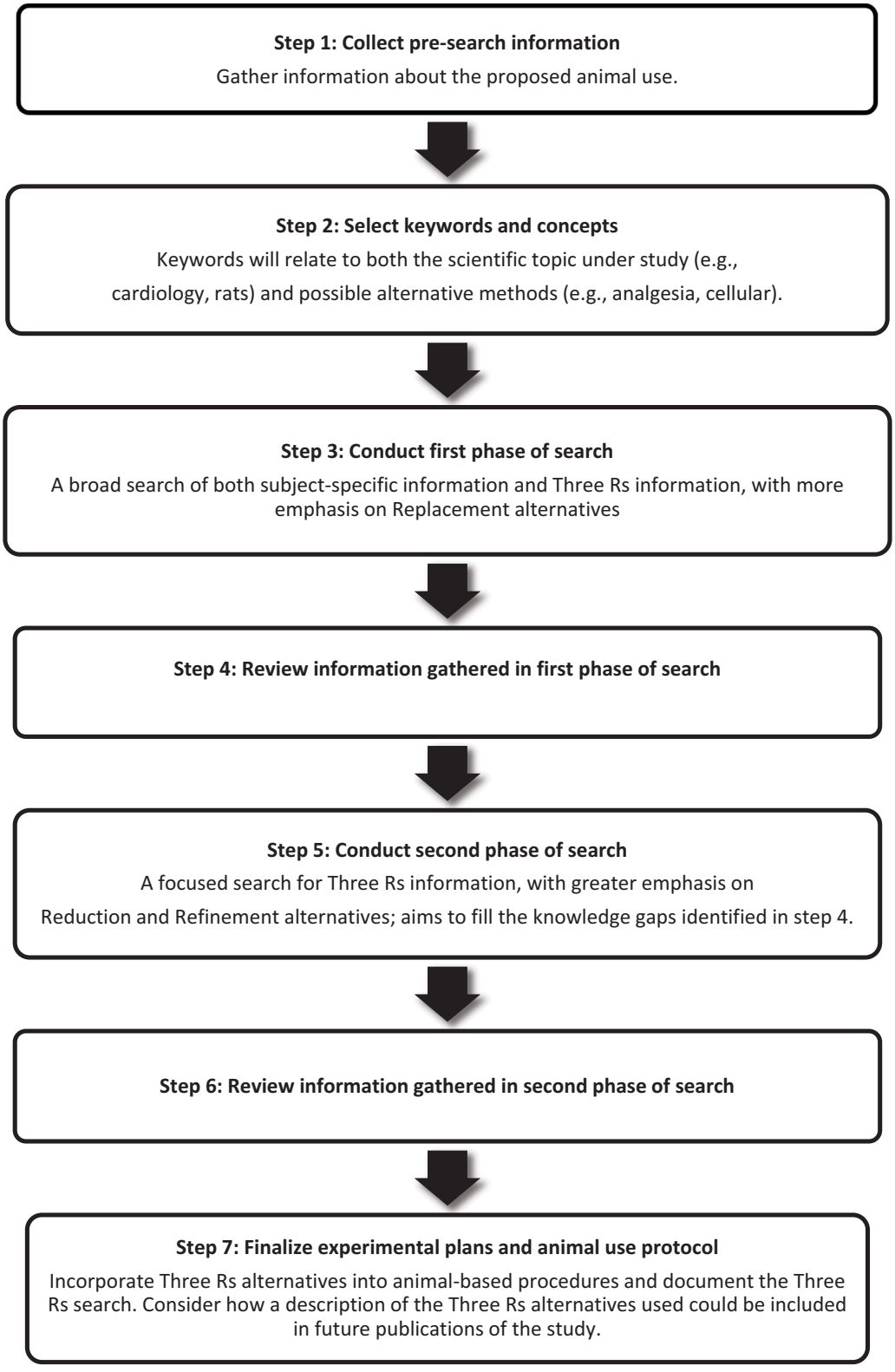

Figure 1: CCAC Three Rs Search Guide step-by-step strategy

experiments proposed and funded include studies of tissues isolated from different ages of wild-type and transgenic mice which model Huntington's disease, as well as studies aimed at reducing the expression of the mutant via gene therapy. Following the successful grant submission, the investigator needed to submit an animal care and use protocol to the local ACC and indicate how the Three Rs had been considered. Using the Three Rs Microsite, the Search Guide and the animal use protocol worksheet, the investigator was prompted

Information review

Did the Three Rs search determine any possible Replacement alternatives?

Replacement alternative category	No	Yes	If yes, describe and/or note citation
Absolute replacement			
Relative replacement			
Other:			

Did Three Rs search determine any possible Reduction alternatives?

Reduction alternative category	No	Yes	If yes, describe and/or note citation
Experimental design			
Sample size calculation			
Animal model selection			
Telemetry			
Animal supply strategy			
Data sharing strategy			
Animal re-use strategy			
Other:			

Did Three Rs search determine any possible Refinement alternatives?

Refinement alternative category	No	Yes	If yes, describe and/or note citation
Animal handling			
Animal housing			
Anesthesia			
Analgesia/pain management			
Blood & tissue sampling			
Humane endpoints			
Welfare assessment			
Humane killing			
Other:			

Figure 2: Excerpt from the CCAC Three Rs Search Guide animal use protocol worksheet

to consider the proposed animal experiments with respect to the animal model, proposed procedures on animals, potential causes of pain and distress to the animals and any known species-specific considerations. This investigator was already familiar with all of the potential models of Huntington's disease including *in vitro* models of specific intracellular functions, cell lines and primary cell culture models, and the range of animal models including nematodes (*C. elegans)*, fruit flies *(D. melanogaster),* zebrafish (*Danio rerio*) and a variety of transgenic mouse lines and chemically-induced models of Huntington's disease in which mice, rats and primates receive intracerebral excitotoxins to induce cell death in specific groups of neurons in the brain. The choice of animal model in the original grant application had been selected as the best available model that could fulfill the research objectives within the expertise and technical skills resident in the investigator's laboratory. At this point, the investigator was unlikely to radically change the animal model as peer-review of the grant had provided support for the experiments and animal models proposed. However, the Three Rs search led the investigator to realize that collaboration and the development of alternative models may be possible for future grant applications. Investigators who are not familiar with information on alternative models within their own field or within related fields would find such information using

simple search strategies combining model organism names with the name of a specific disease or more general terms such as neurodegeneration. The Three Rs Search Guide and listed resources give detailed information regarding information search strategies. Time constraints and the familiarity of the investigator with search strategies and the general literature will no doubt influence the thoroughness of the search. Over time, repeated Three Rs searches linked to ongoing research will likely encourage the consideration of alternative replacement models.

Searches for ways to reduce and refine animal use are complex as the searches are not necessarily disease- or species-specific. Information given on the CCAC Three Rs Microsite may prompt investigators to consider different means to reduce the number of animals used and refine procedures for the animals that must be included in a study. At this point, an investigator is likely to read published literature in detail to support best practices to generate high quality data from the minimum number of animals and then consult with the veterinarian and animal care staff to include the current standards of housing and care. In the case study example, the investigator had selected a specific strain of transgenic Huntington's disease mice that show a phenotype involving progressive neurological degeneration over a period of 12 weeks. These animals have reduced fertility and exhibit signs of motor and cognitive impairment that are apparent by about 4 weeks of age and progress over the next 8 weeks. Generally, the animals have reduced muscle mass, have progressive loss of motor skills and will die between 12 and 15 weeks of age. Having learned as much as possible of the a particular animal model's needs and life history, the investigator could ask specific questions of animal care and welfare specialists and search the literature to devise methods to derive the maximum information from groups of animals and refine animal care. Specifically, the investigator found literature regarding standard behavioural tests of motor function and statistical analysis that are currently accepted for this model, descriptions of changes in animal function that would require modifying diet and husbandry, procedures for surgical protocols and follow up care, timing of euthanasia that relate to humane endpoints, etc. In the Search Guide, questions included for information synthesis appear towards the end of the animal use protocol worksheet. Some investigators will likely consider these questions while searching for the information as opposed to collecting the data and then considering how the information addresses these questions. The worksheet and questions encourage investigators to search for and integrate data using their individual system of logic that fits with their experimental goals.

Each Three Rs search related to research will be highly unique depending on the research question, the investigator and the constantly changing base of knowledge related to the field of interest. As such the iterative process of designing experiments, while seeking funding and carrying out Three Rs searches for animal use protocols, will continue to co-evolve allowing for ongoing consideration of the best ethical use and care of animals to achieve research excellence.

Case study: integrating a Three Rs search in teaching protocols

In contrast to research, teaching goals may remain relatively constant over years or even decades. Instructors, who have defined pedagogical goals and attained curriculum approval, may be less inclined to carry out an ongoing consideration of how to include Three Rs-alternatives into updated animal use protocols. However, the Three Rs Microsite has links to a number of databases that can be readily consulted to look for alternatives to replace absolutely or to allow for fewer numbers of animals to be used if teaching goals can be complemented with non-animal resources. During the submission of animal use protocols for teaching, an instructor can easily consult the databases and use the worksheet to collect information regarding possible alternatives and supplementary teaching material.

For example, a long-standing course to teach principles of pharmacology includes lectures and laboratories. Over time, rodent experiments have replaced experiments that once used dogs and cats, and cell-based assays have replaced some experiments that used tissue from animals purchased for laboratory exercises. However, due to the nature of the complexity of living systems, some laboratory exercises that illustrate animal behaviour or interactions between body systems in response to drugs remain. A simple search of pharmacology subsections within several of the data bases of Alternatives linked to the Three Rs Microsite revealed video resources, computer simulations and tutorials that could reduce animal use. Some of these resources are available without charge, while others have a significant cost. The Three Rs search can be used for future planning and to request funding to support the acquisition of teaching resources that over time will lead to ongoing replacement and reduction of animals in teaching labs. Therefore, although the teaching objectives do not change rapidly, the Three Rs Microsite can readily promote ongoing consideration of animals used in teaching.

Discussion

As part of preparing the CCAC Three Rs Search Guide, three other search strategies were reviewed. The Focus on Alternatives (FoA) *Early planning poster for a project that might involve animals* is a one-page visual overview of a decision-making strategy for considering alternative approaches when designing research projects. [10] However, it did not contain sufficiently detailed guidance to meet the CCAC goal of facilitating Canadian investigators' alternatives searches.

The University California Center for Animal Alternatives (UCCAA) *Search for Alternatives: A step-by-step approach to an alternatives search* is a two-phase search strategy designed to meet the animal welfare legislative requirements of the United States Department of Agriculture (USDA).[11] The CCAC Search Guide follows the UCCAA approach in order to lead investigators through a defined search process, in particular, we incorporated the UCCAA step of pre-search information collection.[12] However, the emphasis of the UCCAA strategy on meeting the requirements of US legislation and the assumption that an information specialist (IS) would conduct the search meant that the search steps were structured to familiarize the IS with the research and to facilitate communication between the IS and the investigator, making the remaining steps unsuitable for Canadian purposes. Thirdly, the search is structured to look for refinements and reduction in the first phase and replacement in the second phase, which does not reflect the CCAC requirement of sequential application of the Three Rs (i.e. replace, reduce, refine). The FRAME (Fund for the Replacement of Animals in Medical Experiments) search *How to search for information: A guide to searching for alternatives to the use of laboratory animals* was also reviewed.[13] The FRAME strategy contains detailed general information on database and internet searching and divides the alternatives search into two parts: a subject-related search followed by a Three Rs-related search. However, this strategy also did not completely fit with Canadian Three Rs search requirements.

We recognize that there are some limitations to the CCAC Search Guide. Firstly, the search methodology is designed for research use and may need some adjustments to fully support testing or teaching alternatives searches, beyond providing a portal to readily access information. In addition, we did not include an example, or tutorial, of a completed alternatives search using the strategy and a hypothetical (or real) research protocol. These types of examples are available for both the UCCAA and FoA searches.[14-16] However, some investigators have suggested that examples can be limiting. It may be more useful to find a means of stimulating the necessary thought processes for investigators, each time that a Three Rs search is needed.

The creation of the Search Guide and the Microsite have provided the CCAC with an opportunity to communicate new Three Rs information to investigators—beyond what is written in CCAC guidelines documents. It also provides greater opportunities for all CCAC constituents to contribute information outside of the formalized CCAC guidelines development process. In the course of developing these tools, new questions about how and when to begin searching for Three Rs alternatives have been opened for the CCAC. For example, is the best way to organize information by each "R", or is there a more intuitive and user-friendly way?

In addition, in the Canadian oversight system, considerable responsibility is devolved to the local ACC, where the ethical review of protocols occurs. Thus investigators are only required to show evidence of having searched for Three Rs alternatives when submitting their animal use protocol to an ACC. This is late in the research planning process and can be frustrating for both investigators and ACC members alike when the ACC requests evidence that the work cannot be accomplished without the use of animals, or questions the experimental design. As the research case study used in this paper has shown, it is more relevant for a Three Rs search to be carried out at an earlier stage in the research process, such as grant writing and programme of work planning. Through familiarity with the CCAC Three Rs Search Guide, it is envisaged that Three Rs searches will become part of the iterative process (i.e. funding, seeking ethical approval, carrying out studies, reporting and publication) for Canadian investigators.

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Journal editorial policies as a driver for change – animal welfare and the 3Rs.

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Abstract
Scientific journals are instrumental in the communication of knowledge and can play a key role in encouraging good scientific practices. With respect to animal research, journals can exert a strong positive influence on standards by insisting on adherence to sound ethical principles, best practice in animal housing and husbandry, and by publication of detailed 3Rs information. To this end, explicit editorial policies on the publication of animal research, and clear instructions to authors, are essential. Analysis of journal editorial policies over the past four years has shown that a large proportion of journals publishing animal research have no such policy or instructions. Those that do, vary widely in their requirements. We have therefore developed a set of guiding principles, and a model editorial policy that journal editors and publishers can adopt. The model includes requirements for proper ethical review, application of the 3Rs, adherence to published good practice guidelines, and the publication of details crucial to the dissemination of good practice, such as experimental design and analysis, housing and care arrangements, and pain management. To ensure effective dissemination of this kind of information, journal editors need to require authors to include it in the papers they submit for publication. This will provide readers with a greater wealth of knowledge, and lead to improvement and greater consistency in standards around the world, as well as stimulating an informed discussion of the ethical issues that are integral to the use of animals in research.

Keywords: publication policies, journals, 3Rs, information

Scientific journals constitute the most important medium for the publication of research. Publication is vital to scientists not only as a means of disseminating the results of their research, but also to provide evidence of their productivity, on which their ability to maintain and attract financial support depends. Journals therefore have the opportunity to exert considerable influence on research standards, through their editorial policies and systems of peer review. In those fields where animals are used, we believe that they have not only the opportunity, but also a responsibility to help disseminate and develop good practice, facilitating the uptake and implementation of the 3Rs. More broadly, journals can contribute to promoting more humane science by encouraging thorough ethical evaluation of animal research projects, the informed discussion of ethical issues, and greater openness and transparency about the use of animals in science.

Guiding principles

We have developed a set of guiding principles that we would like to see journals applying to their publication policies. We believe that any journal publishing primary data originating from the observation and/or investigation of any non-human animal should:

- acknowledge that the use of animals in scientific procedures raises serious ethical and welfare issues;
- define the nature of research that editors consider is acceptable/unacceptable for publication on ethical grounds (for example, some journals will not publish research causing substantial suffering that has not been alleviated);
- request confirmation from authors that research has undergone a rigorous harm-benefit analysis as part of an ethical evaluation, and that animal welfare and other 3Rs issues have been properly addressed;

- clearly explain the information that authors need to include in papers for the research to be accepted for publication and make these instructions easily accessible (for example the level of detail that is required in the methods section on animal numbers, species, sex, husbandry and care practices, and refinements);
- be prepared to publish sufficient information on experimental design, the 3Rs and animal welfare in order to help disseminate this to the wider scientific community;
- require adherence to the editorial policy as a stipulation for publication - this is essential for any policy to be taken seriously;
- require reviewers to make sure the above points are taken into account in the papers they review.

Current practice – RSPCA Annual survey

We were concerned that scientific journals could go much further in exploiting opportunities, or discharging their responsibilities, to improve standards in research involving the use of animals. Therefore, we began an annual survey of journal publication policies to assess whether, and how well, these issues were being addressed. The first survey was conducted in 2007, using data from July 2005 to June 2006. The results of this survey, together with full details of the methods employed have been published in the American Journal of Bioethics[1]. Subsequent surveys were conducted in 2008, 2009, and 2010.

A PubMed search was conducted to identify primary research articles, in English, that involved the use of animals. To allow for delays entering articles into PubMed, articles published between July and June were extracted in the following January. Journals which had published 4 or more relevant articles within this period were identified, and a random sample taken.

The numbers of articles and journals found are shown in Table 1, together with the number of journals sampled for publication policies in each year. Since starting the survey, the number of relevant journals has increased 1.8-fold, and the number of relevant articles 2-fold. Some journals appeared in more than one of the annual samples but to date we have surveyed the publication policies of 868 different journals, which is equivalent to 40% of all relevant journals included in the 2010 survey.

Publication policies of the journals in each sample were collected directly from the journal's website and were largely in the form of 'Instructions for Authors', 'Ethical policies' and other relevant statements. The journal policies were then scored according to the scheme shown in Table 2. Specific mention of animal use in a policy was taken as an acknowledgement that animals are used in the research the journal publishes, even if the policy did not impose any significant requirements on authors; many journals fail to mention animal use at all.

Where adherence to specific guidelines, codes of conduct or legislation was required, most commonly the UK (Animal Scientific Procedures Act, 1986), European legislation (EU Directive EC86/609) and the ILAR Guide for the Care and Use of Laboratory Animals, no assessment was made of the guidance they refer authors to. With regard to prior ethical review, requiring a statement confirming approval by an IACUC or animal care committee was not counted because these committees are not required to conduct an ethical review, by which we mean a full harm/benefit analysis.

Recently, two sets of guidelines (GSPC[2] and ARRIVE[3]) have been published which address, in detail, the issue of what information on the design and conduct of animal experiments should be required by editors. Our survey did not attempt to assess the extent or nature of the information requirements given in journal policies. A point was awarded simply for recognition of the need to provide some basic detail, such as species, animal numbers and novel refinements of husbandry or procedures.

Where no policy relating to animal use was found on the journals website, an attempt was made to contact the editor by e-mail, telephone and/or by letter, asking where any publication policy could be found. If no relevant policy could be identified, the journal was scored zero.

Table 1. The number of articles and journals identified and surveyed in each period, from July of one year to June of the next. Relevant articles are primary research reports involving animal use (not reviews). Some journals appeared in more than one of the samples. The total number of different journals sampled over four years was 868.

Year	Number of articles	Number of journals	> 4 relevant articles	Sample size
2005/6	62,337	1,691	1,152	288
2006/7	67,457	2,164	1,444	299
2007/8	121,436	2,342	2,046	316
2008/9	125,280	3,048	2,131	320

Table 2. Scoring criteria. The editorial policies of journals sampled in the survey were scored according to this scheme.

	Short title	Score
Main criteria:		
Mentioning the use of animals in research and testing.	Mention only	1
Requiring adherence to specific guidelines, codes of conduct or legislation relating to research involving animals, and providing links to them.	Link to guidelines	1
Stating that adherence to the relevant policy was a precondition for publication	Policy adherence	1
Having an overall considered, positive statement regarding animal welfare or the ethics of animal use.	Other statement	1
Additional points were awarded for specific requirements that:		
Maximum possible implementation of the 3Rs is demonstrated.	3Rs application	1
Animal housing and care followed contemporary good practice.	Housing & care	1
Appropriate anaesthesia and analgesia was used to minimise discomfort, distress and pain.	Minimising pain	1
Humane endpoints were defined and implemented.	Humane endpoints	1
A requirement that the research had undergone prior ethical review.	Ethical review	1
Euthanasia was carried out according to best contemporary practice.	Euthanasia	1
All information that is suitable for publication, such as species, strain and numbers of animals and other pertinent details including refinements in husbandry and procedure are included in the article.	Details provided	1
Maximum score		**12**

Survey results

The results of the four surveys are summarised in Figure 1. The average scores for all the journals sampled were 1.27 in 2005/6, 1.13 in 2006/7, 1.40 in 2007/8, and 1.31 in 2008/9. This is extremely disappointing trend, and indicates no improvement over the 4-year period.

According to our criteria, journals can score a maximum of 12 points, although to date none have scored higher than 9 (achieved by 4 different journals). Over 40% of relevant journals had no policies relating to the use of animals in the research they publish. Enquiries to these journals met with much openness, interest and encouragement from the editors or their publishers. A number of journals have either adapted, or are in the process of adapting their policies to include details relating to the use of animals as a direct result of our enquiries. Furthermore, some of the journals assessed in more than one year changed their policies and showed an improvement in their survey scores from previous years. There was only a slight decrease in the proportion of zero scores over the four years of the survey, but this may reflect the fact that the majority of journals included in the survey are different each year.

The frequency with which points were awarded for specific criteria was analysed for 3 of the survey periods; 2006/7, 2007/8, and 2008/9. The results are shown in Figure 2. This shows that where journals had a relevant policy, most were likely to:

- mention that animal use should be conducted legally, or to unspecified guidelines;
- ask that research is conducted according to specific legislation or guidelines;
- ask that the research has undergone an ethical review;
- ask that distress, pain and discomfort be minimised;
- make some other positive statement relating to animal welfare or the use of animals in research;
- require adherence to their publication policies as a condition of publication.

The requirement for the conduct of animal experiments according to specific legislation or guidelines is important, but legislation and guidelines can be very variable in scope, level of detail, and standards required. Merely citing guidelines therefore does little to ensure that a robust ethical review has taken place, or that the 3Rs have been implemented. In our opinion, authors should be specifically asked to

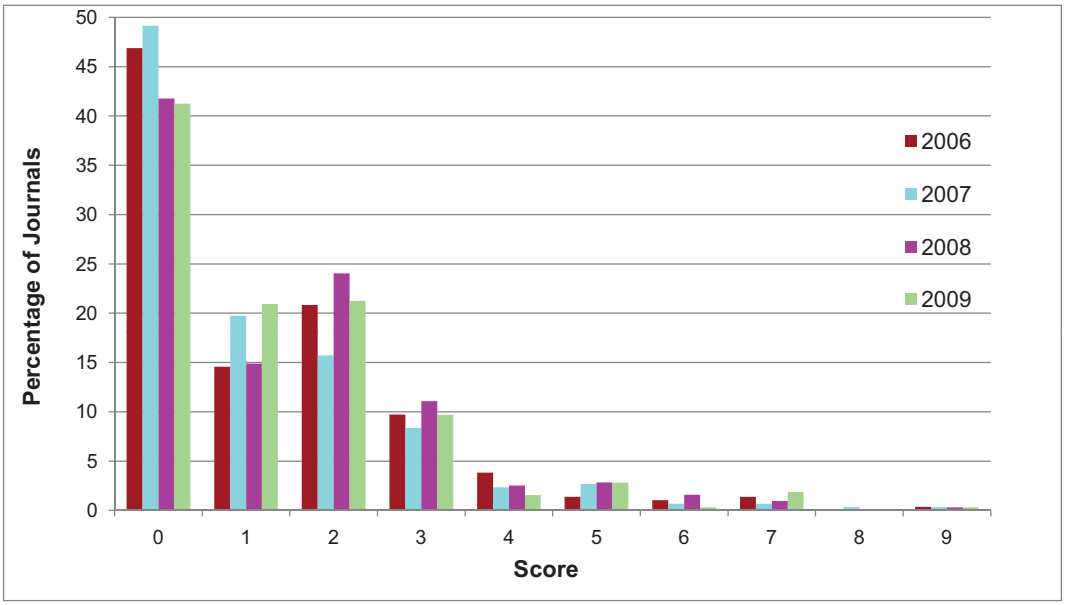

Figure 1. The percentage of journals within the samples for the four years of the survey which either had no stated policy on the publication of research involving animals (score 0) or which had policies which were awarded the score shown, according to the criteria given in the text (no journal scored more than 9 out of a possible 12).

confirm that research has undergone a rigorous harm-benefit analysis as part of an ethical evaluation, and that animal welfare and other 3Rs issues have been properly addressed.

With regard to the 3Rs, only 3 out of 694 journal policies analysed were found specifically to require adherence to the 3Rs of Russell and Burch. However, an additional 3 journals required application of replacement, reduction and refinement, without referring to them in those terms. A further 8 policies covered 2 of the 3Rs, and 2 mentioned only one R. Requirements relating to the inclusion of essential information such as species, strain, and housing conditions were found in only 5.4% of policies.

These results show that there is enormous variability between journals with regard to editorial policies on animal research. This is likely to reflect variations in the level of awareness of animal use

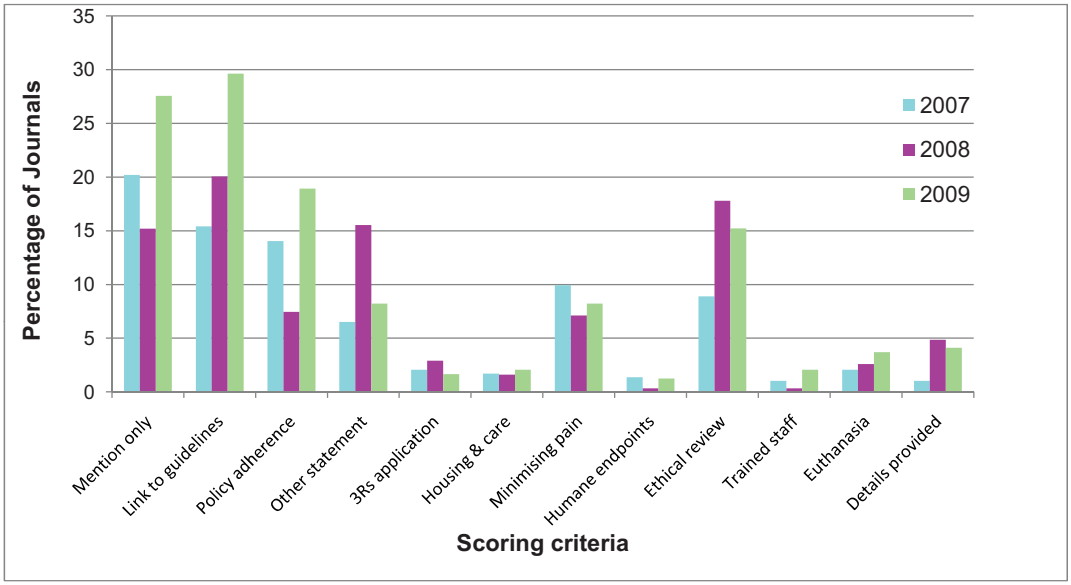

Figure 2. The percentage of journals in survey years 2007-2009 which were awarded a point for the criteria given in the Table 2('short title' shown). 'Mention only' means that animal research was mentioned in the journal's instructions to authors but the policy satisfied none of the other criteria.

between individual journal editors, the importance of animal research in their field of publication, and many other factors. However, the best examples we found demonstrate that it is possible and practical for journals to have workable publication policies relating to the use of animals in the research they publish. Lack of awareness of the value and importance of such a policy is one obvious obstacle that can be addressed.

Developing best practice guidelines

The lack of a relevant publication or editorial policy, 'instructions to authors', or set of author guidelines which journals can easily adopt 'off the shelf' is a major obstacle to progress. It is difficult to envisage a single document that would be appropriate for all the different areas of animal research that journals are likely to encounter. However, we have produced a basic good practice model 'instructions to authors' that journal editors could use as a point of reference when reviewing their own publication policies (Table 3). This provides a workable example of a publication policy that would adhere to the principles described above and achieve a maximum score in our survey.

More information can be found in a factsheet[4] that we have produced for journal editors, which has been widely distributed and can be downloaded directly from www.rspca.org.uk/scientificjournals. If journals adopted this policy it would represent a significant step forward. In addition, it is also shown by others (GSPC[2], ARRIVE[3]) that there is a clear need to develop and integrate specific recommendations on information requirements into the author instructions/ guidance provided by journals.

Plans for the future

Growing recognition of the ethical issues surrounding research involving the use of animals and the results of our survey has resulted in increasing interest in the development of editorial policies on animal research among journal editors and publishing organisations. Our correspondence with individual journal editors has also highlighted a number of areas of research that raise specific animal welfare concerns (for example pain research, the use of cancer models, or studies involving wildlife), for which more specific guidance for journals would be welcomed and we are providing on a one-to-one basis.

We also believe that reviewers of scientific papers have a vital role to play in ensuring that sound policies on the publication of animal research are adhered to. However, there is very little guidance for reviewers about what journals expect of them when reviewing papers, especially with respect to the ethical issues and animal welfare. We therefore plan to produce a leaflet on these issues for peer reviewers of animal research.

Table 3. Good practice model.

Good practice model 'instructions to authors'
'Journal X is committed to ensuring that the research it publishes gives full consideration to animal welfare and ethical issues. Papers will only be accepted for publication if authors can confirm that: • research conforms to the standards set out in contemporary best practice regulations or guidelines as specified by the journal (All journals should decide what guidelines they consider to be contemporary best practice in their specific area of animal research and provide direct links to these within the policy); • the research proposal went through a process of ethical review prior to the study commencing; this should include a weighing of the likely adverse effects on the animals against the benefits likely to result from the work; • the potential for application of the 3Rs was rigorously researched prior to starting the studies, and every opportunity was taken during the course of the study to implement each of them; • animal husbandry and care was in accordance with contemporary best practice; • all individuals involved with the care and use of animals were trained and skilled to an acceptable level of competency; and that • appropriate anaesthesia and analgesia were used to minimise pain and distress, human endpoints were defined and implemented where appropriate, and , with euthanasia carried out according to contemporary best practice. **Manuscripts for publication should contain details of:** • Species and, where appropriate, strain of animal • Total number of animals used throughout the study • Experimental design including statistical design and analysis • Other pertinent details relating to the lifetime experience of the animals, including housing and care, refinements to experimental procedures to reduce suffering' pain management, humane endpoint,; and euthanasia methods.'

Conclusions

The surveys we have carried out have shown that many journals do not have any written policy on the publication of research involving animals, and that existing policies vary greatly in content. Very few approach our ideal good practice model. We recognise that journals may operate publication policies but just not state this explicitly in their author guidelines. Nevertheless, we believe that, if the general standard of ethical review and reporting is to be improved, explicit policies need to be developed and made readily available to authors by all relevant journals. We therefore hope to continue working with individual journal editors and publishing organisations to ensure that any journal publishing research involving the use of animals adopts editorial policies tailored to their own particular publications. By developing journal policies, we believe that significant advances can be made in the welfare of the many animals used in research each year.

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3R compliance, culture and consequenses

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Abstract
Compliance describes commitment to regulations or standards. Animal experiments raise ethical questions and compliance to regulations is essential for legitimacy. The 3Rs, an acronym for Replacement, Reduction and Refinement, help to legitimize animal experiments. 3Rs are implemented in rules and regulations, such as the European Directive on the protection of animals used for scientific purposes. This presentation focuses on human factors in compliance to the 3Rs. Research on organizations and changes in management has shown that organizational culture is just as important for changes in human behaviour, as are formal rules and regulations. An important question is whether the implementation of 3Rs in regulations causes significant changes in behaviour and compliance to the 3Rs. Moreover, can integration of 3R values in the organizational culture promote 3R as a strategic tool in problem solving and decision-making? Understanding both compliance and organizational culture is significant for implementing the 3Rs, because human behaviour is a crucial key to success. This presentation gives an introduction to the terms compliance and organizational culture, and discusses them in the context of 3R integration.

Keywords: 3R, compliance, culture, communication, knowledge transfer

Introduction

Animal experiments raise ethical questions and compliance to regulations is important for legitimacy of the experiments using animals. The 3Rs, an acronym for Replacement, Reduction and Refinement[1], help to legitimize animal experiments. The 3Rs have been implemented in rules and regulations for use of animals in research, like in the European Directive on the protection of animals used for scientific purposes (2010/63/EU), where it is stated that "member states shall ensure that wherever possible, a scientifically satisfactory method not entailing the use of live animals shall be used", that the "number of animals used in projects is reduced to a minimum" and finally refinement to "eliminate or minimize possible pain, suffering, distress or lasting harm"[2].

This clear statement creates expectations of a change to increased compliance to the 3Rs, so that no animals are used or suffer unnecessarily. Many organizations have welcomed the very clear demand on 3R implementation in the directive. However, experience on changes in other organizations has shown that change in formal regulation alone is not sufficient for substantial changes to happen[3]. There are informal forces and mechanisms resisting changes anchored in organizational culture, which resist to change[3].

Compliance

Compliance describes commitment to regulations or standards[4]. In medical treatment, compliance means to what degree the patient fulfills the treatment according to the prescription of the physician[5-7] or veterinarian[8,9]. In risk-based activities, like in the aircraft, nuclear and petroleum industries, compliance to safety rules is important to avoid unwanted incidents or accidents. The term "compliance" is somewhat controversial and provokes negative feelings because non-compliance indicates disobedience[6] or opportunistic behaviour[7]. "Concordance" has been suggested as a replacement term for compliance by the Royal Pharmaceutical Society[10]. However, as the term compliance is so well established, it will be used in this text.

Compliance can be understood from a relational as well as a competence perspective. The relational perspective includes the paternal and the cooperative relationship. In the paternal relationship, one part is the superior, providing a solution to an inferior part. In the collaborative relation, the involved actors agree on a solution together. Studies on compliance to medical

treatment show that the cooperative relationship increases compliance to medical treatment[11,12].

Compliance can also be understood from a competence perspective[13]. Rule-based compliance is simply following a description of a standardized procedure. This can be possible for standard operating procedures (SOP). Applying a wrong rule or misapplication of a rule can cause failure, or lack of compliance.

Knowledge based compliance involves making the right decision in the right situation based on knowledge of alternative solutions. Selecting the correct painkiller for a specific experiment, without inducing adverse effects or bias, is an example of knowledge-based 3R compliance. Knowledge based compliance is necessary when the solution is not clear once and for all. Knowledge based failure can be caused by limited capacity or incomplete knowledge or occur in unfamiliar tasks that demand active problem solving.

Skill-based compliance is not only dependent on knowledge, but also on practical skills to perform the action in the correct way at the critical moment. Handling of animals is an example of a common and necessary procedure that causes physiological responses in the animal[14]. Correct handling demands not only knowledge of the right way to handle, but requires also the practical skills to perform handling correctly. Practical skills and experience, achieved by practical training, are seldom articulated or verbalised. "Tacit knowledge" is a term for experience-based, not formalized and non explicit knowledge[15]. Tacit knowledge is difficult to communicate by means of writing it down or verbalizing it, and is therefore difficult to report in publications or describe in ethical applications. This is especially relevant for several refinement strategies, which makes them therefore difficult to formalize[16]. And when something is not formalized, it then becomes difficult to document compliance.

Slips, lapses or inattention are examples of lack of skill-based compliance and they occur in routine and well-known tasks. Ellen Langer, Professor in the Psychology Department at Harvard University, pinpoints routines as a major source of lack of attention or mindfulness[17]. According to Langer, standardized procedures foster human failure. On the other hand, there are managerial theories that favour the improvement by continuous improvement of standardized procedures, like the Japanese Kaizen concept[18]. An important success factor is the ability to focus on the task independent of how many times it has to be repeated. This is the topic in Robert Pirsig's famous book "Zen and the art of motorcycle maintenance"[19]. We find a western approach to continuous improvements in the Plan-Do-Check-Act (PDCA)-circle which is a central moment in ISO 9001 accreditation[20]. From the competence perspective, it is clear that compliance is more complex than simply following the rules.

With 3R compliance, we understand planning and performing animal experiments in accordance with the principles of the 3Rs[21]. The content, value, or boundaries of application of 3Rs vary according to context or conditions and is not fixed once and for all. It was also demonstrated in a survey among researchers in academia that the 3R search and implementation is quite complex[22]. It may therefore be necessary to use other approaches than simply following the rules and regulations to study 3R compliance, and understanding of the organizational culture is therefore fruitful.

Organizational culture

Organizational culture represents a pattern of basic assumptions that are created or developed by a social group as they experience to master their problems[23]. This strategy works appropriately when it is regarded as "true" and is taught to new members of the group as the right way to understand, think and feel concerning problem solving[23].

Organizational culture modulates behaviour and fills the gaps where formal laws and regulations do not exist[24]. The norms and values of the organizational culture tell what is right and what is wrong, and this pattern is stable and valid over time and in different situations.

Organizational culture represents a pattern of basic assumptions that help to form a common way for group members to understand and solve problems. The basic assumptions are not necessarily rational or optimal for problem-solving, but they represent the values and norms of the group and set the standard for the group's preferred strategy for problem-solving. It is important to know the basic assumptions of a group when implementing the 3Rs.

One basic assumption might be that the 3Rs are mainly another bureaucratic exercise and an obstacle to research. This basic assumption does not promote willingness towards 3Rs' implementation. An alternative interpretation is that the 3Rs are a really efficient tool to promote good science[25]. This basic assumption will ease 3R integration.

Integration of the 3Rs into legal regulations alone does not guarantee behaviour in accordance with 3R values and norms. 3Rs must be experienced as useful to be completely integrated in the culture. Cultural artifacts are observed phenomena that reflect intrinsic standards about values and norms. With 3R culture we understand a pattern of basic assumptions within a

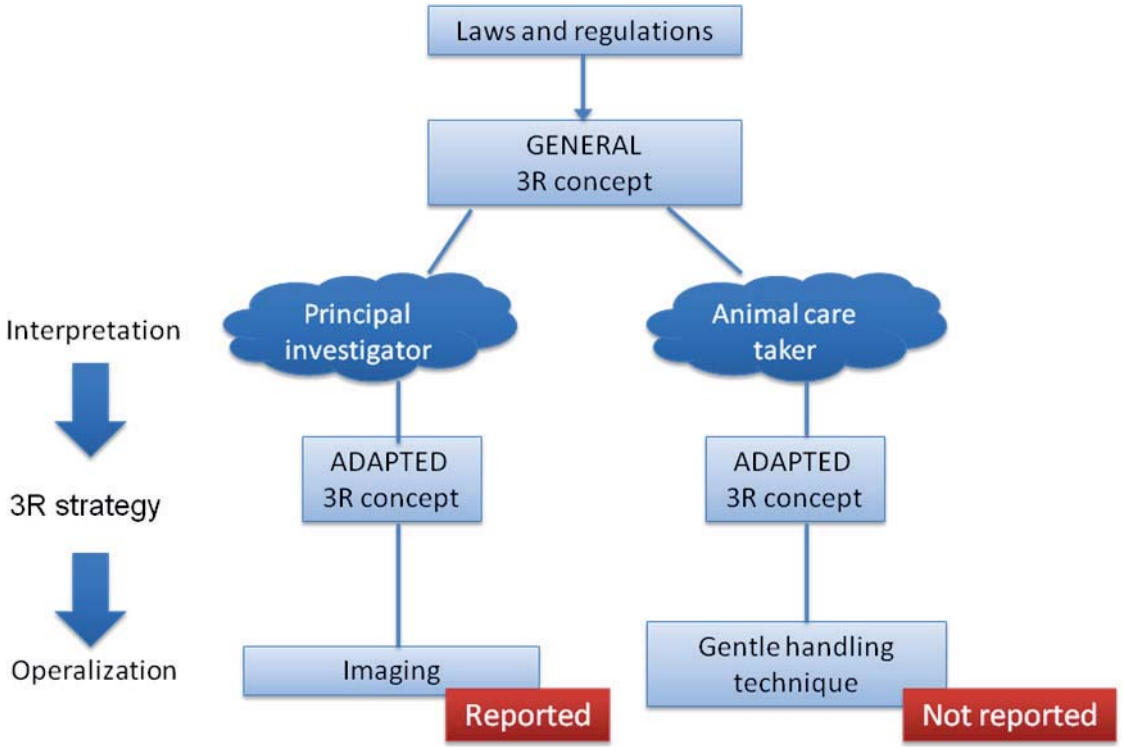

Figure 1. The general 3R concept is interpreted into an adapted concept that is suitable and relevant for the different participants before it is taken into action (Imaging and animal handling are taken as practical examples)

group on how best to comply with the 3Rs and this can be described by studying cultural artifacts[21].

Consequences for 3R compliance

European Directive 2010/63/EU on the protection of animals used for scientific purposes literally and formally states a clear demand for compliance to Replacement, Reduction and Refinement strategies[2]. Research has shown that organizational culture is just as important for behavioural changes as formalized rules and regulations[24] and organizational culture conserves values and norms over time. Managers can influence the evolution of the culture by fostering desired values, but they can never control culture[24]. How can managers influence an organizational culture so that 3R strategies are in the frontline in problem solving and decision-making?

There are two models for how culture can be changed. The economic model (model-e) emphasising structural changes decided by the management as important for cultural change, because people are mixed in new social groups where (old) basic assumptions are challenged and questioned. The organizational model (model-o) has more trust in changing basic assumptions through education and cognitive mechanisms. Selection of the best model depends on which organization it is that has to be changed[26]. Knowledge-based organizations (like academia), will often resist changes dictated by management, and they will only support changes they "believe in" themselves.

Lessons learned from compliance studies have shown that the cooperation approach is most efficient and should therefore be the preferred relationship[11,12]. Cooperation demands flexibility and good communication skills. It might be a pedagogic problem to promote 3R initiatives in a way that makes sense and motivates the researchers, because people do not always speak the same professional language. Researchers might use other words for 3R strategies and compliance to 3Rs, and they might also have other motives than ethics[16]. However, the fact that we do not use the same language does not say that we do not share the same governing values[27].

Scientists might feel overwhelmed by the bureaucracy they have to overcome to do research. In the worst case, researchers interpret the 3R evaluation as a bureaucratic hindrance they have to avoid or use least possible effort on. This may be caused by bad experiences with earlier bureaucracy. In that case the 3Rs are regarded a problem - and not a tool for problem solving. "People work best for objectives they themselves believe in"[28], and therefore, taking the paternal, superior position does not promote good cooperation and compliance. The organization must

therefore foster and nurture a culture for cooperation. Experience from the compliance studies in medical treatment also shows that we should strive for the collaboration relationship[5-7, 30].

In many academic institutions, animal houses are organized as centralized core facilities. There are several advantages with this model, including assembling competence available for the researchers. Also specialized 3R resource centres offer services and competence to researchers. However, segmentation in different organizations might foster "us-them" attitudes and this is not a good foundation for collaboration. Involving animal staff as equally important partners in specific research projects (matrix organization) increases ownership, commitment and information flow. As an active part demonstrating that 3R strategies contribute to efficacy and better quality, the likeliness to use the 3R approach for problem solving and decision-making on later occasions increases. The 3R values can then integrate into the fundamental values and the organizational culture.

Not all information fits the format of scientific publications. IMRaD is an acronym for Introduction, Methods, Results and Discussion. It relates to the standard main structure of a scientific paper, which typically includes these four sections in this order and many scientific journals prefer this main structure for their articles. A main explanation for the success of the quite rigid and superficial IMRaD structure is found in the modern researchers' need for speed when reviewing literature in their field, as the format allows the reader to pick those parts of the article that is of particular interest. Fixed templates or standards, like IMRaD, affects the way we plan, perform and report experiments and especially what researchers emphasize. In order to improve compliance to the 3Rs in publications, new guidelines have been published (ARRIVE guidelines, Gold Standard Publication Checklist)[31].

Scientific papers are only one way of knowledge sharing. Knowledge management systems refer to information technology (IT) based solutions for creation, capture, storage and dissemination of information in organizations. However, there seems to be an excessive trust in handling knowledge like any material substance that has to be acquired and distributed. Knowledge also involves interpretation and integration into earlier experiences. Story telling, gossips and observation of each other's work is just as important for knowledge sharing. Innovation narratives are cultural mechanisms powerful for translating ideas across the organization so they become comprehensible and appear legitimate to others[29].

It might seem as a paradox, but the sum of knowledge in an organization is usually less than the sum of individual knowledge[32]. This might be because of dysfunctional communication patterns that hinder the potential synergy effect of people with heterogeneous professional backgrounds. Organizational culture is important for improving communication patterns and knowledge exchange in an organization.

The different participants roles and level of project planning will influence how they interpret and take 3R into action (operalization). A challenge for 3Rs integration into organizational culture is that the 3R principles represent global values that must be interpreted before they can be applied in a specific context. The global values of the 3R must be adapted into specific behaviour suitable for the particular context (Fig 1). Interpretation and adaption depends on an individual's previous knowledge, role and particular needs. A researcher and a research technician have different roles and different needs in a research project and therefore their interpretations of the 3Rs may differ. A principal investigator might use imaging as a way to reduce animal numbers and refine experiments, while an animal caretaker on the other hand might use a gentle handling technique to reduce harm to the animals. The use of imaging will be included in the publication – however not necessarily as a 3R strategy. Gentle handling is more difficult to make verbally explicit and will most likely not be reported, because this kind of information is not commonly reported in scientific papers or ethical applications. The interpretation and adaption of the 3Rs will influence what information will finally be reported. Not all these adapted actions are suitable for publication, however this does not mean that it is irrelevant from the animals perspective. Moreover, there are more than 100 3R databases that have assembled relevant 3R information. The huge number of such databases which each has a different make-up, make it a hurdle to do a proper 3R search[22].

The content, value, or boundaries of application of the 3Rs vary according to context or conditions and is not fixed once and for all. Neither the bottom line nor the top line are uniquely defined. Relevant or important information does not always fit the format of publication. It might therefore be necessary with a broader repertoire of approaches to collect information on 3R operalization and make it easily available. Study of organizational culture is therefore a fruitful approach to review and evaluate 3R implementation and how this can be improved.

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Advocating the 3Rs through evidence-based guidelines that promote the wellbeing of animals used for scientific purposes

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Abstract

The *Australian Code of Practice for the Care and Use of Animals for Scientific Purposes* (the Code) governs the use of animals for research, teaching and product testing in Australia establishing a framework for ethical practice. The principles of Replacement, Reduction and Refinement (3Rs) inform the processes for ethical review, approval, conduct and monitoring. The participation of community and animal welfare representatives is integral to these activities although some animal protection groups argue that more measures are needed for effective implementation. Since the nexus between animal welfare and scientific outcomes is fundamental to the Code, an evidence-based approach is essential for a sound, case-by-case consideration of opportunities to implement the 3Rs. Evidence-based guidelines on special topics have been developed to assist investigators and members of Animal Ethics Committees to implement the Code. These are living documents being regularly reviewed to ensure they reflect current knowledge.

The *Guidelines to Promote the Wellbeing of Animals Used for Scientific Purposes* (2008) inform the critical development and implementation of strategies to achieve the 3Rs focusing on practical strategies to identify, minimise and manage pain and distress; fact sheets on specific research procedure also are included. The *Guidelines for the Generation, Breeding, Care and Use of Genetically Modified and Cloned Animals for Scientific Purposes* (2006) address specific animal welfare and scientific issues and the *Guidelines for the Housing and Care of Animals Used for Scientific Purposes (2003-2011)* provide a detailed, evidence-based approach to the husbandry of rats, mice, guinea pigs, rabbits and sheep.

Keywords: Australia, guidelines, wellbeing, GM animals, housing.

The *Australian Code of Practice for the Care and Use of Animals for Scientific Purpose*[1] (the Code) is the key national policy governing the use of animals for such purposes in that country and is incorporated by reference into relevant animal protection legislation. Through the establishment of an ethical framework for decision-making and the processes for ethical review, approval and monitoring, the Code promotes the principles of the 3Rs. The Code emphasises the responsibilities of all involved in the care and use of animals noting that this embraces a duty of care that, *inter alia*, demands a commitment to promote the wellbeing of the animals involved.

The scope of the Code includes all live, non-human vertebrates and 'higher order' invertebrates, such as cephalopods encompassing all aspects of the care and use of these animals in research, product testing or teaching activities in the fields of medicine, biology, agriculture, veterinary, environmental and other animal sciences.

The Code is not a prescriptive document, but rather sets out principles establishing criteria against which the evidence put forward to support a decision to use animals can be tested on a case-by-case basis. This approach permits a set of common principles to be applied to a wide range of circumstances. In this way there is a flexible approach that focuses on an in depth review of the specifics of a particular project and supports the incorporation of new knowledge relevant to promoting animal welfare on an on-going basis. A case-by-case treatment optimises the opportunities for practical realisation of the 3Rs and enables animal welfare outcomes in the context of scientific activities that would not be possible with a more prescriptive document. Clearly, the quality of the supporting evidence is important to facilitate robust ethical

review. Whilst an evidence-based approach underpins the application of the principles of the Code, this needs to be grounded on a critical review of current knowledge so as to achieve both animal welfare and scientific goals; a recognition of the pivotal relationship between these goals being a foundation principle of the Code since it was first published in 1969.

Guidelines to support the implementation of the Code have been developed to provide contemporary, relevant information. As with the Code, these are living documents that are reviewed and updated from time to time so as to reflect advances in scientific knowledge or to address emerging issues that represent particular ethical dilemmas. This paper outlines three recent publications each of which is based on a comprehensive analysis of the published literature and was submitted to both international peer review and public consultation prior to publication. These guidelines address key animal welfare questions relevant to the implementation of the Code namely, the promotion of wellbeing, the housing and care of animals and the particular issues concerning the welfare of genetically modified (GM) animals.

Promoting wellbeing

The avoidance and minimisation of an animal's experience of pain and distress has been a guiding principle of the Code since its inception. Further, the need to address ways by which the comfort of animals is supported also has been acknowledged. Although the notion of wellbeing was recognised, it was not until the most recent revision in 2004 that this was given prominence and the need to inform this through practical guidance has been acknowledged.

The 2004 edition of the Code broadened the concept of animal welfare to encompass an animal's range of experiences from a positive state of wellbeing to a negative state of distress; a state of wellbeing being the default position with the aim of promoting positive experiences and, if the experience of pain or distress is justified as integral to the research plan, then that occurs to the minimum extent necessary.

These developments reflected a growing recognition of the need to expand considerations of animal welfare to include not only elimination or minimisation of pain and distress but also to take into account the broader perspective of quality of life and whole of life experiences. In this context, the notion of wellbeing and its implications for animal welfare are central.

The concept of wellbeing refers to the complex and dynamic relationship between an animal and all aspects of its environment. It is an internal state that, at any given time, reflects an animal's perception and experience of its situation responding to sensory inputs from both its internal and external environment[2]. As defined in the Code, *wellbeing* implies a positive mental state, successful biological function, positive experiences and freedom from adverse conditions and thus relates to evidence of how an animal is coping with a given situation and a judgement as to how it feels in these circumstances. Whereas *distress* is defined as the state of an animal that has been unable to adapt completely to stressors and manifests as abnormal physiological or behavioural responses. Distress can be acute or chronic and may result in pathological conditions. Whilst distress is not necessarily associated with pain, the reverse is not so: *pain,* being defined as an unpleasant sensory and emotional experience associated with actual or potential tissue damage that may elicit protective actions, results in learned avoidance and distress and may modify species-specific traits of behaviour, including social behaviour.

Concerning pain and distress, the Code concludes that, unless there is evidence to the contrary, we must assume that an animal's experience is in a manner similar to humans, and requires that:-

- All projects involving animals are designed to avoid or, if that is not possible, minimise both pain and distress;
- Investigators must provide pain management appropriate to the species being used, the procedure to be undertaken and the circumstances under which the research will be conducted;
- The planned end point must be as early as possible to avoid pain and distress; and
- Alleviation of pain and distress that has not been anticipated must be addressed promptly and takes precedence over the completion of the project.

Strategies that promote wellbeing and minimise pain and distress are essential to achieve the goals of the Code; thus the *Guidelines to Promote the Wellbeing of Animals Used for Scientific Purposes*[3] were developed by a Working Party of Australia's National Health and Medical Research Council (NHMRC) as a comprehensive and practical guide to support these goals. Based on current scientific knowledge the guidelines focus on practical issues, providing background material about wellbeing, stress, distress and pain and proposing strategies to identify, minimise and manage pain and distress[4]. To achieve the goals of the Code, it is argued that not only is it necessary to manage the pain or distress that may be associated with a specific research procedure, but it is also necessary to identify and manage the range of stressors an animal may experience as part of its daily living conditions, including its social environment, as any unintended adverse effects on wellbeing may affect the validity of

research results and the number of animals required to achieve a scientific objective.

The guideline comprises three parts: -

A. Provides a background to the document with an overview of the scientific basis for the concepts of wellbeing, stress, distress and pain. The physiological and behavioural indicators of wellbeing, stress, distress and pain are discussed and the effects of an animal's wellbeing on scientific outcomes are reviewed.
B. Covers the basic strategies for -
   - Planning protocols to identify risk of pain and distress;
   - Conducting research to manage risk; and
   - Reviewing protocols to minimise pain and distress in future work.
C. Comprises a series of Fact Sheets that deal with specific research protocols.

In planning a research project it is contended that the strategic application of the 3Rs through a critical evaluation of the supporting evidence is fundamental to promoting the wellbeing of animals. If it is agreed that a proposed project has scientific value, then, first and foremost, there needs to be clear evidence that the use of animals is justified and if that be so, the principles of Reduction and Refinement guide subsequent decisions in the planning and conduct of a project.

When the use of animals is justified, the guidelines recommend that in the planning stage for each project, there is a risk analysis of all potential causes of pain and distress and the development of a strategy to assess, minimise, and monitor such risks. In this context, the need to identify those clinical signs or observations that will be used to assess wellbeing, predict the impact of treatments and indicate the need for intervention, including humane endpoints, is emphasised and the value of pilot studies to inform these decisions is highlighted.

The management plan developed through this process will be validated in the conduct of the project. Recognising that specific details will be tailored for a particular protocol, the guidelines advise that, as a general approach, animals should be monitored so as to manage both predicted effects and unforeseen complications and, when necessary, to provide adequate pain management and palliative treatment. The need to promptly ascertain the cause of unforeseen complications is stressed noting that this may necessitate modification to the protocol or the monitoring strategy.

The importance of undertaking a regular review of strategies is highlighted, especially in those circumstances involving a new procedure or where the effects of a treatment are unclear. The importance of the review process so as to ensure optimum animal welfare outcomes and to implement refinement of procedures, where possible, is emphasised.

The Fact Sheets were developed to consider specific, common research procedures such as,

- Administration of substances
- Behaviour modification
- Collection of blood and other biological specimens.
- Humane killing and euthanasia
- Production of polyclonal antibodies
- Surgical procedures
- Tumour Induction
- Wildlife Research

Each Fact Sheet follows a similar format including a discussion on the purpose of the procedure, what is involved, the essential animal welfare and scientific issues and management of predicted impact.

Housing & care

Whereas much of the public debate about the welfare of laboratory animals has focused on the effects of experimental procedures, the impact of their living conditions on the welfare of these animals also has been recognised (for example, the series *Comfortable Quarters for Laboratory Animals*, first published in 1955) and it has been argued that, as poor or inadequate living conditions impact throughout their lifetime, these are significant animal welfare concerns[5,6].

Although the housing provided for laboratory animals is designed and managed so that environmental variables such as temperature, humidity, air quality and lighting are carefully controlled, Russell and Burch[7] questioned the potential consequences, especially to their psychological health, when animals are housed under conditions where they do not have control over or choices within their living space. For example, providing external, mechanical temperature controls and not enabling animals to create or control their microclimate or limiting their options and choices in social interactions, may limit or frustrate expression of species-specific behaviours. To some extent these issues are being addressed with the provision of more complex physical environments, but, as noted in a recent report, there are few studies where the efficacy of these changes has been validated in terms of addressing species-specific needs[8].

As a general principle, the Code requires that the design and management of an animal's living conditions meet species-specific requirements and that the provision of care be based on their behavioural and physiological needs. However, as

with the general approach in the Code, these are statements of principle and it is left to the researchers, managers of animal facilities and members of Animal Ethics Committees to agree on the details for implementation. The NSW Animal Research Review Panel recognised a need to assist these deliberations and about ten years ago began developing evidence-based guidelines for the housing and care of commonly used research species. The goal being, within the intent of the principles of the Code, to provide research establishments with guidelines that document the evidence for good contemporary practice that would promote good science and provide a benchmark against which housing and husbandry practices could be assessed.

To date, guidelines that address the housing and care of mice, rats, guinea pigs, rabbits and sheep have been developed based on a comprehensive literature search of refereed scientific journals and reference books[9,10,11,12,13]. Significant background information was documented and used to develop principles and recommendations, citing relevant literature and noting areas where information was lacking or equivocal.

For each species, findings are grouped into topics including: species behaviour; cage or enclosure design; animal care and management (including social environment, environmental enrichment and nutrition) and environmental variables (including light, temperature and ventilation). Within each topic, a number of principles is presented, each of which is supported by citations from peer-reviewed literature. These principles are then followed by concise recommendations creating a format that is both scientifically robust yet easily accessible to lay persons such as Animal Ethics Committee members.

In formulating recommendations, particular attention has been paid to elucidating evidence that informs how living conditions should be designed and managed to meet species-specific physiological and behavioural needs. For example, evidence indicates that the living area for guinea pigs should incorporate open spaces interspersed with shelters so that animals can rest or hide. Consequently, the use of permanent shelters, such as a section of PVC pipe or plastic hiding boxes, in their enclosure and providing materials, such as straw, hay or wood wool, that can be used to form temporary tunnels provide opportunities for these activities are recommended[10]. In the case of sheep, given the evidence of their social behaviours, their strong motivation to synchronise behaviours, such as resting, and that rest periods are important for effective rumination, the potential effects of housing conditions and space allowances on social behaviours needs to be taken into account . Thus the importance of enabling visual and auditory contact with other sheep and providing sufficient space so that all animals in a group can synchronise rest periods are highlighted[12].

GM animals

The *Gene Technology Act* (2000) is national legislation under which institutions are accredited to use GM technologies. This legislation also mandates the establishment of several committees to advise the Gene Technology Regulator, one such committee being the Gene Technology Ethics Committee (GTEC) that has published A *National Framework of Ethical Principles*[14] as a "*national reference point to promote on-going dialogue on values and ethical principles relevant to the development of gene technology*". This National Framework discusses nine ethical principles, including one to minimise the risk of harm or discomfort to humans or animals. This Ethical Framework complements the principles of the Code.

To provide additional guidance on the special ethical and welfare issues relevant to the use of GM animals, the *Guidelines for the Generation, Breeding, Care and Use of Genetically Modified and Cloned Animals for Scientific Purposes*[15] were developed by the Animal Welfare Committee of the NHMRC, in consultation with GTEC. These guidelines complement the Code and, recognising that GM animals may have specific welfare needs which will extend over their lifetime and into subsequent generations, aim to promote the 3Rs in the management of these animals[16]. The guidelines apply to all GM animals including laboratory species, livestock and companion animals.

The GM guidelines highlight a range of ethical and welfare issues, discuss matters in relation to monitoring and reporting and include specific examples of ways by which the 3Rs can be promoted. Acknowledging that the ethical and welfare issues around the development of new GM lines have been comprehensively addressed in other recent publications[17,18,19], these matters are not discussed in detail in this document.

The GM guidelines identify significant ethical concerns in relation to the production of large numbers of animals to achieve a desired phenotype, high culling rates, the effect of GM on the integrity of the animal including its interaction with con-specifics and the environment and the unpredictability of phenotypic expression. Further the possible tensions between the goals of Reduction and Refinement is noted as an emerging issue.

A number of specific animal welfare issues, which follow from these ethical issues, are highlighted and ways to ameliorate or manage the impact on the animal discussed. In this regard, the impact of techniques used to produce and monitor GM animals, and gene modifications with the potential

to disrupt physiological processes or resulting in a poor fit between the new strain and its environment such as the expression of modified or deleted genes or interactions between gene products, are noted as major animal welfare risks that are compounded by the unpredicted nature of these effects.

The GM guidelines emphasise the key roles of monitoring and reporting to ensure effective management of the welfare of these animals noting the need for a whole of life approach. Although it is recognised that the intensity, nature and frequency of monitoring will need to be tailored to the circumstances, nevertheless, it must include steps to actively seek identification of adverse events needing to cover both anticipated and unexpected outcomes.

Comment

Although the need for and benefits from the use of evidence-based information to guide decisions as to if and how animals are used for scientific purposes are recognised and advocated, particularly in this context, the development of guidelines through an evidence-based process is not without challenges.

The concept of evidence-based practice has been widely adopted in human medicine as the basis for setting benchmarks for quality improvement in health care and for informing best clinical practice. In essence it is a process of critical and systematic analysis of scientific evidence relevant to a particular question or set of questions that is applied either in the development of guidelines or to individual clinical decision-making. A key element of this process is an assessment of the strength of the scientific evidence particularly in relation to its validity, quality, and relevance to the question under consideration.

Experience in the development of the guidelines discussed revealed that not only is there a lack of scientific evidence to inform decisions in many key areas, but, in many circumstances, notably where there are limited studies, data can be contradictory or inconclusive. These difficulties have been identified by others seeking to develop similar guidelines in relation to the welfare of laboratory animals. For example, when the Institute for Laboratory Animal Research (ILAR) hosted an international workshop to discuss the development of evidence-based guidelines for laboratory animal care, workshop participants acknowledged the need to critically review and apply the available scientific evidence, but identified a number of significant knowledge gaps in relation to species-specific data[20]. Similarly, knowledge gaps were identified as a significant issue in two ILAR reports on the recognition and alleviation of pain[21] and distress[22].

Although the term evidence-based is now widely used in discussion and reporting of guidelines relating to the care and use of animals for scientific purposes, most often an evidence-based process is not possible but rather a science-based approach is used whereby scientific evidence is cited to support recommendations. Unlike many situations in human health care where there is a significant body of research and scientific evidence available for analysis, the lack of relevant scientific publications in relation to the care and use of animals in science is a significant limitation. This is of some concern given the number of animals used in research worldwide, the substantial investment this entails and the close connection between animal welfare and scientific outcomes. Although all the guidelines discussed here are based on peer-reviewed scientific evidence, only in the species-specific housing guidelines[9,10,11,12,13] has there been an attempt to assess the strength of the evidence. Never the less, in circumstances where evidence from the scientific literature has been lacking, the guidelines have noted this and, where necessary, recommendations are based on a professional assessment of current best practice.

Such guidelines must be living documents that reflect advances in scientific knowledge in a timely manner; identification of areas where more data are needed or where new evidence leads to a different conclusion is a positive outcome indicative of the benefits of the process. However, these documents are not for the scientific community alone, but also are used by members of Animal Ethics Committees, including community and animal welfare members, to inform their decisions and will be viewed by the wider community, especially those with concerns for animal welfare. In these circumstances, for those who reference these guidelines their confidence in the recommendations provided will often be based on a perception of certainty in the scientific evidence and conclusions, whereas the strength of an on-going evidence-based process is a recognition that evidence and knowledge evolve, that there is an element of uncertainty in conclusions reached and that new evidence may emerge that leads to contrary conclusions.

Whilst from a scientific perspective an evidence-based approach will better inform and achieve animal welfare and scientific outcomes, there is a risk that the outcomes of this process will not sit so comfortably with those who wish to see guidelines used in a prescriptive manner with a high level of certainty. Such tensions are inevitable especially in these circumstances and can lead to disagreements between the parties involved. Clearly, steps that avoid such misunderstandings are important to achieve the benefits of an evidence-based approach.

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Animal welfare in Asia: the AAALAC international experience

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Abstract
With the accelerating pace of animal-based research being outsourced to Asia, as well as the heightened support for in-country biomedical research by the governments of several Asian nations, interest in laboratory animal care and use standards in this part of the world and laboratory animal welfare in particular, is also intensifying. Findings identified by AAALAC International's Council on Accreditation over a six year period were collated on both a global basis and for the Pacific Rim region (with emphasis on Asia). The findings were categorized as mandatory deficiencies, representing issues that must be corrected for the animal care and use program to be of sufficient quality to be fully accredited by AAALAC International, and suggestions for improvement, the correction of which assists the program in its path of continuing improvement. These data demonstrate some differences in the top three animal care and use program deficiencies occurring in Asia compared to those occurring on a global basis. While globally, and in Asia, the Institutional Animal Care and Use Committee is identified as one of the top three mandatory deficiencies, in Asia, in contrast to the global trend, Animal Environment and Veterinary Care also make the top three deficiency list. The impact of these differences on animal welfare, and potentially the quality of research results, is discussed in the context of the expansion of biomedical research in Asia and the concomitant increase in audits conducted by pharmaceutical companies outsourcing to this region as well as in on-site assessments conducted by AAALAC International.

Keywords: welfare, Asia, AAALAC, accreditation

Reports regarding the increase in outsourcing of manufacturing, as well as research and development, in numerous industries are evident daily. Animal-based research is no exception to this trend. Less than 2 years ago, Steve Snyder reported in *Contract Pharma*[1] that economic indicators strongly suggested that preclinical outsourcing would continue to expand. Estimates of approximately a 12% increase in outsourcing between 2001 and 2011 have been suggested,[2] and within the pharma sector, estimates of almost 50% of revenues for the mid-pharmas are projected to be derived from products discovered outside of the United States.[3] It is estimated that China alone has more than 300 Contract Research Organizations (CROs), and that the number of partnering deals grew by 41% in 2009[4]. The economic downturn has impacted these estimates slightly. For example, in 2008 respondents to Contract Pharma's annual outsourcing survey indicated that 72% expected outsourcing spending to grow in 2009. Thirty-nine percent of respondents to a slightly differently framed question indicated that they expected to increase their outsourcing spending in 2010, while 33% indicated that they would spend less than in 2008 and 25% reported they would not change their outsourcing spending level.[5, 6] When asked how likely the respondents were to outsource a project to Asia in the next year, the 2009 data showed an increase in the percent that definitely or probably would (30% in 2008 vs. 35% in 2009) and a reduction in the groups answering they definitely or probably would not (46% in 2008 vs. 43% in 2009). International collaborations between academic institutions are also increasing in frequency. A case in point is the Duke University-Peking University Center on Global Health. And, Yale University hosted a 2010 symposium on U.S.-China Life Science Industry Collaborations. Regardless of the precise yardstick used to measure the expansion of preclinical work conducted outside of a company's own facilities and international academic research projects, it is clear that external sourcing and collaboration will remain an integral component of animal research.

Recent problems identified with products (e.g., melamine in pet food, toothpaste containing ethylene glycol, lead paint on children's toys, and counterfeit

pharmaceuticals)manufactured in Asia, and in particular in China, have led to an understandable public skepticism regarding quality assurance measures in some overseas markets. An offshoot of this in the life sciences research sector is concomitant concern regarding the level of welfare afforded research animals. As Snyder noted,[1] "Let's hope we don't see a catastrophic failure due to a quality crisis." Indeed, in the face of increasing globalization of animal-based research, harmonization of animal care and use standards and practices becomes essential.

Influences on animal welfare perspectives

Several factors may play a role in the regulatory framework for and attention paid to laboratory animal welfare. For example, the economic development of the country can have a significant impact on how many resources are allocated to ensuring the research animal's environment is appropriate, that trained and qualified individuals manage the program, and that quality assurance is sustainable. Heating, ventilation and air conditioning systems; mechanical cage washers; appropriately designed and manufactured animal caging; and other infrastructure elements come at a high cost. Also, recruiting top tier professionals requires a firm commitment to adequately resourcing the animal program.

Second, the religious and cultural context of the country where animal research is performed may influence animal treatment. Traditions of Confucianism, Taoism, Jainism, Hinduism and Buddhism variably speak to a relationship between human beings and non-human animals. Some reflect the philosophy that humans are superior to other animals and view animals as a source of food, labor, and utility. In such a perspective, a person treats an animal with kindness "not because of their inherent value but as a reflection of one's own refinement as a human being".[7] Other schools of thought, particularly those that emphasize reincarnation, place value on animals as a component of the human-animal continuity. However, in addition to religious influences, societal mores—customs, teachings, etc.—can affect commonly held opinions regarding acceptable care and use of animals.

A third potentially significant influence on an individual's concept of animal welfare is that person's exposure to other cultures and philosophies. This exposure could occur through websites with an emphasis on laboratory animal medicine and science or animal welfare in general; published literature; and visits to other countries. It is recognized, however, that language barriers may be an impediment to an individual's ability to take full advantage of the vast array of print and on-line resources available pertaining to laboratory animal care and welfare. A significant amount of this information is published in English, and many terms may be more technical in nature, and thus not readily understood by an individual whose primary language is not English. While translations of some documents are available (e.g., the *Guide for the Care and Use of Laboratory Animals*),[8] such translations are complex and often costly, and the documents may be updated or revised, thus rendering the translation outdated (e.g., the 2011 edition of the *Guide*). Also, accuracy of the translation must be assured. While exposure to animal facilities outside of one's own country is extremely informative, the cost and logistics of setting up such travel can be prohibitive. However, the value of such interactions cannot be understated. Indeed, China's "sea turtles" (individuals who train outside of China and then return to the country with specific expertise) is an apt model for this approach.

Thus, as one views the global laboratory animal research field in its entirety and across borders, several potential differences in key program elements may arise. Critical areas to attend to include animal procurement, transportation of the animal from the vendor to the place of study, the provision of adequate veterinary care, the training and competency of the personnel associated with the animal program, the animal's environment (both in the primary enclosure and in procedure areas), and the method of review and approval of the proposed work (i.e., ethical review). Each of these program elements may be influenced by the economic, religious and cultural experience of the personnel at the institution. While there is no single approach to ensuring quality laboratory animal welfare, fundamentally there must be agreement (harmonization) regarding critical practices and procedures.

A glimpse of laboratory animal welfare in Asia

To provide a complete review of the laboratory animal welfare regulations and customs would exceed the scope of this paper. However, some points are worthy of note when assessing the status of laboratory animal welfare in Asia.

For example, in Japan an Institutional Animal Care and Use Committee (IACUC) reviews animal research protocols and submits a report to the Director of the institution, who then determines if the work will be done. In the preparation and review of the protocol, the Three Rs should be considered and humane endpoints should be identified, as appropriate to the study.[9] Hachisu[10] has noted that the Japanese "…are now more interested in toys for animals and improving cages sizes, thinking of what toys are best for which animals and how cage sizes should not limit animal activities."

In Korea, alternatives to the use of animals must be considered in the development of an animal research protocol.[11] It is also required that the minimum number of animals necessary to achieve the research goals be used. Further, steps must be taken to minimize pain experienced by the animals. Protocols must be reviewed by an Animal Experimentation Ethics Committee, and appropriate qualifications of individuals performing experiments must be assured.

Singapore has developed an extensive set of guidelines[12] that was adapted from the *Australian Code of Practice for the Care and Use of Animals for Scientific Purposes* (National Health and Medical Research Council, Australia); the *Guide to the Care and Use of Experimental Animals, Volume 1* (2nd Edition) (Canadian Council on Animal Care, 1993); the *Good Practice Guide for the Use of Animals in Research, Testing and Teaching* (National Animal Ethics Advisory Committee, New Zealand); the *Guide for the Care and Use of Laboratory Animals* (National Academies Press, Washington, D.C., USA, 1996); the *Public Health Service Policy on Humane Care and Use of Laboratory Animals* and the ARENA/OLAW *Institutional Animal Care And Use Committee Guidebook* (Office of Laboratory Animal Welfare, National Institutes of Health, USA). The Guidelines are divided into three sections: "Guiding Principles for the Care and Use of Animals for Scientific Purposes," "Guidelines for Institutional Animal Care and Use Committees," and "Training Guidelines." The composite guidelines address standards for animal housing and management, animal procurement and transportation, veterinary care, IACUC functions, as well as personnel training and qualifications.

A *Newsweek Magazine* article[13] entitled "It's China's World; we're just living in it" highlighted the pivotal role China has assumed in major global issues, including trade, climate change, currency and technology. Similarly, China has assumed a prominent role in life sciences research. In China, the Ministry of Science and Technology (MOST) is the responsible government agency for establishing regulations pertaining to the conduct of research. Kong and Qin[14] have summarized the progression of reference to animal welfare in Chinese regulations. Reference to research animal welfare dates back to 1988 in the Statute on the Administration of Laboratory Animals, which essentially addressed animal welfare through the provision of nutritious food, potable water and qualified personnel working with the animals. Several years later, in 1997, mention is made of the Three Rs in MOST documents. Since 2005, the guidelines published by MOST have increasingly strengthened recommendations related directly to laboratory animal welfare, to include a requirement for provincial ethical review of proposed work with animals and regulations addressing husbandry, use and transportation issues. The 2006 regulations include noncompliance with laboratory animal policies as one of six "dishonorable behaviors" which can result in disciplinary action. The Chinese are actively hosting symposia to disseminate training in IACUC function, veterinary care roles, environmental enrichment and other related subjects throughout their research community. Examples include the annual China Pharmaceutical R&D Summits; the "Shanghai Laboratory Animal Welfare Sharing Conference" co-hosted by the Office of Shanghai Administrative Committee for Laboratory Animals, Global Research Education & Training (GR8) and AAALAC International; Peking University Workshops; and others.

Asia is not immune to attention from animal rights/protection organizations. For example the Japan Anti-Vivisection Association has targeted the use of animals for testing, education and research. PETA Asia-Pacific advocates ceasing animal experimentation on its website. The Chinese Animal Protection Network addresses many animal issues including what they refer to as "academic research of animal ethics" and an

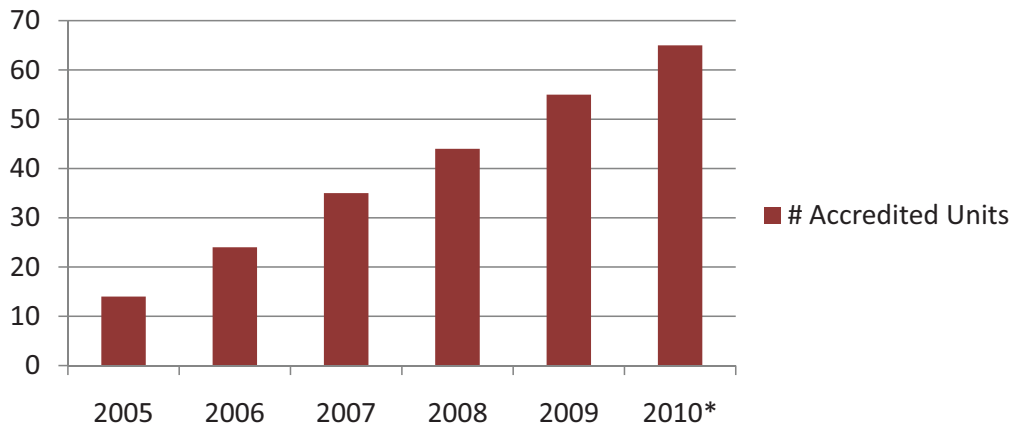


Figure 1. Trend in number of accredited institutions in the Pacific Rim region. (*Data for 2010 incomplete at the time of publication.)

"awareness campaign of lab animal protection" and in 2008 organized the first World Lab Animal Day in China.

AAALAC International's experience in Asia

Approximately 30% of new applications for accreditation of animal care and use programs by AAALAC International are submitted by institutions located in the Pacific Rim. Indeed, the increase in accredited institutions in this region of the world has been steady (see Figure 1). Accredited institutions are located in several Asian countries, including China, Indonesia, Japan, Korea, the Philippines, Singapore, Taiwan, Thailand and Vietnam.

Periodically, AAALAC International evaluates the number of deficiencies identified during the on-site assessments of institutional animal care and use programs. Findings identified by AAALAC site visitors are categorized as mandatory items for correction or suggestions for improvement. A mandatory item is a deficiency which must be corrected for Full Accreditation to be awarded or continued. A suggestion for improvement is an item which the Council on Accreditation feels is desirable to upgrade an already acceptable or even commendable program. Globally, the three most commonly identified mandatory deficiencies are related to (in rank order): 1) occupational health and safety program; 2) IACUC; and 3) heating, ventilation and air conditioning system performance. In the Pacific Rim region, the three most commonly identified mandatory deficiencies are related to (in rank order): 1) Institutional Animal Care and Use Committee; 2) animal environment, housing and management; and 3) program of veterinary care. Figure 2 shows the percent of mandatory items and suggestions for improvement during the period of 2004 though 2009 identified in the Pacific Rim region.

Institutional Animal Care and Use Committee

As noted above, IACUC operations were the most commonly identified mandatory deficiency in the Pacific Rim region. Globally, an analysis of the specific concerns noted during accreditation site visits indicates that protocol review issues, concerns regarding the semiannual program reviews and facility inspections, and IACUC policies were the three most commonly identified mandatory items for correction and suggestions for improvement. These were followed by (in order) composition and participation of the Committee, documentation of Committee activities, and assurance of appropriate training and qualifications for personnel working with animals (see Figure 3).

This varies somewhat from the specific IACUC issues identified in the Pacific Rim region. In this region, the most frequently identified mandatory item for correction was associated with the semiannual program reviews and facility inspections, followed by the composition and participation of members of the IACUC, and documentation of IACUC activities. In the Pacific Rim, there were only minor differences in the frequency of observations across all of the elements of IACUC function evaluated by AAALAC International and classified as suggestions for improvement (see Figure 4).

Animal Environment, Housing and Management

A comparison of the trends observed by AAALAC International globally versus just the Pacific Rim related to animal environment, housing and management indicates that for both, the most frequently observed mandatory deficiency is related to sanitation and maintenance (see figures 5 and 6). In the Pacific Rim, the number of deficiencies pertaining to animal cage space recommendations was equivalent to the sanitation/maintenance deficiencies. Also in the Pacific

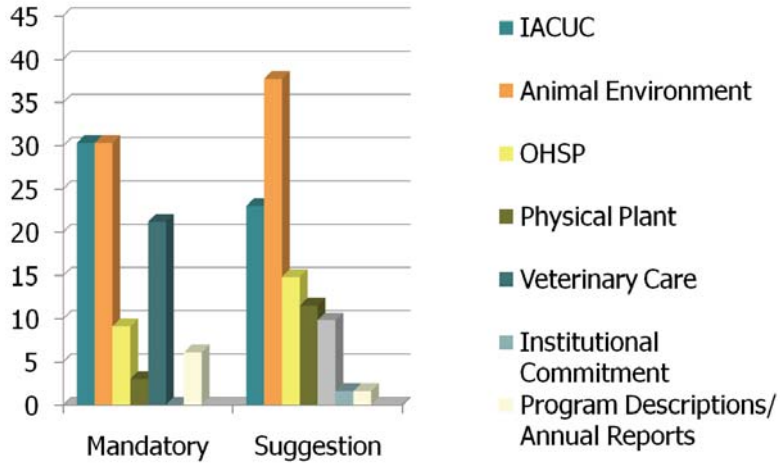


Figure 2. Percent Mandatory Items and Suggestions for Improvement for the Period 2004-2009 in the Pacific Rim Region.

Figure 3. Percent IACUC findings, globally, for the period 2003-2008.

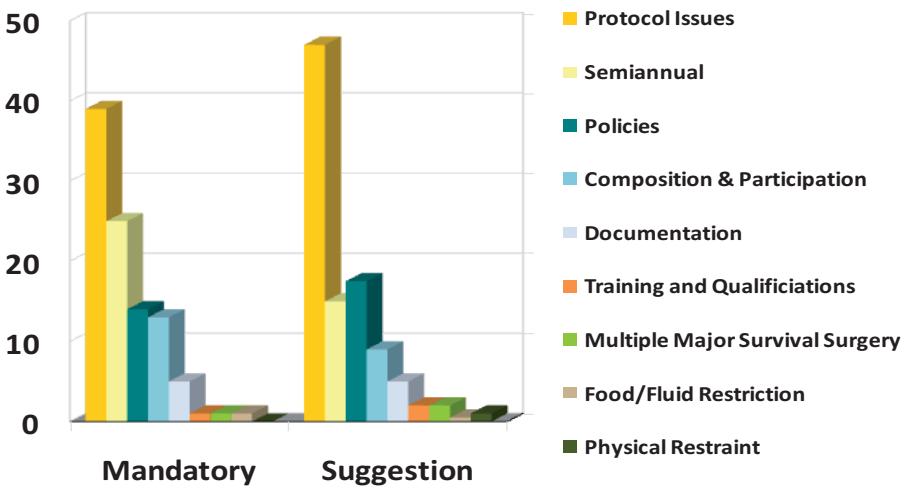
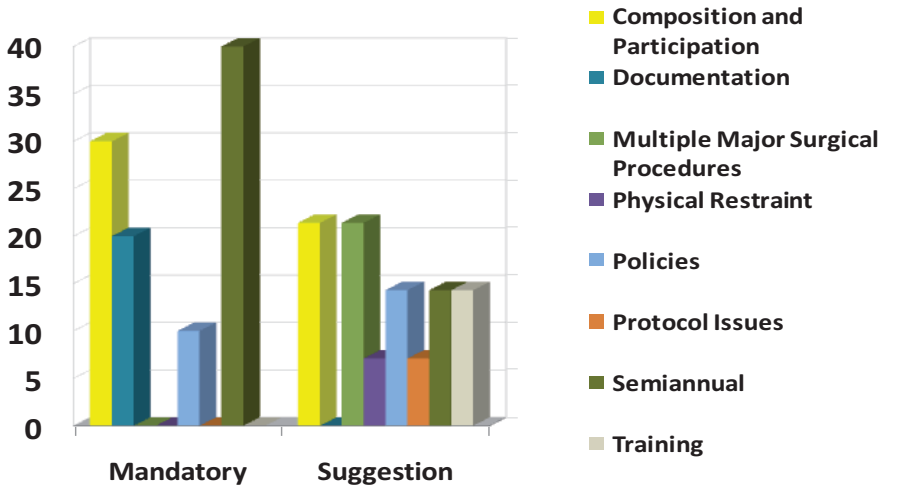


Figure 4. Percent of each element pertaining to the IACUC reviewed by AAALAC International, categorized as mandatory items for correction or suggestions for improvement, for the period 2004-2009 in the Pacific Rim Region.



Rim region, the behavioral management program was the second most commonly observed deficiency, as compared to animal housing from the global data. Globally, space recommendations were the third most frequently observed deficiency.

Program of Veterinary Care

Quite consistently, globally, AAALAC International has observed a very low percentage of mandatory deficiencies related to the program of veterinary care. Since 2003, this number has ranged from less than 5% of all deficiencies observed to approximately 8%. Similarly, very few suggestions for improvement have been cited by AAALAC International regarding the program of veterinary care (see Figure 7). Of those very few instances, the most frequently identified issue related to the overarching program of veterinary care (i.e., at the institutional level), followed by the key aspects of the preventive medicine program (surveillance, diagnosis, treatment and control of disease) (see Figure 8). The adequacy of the overarching program of veterinary care was also the most frequently identified mandatory deficiency in the Pacific Rim region, with the second most common mandatory finding also being the aspects of the preventive medicine program related to surveillance, diagnosis, treatment and control of disease. In the Pacific Rim, the issues tended to center on the Attending Veterinarian having limited training and experience specific to laboratory animal medicine, with resulting inadequate involvement in and oversight of several aspects of the program.

Conclusions

There is no doubt but that Asia is increasingly becoming a focus for the conduct of animal-based biomedical research. With appropriate precautions, the current economic environment offers an opportunity to significantly improve the welfare of research animals around the world. It is important that we work with our Asian colleagues to promote training in laboratory animal medicine and science in those countries where such training is absent or minimal. We must also seek

Figure 5. Comparison of the percent each element of Animal Environment comprising the total mandatory deficiencies observed in this program component globally.

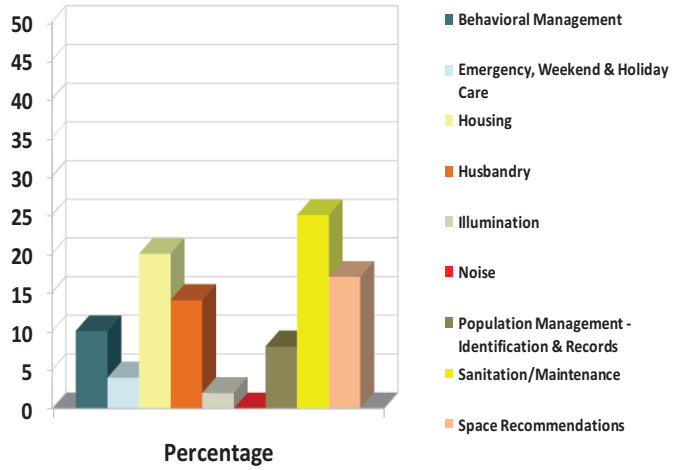
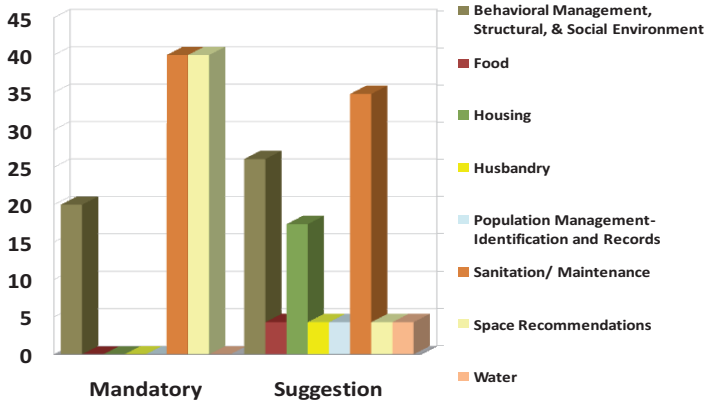


Figure 6. Comparison of the percent of each element of Animal Environment, Housing and Management comprising the total mandatory deficiencies and suggestions for improvement observed in this program component in the Pacific Rim region.



ways to enhance the availability of veterinary drugs and medications in some Asian countries, as well as emerging animal facility technology. The expertise and credibility of the veterinarian are pivotal to that individual having a prominent role in ensuring laboratory animal welfare. With the appropriate tools and stature in the institution, the veterinarian can ensure a suitable animal environment, ensure adequate attention is paid to minimizing pain and distress of research animals, play a necessary role on the IACUC in the review of protocols and in the semiannual reviews, and have an appropriate advisory role to the occupational health and safety program. Some countries in Asia, such as Japan and Korea, are very advanced in the training afforded laboratory veterinarians, and both countries have board certification specialty programs in laboratory animal medicine (Japanese College of Laboratory Animal Medicine and Korean College of Laboratory Animal Medicine). It is important to resist viewing Asia from a homogeneous perspective. Rather, it is a very diverse region overflowing with energy, talent and potential.

Acknowledgement

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Figure 7. Comparison of the percent of each element of the Program of Veterinary Care comprising the total mandatory deficiencies and suggestions for improvement observed in this program component globally.

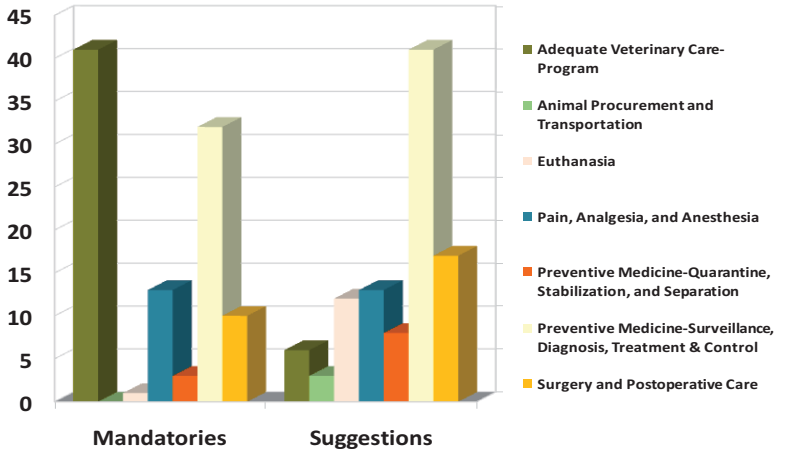
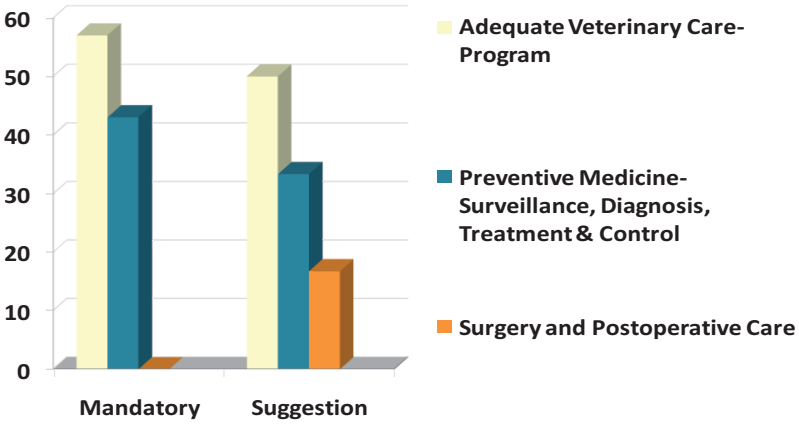


Figure 8. Comparison of the percent of each element of the Program of Veterinary Care comprising the total mandatory deficiencies and suggestions for improvement observed in this program component in the Pacific Rim region.



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Workshop: How to prepare the animal ethics part in (EU Commission) FP7 research applications

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Abstract
Applicants for EU FP7 projects are asked to meet certain scientific and ethical standards regarding any proposed animal use. The workshop dealt with ethics scrutiny, covering each of the Three Rs – replacement, reduction and refinement - in detail, so that those attending would be more aware of what is required and what the reviewers will be looking for.
This workshop aimed to help participants provide suitable information for ethical assessment, given that FP7 reviewers of project applications are asked to make judgements on the following.
a) Were the ethical aspects of the proposed research well described in relation to its objectives?
b) Were the ethical aspects of the proposed research well described in relation to its methodology?
c) Were the ethical aspects of the proposed research well described in relation to the possible implications of its results? (with a note that the scientific evaluation should not be revisited).
The objective was for participants at this workshop to understand what is needed for ethical assessment and to be able to show how ethical considerations had been considered in a research grant application.
The workshop was structured around the Three Rs, i.e, Replacement, Reduction and Refinement (collectively called "alternatives"), these being the main ethical considerations. A fourth section covered other issues such as harm benefit analysis, training in ethics and ethics review, training and competence in techniques, responsibilities, euthanasia, and maximising benefits. The workshop was interactive and each of the Three Rs sections included a group discussion with feedback.

Keywords: FP7, replacement, reduction, refinement

Replacement

The first ethical question is whether animals need to be used at all, so an application should discuss possible replacements. Russell and Burch, in formulating the Three Rs concept in 1959, considered replacement as any scientific method using non-sentient material that replaces use of animals in experiments or tests. "Non-sentient" is usually taken to include creatures whose nervous systems are insufficiently developed for them to experience pain and "animals" usually taken as "living vertebrates" - so use of insects instead of mice would be a replacement – but as Cephalopods will be within the scope of the revised EU Directive, a method using them would not be regarded as a replacement alternative in EU assessments.

Replacement can be considered at two levels:

Complete replacement, where the method does not require any animal derived material, e.g., the Ames test which uses bacteria for screening for mutagens, and

Incomplete replacement, in which the method requires biological material obtained from living or killed animals or uses embryonic stages or invertebrates. An example would be using orientated brain slices instead of the whole animal for studies on the visual system.

As with these examples, most replacements are very procedure or objective specific, so to be sure there is no replacement available each section of the work plan needs a clear objective, i.e. what the research is hoping to achieve. Depending upon that objective, it may be necessary to consult a variety of "alternatives" databases and undertake literature searches on how

the objective might be achieved. This is not a simple matter as there are many websites to consult, e.g.

- Altweb – global clearinghouse for information on the 3Rs http://altweb.jhsph.edu/resources/links.html
- DB-ALM - http://ecvam-dbalm.jrc.ec.europa.eu/
- Go3R - PubMed based http://www.go3r.org/
- National Centre for the 3Rs (UK) www.nc3rs.org.uk/informationportal
- AnimAlt-ZEBET http://www.dimdi.de/static/en/db/dbinfo/zt00.htm
- Alt.Tox - advancing non-animal methods of toxicity testing through online discussion and information exchange http://alttox.org/
- Norwegian Reference Centre for Laboratory Animal Science and Alternatives http://oslovet.veths.no/norina
- Fund for the Replacement of Animals in Medical Experiments http://www.frame.org.uk

However there are also general replacement possibilities to consider.

- Computer models: Physico-chemical characteristics can be used to predict mechanism of action or toxicity. If sufficient *in vivo* data is available simulation models can be used to explore possible interactions between components of a body system or effects of substances.
- Human volunteers, human tissue or tissue fractions: These possibilities may be particularly important to consider for non-human primate work.

Hence in addition to well-stated objectives and some indication of the searches undertaken, or references to show the group was itself developing replacements, an application should consider against each objective why animals have to be used. This should include the absence of feasible non-animal alternatives and reasons why humans or human tissue could not be used.

In group discussion it was pointed out whole body studies are difficult to replace and some research, e.g. behavioural research, self-evidently needs the whole animal. There were concerns that some potential replacements were not reliable and some not adequately predictive. Other points were that animal studies were needed to confirm computer model data, that if a replacement were not possible, using animals of lower neurophysiological sensitivity should be considered, and that if animals are needed to meet the objective one should consider a comparable objective for which animals may not be needed.

Reduction

The numbers of animals involved in experiments can be minimised

- by design of the programme or of each experiment
- by minimising variability (good health status, genetic uniformity, minimal stress)
- by collaboration to reduce numbers of experiments – and full publication
- by maximising use of tissue, and genetically altered animal lines
- by re-using animals

So an application should include an explanation of how the programme of work is designed to minimise the use of animals, some indication that the experiments will be designed in accord with fundamental principles and to minimise numbers, and consideration of how variability will be minimised. There should also be discussion of possible collaboration to avoid needless repetition of experiments (or false trails!) by different groups, to maximise use of the tissue from the animals or, where applicable, of genetically altered animal lines, and to facilitate re-use between programmes of animals which have suffered little adverse effects.

The aims of the proposed programme should be clear and evidence given of an adequate literature search of possible different approaches (including for ways of reducing numbers and severity, not just scientific novelty). The programme should be staged around some specified review points at which improvements in methods could be discussed, the numbers needed reappraised in the light of experience, or the need for further experiments reconsidered. Ideally it would start with the least severe experiments, obtaining as much information as possible on these before proceeding to more severe studies. It should include pilot experiments (trials with small numbers that allow note of adverse effects, time to treatment effect, sources of variability, technical problems, etc.), as these guard against over-optimism and so save avoidable wastage of animals, and the adverse effects noted can help set severity endpoints and the time when the relevant effect is observed is useful for setting objective-related endpoints.

In the subsections of a work plan some discussion of the experiments involved would be expected and the points that someone knowledgeable in experimental design might be looking for are:

- a clear statement of the experimental objective for each set of experiments,
- some discussion of reasons for choosing the different designs proposed for the experiments in the work plan and why these were regarded as the most efficient,

- an indication of how the numbers needed would be determined (e.g. power analysis or use of the resource equation) and
- text that gives confidence that the proposers know how to design and analyse experiments properly (i.e. would keep to the fundamental principles of adequate replication, suitable controls and proper randomisation).

Recent surveys of published studies show that none of this can be taken for granted.

Group discussion provided some further points on good practice:

- Minimise wastage of animals not used for providing data (especially in breeding programmes) and of animals that are not reported e.g. due to technical failures, unsuccessful experiments
- Use animals as their own control or take serial measurements on same animal, e.g. by imaging, non-invasive techniques, rather than serial kill
- If several animals are needed to give sufficient material for a data point, more efficient (e.g. low volume) assaying can reduce numbers
- Use of other studies using same methods, but not same objective, may avoid need for pilot studies.
- Re-use of same animals (after suitable washout period and provided there was no surgery except that which is preparative for both uses)
- Increase the data obtained from an animal, e.g. by an experiment meeting more than one objective
- Reduce variability by screening out unsuitable animals (provided the bias this causes is recognised)
- Use non-animal pre-screens to weed out drugs that won't work
- One control group can serve for several comparisons, and a large control group can mean smaller numbers in experimental groups.

Refinement

This could be considered as not only lessening the severity of procedures but also enhancing animal well-being to obtain reliable scientific measures. This would be through housing, husbandry and care, and health status, all of which can impact on the scientific measures.

Researchers need to ensure that:

a) the animals' husbandry would be in compliance with the revised Appendix A of the Council of Europe Convention, http://conventions.coe.int/Treaty/EN/Treaties/PDF/123-Arev.pdf
b) the health status of the animals would be appropriate to the scientific objectives and not affect the scientific outcomes
c) any deviation from Appendix A, or from a high health status, would only be with good scientific justification.

They need to explain how they might avoid or alleviate any adverse effects, how any adverse effects, e.g. pain, distress, in the animals would be recognized, how the severity of the procedures would be evaluated, taking into account intensity and duration of any suffering and the numbers of animals affected, and why less or non-invasive methods would not suffice. For example, magnetic resonance imaging (MRI) or positron emission tomography (PET) may be appropriate and microarray techniques - genomics, phylogenomics, proteomics, metabonomics - provide a wealth of information and can reduce sampling and the degree of toxicity or physiological disturbance needed to detect biologically significant changes.

It is also important to discuss the possible impact of adverse effects on the scientific outcome measures, to consider what humane endpoints (in terms of recognisable clinical signs) are to be implemented, when and by whom, how the training and competence of personnel working on the project will be assured and the likelihood of technical success.

Refinement should also be part of the experimental design. Generally lesser severity studies should come first in a programme of experiments and minimal severity testing should be used before exposing animals to more invasive or more painful procedures. For example, when potential anti-cancer agents are being studied, test the therapeutic effect on small tumours before large ones, when potential analgesics are being trialled use minimal stimuli first and prefer escapable stimuli for which the animal determines its own tolerance. As indicated above, pilot studies show opportunities for refinements as well as contributing to reduction, and are useful for showing suitable end-points. Death as an end-point is rarely necessary, although anaesthetic safety margin might be one exception. Suitable biomarkers e.g.metabolites detectable by urinanalysis can be used to provide humane end-points) . Sometimes good use of historical data can help not only to reduce the number of control animals needed but also overall severity.

The work should allow for the introduction of improved husbandry, improved methods of anaesthesia, analgesia, and euthanasia, and dosage by substances with lesser adverse effects. Researchers may be concerned that such changes adversely affect the research, and it may be advisable to include pilot experiments to check this and provide a link to allow back-comparison with previous results. With some more sophisticated techniques, such as radio-telemetry, the value of lesser disturbance during data

collection and obtaining more data from the same animal has to be balanced against the cost and harm of instrumentation.

The groups reiterated the importance of the training of personnel, especially training of scientists in the Three Rs, and of refresher courses on new methods of exchange of information on refinements and experience with experimental procedures between groups, of enrichment possibilities and so on, and of showing this in the application. It was important to include animal care staff within the research team, to consider the whole life time experience of the animal, to ensure compatibility in group housing, and to have quality assurance in the animal house and institutional arrangements to improve care. There should be a range of strategies for refining, including training of animals to reduce stress, appropriate selection of experimental individuals, and selection of the strain that would have least severity. The Three Rs should be in the scientific methods sections of publications, and regulators who question refinements challenged.

Harm-benefit analysis

In addition to consideration of the Three Rs scientists should discuss why the likely benefits of the proposed work are considered sufficient to merit the extent of suffering and distress involved. There are several published approaches to aid this harm-benefit analysis, such as Bateson's cube or semi-quantitative evaluations. Even if minimal numbers and lowest severity can be confidently expected, it does not mean the work is worth doing!

Applicants for FP7 monies should be aware that the proposal must pass EU ethical review if it is to be funded, even if it has an excellent scientific review. The ethical guidelines will need updating after the revision of Directive 86/609 has been finalised, and the extent to which ethical review will be done at national rather than EU level is currently uncertain. They should check at CORDIS http://cordis.europa.eu/fp7/ethics_en.html for up-to-date details of what should be included in an application

Summary

- Give clear, well-stated programme aims and experimental objectives
- Indicate the steps taken to seek replacement alternatives and refinement possibilities
- Explain how the programme of work is designed to minimise use of animals and overall severity
- Discuss how the individual experiments will be designed to meet the objectives with efficient use of animals, minimising numbers and variability, and with procedures of lowest severity
- Consider the approaches to be taken to improve the whole life-time experience of the experimental animals and the enhance their well-being
- Provide a harm-benefit analysis for the proposed work

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Workshop: Systematic reviews, including the enhanced 3Rs search

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Keywords: systematic reviews of animal studies, 3R implementation, quality improvement

Introduction

Systematic reviews (SRs) of animal studies are a new research topic within laboratory animal science. They can be described as a scientific way of analysing existing literature on a specific topic before the start of new animal experiments. Such an evidence-based approach to research is not common within laboratory animal science, even though this seems essential for carrying out ethically and scientifically sound animal experiments and for working in accordance with the 3Rs. Much can be learned from the medical field, where 25-30 years ago evidence-based medicine, including systematic reviews, was introduced. This introduction eventually resulted in high quality human studies. In the oral session on SRs, Prof. R. Scholten (Head of the Dutch Cochrane Centre) nicely illustrated the parallels between clinical and animal research and the great advantages SRs can have for laboratory animal science as well. We believe the introduction of evidence-based laboratory animal science and of SRs of animal studies has great potential for improving the quality of animal studies and for implementing the 3Rs.

The goal of the workshop was to introduce the new research topic 'systematic reviews of animal studies' within the field of laboratory animal science and to discuss the advantages and disadvantages of SRs. Before the workshop, there was an oral session on systematic reviews, meant to introduce the topic.

Workshop

Provocative statements

A total of 24 participants attended the workshop. As a warm-up, each participant received an orange baseball cap (stating SR FELASA 2010). This cap was used for a game called: 'cap on, cap off'. Provocative statements were given by the moderator and per statement each participant had to indicate whether he/she agreed (cap on) or disagreed (cap off) with the statement. The provocative statements were based on the oral session. The statements used are given below; between the brackets are the number of participants who agreed with the statement.

- We need library experts to do a proper literature search (18).
- The trial register of the Cochrane collaboration should also encompass / register animal studies prospectively (13).
- Scientific journals are not interested in publishing details of animal studies (8).
- Due to publication bias, the health of human patients is unnecessarily compromised (16).
- For ethical reasons, all publication on animal studies should be in the "open access" (12).
- 3R literature studies can be stopped, as the 3R information comes through personal networks (0).
- Funding bodies must demand and pay for SRs (18).
- Only a change in organisational culture can prevent that the 3Rs are used as "window dressing" only (14).

The statements led to a lively discussion and it became clear that the topic of the workshop 'Systematic reviews of animal experiments' was rather new to most participants. Furthermore, some interesting topics emerged. One of these topics was the question whether human and animal ethics committees should meet and/or exchange information. Another topic was whether a laboratory animal expert on medical ethics committees would be a good idea. In Canberra, this appeared to be a tradition. Another sensitive topic was open access for all publications.

Group discussion

After the warming-up session, the workshop continued with a session in which the participants discussed the following questions:

1. Do you think SRs are useful? Please mention arguments in favour and against.
2. What are the practical and methodological obstacles to perform SRs?

The 24 participants were divided into 3 groups. In each group, one of the participants was familiar with (Cochrane) systematic reviews of clinical studies. This was a great help, since, during the first part of the workshop, it had become apparent that SRs are really new and an unknown topic for most participants. The participants were first asked to think about the questions individually and write down their answers on an answering sheet. Then the groups were formed. Each group had a chair and a secretary (reporter). The following format was used: firstly, each person tells his/her individual answers. Then the group discussion starts and finally the group's opinion is prepared. In a concluding plenary session, the answers to the questions were collected from the three groups and captured in key phrases. These are the brief answers given by the participants:

Answers to question:

1. Do you think SRs are useful?

Arguments in favour of SRs: Appear to be useful; Proven methodology; To learn from what others did in the past; To get picture clear and get new knowledge; Strengthen your conclusions; To back up choice of animal model; Increase quality and efficiency; Avoiding bad studies; Increase openness and transparency; Better identification of best practice; Prevent duplication of experiments; Decrease costs by prevention of unnecessary studies; Avoid adverse effects in human patients.

Arguments against SRs: Only useful when you make decision based on rationality (SRs can still be subjective); Investment of time and money; Publication bias is hard to detect, so do not blindly believe in SRs; Without Cochrane-like organisation it is not worthwhile doing.

Answers to question:

2. What are the practical and methodological obstacles to perform SRs?

Publication bias; Skills and education are needed; Resources: money, time & personnel; Access to databases; How to work with the databases; Deficient information in publications; Heterogeneity in studies; Lack of efficiency in finding primary studies; Protection of intellectial property by industry and academia; Level and type of expertise needed to perform SR: ideally, expert in biological content, expert in SR methodology and expert in primary studies methodology.

Conclusions

Many arguments in favour of systematic reviews were given including scientific, ethical and animal welfare arguments. The topic appeared to be very new and unknown to most people in the field of laboratory animal science. From the workshop, we learned which topics the participants were concerned about and to which topics attention should be paid in order to successfully introduce systematic reviews. Many practical hurdles were mentioned, which need to be tackled to be able to perform proper systematic reviews. Finally, we can conclude that systematic reviews of animal studies appear very useful and therefore deserve attention within laboratory animal science.

Workshop: Communication to the public about developments in animal research

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Introduction

Is the public aware of the fact that animal experiments are being performed and for what reasons; that alternatives (3Rs methods: Replacement, Reduction and Refinement alternatives) are being developed to reduce animal experiments and to improve science; that scientists also care about animal wellbeing; that animal experiments are in fact a part of day-to-day life and that the results are used for the wellbeing of the public? Where would the public get the information? Can the public develop a well-informed and balanced opinion about animal experiments? These are questions that many scientists, involved in animal experiments, worry about.

Notably, publicly available information on animal experiments often is negatively biased and out of context with reality. This is mainly due to the fact that people dealing with animal experimentation do not see it as their primary duty to communicate with the public. Who should be responsible for communicating facts on animal experimentation? Patient organisations generally decide that their patients should not be burdened about animal experiments, and in particular the suffering involved, that are the bases of their medical treatments. Consumer organisations are often more interested in price and quality than in the fact whether or not animal experiments were involved in the products they test and review. *Non-governmental organizations* (NGOs) do provide information on animal experiments, but this information often is doubted to be correct and balanced. Scientist and research organisations seem reluctant to inform the public about animal experiments.

From several studies, it could be concluded that, generally, the public becomes aware of animal experiments at the very moment they are interviewed about this topic or watch an item on TV[1]. Being confronted with the topic, most people then acknowledge that they have insufficient information to form an opinion; 42.5% don't have enough information on animal experimentation and how animals are treated; 76.6% find that there should be more transparency and public participation in determining when and how animals are used in experiments; 70.8 % believe that scientists and industry using animals do not currently provide sufficient information. These are results from a broad public consultation on animal experiments that the European Commission performed in 2006[2].

Notably, it seems that the designers and/or performers of the experiments are not very active in providing the public with information on animal experiments, and, moreover, the public is not aware of the attempts being made. For that reason, a workshop was organised during FELASA 2010, to discuss communication to the public about animal experiments and the 3Rs activities to reduce these. Three panel members, Kirsty Reid, Ann-Christine Eklöf and Jan-Bas Prins, were invited to give their views on how and what to communicate to the public.

Below you will find a summary of their considerations. These are followed by the summary of the subsequent discussions that evolved among the participants of the workshop and the panel. The discussion was chaired by Jan van der Valk and recorded by Frauke Ohl.

The discussions had to be cut short because of time constraints. However, one of the conclusions was that the lively discussion and the topics raised demonstrated the necessity to organise a follow-up. This report is meant as an initiation for the next part of this discussion perhaps already during the 8th World Congress on Alternatives and Animal Use in the Life Sciences in 2011, Montreal, Canada.

Animal welfare - NGO position (Kirsty Reid)

Use of animals in research and public concern

At Eurogroup for Animals, we believe there are serious ethical dilemmas associated with using sentient animals in procedures likely to cause them pain, suffering or distress. The fact that currently animal experiments are considered necessary to scientific research and safety testing does not diminish this fact. A paradigm shift is needed - a change in mindset away from '*how to continue to justify animal use'* to *'how can we ensure we replace animals as soon as possible*'?

Studies show that animal research is a serious concern for European citizens who believe far more needs to be done to protect animals and their welfare. Public opinion is important, as much research is funded directly or indirectly with the public's money and therefore they should have information that allows them to make well-informed judgements. A high percentage considers that they do not have enough information – e.g. Eurobarometer[3].

Communication is key

Openness and transparency are vitally important. Dialogue amongst stakeholders with two-way communication is essential. Constructive debate on 3Rs and animal use can only be accomplished when there is respect and commitment amongst stakeholders and where information provided needs to be accurate and honest.

Eurogroup believes that there is a need for greater openness and honesty about the true impact of scientific use on animals and about all the different purposes for which animals are used. In order to make progress, with both animal welfare and dialogue, we need to separate the rhetoric of debate from the actual facts and real concerns. Stakeholders involved may hold different perspectives:

The scientific community readily talks about the 'benefits' of animal use, but is much less forthcoming about the 'harm' to animals. Some scientists are themselves critical of the real benefits of some uses (by other scientists!), but this does not come across in information to the public. We hear about 'suffering being kept to a minimum', but a glance at the scientific literature or regulatory test guidelines shows that this does not prevent experimental animals from experiencing serious harm. We also hear about 'strict regulation' and 'high standards', yet under the current Directive 86/609/EEC, many Member States lack any proper system of authorisation of animal use or of ethical review. We hear how 'good husbandry and care' is, yet, although it is agreed current standards need upgrading for welfare (and scientific) reasons, pressure means the revised directive allows a 7 year delay before this needs to be done. Perspectives differ, where the definition of 'high standards' depends on your perspective: one person's high is another person's basic minimum!

There is a number of positive examples in the EU where stakeholders are working together to improve animal welfare and 3Rs, which merit good promotion to the public. These initiatives include EU legislation and dossiers where 3Rs are incorporated: the new animal experimentation directive; the European Action Plan on the Welfare and Protection of animals; the Sixth Community Environment Action Program and EU Framework Programs. Other EU Initiatives are the European Centre for the Validation of Alternative Methods (ECVAM); the European Partnership for Alternative Approaches to Animal Testing (EPAA); the European consensus-platform for alternatives (ECOPA); and transatlantic agreements (CAAT-EU) and initiatives.

Effective communication plays a vital part in: ensuring that new legislation reflects the high level of public concern about the use of animals in experiments and applying the treaty which obligates the EU to fully respect the welfare of animals in its research policy; achieving significant increase in funding and resources to speed up the development, validation and acceptance of alternative testing strategies, in particular non-animal testing methods; developing a coherent and comprehensive strategy to phase out animal testing; achieving constructive debate and cooperation involving all stakeholders; and accomplishing improved International co-operation.

Good and open communication between scientist and public is necessary for mutual respect (Ann-Christine Eklöf)

Experimental research is very important to acquire basic knowledge and for development and improvement of health care in our society. When animals are involved in this kind of research, it is necessary to have strong ethical rules and evaluations; but even more importantly, trustful and open dialogues with the public. This communication must be carried out in a way that both the scientific and the public community are satisfied.

The public must have trust in the scientific community. They must trust that we all are carrying out experiments regulated by national, European or International laws. We need to be open and communicate with the public about why and how we are performing research including animal experimentation.

Different strategies can be used, i.e.:

- Openness and transparency are essential, but issues, such as personal security and illegal activities of animal rights extremists, must be considered,

- Internal and external communications and information flow within and between stakeholder groups,
- The strategy must ensure that the right information is provided to the right people at the right time.
- An effective communication strategy is to establish a dialogue rather than simply the one-way transmission of information.

It is sometimes also difficult to identify who has the information and who and what should be communicated. To simplify we could say: everything that is relevant and can be of help for the public to understand, evaluate and establish their opinion is important to communicate.

The emphasis should, of course, be on increasing the knowledge, necessary for both the ethically sustainable, and the scientifically valid use of animals in research – and addressing issues relating to ethical evaluation and cost-benefit analysis. If scientific validity cannot be demonstrated, then there is no sustainable ethical basis for the use of animals in procedures with the potential to cause them pain, suffering, and distress.

The outputs would be framed within the following general assumptions: The scientific community is driven by a desire for good science and good animal welfare. Openness and transparency are essential for successful communication, but issues, such as personal security and illegal activities of animal rights extremists, must be considered.

Finally, to achieve mutual respect of scientists and the public for the research that is carried out which involves animals, we must consider the following:

- ethical evaluation of animal experiments is necessary to achieve mutual respect between scientist and the public,
- ethical evaluation must be carried out so that all involved and the public can trust the decisions,
- good science and research must go hand by hand with animal welfare,
- open communication and transparency is of utmost importance, but should be regarded in view of social factors.

Communication about animal experimentation and testing: an obligation and a two way street (Jan-Bas Prins)

There are four key questions when it comes to communication strategies:

- What do you want to achieve?
- What do you want to communicate?
- How do you research your audience?
- How do you measure your effects?

These questions are general questions and apply wherever effective communication is being discussed. In the case of communicating about animal experimentation, however, answers to these questions appear to be not so universal, but dependent on tradition and social background. Hence global answers are unsatisfactory in a world that is developing into an increasingly 'global society'. Are we then reaching for the impossible with the wish to effectively communicate about animal experimentation from the experimenters' point of view?

One step back is to learn from previous mistakes and from those experiences that have a proven track record. What tends to go wrong in communicating about animal experimentations is among others: stereotyping and generalizing 'the other'; not speaking the same 'language'; communication without communicating; trying to reach everybody with the same message through the same means; and to allow provocations by certain stakeholders to frustrate communicating with others. The last is often used as an excuse for not having to communicate at all. However, one has to be realistic on the one hand and not to be ignorant about possible threats on the other.

More effective strategies include: sharing reasons why it is still necessary to perform experiments with animals, but also the dilemmas that are not that different from those of the public at large; inviting the public to visit your animal centre and show what and how animal experiments are organized and executed; and not to hide behind others.

Evidently, instant results are not guaranteed. More often than not results will only come after considerable effort and time during which the messenger has to earn respect and the message has to find its proper way. Join forces within your institute and beyond and seek professional advice from communication experts. Do not just copy, but learn from others and find the way that fits best in your tradition and society.

Discussion

Following the introductions by the panel members, the discussion followed four main topics.

Why communicate?

It was generally concluded that the public is not well-informed and sometimes even misinformed about animal experiments. Nevertheless, the public has the right to know about the why and how of animal experiments. The scientists are supposed to be the ones to openly communicate about their involvement in these experiments since they design and perform the experiments and, thus, can and should provide the public with honest information. It was mentioned that

scientists are not always aware of the importance of communication about their work to the public.

Many scientists, and in particular the management of scientific institutes, are reluctant to speak about animal experiments, because of the threat of personal intimidation. It may be questioned whether not communicating to the public will take away this potential threat, since information on animal experiments is already available through other means, like, for example, scientific publications. It was suggested to depersonalise the information regarding animal experiments when approaching the general public.

Public opinion about animal experiments is affected by negative emotions (with respect to animal welfare) and biased by negative preconceptions of scientists (they only care about results, not about the animals). Facts provided by scientists have to give the public the opportunity to develop a more balanced opinion on animal experiments and also to develop mutual respect.

What should be communicated?

First of all, scientists should be open and honest about animal experiments. Not only should the possible benefits of experiments be communicated, but also the fact that, in some cases, they may cause suffering in animals. To ensure a careful cost-benefit analysis of experiments to be done, in most countries experiments are weighed on a benefit-harm scale.

As the public seems to think that scientists don't care about experimental animals, it is also important to communicate that the scientists do care about animals as well and, thus, regard animal experiments to be an ethical issue. For that reason, it is crucial for the public to be aware of the fact that 3Rs models (alternatives) are being developed and used by the scientific community and that strict regulations are in place in many countries to avoid redundant experiments and experiments with avoidable harm to the animals. In addition, regulations require optimal housing and care for the animals. The public should be made aware that they, often unknowingly, make use of results from animal experiments on a daily basis, either for safer products or for better medical care. To provide the public with facts, they should also be shown pictures, even if pictures might be misused or put in the wrong context.

Finally, scientists should be careful when communicating about the potential use and importance of scientific results. Unrealistic promises, for instance with regard to health care or the development of 3Rs models, may have a counter-effect and, as a result, scientists may lose the public trust.

How should be communicated?

The most effective way is direct personal communication by, for example, opening the animal facilities for visits by the public and starting up a bi-directional communication. Moreover, the personal social environment – neighbours, family, pub mates, and colleagues – should be perceived as an important forum for every scientist to discuss animal experiments. Science cafés are also suggested as being important events to discuss animal experiments in an informal way, with the scientist not being an untouchable person in the ivory tower, but a human being with his or her emotions and personal opinion about animal experiments. This allows the public to 'personally' meet the scientists and get better understanding of his/her position.

With regard to raising awareness, it was suggested to label products with the information that it was developed by using animal experiments to assess its safety or pharmaceutical effects.

An Internet site is a good source of information, but a static one. People need an incentive to look for information on an Internet site, like an item on radio or TV, or an interview. Modern communication means, such as through the Internet, like Facebook, Twitter, etc, could be explored to communicate about animal experiments and the 3Rs.

Since scientists rarely are communication specialists, and communication specialists rarely are scientists, it is recommended that both should consult each other before engaging in public activity. Pinpoint the person who has the knowledge and skills to communicate at a lay level the facts of animal experimentation to the general public or specific audience.

Finally, it was stated that organisations, where animal experiments are performed, should increase their awareness of the importance of public communication. Often, the management hesitates to communicate about animal experiments, since only the potential disadvantages, but not the potential advantages are seen. It should be realised that information supply is not a one time event, but has to be a continuous process. It is therefore essential for an organisation to create a communication plan to be followed.

Whom to target?

In principle, no difference should be made between people or groups of people when discussing animal experiments. Most people are interested in the mere facts. At the personal level scientists can explain what they do to their direct environment: family, friends, pub mates, etc. Notably, even in the direct working environment, both the management and the staff

should be aware of the fact that and why animal experiments are performed in the company.

At the professional level, it is important to have a communication plan available. Communicating with the press is different from communication with students visiting a scientific project or the animal facilities. In addition, the government and NGOs should be made aware of the animal experiments and activities to reduce them.

The general public will not by itself search for information on animal experiments. If they do, it is often the result of a coverage on TV or radio that for a short time stimulates the discussion. Easily available information, such as via the Internet, should then be available.

Important targets are students at high school. They are involved, dedicated to discuss this topic and also to discuss their findings with their parents and others in their direct environment.

It was also suggested that, during scientific events like the FELASA congress, a symposium should be organised for the public from where they can obtain information on animal experiments and meet the scientists.

Conclusions and suggestions

- It is essential that scientists become more involved with communication about animal experiments and the 3Rs to the general public.
- Every scientist designing and/or performing animal experiments is responsible for good and open communication with her or his direct environment: family, friends and colleagues.
- When communication takes place with other groups in the public (eg. patient organisations, government, general public), it should be done either by scientists who have experience with communication to the specific group of people, preferably in collaboration with communication experts, or by communication experts after a briefing by the responsible scientists.
- Openness, transparency and honesty are the keywords when contacting the public.
- Effective ways of informing the public are guided visits to animal facilities, informing students at high schools and providing these with material for school activities, and contribution to TV and radio programmes.
- Participants have also experienced that science cafés, where scientific topics are discussed in an open and informal way, are effective for communication. In addition to the static websites, new communication means like Facebook, Twitter, etc, could be explored on its usefulness to forward information on animal experiments.
- The final aim should be that the public can develop an independent and well-informed opinion on animal experimentation.

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Basic assumption was that this is scientific research and important in itself - a discourse analytic study on local ethical committees in Finland

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Abstract
Most studies concerning the ethics of laboratory animal use have been surveys on the opinions of the general public. To obtain a more balanced picture of the field, it is important to study also the views of the members of the scientific community working within the laboratory animal discipline. In the present study the subjects were Finnish authorities or members of local ethical committees during years 1995-2005. The interviews were carried out 2-3 years after the local committees had been replaced by the new centralized ethical board system. The method used was semi-structured interview, the theme being how the interviewees spoke about the role and mode of action of the ethical committees. Discourse analysis was used to find out the strategies the subjects used to factualize the practice of animal experimentation. This practice was found to be constructed by seven strategies of factual discourse the main strategies being: 1) importance of scientific knowledge, especially the benefits of increasing knowledge 2) expertise 3) educational role of the committees and 4) progress in animal welfare. Our question is why the interviewees constructed this kind of version of reality within laboratory animal discipline. For instance, ethical issues or value considerations beyond animal welfare issues were left unarticulated. The results are explained by an empiricist repertoire that leaves out value considerations.

Keywords: ethical committees, discourse analysis

Although surveys on opinions of the public concerning animal experimentation are numerous, only a few studies have been carried out on the most important members of the scientific community in this context, experts within the field of laboratory animal practice. Experts are the group that has real power to define the common-sense of the scientific paradigm; the shared values and beliefs, what issues are discussed and what is taken as given.[1] For that reason, it is important to find out how these persons speak about their work and what kind of methods they use to make their work factual. Arluke[2], Orlans[3] and Graham[4] have studied the ethical committees in USA, Michael and Birke[5] in Britain and Borgström[6] in Sweden.

Materials and Methods

In the present study, the subjects were Finnish authorities and members of local ethical committees during years 1995-2005. Four of the subjects were authorities within the laboratory animal discipline and seven members of ethical committees having expertise in laboratory animal science. Thus, altogether eleven persons were interviewed. All the interviewees of the latter group had more than five year's experience of the committee work. They came from local ethical committees of the main universities (Helsinki, Kuopio, Turku, Oulu, Jyväskylä) and one research institute. Semi-structured interview method was used to find out how members of local ethical committees spoke about the role and mode of action of the committees. This method allowed the interviewees to concentrate on the themes they personally found important while ensuring a broad view on the general theme.
The main interview questions asked were:

1) What were the topics of discussion in the ethical committees?
2) Did the topics change during the years 1995-2005?
3) Did the society's atmosphere affect the topics discussed?
4) Was there any change in the application forms during 1995-2005?
5) How many applications were dealt in one meeting?
6) How many applications were rejected yearly?
7) How did the researchers justify their experiments?
8) How did the interviewee find his/her role in an ethical committee?

The interviews were transcripted into written text and analyzed by using discourse analysis. By discourse analysis it was possible to find out different strategies the interviewees used to make the committees' work factual.

Discourse analysis is a part of qualitative research methodology in social sciences. It covers all kinds of ways language is used; formal, informal, written and spoken. Main focus is on speech as action, such as justification, rationalization, categorization, naming and recognition.[7, 8] Our focus was on the ways the language is used to construct reality within the field of laboratory animal science, and to convince the listener of the truth of this version of reality. The main principle of discourse analysis is that language is both constructed and constructive, thus its theoretical home is found in social constructionism. For social constructionism reality is constructed through language and symbols between human relations.[9] This concerns also scientific knowledge; scientific accounts – written or spoken – are not readymade and cannot be applied to every scientific issue, but are rather devices that can be used to reach certain objectives. This means that scientific accounts become accurate and sensible through their cultural context.[10] This was the starting point of our study. The presupposition was that all kinds of facts or information (scientific etc.) are part of a social process. This means that there are no 'naturally given' truths, but what is true is constructed through speech.[7] Our focus is on the rhetorical means the interviewees use to construct reality within their own field.

Because of the interviewees' position as experts their opinions are important within the field of laboratory animal practice as well as to the public and political decision-makers. Their opinions are easily considered as truths and facts. Study of factual strategies is a method of choice when analyzing the speech of experts that actualizes as acts, such as decisions. Factual language used in this context means a way of speaking that gives the audience an undisputable impression of a true version of reality. Factual strategy on the other hand means a certain way of convincing the fact nature of some phenomenon. It does not differentiate the spoken from the true version of reality.[11]

In the present study, the focus was on the means and methods by which the interviewees constructed a certain version of committees' work and on the manners the interviewees used to implement these versions. Our interest was on the truisms the interviewees used when talking about the work of ethical committees and the ways in which they used those truisms in order to make the committees' work factual. While analyzing the material and constructing factual strategies the focus was on specific repetitive statements and words that the interviewees used. For example, in quantification strategy numbers and quantities did not in themselves formulate a quantification strategy, but helped to create an impression of it.

Results

Altogether seven factual strategies were found. These are presented starting from the most frequent appearance in the interviewees' speech.

The strategy of *reliance to importance of scientific knowledge* was the most common in the interviewees' speech. It appeared altogether 29 times. The value of scientific knowledge emerged as a basic assumption that needed no justification. This assumption justified the lack of ethical considerations. Within this framework ethical considerations were unnecessary neither to justify nor to question animal experiments. This strategy actualized in the context of the first interview question (the topics discussed in the committees), to which the interviewees mostly didn't answer but started argumenting about the importance of scientific knowledge and the benefits of animal experiments.

'Fact speech' was central for this strategy. It means that for the subjects things just happen without possibilities to affect them. What the subjects can do is only to accept.[12] For the interviewees scientific knowledge was seen as a truism. An example of fact speech is presented in the next quotation:

1. "*KL: what were the discussed topics?*
"INT: well at least in our committee we didn't think whether it was right to do these experiments / the basic assumption was that this is scientific research and important in itself /this experiment is useful"

The interviewee speaks about a basic assumption that makes other alternatives impossible. Alternatives do not have space nor are needed, as can be seen in the next quotation:

2. "*KL: how did the researchers justify their experiments?"*
"INT: they explained the scientific background what was behind it and that we believe that this receptor affects to this and this/ and this is what we want to study by this way and / it was very scientific the basis so that the study's reasoning came from the scientific way of thinking / our first question wasn't how the world will be saved by this study instead we asked about the scientific problem that was to be solved"

In this quotation, the argument of the importance of scientific knowledge is used to justify the experiment.

In the third quotation, the interviewee speaks about importance of increasing scientific knowledge

which justifies the lack of ethical considerations. Value of scientific knowledge is taken as given.

3. "*KL: why was ethics not discussed?*

" it was so self-evident for that group [committee] *that of course we didn't question the usefulness of the experiment / the starting point for everything was that this is true a new knowledge can be achieved and that itself was enough / the research is almost mostly basic research and it is very difficult to say whether it is useful or not / the supposition is that a new high standard research is valuable in itself"*

Argumentation on scientific knowledge can be seen as a cultural convention and can only be understood through the values and norms predominant in the scientific community.

Quantification was the second frequent strategy; it appeared fifteen times in our material. By using quantification the subjects convinced the thorough work done in the ethical committees. Precise numbers increase the information value of truth.[11] Quantification does not only mean numerals, but also all kind of quantities that emerge in the speech. The interviewees spoke a lot about the duration of meetings as well as the large scale of experts in the committee:

4. " we had very thorough discussions so that the meetings could take even five hours long, so we truly discussed and once we even discussed for two hours whether cutting off head from rats by using guillotine is a significant harm or not"

Another context in which quantification was used was when the committee discussed the pain and suffering that was caused to animals during experiments. Quotation number five is part of the discussion of the rejections of applications. Rejection was rare but some applications and experimental designs raised more discussion than others, particularly when the experimental design was somehow controversial.

5. *"there were also always a couple of applications that caused an extended discussion whether this experiment is essential or whether this same thing has been done earlier or elsewhere"*

Reliance on expertise of the committee members – strategy (14 appearances) was used to make factual the ethical committees as experts and competent to decide on the proposals for animal experiments. Factual speech that is based on expertise concerns convincing somebody by special knowledge.[11] In the present case, it mostly represents the interviewees' own expertise.

6. *"when we were deciding whether this experiment belongs to first (*severe suffering*) or second class, what caused a lot of discussion was especially in the context of first class experiments that was the experiment planned so well that the committee can send it to municipal authorities for final judgment / the committee always wrote a statement to municipal authorities in the case of first class experiment / but we thought that this municipal authorities judgment was kind of a pseudo decision in the sense that if we in the committee had decided something it hardly ever changed in the municipal the authorities hardly ever decided against our view.*

In this quotation, the interviewee convinces the expertise of the ethical committee by naming the municipal authorities' decision as pseudo- decision that gives the interviewers an impression of the ethical committees' expertise in relation to other authorities.

Requirement strategy (14 appearances) was used when discussed about the requirements the committee set for the researchers. By using this strategy, the interviewees constructed an ethical committee as a place to learn and to control the researchers. In the following quotation the interviewee speaks about his role as a person who has to ask for some basic information from the researcher. This quotation is part of speech in which the discussed topic is application forms and their shortcomings.

7. *" I noticed that in the application form some details are important for the funding and this important issue concerning the animals was missing, so I had to separately ask the researcher why he is doing it in this way, you need to say it a bit different way because it's the animal that matters"*

By using c*onsensus strategy* (9 appearances) the need to strive for consensus on the aims and discussions of the ethical committee was stressed. Interviewees' interest was, understandably, to construct a united picture of the committee.

8. *"most people were very willing to attend our meetings --- I would say that, all in all, it unified the researchers and the persons working within laboratory animal discipline"*

In *appealing to other colleagues -strategy* (8 appearances), the discussed topics were acceptance of higher authorities to committee work. This means that the work was justified and made factual by appealing to higher authorities, such as university principals and professors. By using this strategy, the committees' work was constructed as acceptable and in accordance with society's norms. In every culture, there are certain categories of agents who are treated differently than others. They are treated as if they were entitled to know particular sort of things better than others and their arguments are thus given special weight.[13] In the next quotation a university rector is that sort of agent.

9. *"even the university rector found it very important that we had a representative of animal welfare in our committee"*

Reliance to self- perceived –strategy (8 appearances): interviewees appealed to personal

experience that animals were looked after well and that animal welfare has improved. Animal welfare was constructed as a progressive narrative. Narratives are products of social communication. They are not seen as stable structures, but as temporary constructions that are shaped by social norms, contexts and social interests.[14] The following quotation is an example of a progress story:

10. *" I remember one discussion with a researcher when this committee work had just began we were talking about the researcher's thoughts on what he thought while doing these experiments and he said that he doesn't think much about anything, that it is better to think the animal as an object, then it is much easier to do experiments; but nowadays the researchers are in a way forced to think of the animal, because animal welfare is so big an issue these days and it is also allowed for the researchers to think animal welfare; so it has changed and it is part of the experimental design and not any oddity anymore"*

Discussion

Within the framework of social construction theory where reality is constructed through language, what is left unspoken is as important as what is said. It is interesting that in committees whose task was to evaluate justification of experiments on animals ethical issues were left unarticulated. The results indicate that the ethical committees mainly replaced the genuine ethical considerations with from ethical point of view empty strategies, such as importance of scientific knowledge acquired by animal experimentation, expertise and consensus. Reasons for this can be sought from the cultural context the scientists are part of. The interviewees were arguing within the framework of science. This became especially evident in the context of the question concerning the issues discussed in the committees. The question was mainly answered by stressing the importance of scientific knowledge. Scientific knowledge can be seen as a cultural convention in which the interviewees based their argumentation.

Our results are in agreement with the study of Michael and Birke.[5] Finnish agents within the field of laboratory animal science use the same kind of argumentation to justify animal experimentation as those studied in Britain. According to Michael and Birke, persons who work within laboratory animal discipline form a sort of 'core set'[5], which means a group of scientists involved in a continuous dispute on some scientific controversy.[15] They expand this definition by suggesting that scientists are potentially engaged in actively constituting a core set by setting out cultural criteria for membership.[5] The disputable nature of animal experimentation as a tool for acquiring scientific knowledge renders the setting of criteria for core set membership crucial. Acting in this self-constituted core set affects strongly the values of individuals.

Gilbert and Mulkay show that, among scientists, it is typical to formulate a picture of science by using two interpretative repertoires: empiricist repertoire and contingent repertoire.[16] Empiricist repertoire includes a definition of good science and it is used in the context of formal research papers. Scientific work is characterized in very conventional manner; as following from impersonal universal rules that are universally applicable. The advantage of the empiricist repertoire is that it makes scientific knowledge seem self-evident and undisputable. Within the contingent repertoire, scientific work is more dependent on personal characteristics, social ties and group memberships. However, this does not mean that the empiricist repertoire would not exist in the same time. These two repertoires may be considered the framework within which the discourse within the scientific community is formulated. These scientific discourses affect strongly the debate within the practice, e.g. how much weight is given to ethical issues and how ethical issues are argued. In our study, the empiricist repertoire seems predominant. The lack of ethical considerations we observed may be explained by a learnt empiricist discourse that leaves little room for ethical issues.

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Values behind ethical evaluation systems

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Abstract
The cost-benefit analysis of applications submitted for animal experiments is an essential part of laboratory animal practice. However, weighing the benefits of an experiment against the harms caused to experimental animals is a complex task and prone to be affected by personal values. For consistency of evaluation, it is of utmost importance to discuss and agree about the definitions and contents of the concepts used and about the commonly accepted values within the scientific community. In the present study philosophical argumentation analysis method was used to find out the values, principles and theoretical framework behind the following proposals for ethical evaluation systems: Porter 1992, Stafleu et al. 1999, Voipio et al. 2004. Emphasis was on the inherent consistency of each evaluation system and on the nature and validity of the arguments used. Philosophical analysis revealed some common features in the evaluation systems. The starting point was utilitarian, although elements from other theoretical frameworks contradictory to utilitarianism were present. Three main benefits emerged: health, which was valued highest, knowledge and economic benefit. Principles and presuppositions, such as concerning health as a prerequisite of human happiness, were taken as given. Utilitarian ethics aims at minimizing suffering and maximizing happiness for the greatest number of beings. Thus, cost-benefit analysis may seem relatively easy to perform in this framework. However, the utilitarian framework leads us to basic questions such as the nature of human happiness, especially the role of health as a constituent of happiness. For a truly ethical evaluation of animal experiments, these questions cannot be neglected.

Keywords: ethics, cost-benefit analysis, values

Most of the proposals for ethical evaluation systems of animal experiments concentrate on questions like the importance of the research to be done, the capability of the research group to achieve its goals and the ways the 3R principles are taken into account, eg. measures to minimize pain and suffering of animals. Behind these seemingly practical questions basic philosophical questions emerge. What is the theoretical framework within which ethical evaluation systems are being developed and applied? What are the values and principles they are based on? How is experimentation on animals justified within the scientific community? What are we actually talking about when we refer to the benefits of animal experimentation or to the right of animals to be treated in a way that respects their intrinsic value?

It may seem that philosophical analysis within a practical field of science would be waste of time. However, as Voipio et al.[1] stress, without commonly defined and accepted values ethical evaluation is prone to be affected by personal values. Thus, for consistency of evaluation, it is important to discuss and agree about the definitions and contents of the concepts used and about the commonly accepted values within the scientific community. However, consensus on an issue within a community is not as such a sign of its moral acceptability. Philosophical analysis is needed for finding out the existing values and the presuppositions and beliefs behind them and to evaluate their validity. Philosophical analysis may prove useful also in another field; the far too often polarized dialogue between scientists and the groups opposing animal experimentation. The prerequisite for constructive debate within the scientific community, as well as between the scientific community and the public, is that the philosophical framework and central conceptions used are clear for all parties. Actually, even the concept of "animal experiment" is value-laden. While for a researcher an animal experiment may mean a tool for achieving something valuable, such as knowledge, a layperson may see an ethical problem of

trumping something valuable – the life and welfare of living beings.

The values behind practices of animal experimentation within the scientific community have not been studied extensively. Most studies concerning the ethics of laboratory animal use have been surveys on the opinions of the general public. However, mere knowledge of the views of the public or even of the members of ethics committees[2,3,4] does not provide adequate tools for dealing with ethical problems and conflicts of values. Information about current values in the community is but a starting point for their deeper analysis.

The aim of the present study is to find out and clarify the theoretical framework within some proposals for ethical evaluation of animal experiments and the ways in which animal experiments are justified. I will start by analysing two well-known theoretical proposals for ethical evaluation systems – Porter[5] and Stafleu et al.[6] – and one more practical proposal presented by Voipio et al.[1]. The first two proposals, although they are not in use as such, have significantly contributed to evaluation of animal experiments in practice and have the advantage that their theoretical basis is spelled out. The third paper is based on the results of a Nordic-European workshop for ethical evaluation, whose participants were laboratory animal scientists, ethicists and authorities and thus represent a broad spectrum of agents within laboratory animal practice. These papers reflect well the theoretical framework within which ethical evaluation is performed in practice. In the second part of the study, I will concentrate on philosophical analysis of some of the central concepts found in the abovementioned papers

Materials and methods

The following proposals for ethical analysis of animal experiments were analyzed using philosophical argumentation analysis: Porter[5], Stafleu et al.[6] and Voipio et al.[1]. Emphasis was on the inherent consistency of each evaluation system and on the nature and validity of the principles and presuppositions found.

Analysis of proposals for evaluation systems

Within the field of laboratory animal science, the ethical issues concerning the use of animals have traditionally been discussed in the context of practical questions such as welfare of animals or pain alleviation during the experiments. This practical everyday view is also seen in the proposals for ethical evaluation systems for animal experiments[1,5,6,7,8,9]. The starting point is consequentialistic: animal experiments are justified by their expected consequences. Utilitarian balancing of harms caused to animals against the expected benefits to humans or other animals forms the core of the three systems analyzed. Justification of animal experimentation per se is not discussed. Animal use in scientific research is taken as given; only the degree of harm caused to animals in relation to the benefits for humans is discussed. This is understandable within a scientific community where animal use has traditionally been considered an essential part of biomedical research.

Although their evaluation systems comprise clearly utilitarian balancing of harms (or costs) and benefits, both Porter and Stafleu et al. bring in elements from other theoretical frameworks. Porter[5] proposes that "scientists should adopt the Schweitzerian ideal of respect for all life, and become antivivisectionists at heart". Porter creates deliberate tension between the "animal rights" ideal and practice, thus his scoring system is based on deviations from the ideal. He stresses that the ideal should generate a constant pressure to avoid experiments on animals whenever possible.

The concept of "respect for all life" has been borrowed from another theoretical framework, deontological ethics. Within this framework the respect principle implies respecting the intrinsic value of animals. This in turn means that they should not be used instrumentally for the good for any other beings[10]. This principle, which is adopted by animal rights philosophers such as Regan[10], seems to be contradictory to the strictly utilitarian framework that accepts instrumental use of other beings as a tool for maximizing total good or happiness.

What Porter really means by the respect for all life principle remains somewhat unclear. It might be that he refers to what Cohen[11] calls intrinsic value in a broad sense: that animals are valuable in the sense that they are unique beings and cannot be replaced. Thus, loss of any life should be avoided whenever possible. However, instrumental use of animals is still allowed. Perhaps we should interpret Porter by what he says later on his ideal: that its point is to seek to minimize or avoid harm we inflict on sentient beings. This interpretation is compatible with the utilitarian framework and presupposes no rights or intrinsic value for animals.

Stafleu et al.[6] represent another way of rejecting the purely utilitarian stance. Their point is that, unlike in utilitarianism, the considerations to be taken into account in their weighing system are not reducible to one value, such as happiness in utilitarianism. However, it is difficult to see the difference between their weighing of human and animal interests and that of preference or interest utilitarianism. Stafleu et al. present as their fundamental ethical position a principle of respect for persons. Unfortunately, this

principle is not further opened except for a reference to such philosophers as Aristotle, Kant and Rawls and economist and philosopher Amartya Sen. Thus, its content remains unclear. Contrary to utilitarian framework Stafleu et al. ascribe also intrinsic value to animals in order to stress that every animal has a value of its own beside the instrumental value for human interests.

Even if we employ a purely utilitarian system, further definitions are needed. Within utilitarian framework there are differing views that greatly affect the outcome of our evaluations. We should make these explicit and – when defined – use them in a concise way. For instance, the weight given to human and animal interests may vary. Among utilitarian philosophers the stance varies from equal consideration of interests applied by Singer[12] through weak human priority[11,13] to strong human priority[14]. The evaluation systems analyzed here take no clear position in this respect. If we take a look at their scoring systems, it seems that Stafleu et al.[6] and Voipio et al.[1] represent the weak human priority view. It must be noted that the weak human priority principle does not mean that we may treat animals as we like. Cohen[11] stresses that our own moral principles on inflicting pain restrict our use of animals. If we think that causing pain is morally wrong, we should also avoid causing unnecessary pain to animals – provided that we believe that animals are capable of experiencing pain. We have also a moral duty to fulfill the duties of care we have voluntarily taken towards the beings whose welfare is dependent on our acts. Thus, researchers as moral agents have a duty to treat animals well and avoid causing them pain. This principle seems very close to that of the 3R principle of Russell and Burch[15], widely agreed upon within the scientific community.

On the other hand Porter[5], who criticizes the predominant dogma that humans come first and that biomedical research is essential for human wellbeing if not survival, seems to be near to the equal consideration of interests' view. Unlike the others, Porter is not worried about the loss of knowledge as a result of restricting certain types of experiments, but believes in the possibilities to gain knowledge by other means.

All scientific theories, no matter whether biomedical or philosophical, have their flaws. One of the theoretical problems within the utilitarian framework concerns the justification of an act in terms of concepts such as harm or cost and benefit. The interpretation and content of these concepts depends on the values and the spoken and unspoken presuppositions of the community. While these may represent the values of the majority, it is not self-evident that they are based on sound philosophical principles. Thus, it is important to take a closer look on what is considered harm or cost and what are the benefits that justify animal experimentation. In this paper, I will concentrate on the concept of benefit, although what is considered as harm also raises philosophically interesting questions. However, the benefits are of main interest here, because, if the benefits suggested prove to be on an unsound basis, the use of animals in scientific research itself may be questioned.

Philosophical analysis of one of the key concepts: benefit

Health as the main benefit

Most of the philosophical theories concerning justification of animal experiments (eg.[10, 11, 16]) concentrate on applied medical research. Likewise the ethical evaluation systems analyzed here as well as other systems, such as Bateson[7] and Smith and Boyd (8) value health as the highest benefit. This is understandable, because the benefits of medical research are easy to see and justify. However, nowadays a great part of scientific research is carried out for other than medical purposes. Thus, concern of justification of basic research is one of the common factors among ethical evaluation systems. The value of knowledge is stressed, although it is considered lower than that of health.

If we agree in community that the main justification for animal experimentation is the benefit for human health, we must reach deeper and ask what makes health so special among benefits. Why is health so important for us that it justifies harming animals? As long as we use the utilitarian framework as the basis for our evaluation, we should also ask whether the central position we give to health is justified within this framework.

The importance of health benefit is taken as given by Porter and Voipio et al.. Stafleu et al. base their stance on the "*principle of respect for persons whose health and survival are normally necessary for the full and autonomous development of persons*". They refer to population ethicist Norman Daniels[17], who, in his theory on just health care, emphasizes the importance of health in terms of protecting opportunities of individuals.

It is interesting to note that also the philosophers defending animal experiments on the basis of their importance for our health, such as Cohen[11] and Frey[16], tend to take the position of health as the primary benefit as given. Frey emphasizes that practically all conceptions of good life presuppose at least moderate state of health. According to him, elimination of illnesses and lengthening of life are central part of our quality of life and are valued high in the western society. However, as mentioned earlier, appealing

to the current values in society is questionable, as it cannot be taken as given that they are inherently consistent and based on sound principles. It is easy to argue that longevity and health alone do not guarantee a happy life. Not all healthy persons feel happy or are content in their lives. On the other hand, it is possible that severely ill or handicapped persons may lead a good life (e.g.[18]). Furthermore, the fact that something is valued high in our society does not imply that the means we use to pursue it are morally justifiable[16].

Within the utilitarian framework, the ultimate aim that is pursued is happiness. Happiness constitutes a variety of pleasurable things, goods, which are interchangeable, so that the lack of one good may be compensated by other goods. In this framework, health is one of the goods, not the only or the primary good. It is no doubt true that life- threatening diseases, severe chronic pain, or certain types of handicaps endanger the possibilities to pursue one's own goals and may affect strongly the experienced welfare of a person. We may say that within the utilitarian framework a certain level or health is necessary. Thus the health benefit has its place in ethical evaluation of animal experiments. The question remains what would be the level of health needed to justify animal experimentation. As Stafleu et al.[6] observe, not all medical research should be scored equally highly. Some illnesses may even be fought more efficiently with preventive measures than with medical therapy. We must also ask what we actually mean with "health". The conception of health is in part socially determined[17] , thus we should discuss and define the ways it is used within the context of animal experimentation. The central idea of the utilitarian framework is that we are morally required to maximize happiness. Thus, we are also met with questions such as distribution of goods - should we allow painful experiments on animals for therapies that would only benefit a small group of persons, or should we use our limited resources for actions that benefit great numbers of persons?

Economic benefit

Stafleu et al.[6] and Voipio et al.[1] also add economic interests to their list of benefits that should be taken into account. This is not a new feature; also Smith and Boyd[8] refer to economic benefit. Stafleu et al.[6] give economic interest the same value as to knowledge interest, while Voipio et al.[1] refrain from hierarchies. Voipio et al.[1] justify inclusion of economic benefit by improved production, preservation of wildlife as a food source (hunting), effects of society's welfare through employment, and savings in indirect costs of health care. Stafleu et al.[6] observe that economic interests often play an important role when deciding on animal experiments, especially in industry. Including the economic interest as a part of the ethical evaluation system renders the system more practicable and brings this interest more amenable to discussion. Stafleu et al. acknowledge three types of economic interests: industry-related interests (employment, profit and efficiency), national economy and human welfare.

Porter, on the other hand, takes a difference stance. His ideal of respect for life excludes causing pain for profit or curiosity. Thus economic benefit has no room in his system and "curiosity-driven" or fundamental research not directed at health problems or aiming at reducing minor discomfort of human beings is only allowed when costs for animals are low. This fits well to the utilitarian framework.

Both Stafleu et al.[6] and Voipio et al.[1] justify the economic benefit by pragmatic reasons. It is true that the role of economic benefit should be discussed and made visible, if we want to allow economic interests to affect the justification of animal experiments. However, other than pragmatic justifications are needed. Stafleu et al.[6] stress that in their system the benefits considered are not reduced to one common value such as happiness. This might imply that in their system the economic benefit is considered valuable in itself. Unfortunately, the link between economic benefit and respect of persons –principle that forms the basis of their system - is not easily seen. It is true that a certain level of wealth is required so that the basic needs for living are met. The problem is that the economic benefits of industry should be shown to benefit society through, for instance, higher employment and raised salaries, and not only the small group of shareholders. This link is at its best debatable and complicated.

Within the utilitarian framework, the supposed inherent value of economic benefit might be explained by the association psychological view put forward by Mill[19]: through association things that have previously been means of pursuing something valuable gradually grow in the minds of persons valuable in themselves. The prerequisite of this explanation is that the final goal pursued – happiness – can be achieved by means of economic welfare. It is not clear that economic wealth of industry or even of society would guarantee happiness in individual level. Besides, wealth as such does not necessarily contribute to happiness[18]. Economic interests do not fit easily in the utilitarian framework. However, if economical profits were to be accepted as one of the factors contributing to happiness, we should ask the same questions as in connection with the health benefit. When basic needs for food and shelter, rest and spare-time are met, we must define the level where economical benefits cease to contribute to happiness.

Conclusions

Developing ethical evaluation systems in the utilitarian context may seem relatively unproblematic as long as central concepts are agreed upon and defined. In practice, the technical balancing of harms and benefits gets most attention. However, as utilitarian ethics aims at maximizing happiness and minimizing harm, basic questions such as the nature of human happiness and the role of health and other benefits as constituents of happiness cannot be avoided.

No matter which philosophical framework we choose, we should be concise in applying it and should not bring in principles that contradict with that framework. Attaching the different values concerning the justification of animal use within society as a part of a utilitarian cost-benefit analysis is theoretically problematic. Perhaps, if we want to take into account the differing values within society, we should adopt a pragmatic view and turn to contractarian theories. In the contractarian framework, what is considered valuable and how much weight different valuable things are given is defined within the society in a process of social contract. Within this framework, we could include mutually exclusive principles like an obligation to respect animals put forward by the European Commission, the respect for life principle and the notion of importance of human benefit. Of course, also within this framework, the chosen and agreed values should be applied in a concise way. And, unfortunately, even this starting point would not save us from the practical challenge of developing a trade-off system to balance the different values.

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Experimental Models

Non-human primates models: their value, and the current EU perspective on 3Rs (the SCHER report)

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Abstract
In light of the significant developments made in science and biomedical technologies since the implementation in 1986 of the EU Directive 86/609, and stimulated by several reports published by animal welfare non-governmental organizations in recent years proposing a progressive replacement in the use of non-human primates (NHP), a Working Group was established in May 2008 by the Health & Consumer Protection Directorate, made up of permanent members of the Scientific Committee on Health and Environmental Risks (SCHER) and independent experts. The scope of the Working Group was to generate detailed scientific information in the context of biomedical research activities for which the use of NHP was still considered fundamental, and to explore whether alternatives exist to drastically reduce and replace their use in future. The final aim was to support the DG ENVIRONMENT in the co-decision procedure, and the related discussions at both the European Parliament and Council during the current revision of the EU Directive.
The principal aspects considered by the team were as follows: i) the current use of NHP for biomedical projects and procedures, ii) their actual use in terms of absolute and relative (compared to other animal species) figures, iii) distribution of procedures involving their use across EU Countries, and iv) expected future trends. Finally, experimental activities for which alternative models might be implemented with no major impact on the European research and development (R&D) were carefully reviewed, in order to provide the EU Commission applicable suggestions aimed at the implementation of the 3Rs. The report of the Working Group[1], adopted by the SCHER on January 2009, will be summarized and introduced during the presentation.

Keywords: NHP models, EU, alternatives, recommendations

The current use of non-human primates (NHP) for experimental procedures in Europe

According to the most recent EU statistics, around 10,000 non-human primates (NHP) are used on a yearly basis in biomedical procedures within the 25 Member States, representing about 0.09% of the total number of animal used. The vast majority of these are imported from non-EU Countries (mainly Asiatic countries and Mauritius), and nearly 100% are F1 or F2 purpose bred.

Based on the same statistics, NHP are currently used only in exceptional circumstances where no alternative methods are available, no other suitable species are available for the specific purposes of the research, or for procedures expressly required by international legislation for testing the safety or the efficacy of pharmaceutical products and devices (67%). The remaining is used for biological studies of a fundamental nature and for the research and development of products and devices for human (and veterinary) medicine. Research programmes, for which their use is still considered essential, cover mainly diseases affecting the immune system (eg multiple sclerosis), neurodegenerative disorders (eg parkinson, alzheimer etc), infectious diseases (eg human immunodeficiency virus, malaria, tuberculosis, hepatitis etc.) and other emerging serious diseases serious diseases (eg severe acute respiratory syndrome etc).

Testing of safety and efficacy

Safety testing of new pharmaceuticals and other medical products represents one of the major uses of NHPs. Based on definitions of the Declaration of Helsinki regarding human experimentation, the well-being of the human subject should take precedence over the interests of science and society, and clinical trial subject's protection should be safeguarded through risk assessment based on the results of

animal experiments. For these reasons, all the major international regulatory Agencies, including the EMA (European Medicines Agency), require that the safety of a new medicinal product is supported by a variety of non-clinical data prior to the start of clinical studies. The scope of testing is regulated in the EU by Council Directive 2001/83/EEC and its amendments and, worldwide, in the specific Guidelines issued by the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals (ICH).

All these safety guidelines require that the non-clinical studies are performed in "relevant species", and that key studies for risk assessment of pharmaceuticals, such as the repeated dose toxicity testing, have to be performed in two species, one of which must be a non-rodent.

The species should be chosen based on its similarities to humans with regard to pharmacology and pharmacokinetics, including biotransformation. The use of a non-rodent species for the characterization of new medicinal products aims at limiting the uncertainty in extrapolation process from animal toxicity data to the human situation. Such uncertainties are species variation, scaling from small, short-lived animals to large, long-lived species, and use of homogeneous animal populations. Dogs or NHPs are frequently used as the non-rodent species.

Furthermore, for testing of biotechnology derived drugs, the differences in immunogenicity in species other than humans (leading to invalid experiments in some animal species) are also an important reason to select NHPs for toxicity testing.

The most frequent reasons for selecting NHPs as animal species for testing generally are as follows:

- no other species showed the primary pharmacodynamic effect
- had the most suitable metabolism or are the most relevant species with regard to bioavailability
- represents a well established model for pharmaceuticals in the class or the most relevant species for known side effects.

In addition, studies in NHP may be preferred over safety testing in other mammalian species for the following reasons:

- close similarity between the menstrual cycle of NHP females and human females and higher predictor of relevant reproductive effects (eg. NHP are more predictive in assessing ovarian hemorrhagic risks in the case of anticoagulation agents)
- close similarity in the anatomy of the ocular system; in particular the similarity of the "macula lutea" in the eye of NHP and man represents a more relevant model for assessment of specific ocular effects of new pharmaceuticals
- gastrointestinal system less susceptible to vomiting than other non rodents (i.e. the dog) and therefore more predictive on the exposure to the pharmaceutical administered
- species most sensitive for the toxicity of coagulation factors as the clotting system in cynomolgus is highly similar to humans
- several times the most sensitive species for testing the safety of biotechnology-derived pharmaceuticals, as for these products the choice might be driven by specific aspects of the immune system. In particular, monoclonal antibodies (mAbs) are highly specific to their targets and accurate prediction of 'on-target' effects requires testing in a species which shows cross-reactivity, thus frequently requiring testing in NHPs as only species cross-reacting with humanized monoclonal antibodies
- assessment of abuse liability of new central nervous system (CNS) pharmaceuticals, since CNS-active pharmaceuticals with properties indicating stimulant, depressant, hallucinogenic, or mood-elevating effects require an evaluation of abuse liability (EMEA/CHMP/SWP/94227/2004).

Development of vaccines

Finally, several vaccines, including those currently used to protect humans against fatal infectious diseases, have been developed through studies in NHPs. The three major global health threats presently are HIV, malaria and tuberculosis, but new pathogens, such as the SARS virus and avian flu, emerge.

Before new candidates can be evaluated in humans, the efficacy and safety of new vaccines have to be assessed in animals, and, for several infections, NHPs are the only animal species susceptible to the infectious agent, and the proof-of-concept can therefore only be studied in these species. This is the case eg. for smallpox vaccines using exposure to monkeypox as a proof of activity. Furthermore, to understand the mechanisms of protective immune responses induced by candidate vaccines, it is critical to use an animal model in which the immune system closely mimics that of humans. Therefore, the validity and the quality of the induced immune response to the vaccine and their safety still need to be assessed in an animal model that is genetically very close to humans.

In the development of vaccine against **HIV**, preclinical studies in NHPs play a key role. Efficacy data are generated from immunized NHPs challenged with either simian immunodeficiency virus (SIV) or chimeric simian/human immunodeficiency (SHIV)

virus. Macaques infected by SIV develop symptoms very similar to those in humans infected by HIV. In addition, the development of the disease in macaques is predictable from the viral load in the blood in early stages of infection.

Presently, about one third of the world population is infected with Mycobacterium tuberculosis, and more than 1.5 million people die from **tuberculosis** (TB) every year. The current vaccine (BCG) was developed in the beginning of the 20th century, and is still the most widely used vaccine worldwide. However, its efficacy is doubtful and in the last 15 years new strategies to improve or replace BCG have led to several candidate vaccines evaluated in human clinical trials.

As for many infectious diseases, there is no only one ideal experimental animal model for TB, and information has to be gathered from studies in various animal species. Nevertheless, NHPs develop pulmonary granulomas and show an immune response similar to humans. Differences observed in response to vaccination and protection against infection between the two macaque species in response to vaccination and protection against infection may still provide insight into mechanisms involved in protection against tuberculosis.

Malaria is responsible for approximately 500 million cases of morbidity, 10–20 million cases of severe disease and 1–3 million deaths each year. The challenges to develop a successful vaccine are great. There are at least four different species of parasites that may infect people causing malaria, and each species is composed of a number of genetically different strains. In addition, during the course of the infection, the human host is confronted by four distinct life cycle stages of the parasite. Each of these life stages presents new antigens (targets) to the immune system. Human genetic differences can also affect the level of immunity in response to a vaccine. Therefore, a vaccine against *Plasmodium falciparum*, the most serious malaria parasite, must account for the genetic diversity of both the parasite and the human host and provide effective immunity against all different life cycle stages of the parasite.

The owl monkey (*Aotus*) is apparently the only species (besides the chimpanzee) that is susceptible to the human malaria parasite and it is therefore used to test the potential efficacy of human malaria vaccines. The rhesus macaque has also been used to study the immunogenicity of the candidate vaccines, without studying protection against infection.[2] A candidate vaccine developed with the use of NHPs is now in extensive Phase III studies. Although many challenges are yet to be overcome, there is considerable optimism that the development of an effective malaria vaccine is likely.

Hepatitis C virus infects about 170 million people worldwide and is the leading cause of liver failure and transplantation. The search for a vaccine against this disease is complicated by the fact that the only species besides humans that is susceptible to hepatitis C virus is the chimpanzee. In earlier phases of the vaccine development, in vitro techniques and other animal species are often used, and chimpanzees are only used for testing the efficacy in very promising candidate vaccines. Recently, a vaccine capable of eliciting virus-specific immune responses in baboons and genetically altered mice was developed. When testing this vaccine in chimpanzees, the cellular immune responses to the hepatitis C virus climbed significantly within 10 weeks, and the virus in the infected animals became essentially undetectable for at least a year. In Europe, studies with chimpanzees are not performed, and research groups that are studying this virus utilize laboratories in USA and other parts of the world to perform the necessary experiments.

Other research activities

Besides these major areas of use, NHPs are still considered essential for the following research activities:

Neurosciences

Neurosciences include the investigation of the brain activity both in healthy subjects and following disease or injuries in humans and in experimental animals. The main reasons for using NHPs are the close similarities of the brain between these species and human, in terms of the overall cellular and chemical structure, and functional and cognitive abilities. This knowledge may also help to construct new replacement experimental models *in silico* and *in vitro*.

Pain is an area where the use of NHPs is still essential. Basic understanding of its mechanisms and for the development of therapies is one of the most controversial areas of research. In 2008 a workshop was held to discuss the potential and challenges of replacing these activities using human patients and volunteers to replace animals in certain areas in pain research and in the development of new therapies[3] and the result was that NHPs was still a better model, particularly for the research on neuropathic (chronic) pain.

Other major areas of neurosciences cover neural injuries and disease or disorders like epilepsy, cerebrovascular disease, depression, addiction, Alzheimer disease and other dementias, Parkinson's disease, and multiple sclerosis. It was estimated in 2004 that about 127 million European citizens (almost one third of the EU population) were affected by brain disorders. Also for that reason, brain research

received 8% of the life science budget in the European Commission's Fifth Framework Programme of research (FP5, 1998-2002), and 10% of the FP6 budget (2007-2010), a proportion that is likely to grow.

Much of our current understanding of how nerve cells function is based on studies in animals such as the cat, rat and even invertebrates such as squid, but the organisation of nerve cells in complex human brain systems cannot be understood without studies in the similarly complex primate brain. In fact, only because of recent studies in NHPs, the existence of primate-specific developmental features was discovered.

Although experiments using invasive neurophysiological recordings in NHPs raise ethical concern, the understanding of the functional organization of the brain areas involved in vision, sensation, hearing, motor control and cognition in primates and humans made significant progress from such work. An example was the research on primate visual pathways that led to a Nobel Prize in the early 1980s which, as well as elucidating visual centres and the mechanisms in NHPs, went on to discover similar pathways in humans which had not been recognized before.

Today some non-invasive research techniques (such as transcranial magnetic stimulation, TMS) can be used both in humans and NHPs, but some approaches still require that deeper areas of the brain are damaged to reveal their function. In practice, this means that TMS can only be used in humans to study the brain areas near the surface of the skull. In NHPs, deeper areas can be explored using precise permanent or reversible lesions.

Motor control is another area where the use of NHPs has been fundamental for basic understanding of the production and control of arm movements leading to brain-computer interface technologies, which are of major relevance to help alleviate the consequences of brain lesions and spinal cord injuries.

Other neurological diseases such as depression, schizophrenia, attention deficit, hyperactivity disorder, autism, drug addiction and obsessive compulsive disorders all involve malfunctioning of the highly developed primate frontal lobes and their interactions with other parts of the brain. This is also true of conditions such as head injury, Huntington's disease, stroke and some types of dementia which also involve interactions between multiple systems in the brain. Such conditions cannot, or only be partially, be reproduced in non-primate species. Studies on large macaque colonies showed that some individuals, in particular those of low social rank, express behavioural signs comparable with some human depression syndromes. In the study of depression, simple tests for anti-depressant-like activity of pharmacological substances are often based on known classes of therapeutically successful existing anti-depressant agents. Neuroimaging has shown activity deficits in NHPs, comparable with those in humans, thereby supporting their use for further neurobiological characterization and modelling.

A model of Parkinson's disease (PD) in NHPs has been invaluable in studying its pathophysiology. Deep-brain stimulation (DBS) in PD derived from experiments in a NHP model showed that destruction or high-frequency stimulation of certain areas in the brain reversed Parkinsonian symptoms. Over 40,000 patients have now been treated with DBS worldwide, and there are 160 DBS centres in Western Europe. In addition, DBS is showing promise in other brain conditions such as drug resistant cases of depression, obsessive compulsive disorder, and Tourette's syndrome.

Currently stem cell technology opens the possibility to use somatic cells of an individual to repair his own tissue, thereby removing some of the ethical issues concerning embryos and the problem of rejection. These technologies are being currently developed for the repair of brain tissue in Parkinson's, Huntington's, Alzheimer's, in stroke and in spinal cord and brain injuries but will require safety and efficacy testing in NHPs.

Research on sensory and motor systems has led to the new field of neuroprosthetics to restore the severe loss of sensory abilities or movement capabilities in paralyzed patients. The development of brain machine interfaces (BMI) has very much benefited from neurophysiological experiments in NHPs showing that it is possible to use natural brain cortical neural activity to drive computers, robots, and artificial limbs, to restore volitional control of movement to paralyzed limbs, and to compensate for perceptual deficits.

New fields and imaging techniques to assess brain structure and function, such as Magnetic Resonance Imaging (MRI, now routinely used for diagnosis in humans), functional MRI (fMRI), the development of imaging biomarkers involving positron emission tomography, are rapidly developing. However, these techniques still have severe limitations. Microelectrode techniques are more precise both anatomically and temporally. However, fMRI and microelectrode studies should not be viewed as alternatives, but rather as complementary investigative methods. Currently the improvement of molecular and cell biology has reached a point where transgenic NHP models of brain diseases are seriously envisaged. Such transgenic models will, potentially, be of much higher predictability power for human outcomes than rodent transgenic models, but will also incur additional considerable controversy.

Xenotransplantation

The shortage of organ donors for transplantation is a major societal problem and the waiting lists continue to grow due to limited organ supplies. Only a minority of patients, who may benefit from a transplant, will be able to receive one and 10-20% of patients on the waiting list for organ transplants died before donor organs become available. Novel sources of organs may help to reduce this shortage. In addition to treating the terminal failure of organs such as kidney, lung, liver and heart, transplantation could also provide a therapy for patients affected by diabetes and Parkinson's disease, which are also a significant burden on society.

Currently the pig represents the most likely candidate as a source animal for the future application of xenotransplantation and NHPs represent the only useful proof-of-concept species. The immunological incompatibility between pigs and primates is based on a specific immune response (anti-αGal), and only Old World primates (such as baboons and cynomolgus), apes and man possess anti-αGal antibodies. Hence the use of other animal species as recipients would not be subject to the first innate rejection mechanism occurring against pig organs. αGalT-KO rodents have been generated that possess an anti-αGal immune response. However, this anti-αGal response is weak, severely limiting the credibility of results observed in such recipients in experimental models of xenotransplantation.

With reference to the animal models of PD, it has to be considered that the brain pathways for the control of movement are very similar in NHPs and humans, and that damage to the part of the brain that degenerates in PD leads to virtually identical movement disorders in NHPs. The primate is therefore considered the optimal animal model of cell transplantation in experimental PD.

Alternative Models to the use of NHPs

Although in the current biomedical approach animals are only used when absolutely necessary and unavoidable (i.e. when appropriate alternatives are not available) and usually after that extensive studies using computer and *in vitro* methods have already been performed, developing alternatives to animal research is still one of the primary objectives. Nevertheless, there is always a point in the research process where barriers that computers and *in vitro* methods cannot yet help to cross.

Safety testing

In safety testing, regulatory requirements may mandate the use of NHPs since species resembling humans most closely regarding pharmacokinetics and pharmacodynamics should be used for testing and one non-rodent species is to be included. Safety testing of pharmaceuticals also has to be performed along specific guidelines often detailing number of animals/group, study duration, and number of dose levels. In such testing, 3R approaches may be enforced optimizing study protocols and animal care aspects. A total replacement of animal testing for safety is an issue difficult to tackle due to the very slow progress in the area to develop validated alternative models. Arguments against the phase-out of NHPs in safety testing of pharmaceuticals are identical with those used regarding using rodents for toxicity testing, i.e. incomplete knowledge in cell biology and pathophysiology, poor representation of pharmacokinetics by *in vitro* systems, and the absence of departure points (No-Adverse Effect Level, NOAEL or benchmark doses) for risk assessment when using *in vitro* data.

Regarding testing of biotechnology derived drugs, (i.e. highly specific antibodies), in certain cases models such as transgenic mice or homologous antibodies (if the desired target is present) in an alternative species (usually rodents) may be considered. For some antibody targets, transgenic rodents carrying the human pharmacological target may also be applied. This requires, however, that downstream signaling is relevant for humans and that the alternatives are sufficiently characterized. At present, alternative methods are usually considered as additional methods and not as replacement of NHP data by regulators.

Infectious diseases

In infectious disease research and vaccine development, there are currently no ideal small-animal models. For example, for the acute HIV-infection, pathogenesis and antiviral pharmaceutical development and evaluation in the SCID-mice models can be suitable, but immunogenicity experiments cannot be performed. A model for investigating immunogenicity is the Trimera model, where a human immune system is introduced into wild-type mice. However, the viability of the human cell transplant is short and the model can mainly be used for the investigation of short-term immunity. It is difficult to generate a transgenic mouse permissive to infection with HIV, probably since all species-specific factors needed for complete HIV replication in mice have not been identified. Recently, a mouse model (HIV/MuLV) has been established based on the infection by HIV-1 enveloped by a mouse retrovirus envelope. Since the mouse immune system is intact, studies of HIV candidate vaccines and adjuvants can be made over longer time periods as compared with other models. The HIV/MuLV-challenge system is primarily useful for screening candidate vaccines prior to NHP studies.

In parallel, many *in vitro* techniques have also come into use which gave a better understanding about fundamental reactions on cellular level in the immune system. However, these methods today can only be regarded as complementary techniques; there are no alternatives in the investigations of the complex immune reactions in the intact animal model.

Regarding HIV-research, it is possible that a genetically modified mouse strain with a human-like immunity, in which complete HIV replication can take place, may be available within 10 to 20 years. Still, one of the largest hurdles is to know how the immune response in a preclinical mouse model will actually translate into protection in a clinical situation, especially since correlates for efficient protection against HIV infection in humans are not known. It is therefore necessary to continue the HIV vaccine development in NHP in order to learn as much as possible about the immune response. However, a mouse model for HIV vaccine research will never totally replace the use of NHPs, and also from a regulatory point of view it is quite unrealistic that the Agencies may accept efficacy and safety data of the candidate vaccine in a non relevant species before starting clinical trials.

Neurosciences

In the field of neurosciences, MRI is now routinely used for diagnostics in humans. Functional MRI (fMRI) is also widely used in most fields of neuroscience. It is often considered as a possible alternative to replace research using NHP. MRI measures changes in vascular parameters and relies on a link between neural activity and vascular variations and is thus an indirect measure of neural activity. However, fMRI is limited because the knowledge on neurovascular coupling and of its underlying mechanisms has remained incomplete.[4] In addition, by nature the temporal precision of fMRI is low, measuring variations in blood oxygenation levels in the order of seconds, far from the millisecond range at which neural cells process information. Nevertheless, fMRI and other neuroimaging techniques (EEG, MEG) give large scale functional views by being able to record activity or activations from the entire brain. This is not the case with microelectrode techniques, which are much more precise anatomically and temporally. For all the above cited reasons fMRI and microelectrode studies in NHP are complementary. The very promising diffusion imaging techniques (DWI, DTI) use magnetic resonance imaging for the non invasive detection and tracing of neural fibres. However, current shortcomings are lack of anatomical precision, the inability to evaluate the direction of fibres, and the fact that algorithms interpreting signals acquired with diffusion imaging are based on several untested hypotheses.

Computer modelling is rapidly improving and is expected to reach significant importance in the domain of robotics and the development of machines based on neural knowledge. The Blue Brain project is the first comprehensive attempt to reverse-engineer the mammalian brain, in order to understand brain function and dysfunction through detailed simulations. The first phase was reached in 2007 with a complete modelling of a unique rat cortical column of 10000 neurons that required the full computational power of a supercomputer. A realistic model of a primate brain will have to contain up to 100 billion neurons. While this approach is necessary for the advancement of neuroscience, there is no foreseeable time when an artificial model of a primate brain will be feasible.

Based on the presently available science, a total replacement of NHPs in many areas of use, either by other animal species or by non-animal methods, is unlikely to be achieved in the foreseeable future. Progress in the scientific fields should be critically and periodically assessed, but cannot give a specific outlook due to the inherent difficulties in predicting complex developments in science.

In xenotransplantation, the development of artificial organs and tissue engineering may sometimes replace or reduce the need for NHPs. Developments in the field of artificial organs may also help to address the growing problem of donor organ shortage. Currently, artificial organs are mostly extra-corporeal devices, but are not an alternative to organ transplantation. Moreover, functions of organs such as the liver cannot be replicated artificially. Other active fields of research possibly reducing the need for NHPs in xenotransplanation are stem cell research and tissue engineering, but these areas are far away from integration into clinical practice.

Conclusions

Based on the above experimental and scientific evidence, the expert Working Group reached the following conclusions, which were finally adopted by the SCHER:

The use of NHPs has been shown to be essential, but not limited, to the following areas:

- Safety testing of pharmaceuticals to assess potential toxicity in animals to identify unacceptable adverse reactions in humans. For specific biotechnology derived drugs, such as antibodies, NHPs are the most relevant species because of their close similarity with humans
- To understand the pathophysiology of several infectious diseases, where the NHP is the only susceptible species, and to develop safe and

effective vaccines and therapies against these diseases
- NHPs are the only means to learn how the complex brains of primates, humans included, are structured. In addition, NHPs have proven to be the best model of some human brain conditions, have been critical to device current treatments, and therefore are needed to identify and test new therapies
- Only some NHPs show similar immuno-response and rejection mechanism to xenotransplanted tissues as in humans, therefore use of NHPs is fundamental to show tissue functioning and recipient survival.

Recommendations

After considering the reasons for still using NHPs in biomedical research, and the current possibilities to adopt alternative or improved techniques, the expert Working Group defined the following major recommendations:

1. Promoting research on new and accessible technologies for tissue friendly and MRI compatible implants as initiated by NC3Rs
2. Support for a NHP European Network, to provide European laboratories working with NHP access to the information and resources of the network laboratories in order to share expertise, tissue, transportation resources, etc. This would further optimize the 3R goals in European NHP research
3. Promoting the development of accessible databases as developed for neuroanatomical databases. In general, effort should be made to encourage data, platforms, tissues or even animal models sharing through comprehensive databases and collaborative networks
4. Since magnetic resonance imaging (MR) centres are becoming more numerous, improved access for researchers using NHPs to MR facilities may reduce the number of invasive procedures in NHPs or even replace NHP use due to the availability to study certain aspects of neural function in humans
5. Any decision about replacement of NHP use by new technologies should be subjected to a carefully scientific evaluation
6. Promoting the immediate development of biosafety level 3 facilities with housing conditions in full compliance with Appendix A, for increased animal welfare during performance of efficacy studies in vaccine development
7. Negotiation with the USA and Japan for further international harmonization on the requirements for safety testing of pharmaceuticals involving NHPs. The pharmaceutical industry should organize networks to exchange information on NHP use

Additionally, the EU Authorities were invited to carefully consider that a ban of NHP use in Europe would likely translate into moving these activities to other countries, maybe with lower animal welfare standards; whilst publishers, editors of scientific journals, and ethical review board should be urged to critically assess all research involving NHPs submitted for comment, approval, or publication with regard to study design, relevance of the results to obtain benefits for human health or significant progress in the scientific field to avoid conduct and publication of insufficiently justified or inconclusive research involving NHPs.

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Animal models of osteoporosis – important characteristics

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Abstract
As human life expectancy is increasing, age-related diseases, such as osteoporosis, are prevailing. Research into their pathogenesis, prevention and therapy necessitates adequate animal models.
A variety of animal species have been used as animal models in osteoporosis research, which include non-human primates, sheep, pigs, dogs, rabbits and rodents, the latter being the most popular. Although most of these models do not develop fragility fractures which consist the main clinical feature of osteoporosis in humans, bone quality can be evaluated and monitored non-invasively throughout the studies with imaging techniques. Studies on these models can reveal the effect of preventive or therapeutic regimens on bone density, bone geometry and mechanical strength. Rat estrogen-deficient models of postmenopausal osteoporosis, particularly the ovariectomized rat, as well as immobilisation models have been widely used and produced valuable information for clinical application. More recently, research on genetically modified mouse models of osteoporosis and inbred strains of senile osteoporosis are providing new insights into skeletal biology.
There are advantages and limitations of each animal model in its similarities to the human condition. However, detailed knowledge of their specific characteristics, appropriate age, hormonal status and bone site selection, can produce scientifically valid information for human osteoporosis management.

Keywords*:* animal models, osteoporosis, osteopenia, bone loss, ovariectomy

As human life expectancy is increasing, age-related diseases, such as osteoporosis, play a major role in human health and quality of life. Osteoporosis is a bone metabolic disease characterized by loss of bone mass and deterioration of bone microarchitecture, which leads to increased fragility and can result in spontaneous or low energy fractures. According to the definition by the World Health Organization, a person has osteoporosis when his bone mineral density (BMD) has decreased more than 2.5 standard deviations (SD) below the mean BMD of healthy young adults, and osteopenia, when his BMD is less than -1 SD but more than -2.5 SD.[1,2]

It is also called "the silent disease" (or "the silent killer" or "thief") because gradual loss of bone mass is not accompanied by any symptoms. [3-5] Its victims may lose so much bone mass that they may pass the fracture threshold unawares. Spontaneous or low energy fractures may occur during coughing, bending, or lifting light weights. A mild change of posture and small loss of height may sometimes be the only signs, resulting from compression vertebral fractures. Osteoporotic fractures usually occur in areas of bone significantly affected by bone loss, such as the hip, the forearm and the spine. Hip fractures in particular present a major threat to an individual's health, quality of life and health care costs. The impact on health is severe: mortality can reach up to 20% in the 6 months following a hip fracture. The socioeconomic impact is also enormous: Europe's total osteoporosis costs were 31.7 billion euros in 2000, and the projected costs are expected to be 76.7 billion euros in 2050.[6, 7]

Therefore, research on the prevention and therapy of osteoporosis is of major importance and a key objective of biomedical research. Clinical studies have certain limitations:

The disease appears at advanced ages and additionally many years are required for the follow-up of potential therapies. During that time, many patients may drop out of the study due to many reasons, ranging from therapy side-effects, non-compliance, or just not showing up for re-examination, causing the study to end with fewer participants and creating problems in statistical evaluation. In addition to the loss of eligible patients, many subjects from the initial recruitment may have very diverse backgrounds in relation to life-style habits which are known to have an effect on skeletal health, such as diet, exercise and

smoking; they may have other concurrent diseases for which they are receiving medication.

On the contrary, animal studies have certain advantages:

Animals' life cycle is shorter, so osteoporosis can be reproduced and studied faster. They allow follow-up of the same animals, which serve as their own controls and to the R principle of Reduction. Extensive testing of potential therapies is possible, in relation to their efficacy and safety, as well as to the collection of *ex vivo* bone quality results. Additionally, variability is minimized with the study of animals of a homogeneous background and in a controlled environment.

Appropriateness of animal species

Before commencing animal research, it is worldwide required that one should also conduct literature and bibliographic database searches for available alternatives.[8, 9] Researchers should keep in mind that early screening of potential new drugs for osteoporosis therapy can be tested *in vitro,* e.g. on osteoblast cell cultures, before the use of animal models, thereby serving the R principles of Replacement and Reduction. Examination of the potential drugs' effects in these osteoblast cultures (differentiation, development of mineralized nodules) can provide information regarding their future effects on bone *in vivo*.[10] In order to select an animal species as an appropriate model for osteoporosis research, apart from the classical requirements of the appropriateness of a model,[11, 12] the following characteristics should be evaluated:

The hormonal changes occurring during its estrus cycle and if bone loss is produced due to hormonal deficiency. It is important to verify that there are estrogen peaks during the female estrus cycle, to establish their frequency and if bone metabolism is dependent on estrogen levels. There may be cases where estrogen deficiency does not produce significant bone loss. If, however, there is loss and reversal of it with hormone replacement therapy, then it is evident that bone metabolism is hormone-susceptible. The skeleton of animals with infrequent estrus cycles, such as dogs, present resistance to natural or induced estrogen deficiency.[13] The more an animal's cycle is similar to humans, the better its modeling of the human condition will be.

The age at which it achieves peak bone mass. Protocols of induction of osteopenia before peak bone mass is reached are appropriate for the study of the influence of factors on skeletal growth. When protocols are initiated after peak bone mass is acquired, the animal models are appropriate for the study of senile or postmenopausal osteoporosis.[14, 15]

The existence of age-related bone loss. This is a prerequisite for an appropriate model of age-related or senile osteoporosis. In humans, decreased bone mass due to aging, enhanced bone fragility and increased fracture risk characterize the aging skeleton. These features may result from reduced bone formation, increased bone resorption, changes in mineral composition, accumulation of microdamage, from genetic causes and through central regulation.[16] Models of age-related bone loss may present some of the diverse aetiological factors.

The possibility of measuring bone loss. It is necessary to be able to measure experimentally produced bone loss and gain, in order to evaluate preventive or therapeutic strategies. Non-invasive measurements that are applied on humans are also used in animals, such as bone densitometry with dual-energy X-ray absorptiometry, peripheral quantitative computerized tomography and micro-computerized tomography. Additionally, invasive *ex vivo* measurements, such as bone histomorphometry, can be used. It evaluates bone architecture and fragility two-dimensionally, independently of bone mass and provides several static (e.g. number of osteoblasts, osteoclasts, trabeculae, trabecular thickness & separation) and kinetic (e.g. bone formation, mineral apposition rate) indices.[14, 15] Biochemical markers of bone turnover which are used to monitor patients in clinical practice are also used in laboratory animals. It must be noted that their changes are not indicative of site-specific bone loss, but of the entire skeleton. Species-specific bone markers have been produced, but their accuracy and availability do not make them as valuable as they are in human clinical practice.

The existence of spontaneous fractures. Few animal models present spontaneous fractures similar to humans. The study of the animal's age at which they appear is important and determines what is modeled, e.g. *osteogenesis imperfecta* of children or senile osteoporosis. In models that do not present fractures due to bone loss, *ex vivo* bone strength measurements are applied, such as three- or four-point-bending of long bones (tibia, femur), cantilever testing of the femoral head and compression testing of vertebrae.[15]

Animal species which have been used in osteoporosis research are non-human primates, dogs, rats, mice, rabbits, guinea pigs, pigs and sheep. The requirements of drug testing are usually to evaluate potential drugs on one rodent and one large animal model. This article will concentrate on the first four species.

Non-human primates (NHP) are phylogenetically closest to humans and have similar endocrine and digestive systems, as well as bone metabolism. They present monthly estrus cycles similarly to humans and

have demonstrated a similar response to estrogen deficiency that results from either ovariectomy or the administration of gonadotropin-releasing hormone agonists.[13, 17] They acquire peak bone mass around the age of 9-11 years, oligomenorrhea begins in their early twenties and menopause is established in the late twenties to 30 years of age.[13] A major drawback in their use includes their long life-span. Their delayed onset of age-related osteopenia, as well as the slow bone loss after ovariectomy – which becomes statistically significant at 3 months after surgery and reaches steady state at 9 months – also adds to the difficulties associated with their use. Some studies on NHP have focused on loss of cancellous bone (also called trabecular or spongy bone: porous bone tissue with a network of trabeculae, found at the end of long bones proximal to joints, and in vertebral bodies). However, NHP consist the large animal model of choice for the study of remodeling (the process of bone resorption followed by new bone formation) of both cancellous and Haversian (compact) bone, in which they are comparable to humans.[18] Some studies have been conducted on skeletically immature NHP (4-7 years old) before they have reached peak bone mass, which should be avoided. Personnel involved need special training and attention given to potential transmittable zoonotic diseases. There are legal and ethical issues concerning their use. The expenses involved in their long-term housing and use are to be seriously considered as well.

Dogs present two estrus cycles per year. Because their estrogen levels are usually low, rising only twice per year, their bone metabolism does not appear to depend on these changes.[13, 18] They are unsuitable as models for estrogen deficiency / post-menopausal osteoporosis, because studies show inconsistent findings and poor reproducibility regarding bone mass and mechanical properties changes. On the other hand, dogs present both cancellous and Haversian bone remodeling, similar – although more rapid – to humans, and they are suitable models for the study of osteoporosis due to disuse or immobilization.[13, 15] Their relatively long life-span, ethical issues concerning their use and the expenses involved in their long-term housing are also to be seriously considered.

Rats present several advantages over the larger animal species in osteoporosis research: they have a short life-span and osteopenia is rapidly induced after ovariectomy. It was the prevailing animal model used in osteoporosis studies during the years 1966-1998 and its skeleton has been well characterized. [15] They are readily available, relatively inexpensive to acquire and maintain, and their use is accepted by society. They present estrus cycles every 5 days, with estrogen levels peaking for 18 hours every 4 days.[18] Although rats are sexually mature at 2.5 months of age, they are considered skeletally mature at 9-10 months of age when peak bone mass is acquired. [14, 18, 19] If ovariectomy is performed before this age, then cancellous bone loss is observed, due to altered bone growth. This skeletally immature model is appropriate for the study of endocrine, nutritional and environmental factors on peak bone mass.[14, 15] When female rats are ovariectomized after 9 months of age, both cancellous and endocortical (compact bone adjacent to bone marrow) bone loss is observed, due to altered bone remodeling. This skeletally mature model is appropriate for the study of postmenopausal osteoporosis.[14, 15, 19] Rats present site- and age-specific epiphyseal closure times, as well as site- and age-specific bone loss following ovariectomy, which are reliably reproducible. The proximal tibial metaphysis is a site where statistically significant bone loss occurs 14 days after ovariectomy.[19] The mature rat is the small animal model of choice for osteoporosis research, as it closely mimics the human condition, except for its minimal Haversian remodeling which is the main cause of human cortical porosity. The many available protocols to induce osteoporosis (hormonal and dietary interventions, immobilization methods) and the successful application of human routine methods to evaluate bone loss and gain, continue to support its wide use.[14]

Mice models for osteoporosis research are steadily increasing in the last decades, especially with the increase of genetically modified mice and the emergence of the molecular understanding of bone biology. Some of these mouse models present spontaneous fractures, which characterize the human disease. Mice also present estrus cycles every 5 days. Apart from the classical method of the ovariectomy-induced osteoporosis model through estrogen deficiency, osteopenia can be induced with intracerebroventricular infusion of leptin, demonstrating the existence of a central regulation of bone mass by decreasing osteoblastic bone formation.[16] The glucocorticoid-treated mouse model presents many similarities to the human condition, such as an early increase in bone resorption, decreased bone mineral density, increased osteoblastic and osteocytic apoptosis.[19] The senescence accelerated mouse inbred strain SAM/P6 is an excellent senile osteoporosis model, achieving a low peak bone mass and developing age-related osteoporosis with spontaneous fractures. It has been considered that the reduction of osteoblastogenesis observed is due to a change of differentiation of osteoblast progenitors towards adipocytes.[19] Of the many genetically modified mice used, multiple early-onset fractures occur in the osteoprotegerin knockout mice, which present a severe osteoporotic phenotype with an increase of osteoclast number and bone resorption.[16] Spontaneous rib

fractures are observed at the costovertebral junction at six months of age in the metalloproteinase *Zmpste24* knockout mice.[20] *Brtl* is a knock-in mouse model presenting spontaneous fractures during the first few weeks of life, having phenotypic features of human *Osteogenesis imperfecta*, small sized, with low bone mineral density, and bones with weak geometry and brittle failure properties.[21] There are many more genetically modified mouse models with perturbed bone metabolism.

Conclusion

Existing animal models of osteoporosis have already greatly contributed to the current knowledge of the multifactorial aetiology of osteoporosis, as well as to the safety and efficacy of preventive and therapeutic regimens. They will certainly continue to provide more information, together with emerging models, especially in the identification of the genes involved in bone metabolism. Thorough examination of their characteristics and careful selection of appropriate species, age and experimental design can yield valid experimental results.

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Dynamics of neuroinflammation in the macrosphere model of focal cerebral ischemia: an approximation to human stroke patterns

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Abstract

Neuroinflammation (activation of resident glia cell populations, recruitment of blood-derived leucocytes, humoral responses) evolves as a multi-facetted response to focal cerebral ischemia. We previously assessed phagocyte accumulation, a surrogate marker of neuroinflammation, in human stroke by USPIO-MRI. We hypothesize that phagocyte accumulation in the macrosphere model, which mimics arterio-arterial embolism of arteriosclerotic plaque material, the leading etiology of human stroke, may resemble temporal and spatial patterns observed in human stroke. In 15 Wistar rats, middle cerebral artery occlusion was induced by macrosphere model. Key features of post-ischemic neuroinflammation by the means immunocytochemistry of glial activation/invasion of macrophages were assessed at different time-points (24h, 3d, 7d). On day 7, [^{11}C]PK11195-PET-imaging was performed.

In the boundary zone of the infarct, a transition of ramified microglia into ameboid phagocytic microglia was accompanied by an up-regulation of MHC class II on the cells after 3 days. By day 7, a hypercellular infiltrate consisting of activated microglia and phagocytic cells formed a thick rim around the ischemic infarct core, which could also be shown by [^{11}C]PK11195-PET-imaging. The macrosphere model closely resembles the characteristical dynamics of post-ischemic inflammation previously observed in human stroke. We therefore suggest that the macrosphere model is highly appropriate for studying the pathophysiology of stroke in a translational approach from rodent to human.

Keywords: *activated microglia, cerebral ischemia, macrosphere model, neuroinflammation*

Postischemic neuroinflammation is characterized as a multi-facetted response to focal cerebral ischemia involving activation of resident glia and recruitment of blood-derived leucocytes as well as cascades of humoral responses.

Translating rodent research into the situation of human stroke, substantial progress has been made in visualizing aspects of post-ischemic inflammation in man. Post-ischemic inflammation has been repeatedly characterized by [^{11}C]PK11195- Positron Emission Tomography (PET), as well as by Magnetic resonance imaging (MRI) with cell specific contrast agents.[1-4] In contrast to the findings in humans, the spatial and temporal dynamics of the neuroinflammation are much faster in the classical transient middle cerebral artery occlusion (tMCAo) model and that makes it difficult to interpret the results and to translate them into the human situation.[5,6] Accordingly, we searched for an experimental stroke model with particular regard to the dynamics of postischemic inflammation that resembles more closely the human situation.

In the rat "macrosphere model", the intra-arterial injection of defined number of TiO_2 spheres into the middle cerebral artery (MCA) leads to permanent occlusion of the MCA, resulting in large focal ischemia.[7-10] This technique mimics arterio-arterial embolism of "hard" atherosclerotic plaque material as the most frequent cause of human stroke.[9] Moreover, in contrast to the well-established suture model of permanent ischemia, the macrosphere model avoids hypothalamic injury and subsequent hyperthermia, which may be a confounding factor in therapeutic

studies and may influence CNS inflammatory responses as well.[7-10]

We hypothesize that the key features and dynamics of postischemic inflammation in the macrosphere model may resemble temporal and spatial patterns observed in human stroke.

Materials and Methods

Animals

All animal procedures were in accordance with the German Laws for Animal Protection and were approved by the local animal care committee and local governmental authorities. For the studies, male Wistar Unilever rats (n=15) weighing 270-340g were obtained from Harlan Winkelmann GmbH (Borchen, Germany).

For MCAo and PET-imaging, the rats were anaesthetized with 5% isoflurane and maintained with 2.5% isoflurane in 65%/35% nitrous oxide/oxygen. Before starting the surgery, the animals received a subcutaneous injection of carprofen (5mg/kg). Throughout the surgical procedure and PET-imaging, body temperature was maintained at 37.0°C. Ischemia was produced by intra-arterial injection of 4 TiO_2 spheres (Ø 0.315-0.355 mm; BRACE, Alzenau, Germany). After exposure of the left common carotid artery (CCA), internal carotid artery (ICA) and external carotid artery (ECA), the ECA and the pterygopalatine branch of the ICA were ligated. PE-50 tubing was filled with saline and four TiO_2 macrospheres (Ø 0.315-0.355 mm; BRACE, Alzenau, Germany). The macrospheres were advanced via ICA into the MCA by a slow injection of approximately 0.2 ml saline. After macrosphere injection, the catheter was removed, the wound was closed by a suture, and xylocaine-gel was applied.

Following surgery, the animals were transferred to their cages after they had fully recovered from anesthesia. During the experiment, all animals were carefully observed and received intensified care, including subcutaneous injections of carprofen (5mg/kg/12 hours) and saline (5ml 0.9%NaCl/day), as well as local application of xylocaine-gel once a day. For a high calorie diet, animals were fed by baby pap enriched with glucose and cream cheese. Animals were assigned to 2 main groups with respect to different methods for the detection of neuroinflammation.

Histology and Immunocytochemistry

Groups of n=3 animals each were allowed to survive for 24 hours, 3 days, and 7 days after MCA embolisation with TiO_2 spheres before they were decapitated under deep anaesthesia with isoflurane. The brains were rapidly removed, frozen in 2-methylbutane, and stored at -80°C prior to further histological and immunocytochemical processing. Adjacent serial coronal brain sections were cut at 500 µm intervals (slice thickness 10µm) and stained with H&E for the detection of the infarct. To identify parenchymal (resting/activated) microglia cells, a monoclonal antibody against the complement receptor 3/CD11b (clone OX42, dilution 1:1000, AbD Serotec, Oxford, UK, cat-# MCA275R), which is also expressed on macrophages, was used.[15] During activation, microglia cells express the major histocompatibility complex (MHC) type II molecules, which was assessed by staining for MHC class II (clone Ox6, dilution 1:400, AbD Serotec, Oxford, UK, cat-#MCA46G).[11] Activation of microglia cells leads to a complete loss of cellular processes and rounding of cell body typical for phagocytes, which can be identified with

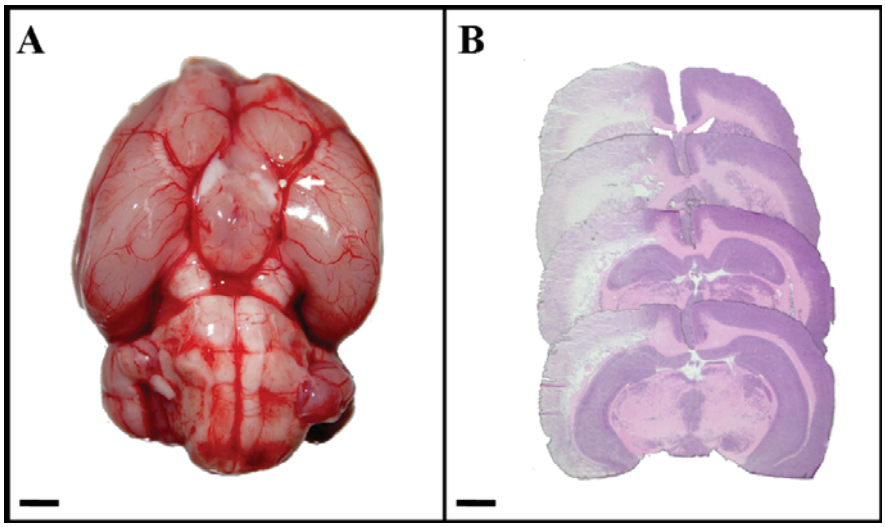

Figure 1. *Characterization of the macrosphere stroke model*
(A) Basal view of the rat brain displaying the cerebral arteries following macrosphere-injection into the MCA. One macrosphere is located in the origin of the MCA and the distal ICA (white arrow). Bar: 5000µm. (B) Representative H&E staining of coronal brain sections demonstrating the extent of the ischemic lesion 7 days after ischemia induction. Objective 1x; bar: 5000µm.

the phagolysosomal marker ED1 (clone ED1, dilution 1:1000, AbD Serotec, Oxford, UK, cat-# MCA341).[11,12] For visualization, the ABC Elite kit (Vector Laboratories, Burlingame, CA, USA) with diaminobenzidine (Sigma, Munich, Germany) as the final reaction product was used.

Imaging procedures

In 6 animals, seven days after induction of ischemia, T2-MRI (4.7T)- and µPET (Focus 220 scanner) -imaging were performed. PET-imaging was performed on a microPET Focus 220 scanner (Concorde Microsystems, Inc., Knoxville, TN; 63 image planes; 1.5-mm full width at the half maximum). During the experiment, anaesthesia was maintained with 2% isoflurane in 65%/35% nitrous oxide/oxygen. Body temperature and respiration rate were monitored by DASYLab 9.0 (DasyLab, Moenchengladbach, Germany). Temperature was maintained at 37°C using a thermostatically controlled water flow system (medres, Cologne, Germany). For PET-imaging, rats received an intravenous bolus injection of [^{11}C]PK11195 (1.1 - 2.5 mCi/rat) via an intravenous line in the tail vein , and emission data were acquired for 30min. [^{11}C] PK11195 is a PET radioligand used to image peripheral benzodiazepine receptors (PBR), which are located on the mitochondrial membrane of the microglia. PBR has a low level of expression in resident glial cells, while its expression steeply increased after activation of those glial cells.

Results

In all animals, hemiparesis could be observed, specifically a lack of extension of the right forelimb. Besides, the animals showed no relevant reduction in their behaviour. In the post mortal inspection of the basal cerebral arteries, one or more macrospheres directly blocked the proximal MCA in all animals (Fig. 1A).

Histology/Immunocytochemistry

H&E staining verified the infarct localization in the MCA territory of all animals (Figure 1B).

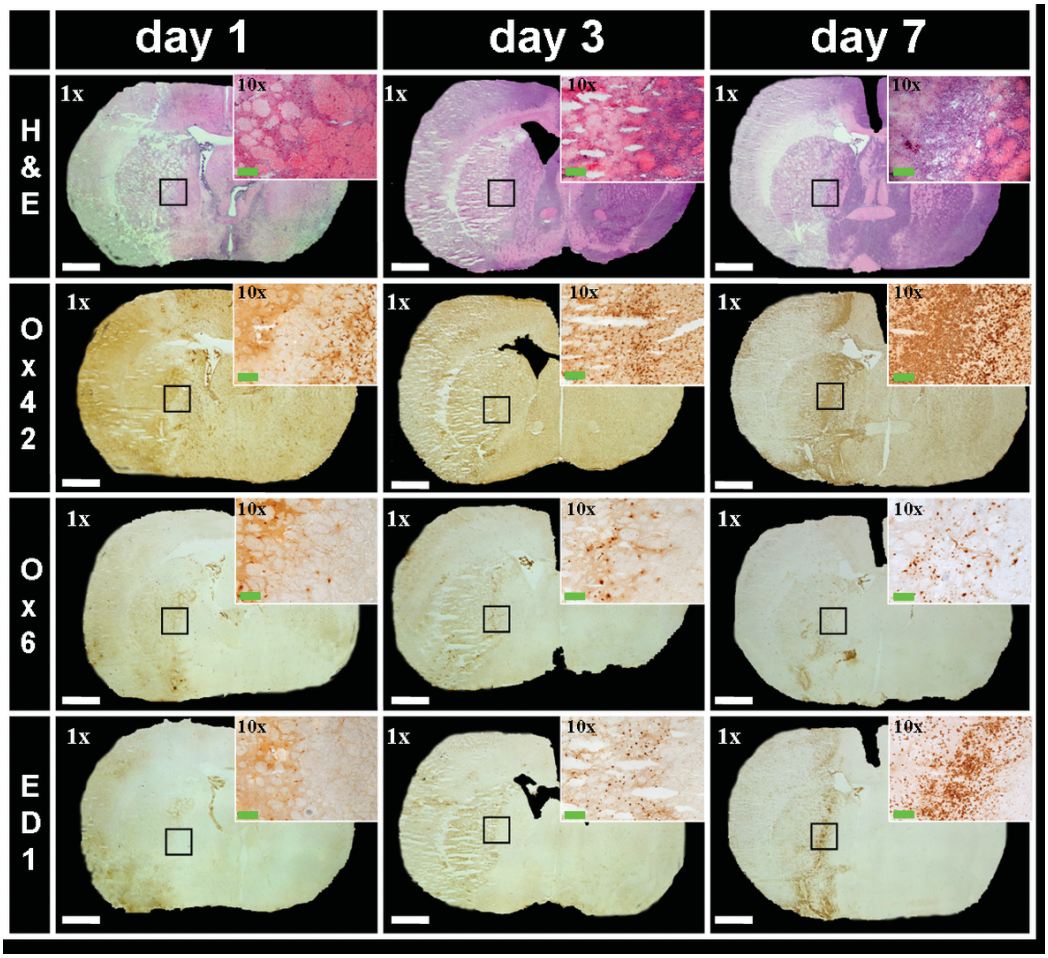

Figure 2. *Infarct demarcation and cellular inflammatory response*
One day after induction of stroke, the infarction is already well demarcated from vital tissue. H&E staining showed eosinophilic coagulation necrosis in the ischemic territory. Around day 7, phagocytic cells containing transformed microglia and hematogeneous macrophages (identified by ED1) developed. Objective: 1x; white bar: 2000µm, green bar: 100µm.

The infarct was increasingly demarcated over time as revealed by H&E and Ox42-staining (Fig. 2). By day 3, marophages/microglia cells (Ox42) started to form a hypercellular rim around the infarct core, exhibiting signs of activation by MHC class II and ED1-staining (Fig. 2). Stellate and amoeboid microglia morphology indicated the transformation of those cells to phagocytes. At day 7, a dense cellular wall of activated and resident microglial cells/macrophages surrounded the ischemic lesion and eosinophilic coagulation necrosis could be observed (Fig. 2). The area of ED1-positive cells (54mm^3±26) extended over 9 to 20% of the affected hemisphere at day 7.

Imaging procedures

[^{11}C]PK11195 was used to image peripheral benzodiazepine receptors (PBR), which are located on the mitochondrial membrane of the microglia. PBR has a low level of expression in resident glial cells, while its expression is dramatically enhanced after activation of those cells. Here, increased [^{11}C]PK11195 binding was mainly observed in the margin of the infarct and only less in the infarct core indicating that the infiltration by microglial cells into infarcted area has not started yet (Fig. 3).

Discussion

In the macrosphere model, the intra-arterial injection of 4 TiO_2 spheres leads to permanent occlusion of the MCA resulting in an ischemic lesion of the MCA-territory. In this study, the extent of ischemic damage obtained by macrosphere embolization corresponded to previous experiments and was comparable to other pMCAo models. In contrast, our pMCAo model was different from the previously described photochemically induced focal ischemia model that produces only a pure neocortical infarction.[13-15]

With respect to the cellular inflammatory response, we observed a transition of ramified into ameboid phagocytic microglia in the boundary zone of the infarct, with consecutive infarct demarcation as late as three days after the induction of ischemia, corresponding to the situation after photothrombosis[13,15] as well as to that after human stroke.[1] Stoll et al. observed the accumulation of phagocytes in the border zone of human infarcts after 5 days.[16] In the ischemic core, morphological signs of macrophages/microglia activation could be detected by day 7, but not at day 1, similar to the photothrombosis model.[13,15] Investigating human stroke, PET studies make use of the peripheral benzodiazepine ligand [^{11}C]PK11195 as a surrogate marker for inflammation to investigate the temporo-spatial profile of microglia-activation and macrophage-invasion.[1-3] Likewise, MRI studies employed ultrasmall supermagnetic iron oxide (USPIO) to study the invasion of blood-borne macrophages into human brain.[4] Human macrophage-invasion/microglia activation started as late as 3 days after onset of ischemia and reached its maximum within one week.[1-3,16] Interestingly, in the macrosphere model activated microglia and migrated macrophages established a particularly dense cellular wall around the infarct core after 7 days.[7] We choose the time-point of maximum neuroinflammation in human stroke, which is 7 days after stroke onset, to investigate

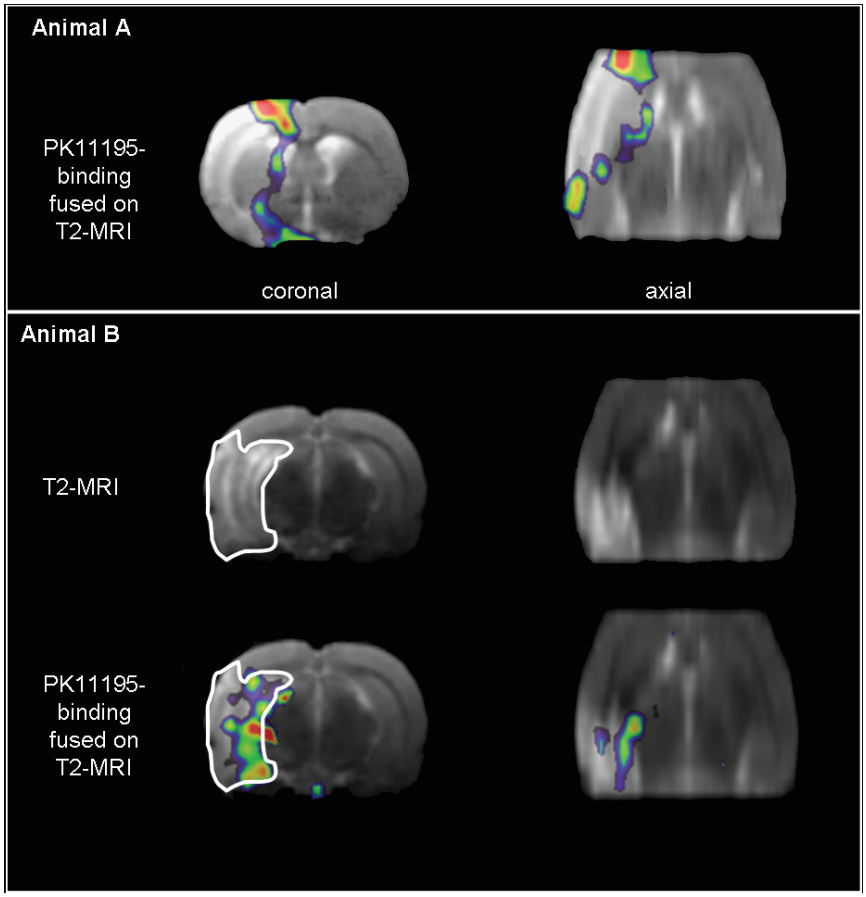

Figure 3. *microglia activation in [^{11}C] PK11195-PET*
[^{11}C]PK11195-PET (colored) is fused on T2-image 7 days after MCAo. In animal A increased [^{11}C]PK11195 binding can only be observed in the margin of the infarct, whereas in animal B the sophisticated infiltration of the infarcted area is visible (upper image: T2-image; lower image: [^{11}C]PK11195-PET (colored) fused on T2-image).

Table 1. Comparison of the inflammatory response in macrosphere model, human stroke and tMCAo

	Macrosphere model	Human stroke	tMCAo
Activation of microglia (maximum)	day 3 (day 7)	day 3 (day 7)[37]	6h (day 3)[17]
Infiltration of macrophages/ microglia in the infarct core	day 7	day 6- day 8[14]	6h[17]
Accumulation of phagozytes	day 7	day 5-8[4]	day 3[17]

the microglia activation in rats. [^{11}C]PK11195-PET could demonstrate that activated microglia started infiltrating the infarcted area at day 7 as seen by immunocytochemistry and in human imaging studies.

By comparing macrosphere model, human stroke and tMCAo, key features of post-ischemic neuroinflammation, e.g. microglia activation, macrophages infiltration throughout the infarct and phagocytic accumulation, showed a similar temporal appearance in the macrosphere model in rats and human stroke, whereas tMCAo in rats leads to a rapid development of inflammation (Table1).

To conclude, the macrosphere model as a model of focal cerebral ischemia resembles closely the dynamics of human postischemic inflammation, particularly imitating the slow time course of human neuroinflammation. Therefore, we suggest that the macrosphere model is superior in reflecting the clinical situation of human stroke. Accordingly, the rodent macrosphere model is regarded most relevant for studying the pathophysiology of stroke.

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Non-invasive imaging technologies: a refinement for musculoskeletal preclinical testing

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Abstract:

The implementation of imaging technologies has dramatically increased the efficiency of preclinical studies, enabling a powerful, non-invasive and clinically translatable way for monitoring disease progressions in real time. They have become increasingly valuable for studying the pathogenesis of bone and joint disorders in animal models and the development of effective therapies. Technical improvements in preclinical systems have provided detailed in vivo anatomical and functional data leading to a more efficient approach for characterizing skeletal tissues. The assessment of the animal models mostly relies on the morphological analysis, which is time consuming and a costly process requiring large number of subjects to be tested through different stages of the disease. The development of non-invasive imaging in preclinical testing has represented a keystone on the refinement and reduction of animal models. The purpose of this review is to describe the imaging modalities generally used for musculoskeletal models, including Magnetic Resonance, Computed Tomography and Positron Emission Tomography as well as Single Photon Emission Computed Tomography. We will emphasize the applications of the different imaging modalities, their technical limitations and identify future challenges. CT provides excellent spatial resolution for visualizing mineralised tissues and it is extensively used in bone disorders. MRI lacks the high resolution of CT, but it remains the modality of choice for assessment of soft tissue structures, although to get a good isotropic resolution long acquisition time is required which is a major drawback as it needs to be performed with the animals under long anesthesia. Nuclear medicine arises as a very promising tool for investigating functional and molecular processes *in vivo* with new tracers becoming available as biomarkers. As a result, the combination of several imaging modalities is often used as a strategy to compensate for the technical limits of each separate imaging technique. Moreover, this approach provides complex data including anatomical, functional and structural information. We thus believe that pre-clinical imaging offers a powerful method for assessing progression of musculoskeletal disease longitudinally.

Keywords: imaging, animal models, bone, cartilage, micro-CT

Introduction: relevance of preclinical imaging in drug discovery

Musculoskeletal disorders impose an enormous social and economic burden on society, and with the increasing population, there is a rising demand for developing effective therapies to overcome functional deficits in patients[1]. Current treatments for bone and joint disabilities arising from trauma, metabolic disorders, arthritic lesions or skeletal cancer often fail to provide more than limited functional recovery in patients and, therefore, there is an urgency to develop new drugs to improve such conditions. The process of discovery and bringing a drug to the market is a long (10 to 12 years) and expensive undertaking, including the identification and validation of a drug targets through the selection of high-throughput screening and profiling in relevant disease models. Shortening this process is critical and can be achieved by improving the characterization of compounds and their effects through more efficacious preclinical testing and by providing earlier and more highly predictive data.

In recent years, new technologies are being developed for imaging small animals and many pharmaceutical companies and research institutions are implementing imaging in their in R&D programs. Preclinical imaging is becoming an important key technology in preclinical testing, enabling a powerful method for monitoring disease progression and testing drug candidates[2]. Its non-invasive approach has represented an important milestone in the refinement of the use of animal models for musculoskeletal studies. For many years, a large majority of the animal studies for bone and joint disorders relied on the postmortem morphological analysis of the tissues, with limitations for studying disease progression in a single animal. Such studies required the use of large number of animals to be analyzed at different time points, leading to an increase in research costs, time of analysis and larger variability between specimens. Undoubtedly, the implementation of non-invasive imaging tools has greatly improved the efficacy of these preclinical studies.

The use of imaging technologies in the clinics has attracted great scientific interest, providing incentives for the development of new preclinical and clinical systems to visualize specific biological processes *in vivo*. A variety of dedicated devices have been developed in this field, including computed tomography (CT), magnetic resonance imaging (MRI), positron emission tomography (PET) and single photon emission computed tomography (SPECT). Each modality has its particular applications, providing excellent tools for assessing anatomical and functional parameters down to the molecular level in animal models of disease. Imaging protocols have been developed to enable good spatial resolution for the anatomical/structural characterization of the targeted tissue and its physiological and functional properties, including blood flow, tissue permeability, metabolism and cellular oxygenation and proliferation. Increasingly, preclinical labs are combining the use of different modalities to allow for a greater efficiency of predictive data generation, complementing multiple physiological and functional parameters or other multiple endpoints from a single animal study. This optimizes greatly the power of such animal studies and the efficacy of experimental readouts.

The objective of this review is to familiarise the reader with the different non-invasive imaging technologies used for assessing musculoskeletal disorders in animal models. We will initially describe the technologies available, mostly focussing on CT; secondly we will review their current application within the musculoskeletal research field outlining their advantages and disadvantages; and finally we will address its implications on the refinement for the use of musculoskeletal animal models and its current challenges and future developments.

Technical aspects of the preclinical imaging modalities

Micro-Computed Tomography (micro-CT): The development of computerized tomography imaging equipment for small animals (micro-CT) has revolutionized the use of animal models in musculoskeletal research[3]. Studies using micro-CT have rapidly advanced providing high resolution and a reasonably fast reconstruction and assessment protocols which overall have led to increased interest from dedicated researchers working on preclinical CT imaging[4,5].

Micro-CT is an x-ray-based imaging modality with a scope for high resolution (advanced systems can go down to an isotopic voxel size of 5-10 µm) in preclinical settings[6]. There are mainly two different µCT systems; one type in which the examined object is placed in the centre and the x-ray detector and radiation source is mounted in a gantry that rotates around it. This set up is the one mostly used in *in vivo* animal scanners. In the second type of CT scanner, the object is rotated within the course of the x-ray beam and the setup allows the free positioning of the sample between the detector and the source, allowing the adjustment of the magnification level. This second configuration is mostly applied in *ex vivo* and custom built systems. There are also differences on the beam geometry of the x-ray source used. Images can be acquired by using either a fan-shape beam in which data is acquired through dynamic acquisition plane by plane or cone beam, which is also called "volume-CT"[7] in which the scanned subject is captured completely (based on the axial extent of the CT field of view) in one rotation, speeding up the imaging process. Different µCT systems set up and beam shapes are illustrated in Fig. 1.

Initially, micro-CT systems were developed for *in vitro* studies, in the form of bench-top systems[8], but further developments have led to the implementation of *in vivo* acquisition systems, allowing the visualisation of the whole body of live animals while maintained under anaesthesia. There are several preclinical micro-CT systems currently available on the market, providing different designs and customized applications. There is an increasing interest in developing multimodality imaging platforms which mostly provides the CT integrated with other modalities such as PET and/or SPECT imaging (Fig.2).

Overall, micro-CT is well suited for anatomical imaging of the skeletal tissue, providing high temporal and spatial resolution (5-10 µm) and reasonably fast acquisition with the limiting factor being the animal exposure to radiation.

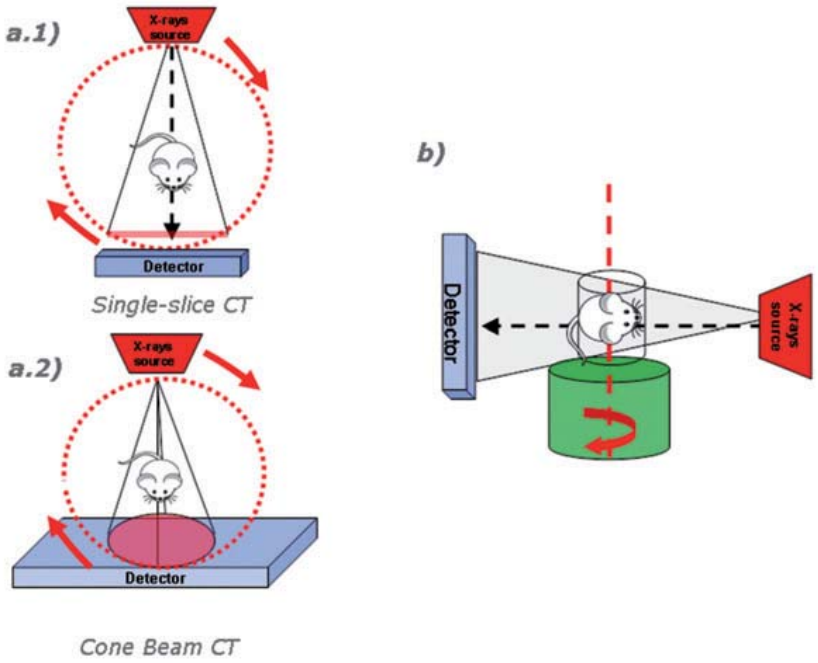


Figure 1. Diagrams showing the different micro-CT designs for imaging animal models. In the systems a) the animal is placed in the centre of the setup and the gantry carrying the detector and the x-rays source is rotated around it. This is the common setup used in *in vivo* preclinical imaging. A.1) in the cone beam CT system (c.1) the angle of the imaging beam (collimator) is increased so that the beam forms a cone. The axial length of this beam is also increased, and the detector used is a flat panel. The advantage given by the cone beam CT image is that the recorded data can be reconstructed to give a 3D volumetric image, compared with the single slice of the conventional CT scan (c.2).
In the system b), the specimen is placed on a stand that rotates within its own axis in the course of the beam.

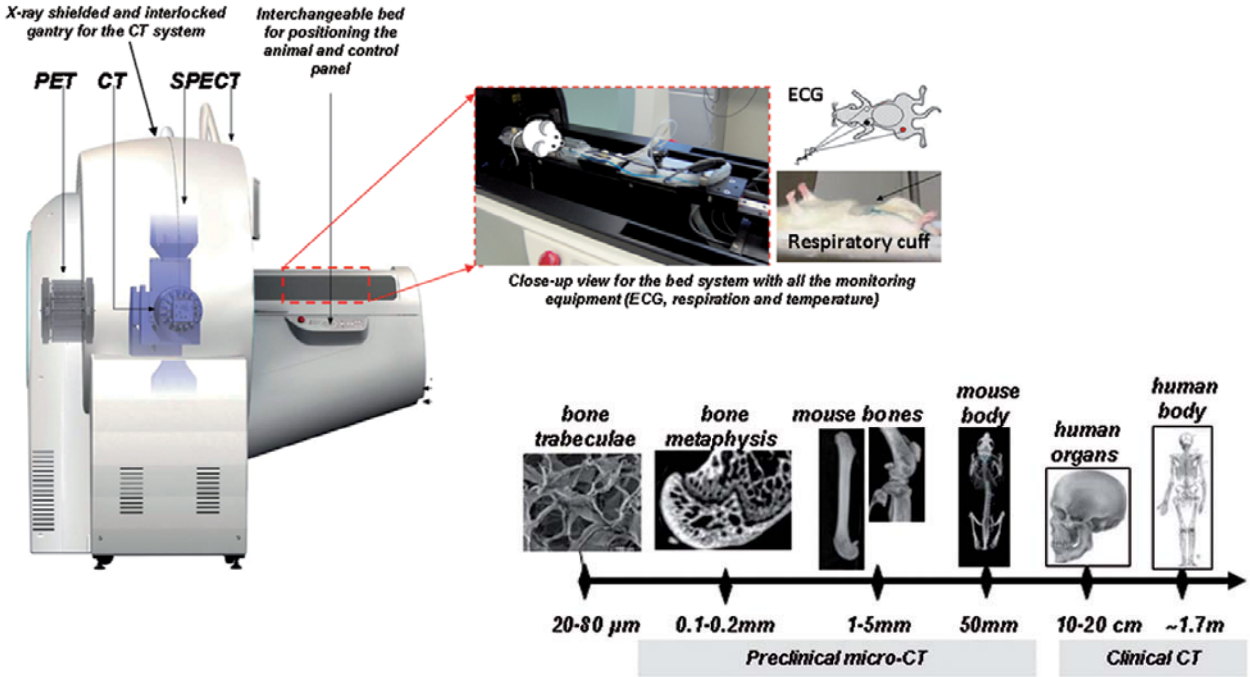


Figure 2. Images showing the multimodality PET/SPECT/CT scanner (Inveon™, Siemens Medical, USA). The animal is placed in an interchangeable bed with an integrated system for monitoring of physiological parameters (respiration, heart function/ECG & temperature) and can then be imaged through the different system by adapting the bed position within the PET and the CT/SPECT gantry. The images show the characteristic respiratory cuff and the ECG electrodes used for rodent preclinical imaging. This versatile system allows unified PET, SPECT and CT data acquisition. The graphic highlights the resolution and bone structures that can be imaged through the preclinical micro-CT system in comparison with the clinical CT's.

Magnetic Resonance Imaging (MRI): MRI remains one of the main imaging applications of choice for assessment of soft tissue structures like tendons, cartilage, menisci, and ligaments in the clinical setting (9) and has also being successfully tested in animal models (mice: ref. 10; rats: ref. 11; goat: ref. 12. rabbit: ref. 13). Magnetic resonance techniques do not use gamma or x-rays, which is an advantage over other methods that heavily depend on ionizing radiation such as CT, SPECT and PET limiting the scan repetition to avoid over-exposition to radiation.

MRI technology uses the magnetic properties of atoms and molecules of tissues to be imaged and their interactions with both a large external magnetic field and radio waves. The proton ^{1}H is the nucleus mostly used for anatomical imaging because of its abundance in tissues (water in the body). MRI technology uses a powerful magnetic field (non ionizing radiation) to align the nuclear magnetization of these hydrogen atoms and then radiofrequency pulses (through RF coils) are used to systematically alter this alignment, causing the hydrogen nuclei to produce a rotating magnetic field that is detectable by the scanner. This signal is then manipulated by additional magnetic fields produced by gradient coils to encode the signal in space dimension[14].

One of the most critical aspects of preclinical MRI is the radiofrequency coil which drives both the signal excitation and reception. Because the coil's sensitivity increases as its volume decreases, animal coils are smaller that humans, improving significantly their signal gains, while still covering the anatomical area of interest. However, the acquisition of a high spatial resolution MR-imaging within an acceptable time remains a challenge. Due to the relatively small size of anatomical structures in rodents, it needs to be performed with the animal under general anaesthesia[15].

MRI provides excellent soft-tissue contrast with broad applicability for anatomical and functional imaging. However, their resolution (50-100 μm) and poor sensitivity for mineralised tissues remains challenging for imaging bone structures in small laboratory rodents, requiring long time acquisition and thus long exposure to anaesthesia.

Micro-SPECT and Micro-PET: Nuclear Imaging technologies

Bone scintigraphy is extensively used in diagnostic orthopaedics to investigate bone lesions. Further innovations, such as single-photon emission computed tomography (SPECT) and positron emission tomography (PET), have allowed the acquisition of medium-resolution, whole-body images of the entire skeleton, with unmatched sensitivity for lesion detection. Importantly, it also provides a 3D localization of the radiation emitted by radionuclide imaging agents or biomarkers. They can provide very high detection sensitivity that can reach nano or pico-molar concentration. These methods are capable of providing good functional information at the molecular level through the onset and progression of a given biological process and /or disease, assessing the relevance of tested drug candidates and monitoring their therapeutic effectiveness[16]. The increasing use of clinical nuclear imaging applications has led to the development of SPECT and PET scanners dedicated for small-animal imaging[17, 18]. To allow for the translation from the animal system to the clinic, these systems have to cope with the small size of rodents; hence they have to achieve enhanced spatial resolution and at the same time high sensitivity for the targeted biomarker.

SPECT systems record gamma-rays directly after radionuclide emission. The system uses a gamma camera to acquire projections data that are detected using a parallel hole collimators. Most of the preclinical SPECT scanners are equipped with multi pinhole collimators to acquire high spatial resolution and detection sensitivity for foci of gamma-emitting tracers within the subject volume. Then a tomography reconstruction of the data acquired is applied, yielding a 3D dataset that can then be manipulated to show any particular axis of the body[19].

PET systems also detect gamma rays but emitted by a biomarker tracer labeled to a positron emitter (e.g. F-18, C-11). These radio-nuclides emit positrons, which causes two gamma photons to be emitted by annihilation in opposite directions and then the scanner detects these dual emissions "coincident in time" providing a higher radiation on a given location and thus higher resolution images (on the range of 1-2mm). However, some state-of-the-art dedicated preclinical SPECT scanners can provide similar resolution capabilities (and even better down to the sub millimeter range)[17]. SPECT radiopharmaceuticals are widely applied in daily practice of clinical nuclear medicine and are significantly less expensive than PET scans. Nevertheless, PET is well suited for small animal imaging and the tracer ^{18}F-FDG, an analogue of glucose, is extensively used as a biomarker of tissue metabolic activity and inflammation. New PET tracers are becoming available as biomarkers for preclinical testing for pharmaceutical and metabolic studies.

PET and SPECT imaging technologies are becoming an increasing part of drug discovery thanks to their high specificity, sensitivity (ρ-nmol) and the ability to quantify the tissue concentration of a given tracer. Their major drawbacks are largely due to the lack of anatomical information and the use of radioactive compounds with limited half-life.

Applications for imaging musculoskeletal preclinical disorders

For the purpose of this review, we will exemplify the application of preclinical imaging modalities to preclinical models of osteoarthritis (OA). This complex degenerative pathology remains as one of the most prevalent musculoskeletal disorders. We will briefly discuss the challenges associated with developing preclinical models for studying the pathophysiology of OA and how imaging technologies can be applied for assessing the changes in the bone and/or cartilage in the joints of these disease models.

Osteoarthritis (OA) is a degenerative condition affecting the cartilage and the underlying subchondral bone in joints[20]. It is the most common form of joint disease in humans (8.5 million people in the UK;[21]), leading to important disabilities to affected individuals and gross economic loss. Despite the large number of individuals affected, no therapies are available to reverse the disease process. There is a driving demand for developing new OA treatments and animal models are critically important for such development. Models of OA have been widely used in large animals (e.g. dog, sheep, goat[22, 23]) and in rodents[23,24], but it remains difficult to identify the model that closely resembles human OA and therefore can better predict translation into humans. Animal models need to balance the demands for rapid screening, decreased heterogeneity, dependance of genetic variation and similar pathogenesis to human disease. Laboratory rodents are preferred as they are more cost-effective models for screening, their genetic background is well described, and they have well regulated handling and housing requirements, allowing better controlled and reproducible *in vivo* studies.

The surgical instability models, in which the biomechanics of the joint is altered, are extensively used for OA studies, as they provide the advantage of inducing a more controlled onset of the disease and higher consistency of disease between animals. Although the severity of disease tends to be highly dependent on genetic background, OA can be induced in any background strain. With many recent studies focussing on the knee joint, the surgical destabilisation of the medial meniscus model (DMM[25,26]) remains one of the most favoured OA models in mice, as it provides extremely good reproducibility and importantly ensures a slower progression of disease.

Because of the difficulties in accurately modelling human features, such as gait movement or pain, and other clinical signs related to OA disorders in animal models, morphological changes remain the main analysis outcome for these studies. The vast majority of studies rely on *ex vivo* analysis of the OA-induced joints, which requires animal euthanasia at

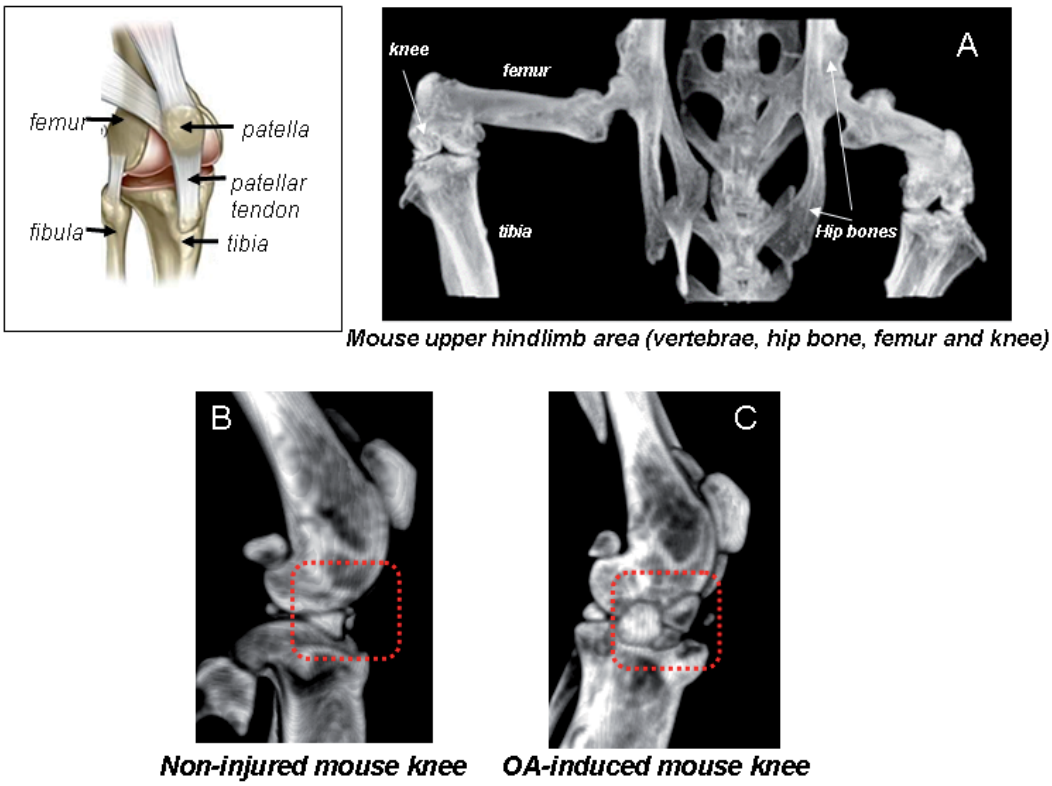


Figure 3. Application of micro-CT for imaging the knee structure: micro-CT images are displayed from 3D surface rendering of the upper region of the hind limb in the mouse (A) and the knee of non-injured m(B) and injured (DMM model) mouse (C). The insets show the region of interest in the medial side of the knee. In the OA model (C), we can see the increase remodelling and hypertrophic calcification. Images were acquired *ex vivo* at 80Kvp, 500uA and with a pixel size of 35 microns.

each desired time point. This critically compromises the possibility to undertake longitudinal studies on individual animals to investigate the pathophysiology of the disease, following the development of osteoarthritis lesions from their onset through later more advanced stages. The implementation of non-destructive imaging modalities, such as CT, allow researchers to create images of the three-dimensional architecture of the joints providing a valuable tool for monitoring the pathogenesis and the progressions of the disease in preclinical models of OA. (Fig. 3)

Micro-CT: This modality is becoming the gold standard for evaluation of bone morphology. Currently micro-CT is extensively used to investigate the structure and density of rodent bone due to its high spatial resolution and high contrast for imaging mineralised tissues. It has the ability to qualitatively and quantitatively assess 3D bone structures, including bone anatomy and density to study bone repair during fracture healing[27], bone resorption, remodelling and regeneration[28,29], bone neoplasm and metastases[30], bone changes influenced by metabolic disorders e.g. osteoporosis[31], among other changes associated with different pathological conditions. The high isotropic resolution can also provide good information on trabecular bone spatial orientation patterns, density, and geometry and growth plate morphology. While much finer details imaging can be achieved in *ex vivo* samples, micro CT imaging has proven very valuable for longitudinal studies. Indeed, a 100 µm isotropic spatial resolution can be effectively achieved within a safe and reasonable acquisition time through longitudinal *in vivo* studies[32], which has proven useful for assessment of bone and knee injury models[33,34]. In Figure 4, we provide an example of different micro-CT images acquired *in vivo* (100 um voxel size) and *ex vivo* (10 to 40 um voxel size) from several mouse bones. For imaging of *ex vivo* samples, the spatial resolution can be close to microscopic images, e.g. 5-10 um, acquired through long acquisition times. However, *in vivo* applications are constrained by the radiation dose to the animals, limiting acquisition time and thus spatial resolution. In particular, for longitudinal acquisitions, with the cumulative dose effect, the radiation exposure (dose and time frame) needs to be considered and it remains an important challenge when acquiring micro-CT images for high resolutions datasets.

Micro-CT imaging has proved very valuable in investigating the changes to the subchondral bone in osteoarthritic knees in mouse models[15,35]. These *ex vivo* studies have provided quantitative and qualitative 2D and 3D visualisation of the subchondral bone morphology and bone mineral density, trabecular bone patterns, meniscus morphology, heterotopic ossification and subchondral cyst formation[36,37]. *In*

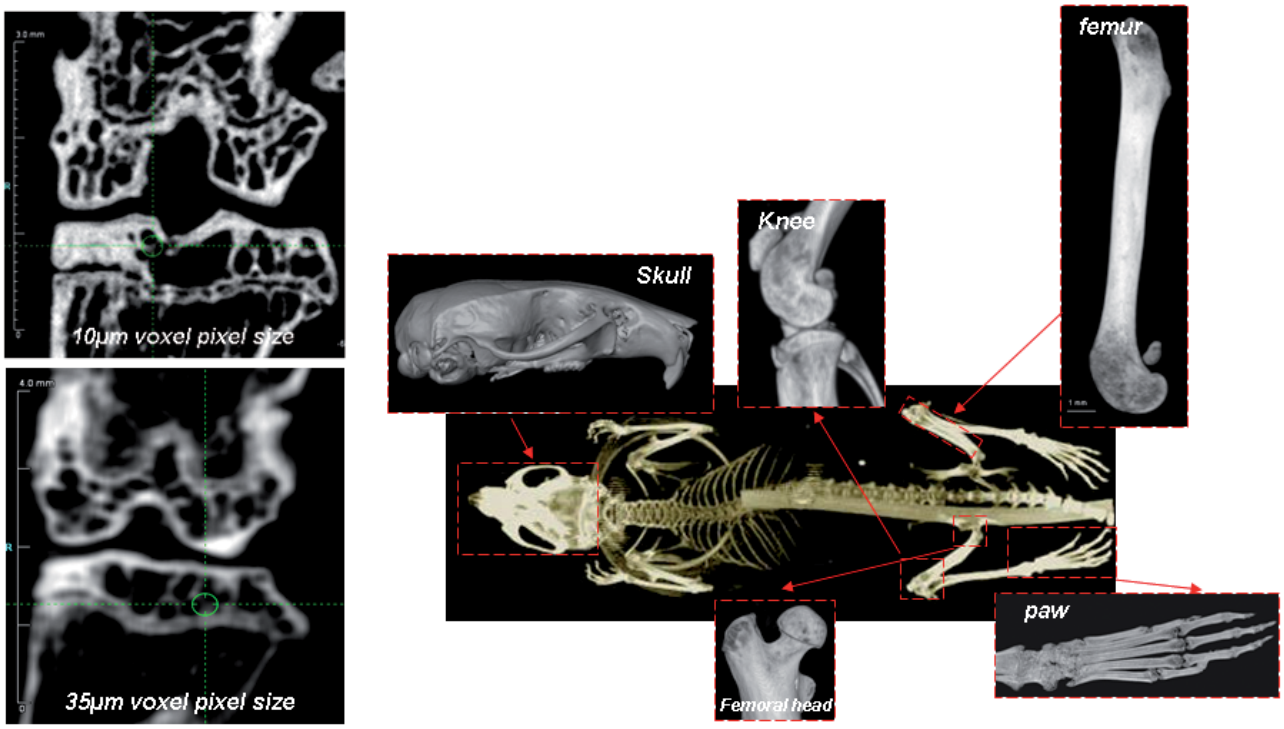


Figure 4. (A) Example of micro-CT images acquired from *ex vivo* mouse knees femurs (coronal views) at different resolutions, 10µm and 35 µm voxel size, displaying differences quality of images to assess the trabecular bone structure. (B) Example of different micro-CT images (3D surface rendering) acquired *ex vivo* from different mouse bone that are generally used for phenotype studies and orthopaedic disorder models. Samples were imaged at 80 kVp, 500uA and with a pixel size varying from 10 to 50 microns.

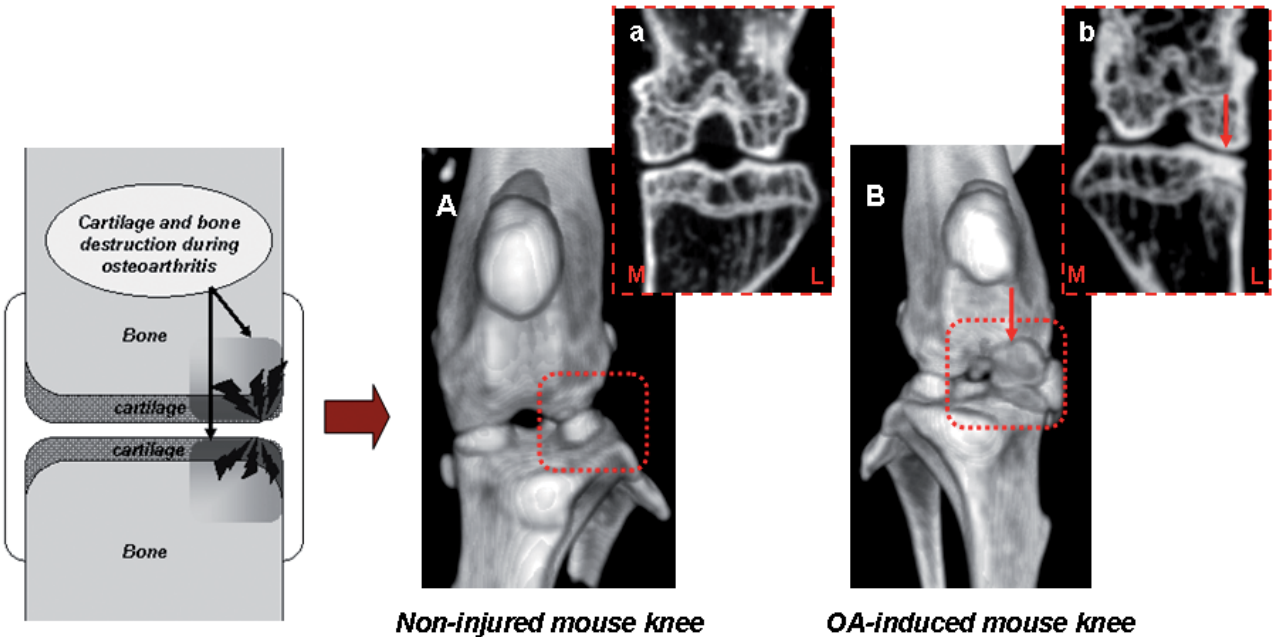


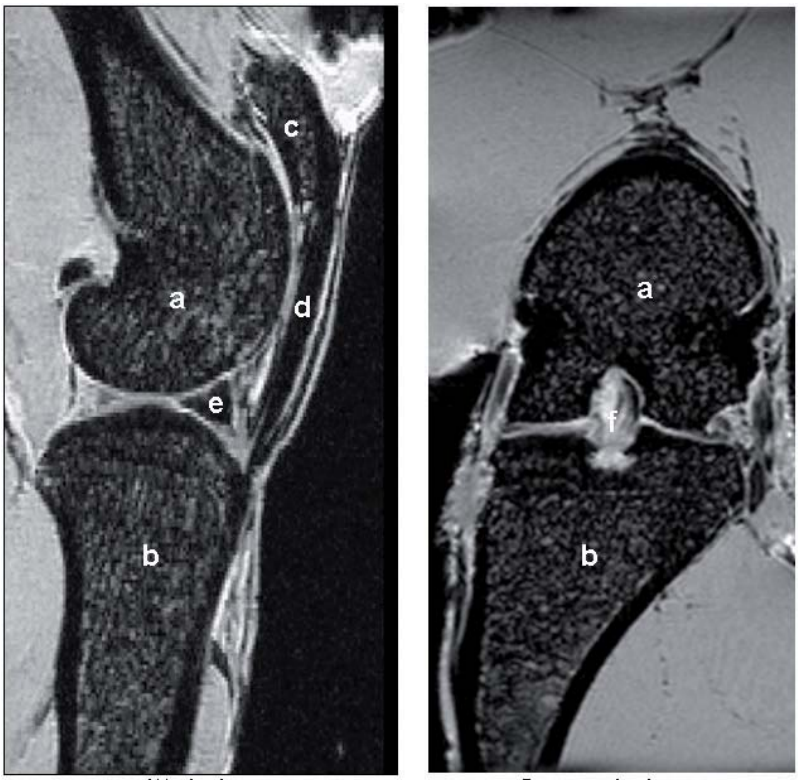
Figure 5. Computed tomography imaging of the knees in a mouse model of osteoarthritis (DMM model). During osteoarthritis the main targeted tissues are articular cartilage and the subchondral bone. Micro-CT images showing 3D surface rendering images and coronal sections of the the knee joint on a DMM mouse model (A) and non injured animal (B). Note the subchondral sclerosis and large hypertrophic calcification areas in the medial region of the OA-induced knee.

vivo assessment of osteoarthritis has been successfully reported in longitudinal rat models of OA[34], reinforcing the potential use of non-invasive *in vivo* micro-CT technology to monitor and validate animal models of OA. Figure 5 provides several micro-CT images of *ex vivo* mice knee with DMM-induced OA and control knees, to characterise the different morphometric features associated with this relevant OA preclinical model.

Micro-CT has very low sensitivity for detecting soft tissues, hence visualisation of the articular cartilage is challenging. To enhance the contrast resolution with the CT imaging, exogenous contrast agenst can be used and there are few studies which have indirectly used these agents to visualise the cartilage in animal models[38,39]. The composition of articular cartilage (biochemical analysis) in rodents has also being investigated by using negatively charged ionic contrast agents; this *in vivo* contrast induced micro-CT is proven valuable for detecting alterations in the cartilage composition and assessment of different stages of tissue degeneration in small animal models of OA[15]. It demonstrated that it is possible to distinguish between healthy and degenerative cartilage. The contrast-enhanced micro-CT can also pick up reasonable good images from other anatomical features, such as ligaments and-non calcified meniscus.While the use of this methodology has great potential as a method for assessing cartilage degeneration in in vivo longitudinal studies, they remain limited in the mouse because of the thin layer of articular cartilage,the small joint space in mice and diffusion variability between contrast agents. Further refinement of the contrast-enhanced micro-CT methods in small rodents need further development befor they can become a valuable diagnostic tool.

MRI: The visualization of bone structure with MRI technology has remained a challenge, due to its largely mineral composition with limited water content (~10%). Advances in clinical MRI for musculoskeletal disorders has provided methodologies for quantifying trabecular bone by indirectly detecting the intesive signal from the surrounding bone marrow which produces a contrast against the low signal from the actual trabecular bone tissue[40]. Some preclinical MRI systems have successfully used similar approach to image trabecular bone in rats[41]. However, it is important to realise that the resolution requirements are more stringent due to the small trabecullar size in rodents reinfocing the need for longer acquisition times. In figure 6, we display some examples of MR images obtained in a 7 Tesla MRI scanner in longitudinal position with a 3D gradient echo technique from rat knees.

The implementation of ultra-short echo-time (UTE) sequences have had a significant impact on the use of MR clinical imaging targeting tissue components with very short $T_{2,}$ such as tendons, ligaments, menisci and periosteum which produce little or no signal with standard sequences[42]. The use of UTE sequences allows a high signal to be detected



sagittal view Coronal view

Figure 6. Example of 7 Tesla MRI images for the rat knee joint. 3D spin echo MR image *(117x114x144 μm)* of a rat knee displaying the anatomical landmarks of the articular joint: a= femur condyle; b=tibia; c=patella; d=patellar ligament; e=meniscus; f=cruciate ligaments.

from these joint-associated structures and has proved very useful for diagnostic purposes. These sequences have also been successfully used clinically for the detection of signal from cortical bone and to study perfusion within the tissue.

MRI is also extensively used in clinics for assessment of articular cartilage; 3D fat-suppressed spoiled gradient echo sequences are generally used to assess the cartilage structure. This sequence has recently been successfully tested to visualised the cartilage in rat knees[43].

Another approach is the use of negatively charged contrast agents, such as gadolinium as markers of ionic transport into the cartilage. Loss of glycosaminosglycans leads to deeper penetration of this contrast agent, which subsequently induces a decrease in T1 relaxation. This technique, named as Delayed Gadolinium Enhanced MRI of Cartilage (dGEMRIC), is validated for clinical diagnosis and has also been reported in preclinical models[44].

The application of these new sequences have greatly benefited the diagnostic use of MRI for musculoskeletal disorders. It provides good visualisation of the main skeletal tissues, of particular interest for detection of tissue changes in osteoarthritis knees, such as subchondral sclerosis, accumulation of synovial fluid, damaged ligaments or patella displacement. However, its application as a regular high throughput preclinical screening tool remains compromised by the inadequate sensitivity for quantitative assessments of cartilage morphological parameters in rodents, and further challenged by long acquisition time and high cost.

Interesting recent developments in MR technology include the implementation of phased-array coils, which, in contrast with the traditional single-channel surface coil, can increase the signal-to-noise ratio providing superior image sensitivity covering a specific field of view. This has been successfully used for imaging the knee joint in rats. In this study, Rengle *et al*.[45] were able to achieve a high spatial resolution of 3D acquisitions with a voxel size 51x51x94 microns^3 with great signal uniformity within the knee joint. The implementation of phase-array coils in MRI operating with high magnetic fields (e.g. 9.4T scanners) could provide very useful non invasive methods for screening musculoskeletal lesions in animal models.

Nuclear imaging modalities:

Few studies with micro-SPECT have successfully reported the use of Tc-99m labelled diphosphonate compounds (e.g. methylene-diphosphonate (MDP; Fig. 7), hydroxymethane diphosphonate (HDP) and hydroxyethylidene diphosphonate (HEDP), to detect changes in bone turnover and cartilage composition in osteoarthritis models in rodents[15,46].

PET imaging using the ^{18}F-FDG tracer is widely used in clinical oncology in musculoskeletal sarcomas and is often used in whole-body scanning to detect metastases. In the preclinical field, this tracer is used in combination with CT imaging in murine models for the study of cancer metastasis to bone, detecting changes in bone structure and metabolism (osteoblastic and osteolytic lesions that are targeted by the ^{18}F-FDG tracer[47]. Similarly, PET scanning with ^{18}F-FDG also has a great potential for assessing fracture healing, as it can provide a direct quantitative non-invasive assessment of the metabolic activity in the region of interest, being very valuable to measure bone repair. This technology has been applied to study bone healing in a rat fracture model[48], providing valuable information in the treatment and prognosis of delayed fracture healing.

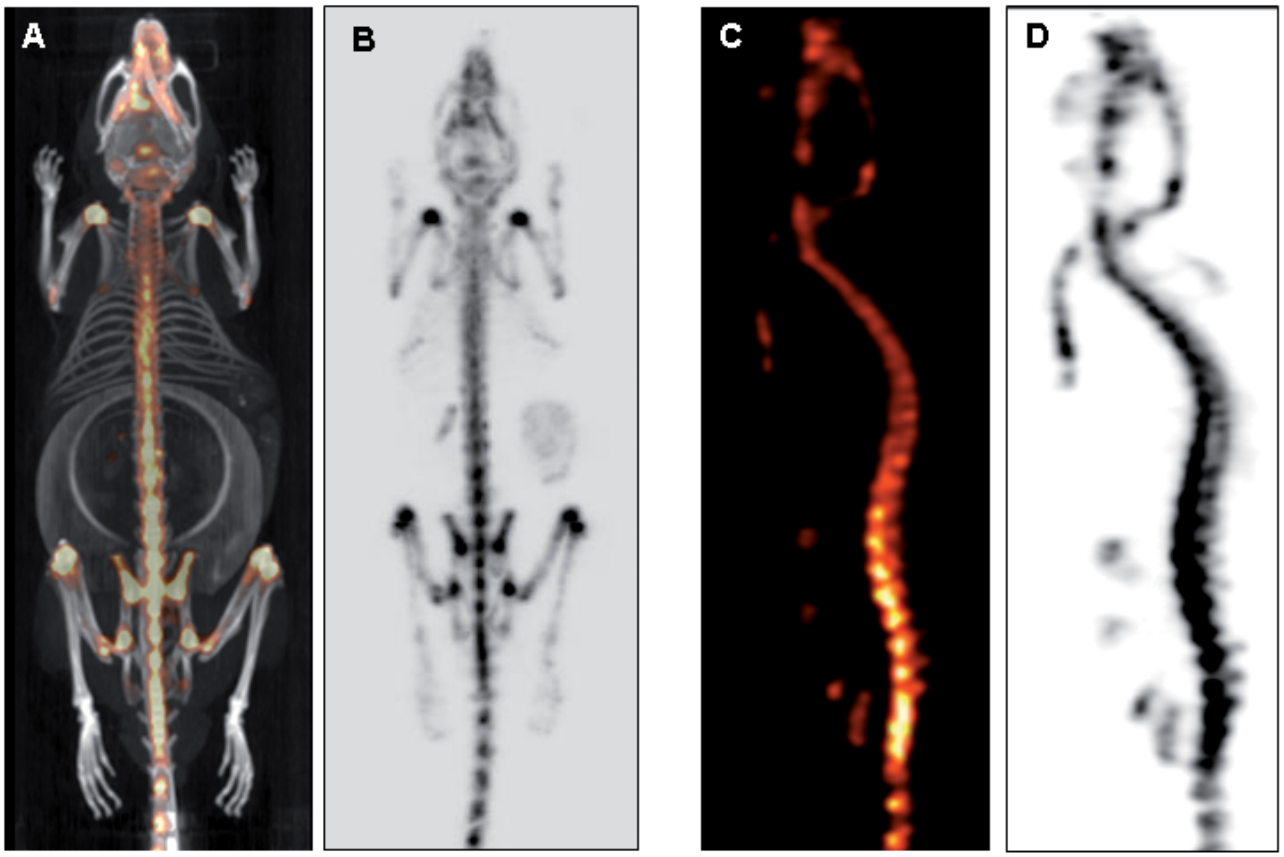


Figure 7. Combination of micro-SPECT and micro-CT images acquired 3hrs post-injection of 99m Tc-MDP (bone targeted tracer) demonstrating *in vivo* bone imaging in a mouse. Micro-SPECT data was obtained through helical acquisition over an axial field of view of 100mm through 60 projections of 30sec. A, B) Full body SPECT/CT and SPECT images, respectively, to detect the high MDP uptake within the joints (knee, shoulders) and the skull. C, D) micro-SPECT sagittal images showing the MDP uptake along the spine.

Another tracer, ^{18}F-NaF is also used as a skeletal imaging agent in PET for fine bone analysis and it has proven very useful in detecting bone microdamage and areas of abnormal osteogenesis[49] in preclinical models.

SPECT and PET technologies offer a great opportunity for refining translational research in musculoskeletal disorders. It is of great benefit in pharmacokinetics studies for drug discovery and for providing functional insights on *in vivo* targeted disorders.

Benefits of preclinical imaging: challenges and future developments.

The implementation of non-invasive imaging technologies has revolutionized preclinical research with great impact on refining *in vivo* protocols, in particular for longitudinal studies, and its overall efficacy for clinical translation. It provides a very useful avenue for a more rapid, efficacious and cost-effective use and characterization of animal disease models. In particular, for the musculoskeletal field, it has proved a good research tool for studying bone structure, and an excellent complementary less destructive approach to the histological or biochemical assays that have been extensively relied on in this field of research.

The application of this non-invasive technology has greatly benefited the refinement and reduction approaches related to animal research[50]. Refinement has been scientifically beneficial in permitting progressive analysis of the development and progression of the disease *in vivo*. This arises from the fact that the imaging process is conducted *in vivo* with the animal under general anaesthesia, minimizing any pain or suffering during the study. This also reinforces the implementation of a regular monitoring system for the animals, with the additional benefit of assessing relevant physiology parameters. Reduction is achieved by implementing repeated imaging sessions in the animal, involving the acquisition of more comparable data within the same subject and also providing a scientific data set using fewer animals.

Most of the imaging techniques require the animals to be immobilized. This can be achieved by imaging the animals under general anesthesia, which helps to restrain their gross motion. Inhalation anaesthetics, such as isoflurane, are generally used in

preclinical imaging units as these can be administered continuously through the imaging sessions allowing for dose calibration to the specific needs of the imaged subject and experimental settings. Anaesthetics are known to alter animal physiology dramatically, causing changes in respiration, heart rate, blood pressure, and temperature[51], which is likely to affect experimental outcomes during imaging. A warming system should be incorporated to help to maintain the body temperature during imaging and a good physiological monitoring system should be implemented. Monitoring systems for laboratory animals are being developed to improve animal restraint and physiological monitoring during imaging refining the procedures and ensuring the standardization and repeatability of the studies.

Another important challenge of the preclinical systems is the small size of the animal specimens. The technology needs to be able to achieve a reasonable resolution and sensitivity in smaller field of views and also account for the movement effects due to the physiological motions of the animals, for example, the movement related to heart beating; the heart rate in mice ranges between 400 and 600 bpm and in rats between 250-400 bpm, compared to the 60-80 bpm for adult humans. Thus, small animal imaging systems have markedly improved their spatial resolution by using gating methods to synchronize acquisitions with the physiological movement interference created through the cardiac and respiratory cycle. Gating (prospective or retrospective, if image acquisition is acquired simultaneously and triggered at some predefined physiological points or process post-acquisition) is already implemented in most of the preclinical *in vivo* imaging systems. The benefits of applying gating during image acquisitions have been well reported in micro-CT imaging of rodents[52].

With the increasing requirements for obtaining high resolution images from different tissues/ organs of interest in small rodents, this may require long and/or repetitive imaging acquisitions. Such protocols, in particular for serial longitudinal studies, are constrained by the cumulative dose effect, the radiation exposure (dose and time frame) and the time that the animal will be maintained under anaesthesia. This remains an important challenge in particular when acquiring micro-CT images for high resolutions datasets, with the cumulative animal exposure to x-ray radiation. The effect of ionizing radiation dose in rodents has been well studied after micro-CT, describing lethal dose, which are typically expressed as $LD_{50/30}$ (whole-body radiation dose that would kill the 50% of the exposed animals within 30 days) in the range between 5-7.6 Gy. The typical x-ray whole body radiation dose for a 3D micro-CT scan reported ranges from 0.017 to 0.78Gy (4). These sub-lethal doses of radiation are unlikely to really compromise using *in vivo* micro-CT, and in particular for musculoskeletal applications, in which it is likely that only a small area of the animal may be imaged and irradiated.

With the growing developments in imaging technologies, with their ability to identify molecular events with adequate sensitivity, specificity and temporal and spatial resolution, there is an increasing trend to combining different imaging modalities to integrated function and anatomical analysis. While PET and SPECT can provide excellent temporal sensitivity for functional procedures through labeled biomarkers, the combination with micro-CT imaging allows accurate spatial correlation to visualize the distribution *in vivo* of the biomarker within the body. Nuclear imaging data can then be co-registered with the anatomical images obtained through micro-CT. In fact, this is one of the major applications of versatile PET/SPECT/CT systems, providing co-registration acquisition of the detection of the nucleotide-labeled biomarker with the entire anatomy in a live mouse. Other multi-modality approach such as PET/MRI are under development with great potential for providing high resolution anatomical data, especially in models where a high soft-tissue contrast is required, with functional studies[53].

The implementation of imaging technologies in animal models has clearly been and continues to be a very useful tool for enhancing our understanding of musculoskeletal diseases and in developing new therapies. Through this review we have exemplified how these technologies have an invaluable justification for driving the refinement and reduction in the field of animal research. With the increasing availability of new tracers as biomarkers to target specific musculoskeletal disorders, we can foresee an important role for PET/ SPECT imaging in translational bone and cartilage research. Similarly, optimization of the micro-CT systems with more sensitive detectors to achieve higher spatial resolution with shorter acquisition times, and reduced radiation dose with improvements in gating acquisitions will reinforce the use of CT imaging. For the MRI, operation in high field strength and further advances in pulse sequence methodology and in coil technology, will provide great improvements in spatial and/or temporal resolution. Undoubtedly, with all the ongoing developments, preclinical imaging will play a key role in contributing to a system level understanding of musculoskeletal disorders and future therapeutic developments.

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Assessment of arthritis in rats with Collagen Induced Arthritis: correlation between thermographic measurements and clinical score.

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Abstract

Thermography is the technique of measuring natural thermal radiation from body tissues of clinical interest. It is safe, requires no control of environmental conditions and non invasive. Our study was designed to evaluate a physiological parameter, hind foot surface temperature, measured with computerized digital infrared thermal imaging, and its association with stages of clinical scores in a rat collagen induced arthritis model. Arthritis was induced with collagen immunization in eight Lewis rats. Four of the animals were treated with dexamethasone as a negative control. Disease progression was monitored by paw edema. On the basis of paw edema a clinical score was given each animal involved in the study. The mean temperature of a hind foot (region covering the metatarsus and tarsus) was compared with a reference area on the back of the same rat. The temperature ratios were compared with the clinical score. The mean hind foot temperature increased with increasing clinical severity in the acute stage of the disease and there was a correlation between temperature ratio and clinical scores. The hind feet were warmer at higher score and lower in normal score. The preliminary data indicates that thermography may be a potent supplemental clinical parameter in the evaluation of the severity of the arthritis in the acute stage and further investigations are underway to evaluate the thermographic assessment of the disease progress and response to therapeutic intervention.

Keywords: CIA, Lewis rats, thermography, arthritis, non-invasive techniques

Non-invasive techniques for evaluation of physiological parameters are becoming more and more used and are contributing to implementation of the 3 Rs. In this study we were evaluating thermography, which is a non-invasive technique for measuring natural thermal radiation from body, in a rat collagen induced arthritis model (CIA). It was hypothesized that the temperature of the affected area (hind foot) would correlate with the clinical score of the arthritis and this technique could be an objective method for evaluate the degree of arthritis and effect of therapeutic interventions.

Animals

Adult female Lewis rats (LEW/Han™Hsd) (n = 8) from Harlan Laboratories weighting approximately 150-180 g were used. The Danish Experimental Animal Inspectorate approved the experimental protocol. The rats were housed (2-3 per cage) in a room under controlled temperature (20±2 °C) and light (lights on 08:00-20:00 h). Food (Altromin 1314) and water were freely available. This study was part of another larger preclinical study for development of a new drug against rheumatoid arthritis in humans. The animals used in this study were the control groups. At the end of the study the animals were anaesthetized with isoflurane and euthanized by cervical dislocation.

Material and methods

CIA was induced to the all animals by injection of swine collagen using a standard protocol[1,2]. Immunization Grade Porcine Type II collagen (CII) (Chondrex cat. #20031) was used for immunizing rats for the induction of CIA. Porcine collagen type II (2 mg/ml) was dissolved in 0.05 M acetic acid by gently stirring overnight at 4

°C. The emulsion for induction of arthritis was prepared immediately before immunization using an electric homogenizer. Equal amounts of collagen (2 mg/ml) and Incomplete Freund's Adjuvant (IFA) (Chondrex cat. #7002) were mixed in an ice-water bath, adding the collagen drop-wise to the IFA while mixing. Mixing was continued until a stiff white emulsion was apparent and congeals instead of dissipating when dropped in water. Rats were immunized with 0.2 ml (200 µg) of porcine collagen type II emulsion (1:1 mixture of collagen and IFA) injected subcutaneously at the base of the tail under isoflurane anesthesia (Forene, Abbott). To ensure a high incidence and severity of arthritis a booster injection with 0.1 ml (100 µg) emulsion was given 7 days after immunization. The rats were divided in two groups (treatment and control); 4 rats in each group. A group of animals were treated with vehicle (sterile phosphate-buffered saline) and another group of four animals were treated with dexamethasone (1 mg/kg intravenously) as control two times a week.

Three days after the last injection of collagen (day 9) and every third day after that the animals were scored clinically on the basis of paw edema and the surface temperature of the rat was measured while the animals were anaesthetized with isoflurane. The clinical score was a standard score system with 0 as unaffected and 4 was heavily affected joints with swelling (Table 1). The total score was calculated as average score on hindlimbs plus average score on forelimbs. An infrared video camera (SATIR-S280, GSAT INC.) was used to monitor the skin temperature pattern (local inflammation response in the joint). The camera spectral range was 7.5-13 µm, and it could measure temperature in the range from -40°C to +160°C at accuracy of ±0.2°C. Temperatures on the hind foots (metatarsal and tarsus) were normalized with the shaved reference area on the back to be sure that the general temperature rise in the rat's body due to collagen immunization did not affect the measured difference in temperature rise in the joints. The mean temperature of each hind foot (left: t_{left} , right: t_{right}) was normalized to the reference temperature on the back (t_{back}) of the same rat, added up and called Temperature Index (TI = $t_{left} / t_{back} + t_{right} / t_{back}$). Normal hind foot temperature was approximately 26.95 °C (TI ≤ 1.5) whereas diseased hind feet had a TI >1.5, where TI=1.5 corresponds approximately to a temperature of 28 °C in the hind foot. Normal temperature of the back was approximately 36±2 °C. All animals were euthanized at the end of the study by injection of pentobarbital.

Table 1. Interpretation of clinical scores. The animals were scored all four feet.

Score	Clinical findings
0	Normal.
0,5	Light redness of ankle or digits.
1	Mild, but definite redness and swelling of the ankle or wrist, or apparent redness and swelling limited to individual digits, regardless of the number of affected digits.
2	Moderate redness and swelling of ankle and wrist.
3	Severe redness and swelling of the entire paw including digits.
4	Maximally inflamed limb with involvement of multiple joints.

All values were expressed as the mean ± standard error of the mean. The number of animals in each group was small, therefore for comparison of clinical score and TI between the two groups at different time points Mann-Whitney test with Sidak correction was used. Differences with *P* values < 0.05 were considered to be statistically significant.

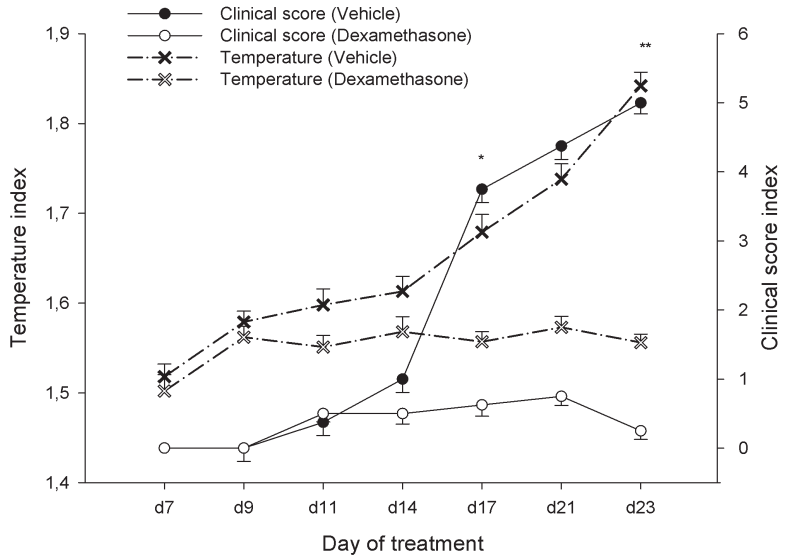


Figure 1. Clinical score and relative temperature (TI) in the hind feet in Lewis rats with CIA rats treated with dexamethasone (open symbols) and in vehicle group (solid symbols). Day of treatment is day after first collagen administration. The animals were treated on day 9. Values are shown as mean ± standard error of the mean. The groups were compared for each time point and the asterisk covers both temperature and clinical score. (* $P < 0.05$, ** $P < 0.001$).

Results

The animals developed arthritis according to expectations in the vehicle group (Fig. 1 and 2a), while dexamethasone treated animals did not develop symptoms or they developed only mild symptoms. (Fig. 1 and 2b). As shown in Fig. 1, no difference in clinical score was seen between groups before day 14, whereas the relative temperature (TI) on day 11 was increased in the vehicle group. There was only few animals included in the study, however comparing clinical score between vehicle and dexamethasone group at day 17 revealed significant difference ($P_{score} = 0.008$). The same comparison between temperatures in the two groups gave also significant difference ($P_{TI} = 0.005$). Examples of thermographic images in a vehicle treated rat at different time points and with different clinical score are seen in Fig. 3.

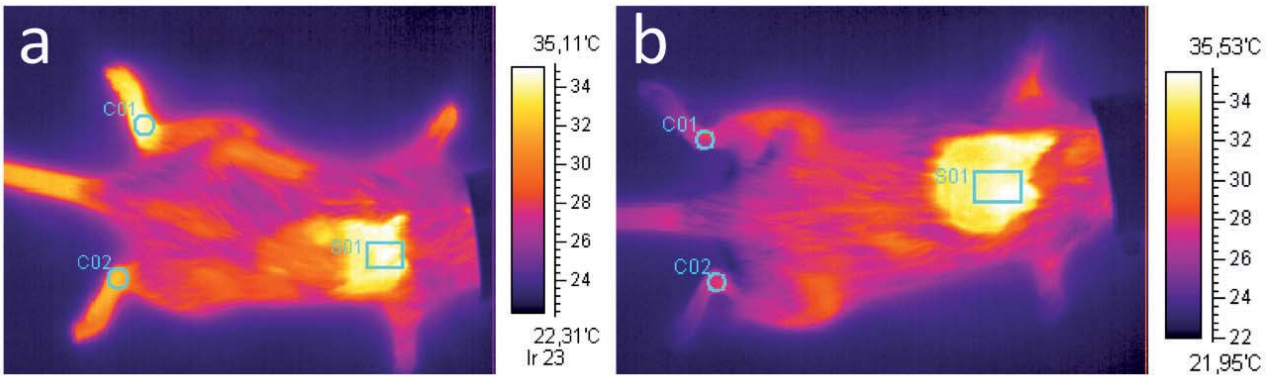


Figure 2. Thermographic images in a vehicle treated (a) and a dexamethasone treated rat (b) at the endpoint, which is at day 23 days after first treatment with collagen.

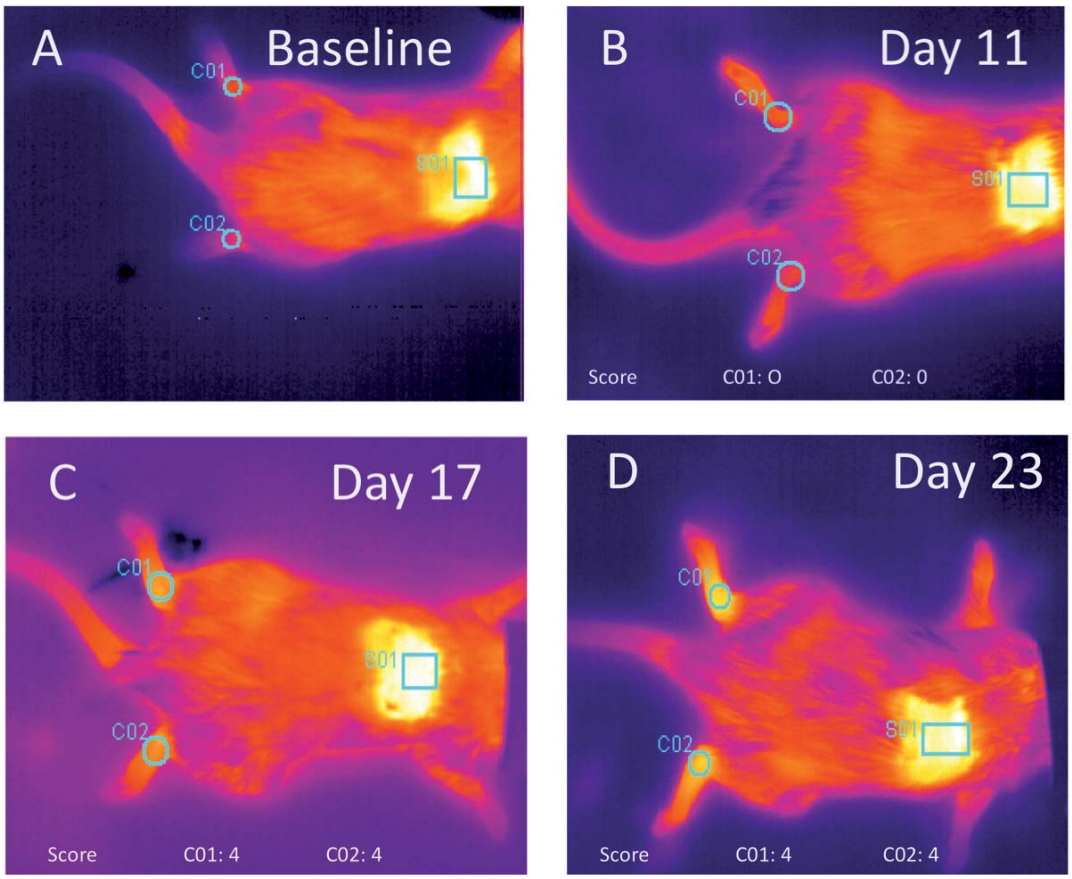


Figure 3. Examples of thermographic images in a vehicle treated rat at different time points (days after 1 immunisation) after the induction of the disease. The clinical score of the individual feet is seen in the lower right corner of each image.

If all the data from start to end of the study was used for the calculation of a median temperature, it was found that the surface temperature on the hind feet was 29.83 °C in the vehicle group and 27.62 °C in the dexamethasone group (difference 2,21 °C). The corresponding temperature indexes (TI) were 1.665 (vehicle group) and 1.591 (dexamethasone group). The study showed a strong correlation between increase in the temperature of the hind feet (TI) and the clinical score (Fig. 4).

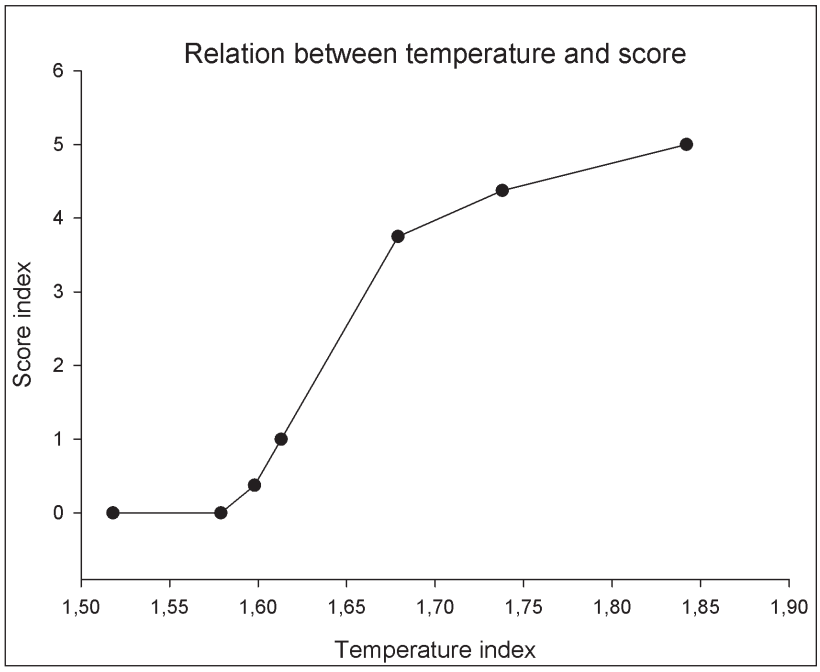
Discussion

Thermographic studies have been performed to evaluate rheumatic diseases of the hand in humans[3, 9] and there have also been several studies using thermography to assess osteoarthritis of the knees and hands[4-6, 9] and the temperomandibular joint[7]. In some of these studies, thermography has been used as a tool to provide objective data on joint inflammation in response to therapeutic interventions. For example, thermography has been used to measure the change in hand joint temperature in response to non-steroidal anti-inflammatory agents[5]. The same investigators evaluated temperature changes in knee arthritis in response to intra-articular steroids[6]. They showed a significant reduction in joint surface temperature 1 week after intra-articular steroid injection in a small number of patients.

Sanchez *et al*. conducted a study[7] using a hand-held thermal imaging device in a preclinical model of general inflammation in an animal model of rheumatoid arthritis. As we did in the present study, they also compared the clinical score with infrared thermography in a CIA rat model. However, in contrast to our approach, they did not include any control group, whereas we used dexamethasone treated rats as control. Furthermore Sanchez *et al*. did not use a shaved area on the back as reference for the temperature measurements as we did. Using the animals own temperature as reference temperature is a more controlled approach since we know that CIA may cause a general temperature rise in the entire body. Normalizing the measured temperature with the reference area on the back will take the general temperature rise into account when evaluating the temperature rise in the joints. Sanchez *et al*. used much higher doses of collagen and this in it self might have increased not only the temperature in the arthritic joints and at the injection site but the body temperature in general. The high dose of collagen together with the lack of a relevant control group in the paper by Sanchez *et al*., makes a comparison with our data difficult.

Although our study demonstrated the stability of thermographic measures, the animals were not monitored every day, leaving some caps between the measurements. The inflammatory process is highly dynamic and changes in the inflammatory stage may vary from day to day. This makes it difficult to follow the disease development exactly in each joint on each animal in our study. It was sometimes observed in one animal, that the temperature in the most clinical affected joint was lower than in the opposite joint, indicating an acute ongoing inflammatory process in the not yet clinical affected joint. This is in accordance with studies[8] in humans where it has been observed that the joint temperature will decrease in the more chronic stages of the disease. Further more pain and stiffness have been shown to be significantly correlated with joint surface temperature in subjects with rheumatoid arthritis[3]. Thus, the evolution to colder joint surface temperatures as the arthritis progresses in clinical severity may indicate that the animals are less influenced with respect to pain during the chronic stage of the disease. This could also be important information in relation to animal welfare considerations.

Figure 4. Correlation between temperature and clinical scores in the vehicle group.



Conclusion

The relative temperature of affected hind feet in a CIA rat model was increased and this effect was inhibited by dexamethasone treatment. The study showed a strong correlation between increase in the relative temperature in the hind feet and the clinical score. The current results suggest that there might be a thermographic response even before clinical signs appear. This indicates that thermography may be a used as a predictive sign for the development of disease, thus therapeutic intervention may be possible at an earlier stage due to earlier diagnosis. Thermography may be a potent supplement to clinical parameters in the evaluation of the severity of the arthritis in the acute and chronic stages and maybe also an indicator of the actual pain. Further investigations are underway to evaluate the thermographic assessment of the disease progress and response to therapeutic intervention

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Specific changes of gut commensal microbiota in a model of indomethacin-induced acute enteritis in rats

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Abstract
Susceptibility to intestinal inflammation has been associated to gut microbial charge. We assessed spontaneous changes of gut commensal microbiota (GCM) in a model of indomethacin-induced acute enteritis in rats bred under different microbiological conditions. Male Sprague-Dawley OFA rats bred under conventional conditions or under barrier conditions and adapted for three weeks to conventional conditions (barrier/conventional) were used. Enteritis was induced by systemic indomethacin administration. Animals were euthanized 4 days later, corresponding to the acute phase of inflammation. Inflammation was assessed through disease activity parameters (clinical signs, macroscopic/microscopic scores and tissue levels of inflammatory markers at necropsy). GCM (caecal and ileal) was characterized using fluorescent in-situ hybridization (FISH) and analysis of 16s rDNA polymorphism. After indomethacin administration, disease activity parameters increased, suggesting an active inflammation, and were slightly higher in conventional vs. barrier/conventional animals. Total bacterial counts were similar across groups. During inflammation, total bacterial counts were unchanged, but similar alterations in specific bacterial groups were observed in both experimental groups, with significant increases in the ileal counts of enterobacteriaceae and *Clostridium*. Caecal microbiota showed similar qualitative changes, although attenuated in magnitude. Bacterial adherence significantly increased during inflammation, with a particular adherence of *Clostridium* spp and *Bifidobacterium* in the conventional group. Animals maintained in conventional conditions or barrier-bred animals adapted to a conventional status had a similar acute inflammatory response to indomethacin. Gut inflammation implies qualitative changes in GCM. Microbiological status of the animals in standard research conditions can be a factor influencing the outcome of intestinal inflammation in experimental models.

Keywords: bacterial adherence, gut commensal microbiota, gut inflammation, FISH, inflammatory bowel disease

Gut commensal microbiota (GCM) contributes to host health, participating in intestinal immune responses, energy and nutrient, and tissue development and repair.[1] However, in certain circumstances, GCM can become detrimental to the host. For instance, it is thought that GCM plays a major role in the pathogenesis of inflammatory bowel disease (IBD), a pathological condition characterized by strong activation of the mucosa-associated immune system due to a complex interaction of genetic, immunological and environmental factors.[2] According to this, several reports have established that the composition of the intestinal microbiota has significant impact on host immunity and influences the course of mucosal inflammation.[1] For instance, an increase of IBD incidence in industrialized countries has been related to the improvement in standards of hygiene and the subsequent changes in GCM.[3] These changes lead to the appearance of abnormal gut immune responses with alterations in the microbiota recognition systems, resulting in an increased susceptibility to gut inflammation and the generation of persistent gut inflammatory states.[4,5]

We have recently described the occurrence of spontaneous adaptive changes of GCM in barrier-bred animals, with controlled microbiological conditions, to standard housing conditions.[5] Following these observations, in the present study we assessed the influence of GCM and its spontaneous variations during the adaptation from a barrier-controlled environment to a conventional environment in intestinal inflammation in a model of indomethacin-induced ileitis in rats. This model has been previously characterized by us and represents a valid IBD model

in which a local inflammation of the ileum appears after systemic treatment with indomethacin.[6] Here, we characterize the indomethacin-induced acute inflammatory response in animals maintained in standard microbiological conditions and in animals breed under barrier conditions and adapted for a 3-week period to standard conditions. In these animals, we assessed changes in disease activity parameters as well as characteristics of the GCM (ileal and caecal) and the incidence of bacterial adherence to the ileal epithelium.

Materials and methods

Animals

Adult outbred male Sprague-Dawley OFA rats (300-350 g), bred and maintained in a barrier protected area with all materials, water, food and bedding sterilized before entering the barrier, were obtained from Charles River Laboratories (Lyon, France). Transport of barrier bred animals was made in filtered boxes to guarantee maintenance of their microbiological status. Thereafter, animals were maintained for a 3-week period in conventional conditions (referred from now on as barrier/conventional animals).

In addition, outbred male Sprague-Dawley rats, bred in conventional conditions in the Animal Facility of the Universidad Autónoma de Barcelona, were used (referred from now on as conventional animals). This conventional colony was established in 1994 from Sprague-Dawley OFA rats from Charles River Laboratories. When in conventional conditions, water, food and bedding were given to animals as facilitated by the commercial provider, without any further treatment. All animals maintained at the university's animal facility were housed in standard plastic cages with stainless steel grid roofs in an environmentally controlled room (20-21ºC, 40-70% humidity, 12 hours light /dark cycle), and received a commercial diet (15.4%,protein, 2.9% fat and 3.9% fibre; SASE, Panlab S.L., Barcelona, Spain) and tap water *ad libitum*. All procedures were approved by the Ethical Committee of the Universidad Autónoma de Barcelona.

Experimental procedures

Intestinal inflammation was induced by administration of two injections of indomethacin (7.5 mg/kg, subcutaneous) 48 h apart, as previously described by us.[6] Control animals received similar treatment with saline (0.3 ml/rat, subcutaneous). Animals were euthanized at day 4 after the first injection of indomethacin, corresponding to the acute phase of inflammation.[6] During this time, clinical signs were monitored every other day (see below, clinical assessment of inflammation).

The following experimental groups were included (n=6-7 for each): 1) indomethacin-treated barrier/conventional rats, 2) vehicle-treated barrier/conventional rats, 3) indomethacin-treated conventional rats, and 4) vehicle-treated conventional rats.

Clinical assessment of inflammation

Clinical assessment of inflammation included appearance of faeces, state of hydration and general condition (including hunch posture, piloerection, motor activity and state of mucous membranes). A total score of 0-9 was assigned to each animal, corresponding to the addition of separate scores (0-3) for each parameter (0: normal, 1: mild alteration; 2: moderate alteration; 3: severe alteration). In addition, body weight changes were also monitored. A macroscopic score (0-9) was also assigned to each animal during necropsy. The macroscopic score was based on: presence or absence of abdominal distension (0: absent; 1: present), presence and characteristics of oedema (0: absent; 1: moderate/localized; 2: clear and generalized), presence of adherences (0: without adherences; 1: some local adherences; 2: extensive and generalized adherences), appearance of the intestine (ileum and caecum) (0: normal; 1: moderate signs of congestion and/or distension; 2: clear and generalized alterations) and appearance of bowel (ileal and caecal) contents (0: normal; 1: reduced content with some fluid and/or mucous; 2: no content, only fluid or mucous, bloody appearance).

Samples collection

Rats were euthanized by CO_2 inhalation. Immediately, a medial laparotomy was performed and the inflammatory state assessed macroscopically as detailed above. Thereafter, tissue samples of the caecum and ileum and faecal content of the same areas (about 0.5 g) were obtained under sterile conditions and immediately frozen with liquid nitrogen. All samples were stored at -20 ºC (faecal samples) or -80 ºC (tissue samples) until analysis.

Histological score

Paraffin sections (5µm) of ileal samples were stained with haematoxylin-eosin following standard histological procedures. Two to four coded sections for each animal were scored for inflammation, in a blinded fashion, by two independent investigators. A histological score based on the epithelial structure (0:normal; 1: mild alterations; 2: moderate alterations; 3:severe alterations), the presence of oedema (0:absent; 1: minor and localized; 2: minor generalized; 3: severe generalized), the presence of ulcerations (0: absent; 1: one ulcer observed; 2: several small ulcers observed; 3: numerous small and large ulcers observed), and

the inflammatory infiltrate (0: absent; 1: mild localized inflammatory infiltrate; 2: moderate diffuse Infiltrate; 3: severe generalized infiltrate) was assigned to each animal (maximal score of 12).

Enumeration of bacteria using fluorescence in situ hybridization (FISH)

For FISH, oligonucleotide probes consisted of a single strain DNA covalently linked with Cy3 at the 5'-end. Probes used were: EUB 338 (5'GCTGCCTCCCGTAGGAGT3') to total Bacteria;[7] NON 338 (5'ACATCCTACGGGAGGC3') to Non bacteria (negative control);[7] BAC 303 (5'CAATGTGGGGGACCTT3') to *Bacteroides* spp.;[8] EREC 482 (5'GCTTCTTAGTCAGGTACCG3') to *Clostridium* Cluster XIVa;[7] LAB 158 (5'GGTATTAGCACCTGTTTCCA3') to *Lactobacillus* spp. and *Enterococcus* spp.;[8] ENT-D (5'TGCTCTCGCGAGGTCGCTTCTCTT3') to enterobacteria;[9] and BIF 164 (5'CATCCGGCATTACCACCC3') to *Bifidobacterium* spp.[10] All probes were obtained from Biomers (Germany).

The procedures followed were identical to those described previously by us.[5] Briefly, frozen caecal and ileal contents were thawed in PBS medium, the suspension obtained centrifuged (1 min, 700 g) and a 1 ml aliquot collected and fixed overnight (4% paraformaldehyde, 4 ºC). Fixed aliquots were stored (-20 ºC) until use.

At the time of analysis, samples were diluted in PBS and disposed on 10-well gelatin-covered slides, air-dried at room temperature and fixed with ethanol (10 min). For hybridisation, slides were incubated overnight in a dark moist chamber with the corresponding probe (5 ng/µl in hybridisation further: 20mM Tris-HCl, 0,9M NaCl, 0.1% sodium dodecyl sulphate pH 7.2, 50 ºC; with 20% formamide for the LAB 158 and NON 338 probes). Except for the BAC 303 probe, which was hybridized for only 3 h at 47ºC and for the LAB 158 probe which was incubated overnight at 47ºC., samples to be hybridized with the LAB 158 probe were pre-treated with lysozime (1 h, 37ºC) prior to the hybridisation. After incubation, slides were rinsed in preheated washing buffer (20mM Tris-HCl, 0,9M NaCl, pH 7.2, 180 mM NaCl for the LAB 158 and NON 338 probe; 30 min, 50ºC), briefly rinsed with milli-Q water, air dried and mounted with Vectashield (Vector Laboratories, Peterborough, UK).

Slides were viewed under oil immersion, using a Nikon Fi 60 epifluorescence microscope equipped with a filter for Cy3. Twenty five randomly selected fields were counted for each sample (in duplicate).

Terminal Restriction Fragment Length Polymorphism (t-RFLP)

T-RFLP analysis of bacterial community was performed in caecal contents following the procedure described by Hojberg et al. (2005).[11] Briefly, a 1497-pb fragment of the 16S rDNA gene was amplified using a 6-carboxy-fluorescein-labeled forward primer: S-D-Bact-0008-a-S-20 (5'-6-FAM-AGAGTTTGATCMTGGCTCAG-3') and reverse primer PH1552 (5'AAGGAGGTGATCCAGCCGCA-3'). Fluorescent-labelled PCR products were purified (QIAquick PCR purification kit columns; Qiagen, West Sussex, UK,) and eluted in a final volume of 30 µl of Milli-Q water. The resultant PCR product was subjected to a restriction with *HhaI* (20,000U/µl) (Biolabs Inc., New England, USA) and fluorescent-labelled terminal restriction fragments (TRF) analyzed by capillary electrophoresis (ABI 3100 Genetic Analyzer, PE Biosystems, Warrington, UK) with a 25-U detection threshold. Determination of the TRFs sizes in the range 50-700 base pairs (bp) were performed with the size standard GS-1000-ROX (PE Biosystems). Data was analyzed and standardized following the method described by Kitts (2001).[12]

Richness was considered as the number of peaks in each sample after standardization. For pair-wise comparisons of the profiles, a Dice coefficient was calculated and dendograms were constructed using the Fingerprinting II software (Informatix, Bio-Rad, CA, USA) and an unweighted pair-group method with averaging algorithm (UPGMA).

Assessment of bacterial wall adherence

Bacterial wall adherence was assessed following techniques described elsewhere.[13] Briefly, a sample of ileal tissue (about 2 cm in length) was rinsed in saline solution, sonicated twice for 60 s and then fixed in 4 % paraformaldehyde for 16 h at 4ºC. Thereafter, tissues were paraffin embedded using standard protocols, sectioned at 5 µm and sections were hybridized following the general FISH procedures described above.

Myeloperoxidase levels

Myeloperoxidase content in ileal and caecal tissue samples was determined as previously described.[6] Briefly, frozen tissues were powdered, weighted and homogenized in lyses buffer (Mini complete protease inhibitor, HEPES 1M, Triton X-100, PMSF 100mM). Thereafter, the homogenates were incubated for twenty minutes at 4ºC, centrifuged at 14000 rpm for 10 minutes at 4ºC and the supernatant recuperate. MPO activity was evaluated in the supernatants using a specific enzyme-linked immunosorbent assay (HyCult Biotechnology, Uden, The Netherlands, limit of detection 1 ng/ml),

Statistical Analysis

Data are expressed as mean ± SEM or median (interquartile range) ± SD (for bacterial counts).

Comparisons between multiple groups were performed using a one-way analysis of variance (one-way ANOVA) or a non-parametric ANOVA, as appropriate, followed when necessary, by a Student-Newman-Keuls multiple comparisons test. Bacterial wall adhesion data were analysed using the Chi-square test. Results were considered statistically significant when $P<0.05$.

Results

Clinical indices and macroscopic assessment of inflammation

During the 4-day period after treatment, control rats, regardless the group considered, showed no clinical signs of inflammation and a linear increase in body weight (Fig. 1A). Conversely, during the same period of time indomethacin-treated animals showed a progressive reduction in body weight (Fig. 1A). Similarly, indomethacin-treated groups showed a progressive increase in their clinical scores for the same period of time (reflecting a worsening in the general state of the animals: piloerection, reduced activity, hunch posture and chromodacryorrhea) (Fig. 1B).

At necropsy, macroscopic assessment of the abdominal cavity and the gut in control groups revealed no signs of intestinal inflammation (Fig. 1C). However, in indomethacin-treated groups, macroscopic scores increased significantly over control values (Fig. 1C). Main alterations observed included abdominal distension, adherences and distension/congestion of the intestine.

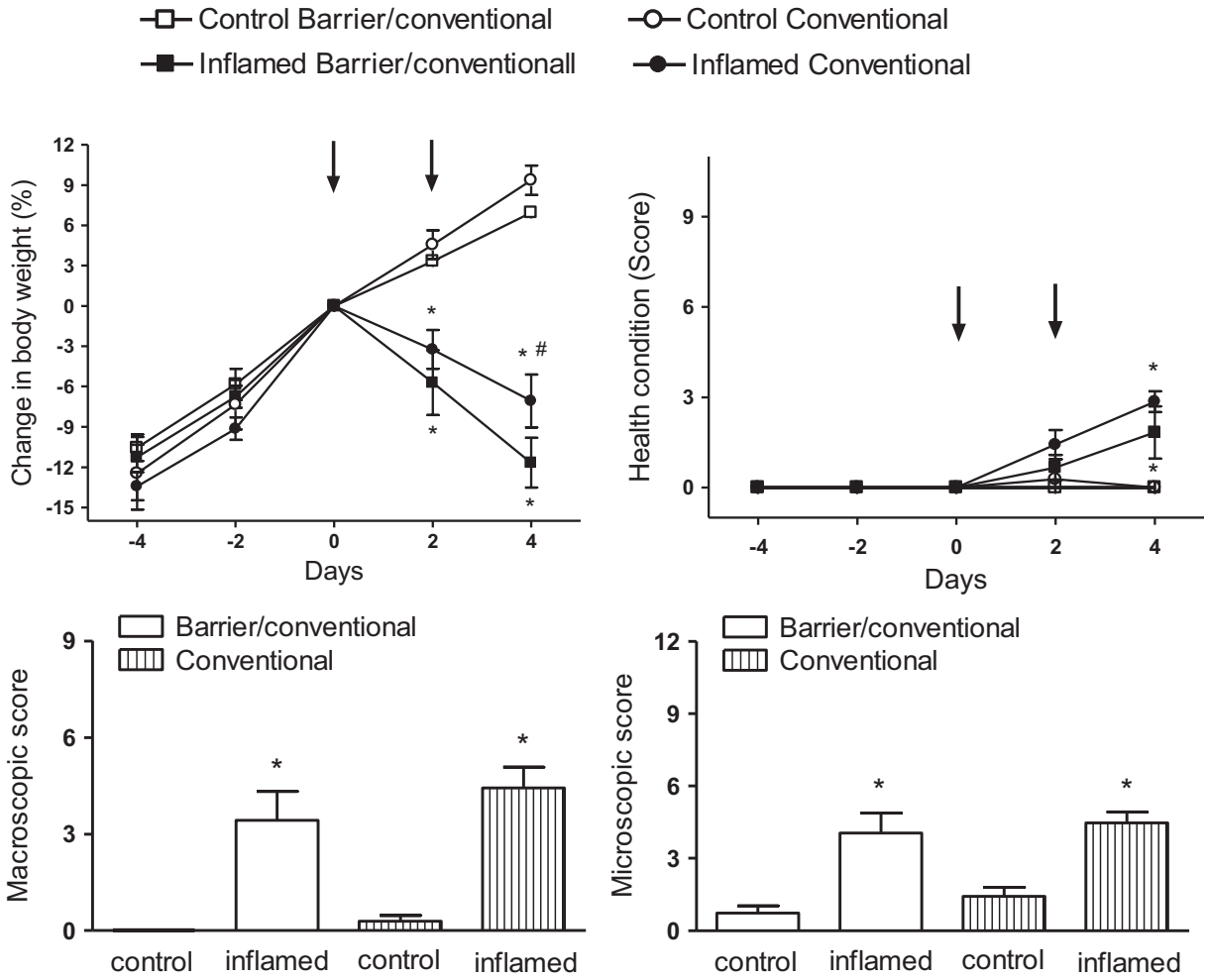


Figure 1. Disease activity parameters in barrier/conventional and conventional animals in control conditions or after indomethacin-induced intestinal inflammation. A: Time-course changes of body weight (percent change from day 0, day of induction of inflammation). B: Time course changes in general health conditions scores (see methods for details). C: Macroscopic scores at necropsy (see methods for details). D: Histological scores from ileal samples (see methods for details). Data are mean±SEM of 6-7 animals per group. *: $P<0.05$ vs respective control group. #: $P<0.05$ vs inflamed barrier/conventional group (ANOVA, body weight, and non-parametric ANOVA). Arrows in A and B denote the two indomethacin or vehicle treatments (experimental days 0 and 2).

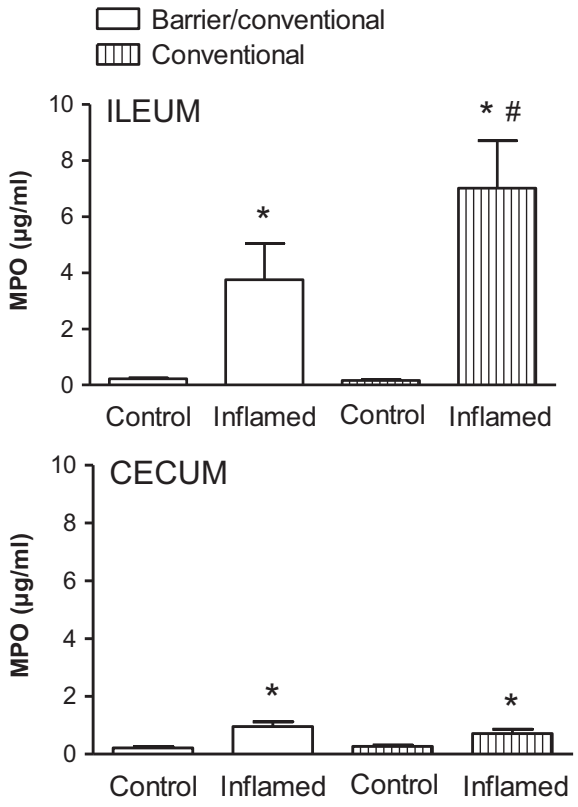


Figure 2. Ileal and cecal levels of myeloperoxidase (MPO) in the different experimental groups, at the time of necropsy (experimental day 4). Data are mean±SEM of 6-7 animals per group. *: P<0.05 vs respective control. #: P<0.05 vs inflamed barrier/conventional group (ANOVA).

Microscopic assessment of inflammation

Vehicle-treated rats, regardless the group considered, showed no signs of intestinal inflammation, with essentially normal histological features. On the other hand, indomethacin-treated groups showed microscopic signs of inflammation and microscopic scores were significantly higher than those in control animals. The more common histopathological changes observed were alterations of the epithelial structure, with variable degree of destruction of the villi, and the presence of local or generalized inflammatory infiltrate. Ulcers were observed only in 1 out of 5 animals (20%) in the barrier/conventional group and in 3 out of 7 animals (43%) in the conventional group.

Total inflammatory scores were of similar magnitude in conventional and barrier/conventional animals (Fig. 1D). One animal of the barrier/conventional group was not scored because of technical problems during the processing of the tissue.

Myeloperoxidase levels

As shown in Fig. 2, in caecal and ileal samples from barrier/conventional and conventional control animals MPO levels were similar, and relatively low, regardless the area considered. Indomethacin treatment resulted in significant increases in MPO levels. In the caecum, MPO levels were increased by 5-fold (P<0.05) and 4-fold (P<0.01) over controls in the barrier/conventional and the conventional groups respectively (Fig. 2). Similarly, in the ileum, MPO levels increased by 17-fold (P<0.05) and 40-fold (P<0.001) over controls in barrier/conventional and conventional animals, respectively (Fig. 2). Interestingly, ileal levels of MPO were higher in indomethacin-treated conventional animals than in barrier/conventional indomethacin-treated animals (P<0.05) (Fig. 2).

Characterization of the luminal microbiota by FISH

Total ileal bacteria, determined by FISH as EUB338-positive cells, was higher in the control conventional group compared with the control barrier/conventional group (P<0.01) and, in both groups, showed a tendency to increase during inflammation (Table 1). However, in the caecum, total bacteria were similar in all experimental groups (Table 1). Four days after the induction of inflammation the overall composition of

Table 1. Ileal bacterial counts, as determined by FISH, in the different experimental groups.

	Barrier/ conventional Control (x10^8 cells/ml)	Barrier/ conventional Inflamed (x10^8 cells/ml)	Conventional Control (x10^8 cells/ml)	Conventional Inflamed (x10^8 cells/ml)
Total bacteria	13±5	67±47	31±3 #	76±30
Enterobacteriaceae	0.02±0.005	15±10 $	0.1±0.05	28±13 *
Bacteroides spp	0.04±0.002	20±13 $	0.8±0.4 #	19±11 &
Clostridium cocoides Cluster Iva	ND	2±0.4 *	ND	12±3 * #
Bifidobacterium spp	0.06±0.03	0.7±0.3 *	4±1 #	4±2
Lactobacillus and *enterococcus* spp	5±2	2±1	8±2	2±1 *

*:P<0.05 vs respective control. #: P<0.05 vs same treatment in barrier/convntional group.$: P=0.08 vs respective control. &: P=0.06 vs respective control (non-parametric ANOVA).
ND: Not detected

Table 2. Cecal bacterial counts, as determined by FISH, in the different experimental groups.

	Barrier/ conventional Control (x10^8 cells/ml)	Barrier/ conventional Inflamed (x10^8 cells/ml)	Conventional Control (x10^8 cells/ml)	Conventional Inflamed (x10^8 cells/ml)
Total bacteria	273±48	273±52	298±33	246±37
Enterobacteriaceae	0.02±0.003	20±9 *	0.03±0.003	33±10 *
Bacteroides spp	11±2	45±10 *	14±1	36±12
Clostridium cocoides Cluster Iva	47±7	31±14	39±7	48±12
Bifidobacterium spp	1,6±0.7	4,6±3.0	3,1±1.5	5,7±1.9
Lactobacillus and *enterococcus* spp	6,9±2.8	3,9±2.0	3,8±0.9	12,8±4.7 * #

*:P<0.05 vs respective control. #: P=0.06 vs Inflamed barrier/conventional group (non-parametric ANOVA).

the luminal microbiota changed slightly in the caecum and more strikingly in the ileum.

In control conditions, luminal ileal microbiota was characterized by significantly higher counts of *Bifidobacterium* spp (BIF164 probe), *Bacteroides* spp (BAC 303 probe) and enterobacteriaceae (ENT-D probe) in the conventional group compared with the barrier/ conventional (Table 1), while the *Clostridium coccoides-Eubacterium rectale* group (*Clostridium* cluster XIVa, EREC 482 probe) were undetectable in either group. During inflammation, and regardless the experimental group considered, a general increase in all bacterial groups determined, except *Lactobacillus* spp and *Enterococcus* spp, was observed. The main finding was the appearance of the *Clostridium coccoides-Eubacterium rectale* group (*Clostridium* cluster XIVa, EREC 482 probe) at levels comparable to those of the other bacterial groups assessed (Table 1).

Composition of the luminal caecal microbiota was similar in the conventional and de barrier/conventional control groups (Table 2). During inflammation, similar quantitative changes in the microbiota were observed in conventional and barrier/conventional animals (Table 2). The main changes were observed in Gram negative bacteria (*Bacteroides* spp and enterobacteriaceae). The major variation was observed for enterobacteriaceae population (ENT-D probe), which significantly increased during inflammation in both experimental groups (Table 2). On the other hand *Bacteroides* spp (BAC 303 probe) increased significantly in the barrier/conventional group while only a moderate increase, without achieving statistical significance, was observed in the conventional group (Table 2). Among Gram positive bacteria, only *Lactobacillus* spp and *Enterococcus* spp (LAB 158 probe) showed an increase during inflammation in the conventional group (Table 2).

Ecological characterization of the luminal microbiota: t-RFLP analysis

The similarity indexes of the t-RFLP profiles, illustrated in the form of a dendogram, of the caecal microbiota in the different experimental groups are shown in Fig 3A. Overall, the dendogram obtained showed two clearly separate clusters corresponding to the barrier /conventional and conventional groups in control conditions. The inflamed groups, either conventional or barrier/conventional, were partially overlapped forming a third, intermediate, cluster (Fig. 3A).

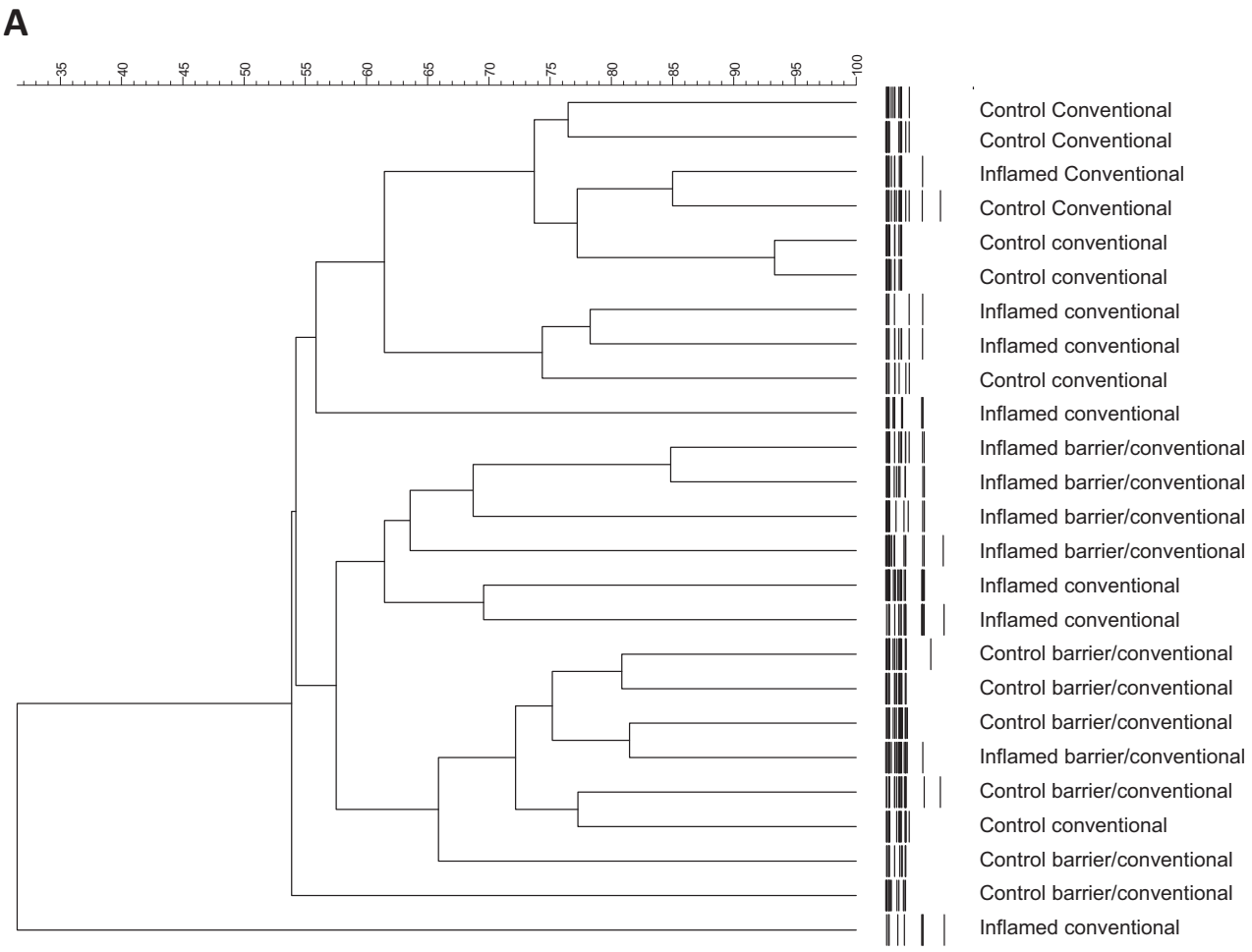
Nevertheless, the overall biodiversity of the microbiota was similar in the four experimental groups, with an average number of t-RFs (taken as a measure of biodiversity) witch varied from 14 to 35 among the different experimental groups (Fig 3B).

Bacterial wall adherence

A DAPI staining showed the presence of bacteria adhered to the ileal wall in all experimental groups (data not shown). Table 3 shows the incidence of wall adherence for the bacterial groups determined by FISH in the different experimental groups. In control conditions, only *Lactobacillus* spp and *Enterococcus* spp (LAB 158 probe) were adhered to the ileal wall. However, during inflammation the incidence of bacterial adherence increased and practically all bacterial groups assessed were found, in different degree, adhered to the ileal wall, regardless the experimental group considered. During inflammation, the more striking findings were the high incidence of adherence of enterobacteriaceae (ENT-D probe) (85-100 % of incidence) and the fact that adhesion of *Clostridium cocoides* Cluster IVa (EREC 482 probe) was only observed in the conventional group (5 out of 6 animals) (Table 3).

Discussion

In the present study, we assessed gut inflammatory responses and simultaneous changes in GCM in a model of indomethacin-induced acute enteritis in rats bred in different microbiological conditions. Results obtained show that severity of indomethacin-induced acute intestinal inflammation was comparable in animals maintained in conventional conditions and



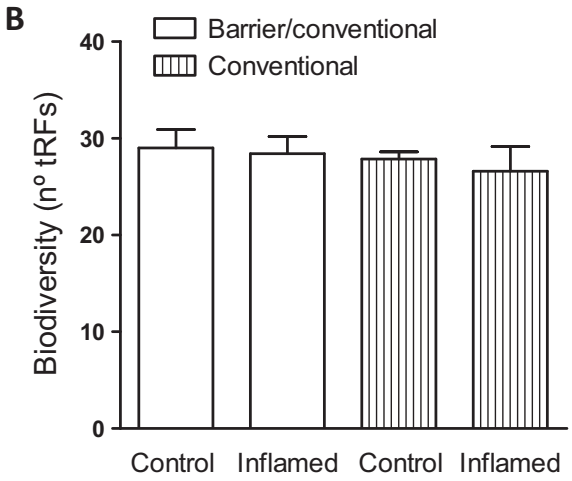


Figure 3. A: Dendogram illustrating the clustering of the different experimental groups according to the t-RFLP banding patterns obtained from the analysis of the cecal microbiota. Notice how control groups are separated (conventional in the upper part and barrier/conventional in the lower part of the dendogram), suggestive of a different microbiota, while inflamed groups have an intermediate position, thus indicating similar inflammation-associated changes in GCM. B: Overall biodiversity among the different experimental groups, as determined from the f-RFLP analysis.

animals bred under barrier conditions but adapted to conventional conditions for a 3-week period. However, GCM, particularly as it refers to the Ileum, was different in these two experimental groups and was further modified during acute inflammation.

During the last years, the focus of microbial etiology has shifted from infectious to commensal agents. A substantial body of evidence has accumulated suggesting that commensal microbiota plays a key role in the development of intestinal immune and inflammatory responses and has, therefore, been included as an important pathogenic component of IBD.[14,15] Total microbial count in the caecum was similar in animals bred in conventional conditions and in animals bred in barrier conditions adapted for 3 weeks to conventional conditions (barrier/conventional), and within the range previously described for the same experimental conditions.[5] However, total ileal microbiota was reduced in number in barrier/conventional animals. This might reflect local differences in the adaptation process of the microbiota between ileum and caecum. As previously described by us,[5] adaptation to conventional conditions of barrier-bred animals implies a qualitative shift in microbiota from the original implantation bacteria towards a conventional-like situation. This was

Table 3. Incidence of bacterial adherence to the ileal wall in the different experimental groups.[a]

	Barrier/ conventional Control	Barrier/ conventional Inflamed	Conventional Control	Conventional Inflamed
Enterobacteriaceae	0/5	5/5 **	0/6	6/7 **
Bacteroides spp	0/5	3/6 +	0/7	1/7
Clostridium cocoides Cluster IVa	0/5	0/5	0/6	5/6 ** #
Bifidobacterium spp	0/6	1/5	0/5	4/6 *
Lactobacillus and *enterococcus* spp	2/5	1/5	4/6	5/7 #

[a]:Results are expressed as positive cases/total number of rats.
*: P<0.05 vs. respective control. **: P<0.01 vs respective control. +: P=0.06 vs respective control. #: P<0.01 vs Barrier/conventional inflamed (Chi-square test).

particularly evident in the caecum, where microbiota was essentially identical between conventional and barrier/conventional animals. However, in the ileum, the microbiota was quantitatively different, being the relative abundance of *Bifidobacterium* spp, *Bacteroides* spp and enterobacteriaceae higher in the conventional than in the barrier/conventional group. These differences in microbiota composition were corroborated by the t-RLFP analysis. Although performed only for the caecal microbiota, the t-RFLP analysis clearly grouped barrier/conventional and conventional animals in two separate clusters in the corresponding dendogram (Fig. 3A). In general, these variations in microbiota agree with our previous observations in similar experimental conditions.[5]

The most interesting observation is that microbiota changed both qualitatively and quantitatively during acute gut (ileal) inflammation. Microbial changes were observed both in ileum and caecum, although were more prominent in the ileum, corresponding to the primary site of inflammation in the indomethacin model. The main change in the caecum was an increase in the cell count of enterobacteriaceae, which was similar in conventional and barrier/conventional animals. On the other hand, in the ileum, *Bacteroides* spp and enterobacteriaceae increased and *Clostridium* cluster XIVa became a very significant component of the microbiota, while being undetectable in control conditions. Interestingly, as mentioned above, during inflammation total bacteria was not altered in the caecum, while in the ileum showed a tendency to increase in both conventional and barrier/conventional animals. Overall, these observations agree with data suggesting that during pathophysiological states gut microbiota changes both qualitatively and quantitatively.[16] Moreover, this suggests that indomethacin-induced gut inflammation does not imply an indiscriminate bacterial overgrowth, but a selective imbalance of the normal microbiota. This agrees with previous observations in several models of gut inflammation, suggesting that inflammation results in selective changes of GCM.[17-20] However, it is worthy to mention that the significant increase in the counts of *Bacteroides* and *Clostridium* contrasts with previous reports indicating that these bacterial groups were relatively rare during indomethacin-induced enteritis in rats.[20]

Enterobacteriaceae, and specifically *Escherichia coli*, have been associated with the aggravation of colitis.[21] Interestingly, the enterobacteriaceae genus was increased during inflammation, with conventional animals showing a tendency to have higher counts than barrier/conventional. Moreover, bacterial adherence data showed that virtually all inflamed animals were positive for Enterobacteriaceae adherence. These observations also agree with previous data demonstrating a direct involvement of *E. coli* in indomethacin-induced enteritis in rats.[6,17] Likewise, *Bacteroides* spp and *Clostridium* spp (cluster XIVa) have been associated with gut inflammation.[22,23] In general, this agrees with the prominent increase observed in these groups after indomethacin treatment and also with the presence of significant bacterial adherence in inflamed animals. Overall, these observations indicate that these bacteria may have a role in the inflammation process, as previously suggested.[17,22,23]

Indomethacin administration induced an inflammatory response that was evident in the ileum and less clear in the caecum. This agrees with the responses previously characterized by us using this model of enteritis in rats, which results in the induction of ileitis.[6] Disease activity parameters evaluated suggest a comparable inflammatory response in conventional and barrier/conventional animals, although some parameters evaluated were slightly higher in conventional than in barrier/conventional animals (i.e. health condition, macroscopic score or ileal MPO levels). Many adaptive differences might account for this slightly higher response in conventional animals. The variations in gut microbiota, particularly within the ileum, might be one the factors contributing to this different susceptibility to inflammation. This agrees with a growing body

of evidence suggesting that gut microbiota is a key component modulating inflammatory responses within the gut.[2,24,25] In general, a restricted, or even absent (such as the case of germ free animals), gut microbiota is regarded as a protective condition against inflammation while a rich and diverse microbiota is considered as proinflammatory.[26] Our results partially agree with this view. In the present experimental conditions, conventional and barrier/conventional animals showed a similar ecological diversity, as established by the t-RLFP analysis. However, conventional animals showed a clear tendency to have a higher number of total microbial counts when compared with barrier/conventional animals, and had higher counts of the main bacterial groups characterized, namely, *Bifidobacterium* spp, *Bacteroides* spp and enterobacteriaceae. Together with the disease activity indices recorded, these observations might indicate higher susceptibility towards inflammation in animals bred and maintained in conventional conditions versus animals bred in barrier conditions and adapted for a short period of time to conventional conditions. This agrees with previous data showing that intestinal inflammation was aggravated in mice adapted to conventional conditions versus those maintained under controlled conditions.[27]

In summary, we showed that indomethacin-induced acute ileitis, in rats bred and maintained in conventional conditions or in rats bred in barrier conditions briefly adapted to a conventional microbiological environment, is associated to specific changes in GCM and bacterial wall adherence. Interestingly, animals bred in conventional conditions showed a tendency to have a worse inflammatory response when compared to adapted animals and also showed a higher incidence of bacterial adherence. These observations confirm a role of GCM modulating intestinal homeostasis and its involvement in the pathogenesis of IBD. Furthermore, differences between conventional and barrier/conventional animals suggest a certain imprinting in barrier animals, likely associated to the characteristics of the originally implanted microbiota, that might modulate inflammatory responses, despite the adaptation to a new microbiological status. These observations indicate that microbiological conditions of the animals has an impact in the outcome of studies on gut inflammation and that standardization of the microbiological status of the animals might help to understand differences among gut inflammation studies.

Acknowledgements

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Improvement of a historical animal model for Crigler Najjar Type I syndrome: development of the normobilirubinaemic *JJ* genotype as a true control for the Gunn jaundiced rat

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Abstract

In 1938, Gunn described in a Wistar rat stock a recessive mutation resulting in jaundice. Further characterization ascribed the loss-of-function mutation to the bilirubin UPD-glucuronyl transferase enzyme, thus providing an animal model for the human inherited jaundice: the Crigler-Najjar syndrome. The Gunn genotype is indicated by the letter *j* for jaundice: *J* stands for the wild-type dominant allele and *j* for the mutant recessive one.

At present, many strains of the Gunn rat exist, in which animals are kept in the *jj* and *Jj* state, while the original *JJ* genotype has been lost. We purchased Gunn rats from a commercial vendor, but without receiving clear information about the original *JJ* strain; after the initial trials, we concluded that neither the Wistar stock nor the *Jj* Gunn rats - which appear normobilirubinaemic – were suited as controls. In this work, we describe the development of the normobilirubinaemic *JJ* Gunn rat through a process of multiple in-crosses and refer its characterization in terms of biometric and reproductive data. The potential of the novel *JJ* control for jaundiced rats in biochemical and clinical studies of bilirubin metabolism is also discussed.

Keywords: rats, hyperbilirubinaemia, Gunn control strains

In 1938, Gunn described a recessive mutation resulting in jaundice in a Wistar rat stock[1]. Further characterization ascribed the loss-of-function mutation to bilirubin UDP-glucuronyl transferase, thus providing an animal model for human inherited jaundice or Crigler-Najjar syndrome[2]. The Gunn genotype is indicated by the letter *j* that represents the mutant recessive allele causing jaundice, while *J* indicates the wild-type dominant allele. Because of the difficulty to distinguish *JJ* from *Jj*, being both nonicteric and in order to maximize the production of jaundiced *jj* animals, the Gunn strain was maintained by mating *Jj* females with *jj* males since the beginning. This mating system has led to the loss of the original wild type *JJ* genotype and, consequently, to the need of generating new congenic Gunn strains. We purchased *jj* and *Jj* Gunn rats from a commercial vendor, but no data were available about the original *JJ* strain; after initial experiments, we concluded that neither the animals from a Wistar stock bred in our facility at the University of Trieste nor *Jj* rats, which appear normobilirubinaemic, were appropriate controls. To obtain a suitable control for the Gunn strain, the normobilirubinaemic *JJ* rat was developed through a process of intercross followed by multiple in-crosses. Herein, we present its characterization in terms of biometric and reproductive data. The potential of the novel *JJ* control for jaundiced rats in biochemical and clinical studies focusing on bilirubin metabolism is discussed.

Animals

The Gunn rats used as founders were purchased from Harlan Laboratories, Inc. (Indianapolis, IN); the colony was established at our facility in 2005 (with 5 *jj* males and 6 *Jj* females) and was maintained by mating hyperbilirubinaemic *jj* males with heterozygous *Jj* females. The breeders were randomly chosen from the population without maintaining strict inbreeding. The hyperbilirubinaemic *jj* Gunn rats, genetically lacking the activity of the hepatic enzyme uridine glucuronyl tranferase 1A1 (UGT1A1 -/-), have a definite yellow tinge, while the *Jj* heterozygotes are white. The Wistar rats used as a control were from our outbred colony (Animal Facility, DSV, University of Trieste, Italy), derived from founders (Wistar Han™) purchased at Harlan Laboratories (S. Pietro al Natisone, Udine, Italy).

Materials and methods

Breeding conditions and procedures complied with EU guidelines (86/609/CE) and current Italian law (decree 116/92): temperature 20°C (2.5); relative humidity 45%-55%; ventilation 10-15 complete air changes per hour; light/dark cycle 12h/12h; pelleted food (Harlan Teklad 2018 and vitamin-enriched Harlan Teklad 2018S) and water *ad libitum*. As animals spontaneously develop the hyperbilirubinaemic phenotype and no experimental treatments have been applied, additional ethical approval was not required. Phenotypic analysis (consisting in careful inspection of the pups for the Mendelian ratio between phenotypes and strict recording of the pedigree and progeny of each breeder) was constantly carried out to compensate for the absence of genotypic characterization.

The normobilirubinaemic *JJ* rat was obtained through one intercross between heterozygous *Jj* animals (1 male and 2 females) followed by multiple in-crosses among animals of filial generations. Animals of both sexes from the F1 filial generations were screened by backcrossing with *jj* males and *jj* or *Jj* females, expecting to find at least some yellow *jj* pups in the case where the parent was heterozygous, and none if the parent was homozygous *JJ*; for each *JJ* backcrossed animal not less than 20-25 pups were considered. Once *bona fide JJ* animals were identified, they were in-crossed (*JJ* x *JJ*) to expand the number of breeders; before continuing the in-crosses, the F2 animals of both sexes were again backcrossed as above to ascertain their genotype. Randomly chosen animals from the F3 generation were mated to start 5 lines; from these, only one line is currently propagated, arriving at F9.

For the 4 different genotypes (*JJ* of the F9 generation, *Jj* and *jj* of the Gunn colony and the Wistar stock), we determined the number of pups/mother and the number of postnatal dead pups/mother for the first and the second pregnancy. A perinatal growth curve was plotted, weighing 49 pups of both sexes at age P2, P4, P7 and P14.

Plasma total bilirubin concentration (TBC) was determined at ages P2, P9, P17 and P60. After isoflurane anesthesia, a heparinized sample of blood was collected by jugular puncture from each single animal. Rats were then sacrificed by decapitation. Bilirubin was measured at the local children's hospital by a diazo-based reaction[3]. All procedures were performed under dim light to minimize photo-oxidation of bilirubin. Statistical differences within the three genotypes (*JJ*, *Jj* and *jj*) and controls (Wistar) were analyzed using a paired students' t-test for ANOVA and a Newman-Keuls post-hoc test.

Development of bona fide *JJ* animals and genotyping through Mendelian backcrossing

From the *jj* x *Jj* pair (Fig. 1), the only possible genotypes were *jj* and *Jj*; the offspring – till postnatal day (P)15 – could be easily ascribed to one or the other genotype on the basis of phenotype (bilirubinaemia, skin color). While jaundiced rats presented a mean bilirubinaemia of 6.60 (1.46) mg/dl at age P60 (*N*=40 males), the *Jj* animals were normobilirubinaemic (mean bilirubinaemia 0.18 (0.06) mg/dl at age P60; *N*=59 females). Therefore, we started with heterozygous breeders (parental P generation - 1 male and 2 females) from the *jj* x *Jj* mating; according to Mendelian rates, we expected that 33% (25/75) of the white progeny would be *JJ* and 66% (50/75) would be *Jj*. In the F1 filial generation, we obtained 3 yellowish pups, immediately culled, and 13 white pups; of these, 6 were males and 7 were females. After 8 weeks, rats were backcrossed with *jj* or *Jj* animals to determine the genotype.

According to these results, we identified 3 males and 2 females as *bona fide* homozygous *JJ* animals (5/13, 30.8%), in agreement with what expected. We then chose one male and one female as founders of the *JJ* strain; the resulting offspring - the F2 filial generation - comprised 5 males and 5 females. All these 10 animals were backcrossed with *jj* males and *jj* or *Jj* females to further confirm the absence of the recessive allele. Two males and two females were chosen as breeders and mated. From the resulting

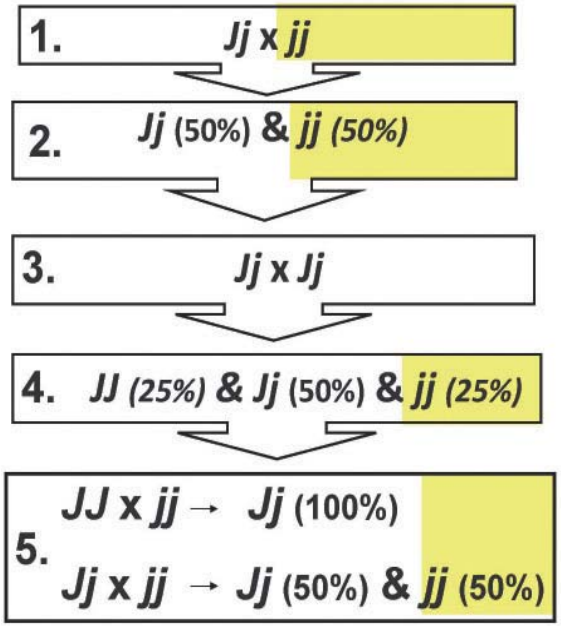

Figure 1. Development of the JJ control strain.
1. Breeding strategy to maintain the colony;
2. Possible genotypes in the filial generation;
3. Intercross to develop the JJ control strain;
4. Possible genotypes in the filial generation;
5. Backcrosses for genotype test.

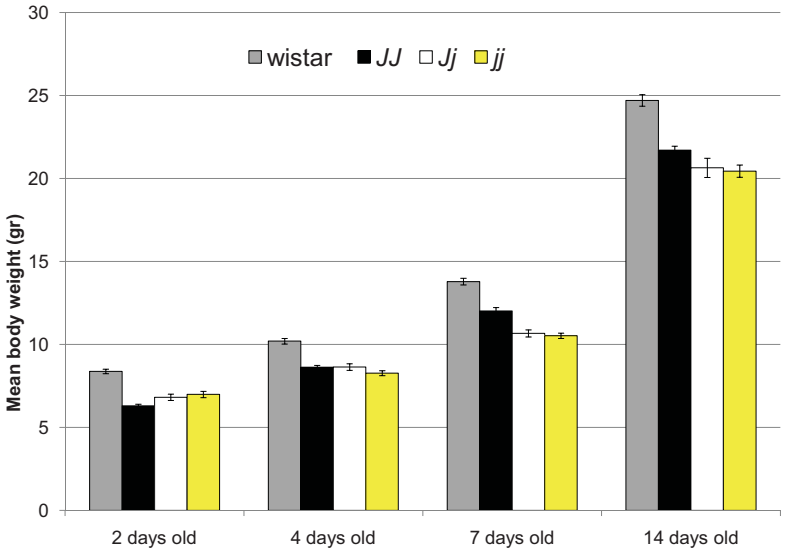

Figure 2. Biometric data (body weight in grams) of the four genotypes. Brackets indicate standard deviation.

offspring (F3 filial generation) 5 lines were derived; all lines were carried on always choosing one couple (brother x sister) for each line. All the 5 lines were kept, at first, in the case that a recessive lethal mutation present in the founders emerged in homozygosity through this strict inbreeding in some animals. At present, we have maintained only one line arrived at F9 - which has proven to be the most prolific.

Results

Reproductive data

According to the Harlan guidelines, the breeding strategy to maintain the Gunn rat colony consists in mating one *jj* male with one *Jj* female. We confirm the data obtained from the vendor as *jj* males are fertile in spite of hyperbilirubinaemia, and *Jj* females are fertile and prolific mothers (83% of pregnant females). *jj* females were also mated for the backcrosses of control, but showed reduced fertility: only 41% of mated females resulted pregnant. Reproductive data of the *jj*, *Jj* and *JJ* mothers are shown in Table 1 together with the data regarding the Wistar females. As expected, both the number of pups per *jj* mother and the number of postnatal dead pups per *jj* mother were significantly different in comparison with the *Jj*, *JJ* and Wistar mothers.

Table 1. Reproductive data of the Gunn (*JJ*, *Jj* and *jj*) and Wistar rats. Newman-Keuls test: **P<0.0005; *P<0.01

genotype		first pregnancy	
		pups/mother	dead pups/ mother
	N	**mean (SD)**	**mean (SD)**
Wistar	58	10.16 (2.94)	0
JJ	40	9.58 (2.25)	0.73 (2.06)
Ji	64	9.53 (2.62)	0.30 (1.19)
jj	8	5.25** (2.38)	1.75* (2.31)
		second pregnancy	
		pups/mother	**dead pups/ mother**
	N	**mean (SD)**	**mean (SD)**
Wistar	48	10.83 (2.86)	0
JJ	12	9.00 (2.05)	0.45 (0.93)
Ji	35	9.74 (2.76)	0.31 (0.68)
jj	1	-	-
		total	
		pups/mother	**dead pups/ mother**
	N	**mean (SD)**	**mean (SD)**
Wistar	106	10.47 (2.93)	0
JJ	52	9.45 (2.20)	0.67 (1.87)
Ji	99	9.60 (2.66)	0.30 (1.03)
jj	9	5.22** (2.22)	2.11* (2.42)

Biometric data

Perinatal body weight curves are shown in Fig. 2. At each age, Wistar rats showed a significantly higher (P<0.0005) weight increase compared to Gunn rats and controls. At 7 days of age, *JJ* rats showed significantly (P<0.001) more weight gain than *jj* and *Jj* Gunn rats. The *Jj* and *jj* rats did not evidence significant differences in the growth rate up to P14.

Total bilirubin concentration (TBC)

As expected, at each age *jj* rats have a higher (P<0.005) level of TBC than the other genotypes (*JJ*, *Jj* and Wistar) (Fig. 3). Concerning the *jj* rats, no significant differences were observed, except for the TBC level reported at P60, which was lower (P<0.01) than that at P17. It has to be noted that *Jj* rats showed a perinatal (P2 and P9) hyperbilirubinaemia significantly (P<0.005) different from the TBC of *JJ* rats at the same age. From age P17 to adulthood, the bilirubinaemia progressively declines in both genotypes (*Jj* and *JJ*) to similar values. TBC values of age-matched Wistar rats, with the exception of a neonatal peak at 2 days of age, were comparable to those seen in *JJ* rats. From age P9 onward, Wistar rats showed a lower (P<0.05) and constant level of TBC.

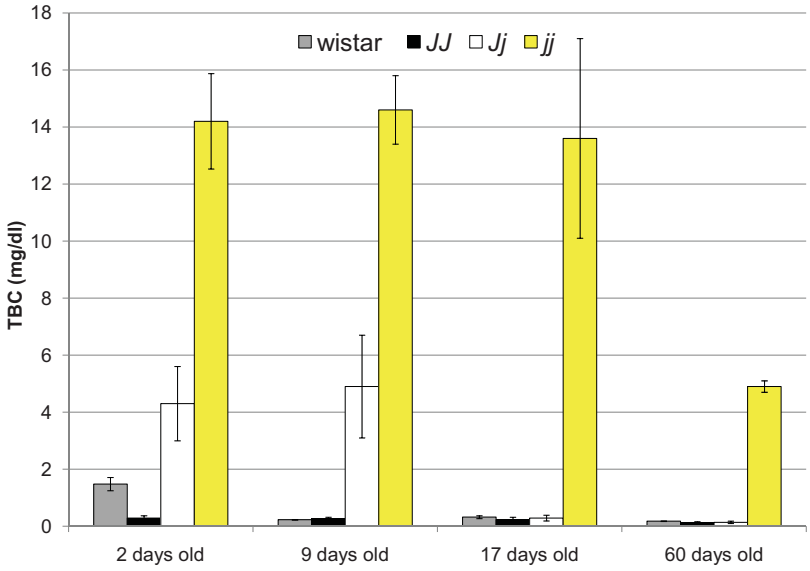

Figure 3. Plasma TBC (mg/dl) during post-natal development. Brackets indicate standard deviation.

Discussion

We present the characterization of a new normobilirubinaemic rat strain, derived from the Gunn jaundiced rat, in which UDP glucuronyl transferase activity has been fully restored. The need for such control derived from the scientific consideration that neither the *Jj* Gunn rat - although normobilirubinaemic - nor the genetically unrelated Wistar rat could be accepted as standard references. In fact, the heterozygous *Jj* animal still carries one mutated, nonfunctional bilirubin UDP glucuronyl transferase allele and the single functional allele does not completely restore normal bilirubin metabolism. Our results concerning the hyperbilirubinaemia observed in young *Jj* seem to confirm this hypothesis. Moreover, the literature reports altered metabolic features concerning the heterozygous *Jj* animals since the preliminary description of the Gunn strain[1,4]. The consideration about the use of the Wistar stock as a control is even more general. Even if the jaundice mutation was described in a breeding stock of Wistar origin (Connaught Laboratories, University of Toronto, Canada)[1], the new mutant strain was subsequently mated according to a monogamous scheme between *Jj* and *jj*[5,6], thus increasing the genetic distance from the original Wistar stock at each generation. The existing difference between the two rats is confirmed by the data collected in this paper.

The perinatal growth curves for the 4 genotypes considered (*jj* and *Jj* Gunn, *JJ* control and Wistar) appeared significantly different, with the "Gunn block" on the low side and the Wistar on the high side. This difference may be ascribed more to the genetic background than to the mutated allele, being the Gunn an inbred strain and the Wistar an outbred stock. Therefore, the use of Wistar rats as controls for the Gunn strain is at least questionable. To bring the mutated allele in a known genetic background, many laboratories started to in-cross *jj* rats with other strains (RHA/N[7], SDGR[8], R/Apfd[9], Wistar-Imamichi[10], LA Wistar[11], ACI/N[12], Wistar-Shi[13]). If on the one side this mating system has made possible the comparison of the *JJ*, *Jj* and *jj* genotypes in a well defined genetic background, on the other side it has generated such a variety of different Gunn strains to bias data reproducibility among laboratories. These considerations moved us to develop the new *JJ* strain.

Since the single point mutation of the bilirubin UDP glucuronyl transferase can be detected only through sequencing, and it was not deemed useful to develop an in-house technique for that, we utilized Mendelian backcrosses with parental *jj* animals and phenotypic analysis to ascertain the genotype of the filial generations. We performed backcross analyses both for the F1 and F2 filial generations to ensure the absence of the mutated allele in the new strain.

Plasma TBC is the most evident finding that reflects the importance of the obtained results. While *jj* animals showed pronounced hyperbilirubinaemia (13.60-14.60 mg/dl) from birth to age P17, declining to a persistent hyperbilirubinaemia (4.90 mg/dl) throughout their life, the *Jj* rats showed a less pronounced postnatal jaundice, that decreased from day 9 of age onwards, reaching at P17 the same values found in Wistar rats. Initially this led us to consider the *Jj* animal as a normobilirubinaemic control, in view of the fact that also the Wistar rat showed a short-lived and mild jaundice at P2, resolving at P9, which occurs in other rat strains as well[14]. On the contrary, the data collected from *JJ* rats show that the animals do not present postnatal hyperbilirubinaemia. To explain that, we hypothesize that hyperbilirubinaemia itself has been a selective factor for the Gunn strain. To cope with it, animals must have invoked some other mechanism(s) in the metabolic pathway of bilirubin allowing normobilirubinaemia in *Jj* animals. When UDP glucuronyl transferase activity was fully restored in *JJ* animals, this enhanced ability to clear bilirubin may have given an increased ability to cope with neonatal jaundice compared to *Jj* Gunn and normobilirubinaemic Wistar rats.

In conclusion, we have produced a *JJ* rat that is suitable as gold standard for experiments involving the Gunn rat which originated at Harlan, and have

demonstrated the unreliability of the use of other unrelated stocks as controls. A possible hereditary mechanism of bilirubin clearance that acts during the first days after birth in Gunn rats is also supposed and deserves further investigation.

Acknowledgements

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Housing and Welfare

Bedding preferences of group-housed female laboratory mice for different bedding materials

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Abstract
Bedding is a very important factor in small rodents' husbandry influencing hygienic, pharmacological, behavioural and consequently statistical parameters. Although a broad variety (different materials associated with various forms and structures) are on the market, there is a lack of information about the preference of group-housed mice. Thus, the aim of this study was to investigate the bedding preference of group-housed female mice, dealing with the structural aspect (shavings versus chips, experiment 1) and with the material aspect (softwood versus aspen wood, experiment 2). Female BALB/c and C57BL/6J mice were kept in stable groups of three. The one-week preference test was performed in a double-cage composed of two makrolon Type IIL cages, connected by a perspex tunnel. Individual crossings between the two cages were detected automatically by the system during the entire experiment. The crossing frequency and the dwelling times animals spent in cages 1 and 2 were calculated automatically. In experiment 1, mice significantly preferred the shavings in all three test combinations (ranking: shaving > coarse-grained chip > fine and medium chips). A significant aspen preference was detected in three out of four test combinations in experiment 2. During both experiments the bedding preferences were more explicit during the resting time of animals. Our results suggest that aspen bedding may be beneficial for female mice and hence should be recommended, ideally with a shaving structure. Should the use of shavings not be possible, coarse-grained chips should be used rather than fine chips.

Keywords: social housing, female mice, bedding, structure, preference test

Bedding materials have been offered for laboratory rodents for a long time. Therefore, it is beyond dispute that bedding materials are as scrutinised as most other items used in animal research.

Most previous studies have focused on bedding characteristics, such as moisture absorbent (urine/faeces), ammonia/hydrogen hygienic quality, dust free and toxicity, or on the influences of bedding materials on experimental results.

Currently, bedding material is further considered to provide animals with a comfortable substrate and allow animals to nest, dig and rest comfortably, due to the concern for refinement. Thus, a lot of commercial bedding (different materials associated with different structures) is available for laboratory rodents. Besides commonly used softwood products there is an increasing range of aspen (*Populus tremula*) products.

By increasing knowledge about animals' needs, preference tests can help to improve animal welfare in laboratory animal husbandry. Choice test has often been performed for evaluating bedding products and for understanding the preference of animals[1,2,3,4]. Various studies have proved the importance of bedding material for laboratory rodents[1,2,3]. Other studies concluded that rodents prefer solid flooring with bedding to wire flooring[2,6,7] or animals prefer a bedding material suitable for nest construction, when nest material is not provided [8,9,10,11].

However, this information is still very limited. Moreover, animals in previous studies concerned with bedding preferences of laboratory rodents were always tested individually. Further, none of these studies clearly distinguished structure-related bedding preferences from material-related ones[5,6,11,12]. As social species like mice are stressed when being isolated[13], data from individually tested animals cannot be transferred to group-housed animals[14].

In order to improve housing conditions for laboratory mice, the present study focused on structure-related (experiment 1) and material-related (experiment 2) bedding preferences of group-housed female mice.

Animals

Female mice of two inbred strains (BALB/cOlaHsd and C57BL/6JOlaHsd, Breeder: Harlan Netherland) were tested, a total of 108 for experiment 1 and a total of 144 for experiment 2. Animals arrived at 3 weeks of age and were kept in stable groups of three during the entire experimental period. From 3 to 5 weeks of age mice were housed in single Makrolon-cages (Type II long) and moved to the test system (described below) at 6 weeks of age. The preference test was carried out following two weeks of adaptation to the test system. The housing conditions were as follows:

- Light/dark cycle: 12h/12h, light intensity: 65 +/- 5 Lux (at DoubleCage-sensor)
- Relative humidity: 55 +/- 10 %, temperature 22 +/- 2 °C, air change: 10-16 times per h

The animals' SPF-status was assured by health monitoring according to FELASA recommendation.[15]

Materials and Methods

The one-week preference test was performed in the DoubleCage test system (see Figure 1). The DoubleCage system was designed in cooperation with the University of Zurich to meet the requirements of our institute. Two macrolon-cages (type II long), cages 1 and cage 2, were connected by a perspex tunnel (length 30 cm, inner diameter 2.4 cm) with a microchip sensor on both sides.

Each cage contained water and food (Altromin N. 1324) *ad libitum* in the lid and bedding (2 cm deep) according to the particular test combination (see Tables 1 and 2). Mice were able to move freely between the two cages during adaptation and preference testing. Since animals were equipped with a subcutaneous microchip, each individual mouse could be detected automatically by the DoubleCage system: Animals' crossings (in seconds, real-time-data) between the two cages were registered by microchip sensors and transferred to the computer. The bedding combinations tested in experiment 1 and experiment 2 are shown in Table 1 and Table 2.

Table 1. Experiment 1: Bedding types in test combinations A-C

Combination	Cage 1 (Softwood)	Cage 2 (Softwood)
A	Shavings	Chips fine
B	Shavings	Chips medium
C	Shavings	Chips coarse-grained

Table 2. Experiment 2: Bedding types in test combinations A-D (Within every combination, the size/ structure of bedding products was identical, except for combination A, due to the fact that no equivalent aspen bedding was available. Aspen shavings were larger, longer and coarser than softwood shavings.)

Combination	Cage 1 (Softwood)	Cage 2 (Hardwood)
A	Shavings	Shavings
B	Chips fine	Chips fine
C	Chips medium	Chips medium
D	Chips coarse-grained	Chips coarse-grained

During the test a personal computer registered the individual number of animals crossing, crossing direction and crossing time (seconds). Thus, crossing frequency and dwelling times for cages 1 and 2 were automatically measured during the entire experiment. Bedding preferences were inferred from dwelling times in cages 1 and 2. Additionally, data were divided into light and dark period (12hours/12hours) and strain for further analyses.

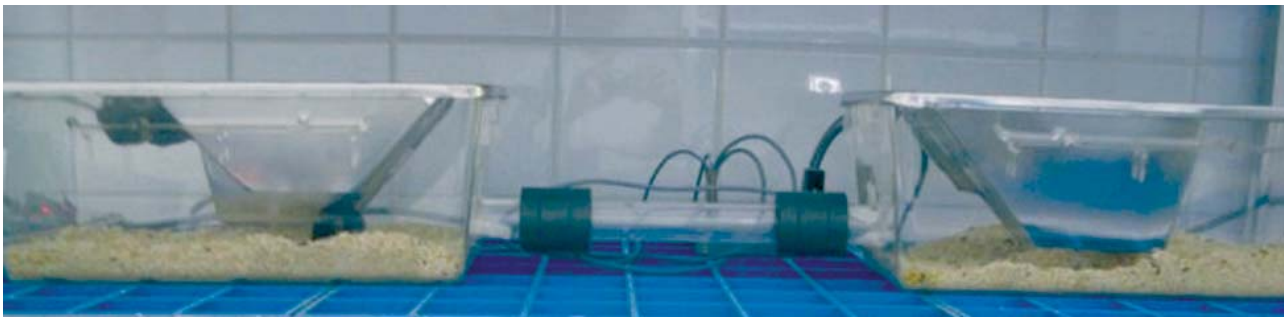

Figure 1. DoubleCage test unit: cages 1 and 2, perspex tunnel with two black sensors

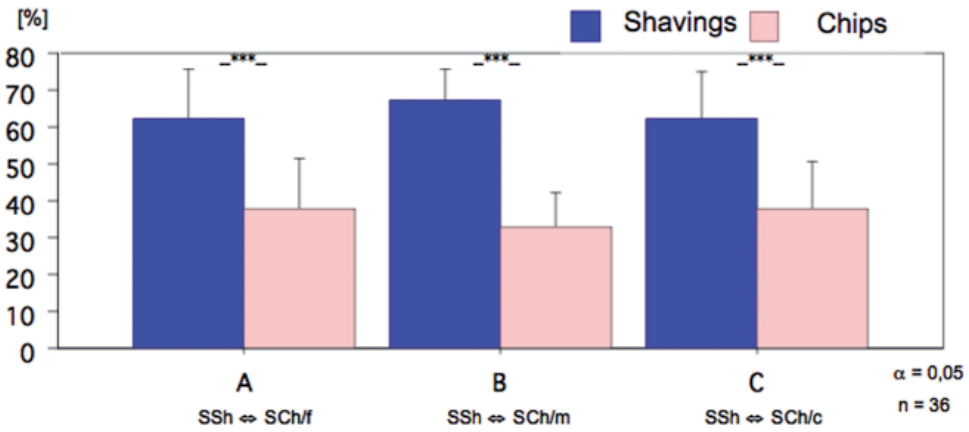

Figure 2. Dwelling times [%] on shavings and chips in combination A-C (***: p< 0.001); S=Softwood, Sh=Shavings, Ch=Chips, f=fine, m=medium, c=coarse-grained)

Data were analysed using the StatView 5.0 computer program. The differences between the bedding materials (dwelling time in cage 1 and cage 2) were compared by paired t-test. Strain differences were detected using ANOVA test with a significant level of 0.05.

Results

Experiment 1:

In each of the three test combinations mice preferred the shaving structure with high significance (p<0.0001 for all combinations, Figure 2). Preference was obvious from the first test day onwards and persisted for the entire week.

Animals spent relatively more time in the cage containing coarse-grained chips than in the one containing other products (Scheffé-test, p=0.0005 compared to medium chips, p<0.0001 compared to fine chips), combined with a higher crossing-activity in combination C. In total, animals spent 70 % of their dwelling time on the shavings. This preference was more explicit during light time (p<0.0001 for all combinations), but it was strong even during dark time (p<0.0001 for all combinations).

In each test combination, C57BL/6J mice showed a higher preference (p<0.0001 for all combinations) than BALB/c mice (p<0.0001 for combination A, p=0.0029 for combination B, p=0.0079 for combination C), but a clear preference for the shavings was proven for both strains.

A total relative ranking of the four tested bedding structures would be: shaving > coarse-grained chips > fine chips= medium chips.

Experiment 2:

For three of the four combinations mice clearly preferred the aspen bedding. There were highly significant differences between softwood and aspen in combination A (paired t-test, p=0.0006), C and D (both p<0.0001). The highest preference was found in C (70.18% of total dwelling time), followed by D (68.56%) and A (62.10%) (Figure 3).

These preferences were present from the first test day and very stable during the entire week. They existed in light as well as in dark phases, with a more distinctive preference during the light period. No preference was found in combination B, neither during the light nor the dark period. In combination A, C57BL/6J mice clearly preferred aspen shavings, while half of the BALB/c mice combined the two materials for resting. Thus, they moved some aspen shavings through the tunnel into cage 1 and built an aspen nest upon the softwood shavings. This led to a statistical strain difference in the dwelling time (Figure 4).

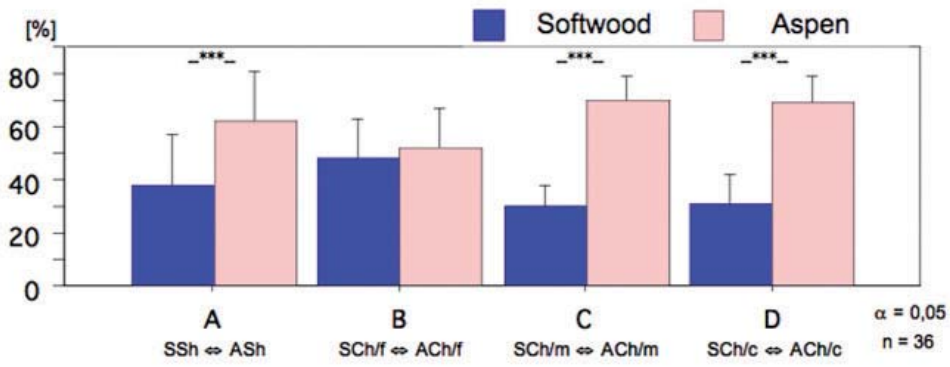

Figure 3. Dwelling times [%] on softwood and aspen in combination A-D (***: p< 0.001); S=Softwood, A= Aspenwood, Sh=Shavings, Ch=Chips, f=fine, m=medium, c=coarse-grained

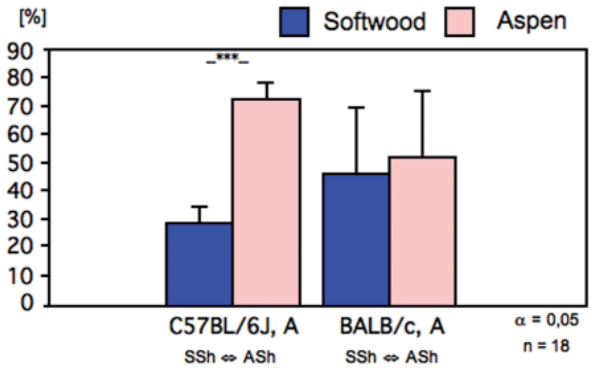

Figure 4. Dwelling times [%] on softwood and aspen in combination A, split by strain (***: p< 0.0001)

Discussion

Various studies demonstrated the importance of bedding material for rodents, especially for resting behaviour[5,6,7,10]. In the present study, group-housed female mice showed clear and consistent bedding preferences during one-week tests. Preference of mice for long, large and coarse particles was already described by previous studies[5,8,10], but in experiment 1 we also could ascertain this for group-housed female mice: Mice clearly preferred the shavings to chips of different particle sizes, especially during the light phases. Although the preference for coarse-grained chips over finer chips was only slight, fine chips do not seem to be recommendable for female mice.

As the present study (Experiment 2) found aspen products were chosen in three out of four test combinations, except bedding with fine chips. This indicates that it can be beneficial for female mice to use aspen bedding instead of softwood bedding. During experiment 2 it was observed that BALB/c used aspen shavings to build nests and led to a strain difference in combination A. This may be due to the coarser structure of the aspen shavings, as they were more shapeable and more like nesting material. Thus, this structure seems consequently more attractive to BALB/c mice. As it has been reported that mice tend to combine different materials for nest building[10,11], providing different bedding materials may be an idea to refine the housing of mice.

In both experiments 1 and 2 mice showed a more distinctive bedding preference during the light period. This fact has already been described for single tested mice and rats[5,6,8] and emphasises the importance of bedding for resting behaviour. According to our observation for nest quality (data not shown), coarser structures such as shavings are more suitable for nest building. This may be the reason that mice stayed significantly longer in the cage with shavings in experiment 1. It would be interesting to study the effect of additional nesting material in this experimental design.

The preferences determined in the present study are only relative ones. Although these were very stable during the entire test week (especially in experiment 1), it still remains open, whether animals will also demonstrate such preferences over a longer period (more than one week). Thus, further long-time preference tests and consumer demand studies are necessary to substantiate our results.

In conclusion, our study suggests that the bedding structure for female mice is at least as relevant as the material. Aspen bedding may be beneficial for female mice and hence should be recommended, but not in fine chip form. Consequently, an aspen product with a shaving structure might enhance the animals' well-being. If the use of shavings is not possible, for example due to technical or hygienic reasons, coarse-grained chips should be used rather than fine chips.

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Effect of housing conditions on behaviour of DBA/2 breeding mice

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Abstract
The impact of housing conditions in different research fields has been studied, but only limited literature has compared the home cage behaviour of breeding pairs under different housing conditions. A previous study has shown that the breeding performance was not significantly affected by environmental enrichment (a nest box, a wood bar for climbing, and nesting material). The aim of the present study was to evaluate the effect of housing conditions on behaviour of paired breeding mice.
Two housing conditions, non-enriched (NE) and enriched (E) housing containing a nest box, a wooden climbing bar and nesting material were studied. A total of 64 DBA/2NCrlBR mice (half of each sex) were used for the experiment. After 4 weeks of adaptation the animals were arranged in breeding pairs and kept in type IIL Makrolon cages, 16 pairs for each housing condition. Breeding mice were randomly recorded by video camera from 11 weeks to 36 weeks of age. Behaviours were defined and analysed after data had been collected. Breeding performance and nest quality of each breeding pair were recorded until 40 weeks of age.
In comparison to non-enriched groups enriched animals spent more time on locomotion, digging, eating/drinking, social interaction, while non-enriched mice groomed, mated and jumped more often than enriched groups. Significant housing differences were only found for locomotion behaviour. The frequency of stereotypic behaviour was decreased due to enrichment (1.64% to 0.19%). The nest quality of the E group was significantly better than that of the NE group.

Keywords: DBA/2 mice, breeding pair, environmental enrichment, home cage behaviour

Enrichment has been used as an experimental tool to study the changes in brain functions, immunoreactivity, medical therapy, interactions between environment and genotype, behavioural performances and other parameters. Currently, environmental enrichment is intended for further improvement in laboratory animal housing. Most studies have focused on the effect of housing condition on non-breeding mice (e.g [1,2,3,4]), only a few studies have stressed the influence of enrichment on breeding mice. A previous study[5] has shown that the breeding performance was not significantly affected by environmental enrichment (a nest box, a wooden bar for climbing, and nesting material, according to Scharmann[6]), this being the case for all rack systems (open rack, ventilated cabinet and IVC rack) provided in the study, even though the coefficients of variation in IVC rack (VR-IVC Charles River, Germany) provided were higher for most variables.

The present study focused on the home cage activity/behaviour of breeding mice under two housing conditions in combination with two rack systems (open rack and ventilated cabinet). During the entire experiment half of the breeding pairs had access to a nest box, a wooden climbing bar and nest material, giving them the opportunity to build nests and climb. Home cage behaviours, breeding performance and nest quality were studied.

Material and Methods

Animals and Housing

Animals: In total 32 DBA/2 mice breeding pairs (Charles River Company, the Netherlands) were used for this experiment. After 4 weeks of adaptation at 10 weeks of age, animals were marked and randomly distributed to the two housing systems with 16 breeding pairs per rack system, 8 pairs each for enriched and non-enriched cages. For synchronisation of oestrous cycles (Whitten-effect) some bedding from the male cages was transferred to all female cages one day before animals were regrouped to breeding pairs.

Environment: The animals were kept in two different rack systems: A ventilated cabinet (Scantainer, Scanbur Company, Køge Denmark) and a normal open rack. Both systems were kept in the same animal room under specific pathogen-free (SPF) conditions at a room temperature 22 ± 1°C, with 55 ± 10% relative humidity, a 12/12 hour light/dark cycle and a light intensity of 120-150 Lux (measured 100 cm above the floor).

Housing: All cages were type II elongated Makrolon cages (32.5 x 16.5 x 14 cm, Charles River Company, Sulzfeld Germany). The non-enriched cages (NE group) were provided bedding, food and water. The enriched cages (E group, Figure 1, according to Scharmann[6]) contained, in addition to the NE group, a nest box (12 x 7 x 4.5 cm), a wooden bar (13 cm x 7,5 cm, pine) for climbing, and nesting material (nestlets, cotton fibre, 5 x 5 cm, EBECO Company, Castrop-Rauxel, Germany).

Food and water: Tap water in drinking bottles and pelleted food containing 22.5% protein, 5.0% fat, 4.5% fibre and 6.5% ash (Altromin No. 1310, Altromin GmbH, Lage, Germany) were given *ad libitum*.

Bedding: 70-80 g wood shavings were used for bedding (Altromin Type 3-4, Altromin GmbH, Lage, Germany). Cages and bedding were changed once a week.

Health monitoring: As infections could be the reason for differences in breeding performance and variance, at the end of the experiment the health of retired breeders was monitored as recommended by FELASA[7].

Observation of nest quality and behaviour

Before the experiment was performed, the observer was trained by other colleagues. Nest quality and behavioural analysis were scored always by one and the same person. Nest quality was classified according to 5 scales, from one (flat, no visible nest) to five (well performed nest, animals were not seen).

Figure 1. Enriched cage for the present study

The observing period was separated into three equal phases. Every cage was recorded for 24 hours during each stage. Behaviour was recorded using a time-lapse video recorder (Panasonic AG-TL550E), 24 hours on a 3-hour tape (1 second of the video time equals 8 seconds of the real time). The behavioural data on videotape was viewed, defined and carried out according to the following ethogram (Table 1).

Table 1. Definition of different behaviour

Behaviour	Definition
Resting:	Lying or sitting without movement (very short or slight movements are not considered as an interruption).
Grooming	Shaking, scratching, wiping or licking its fur, snout, ears, tail or genitals.
Eating	Gnawing food from hopper and bedding.
Drinking	Standing/sitting beside/under water bottle and licking the drinking nipple.
Climbing	Jumping onto cage top, climbing along grill in inverted or hanging position.
Locomotion	Walking, running along, climbing up/down the wooden bar or moving nest material/nest box.
Digging	Digging into bedding or moving bedding with nose, front paws or hind legs.
Social contact	Non-aggressive interaction, including moving and playing with each other.
Mating	Pushing head and forebody beneath a potential mate.
Jumping	Jumping towards the cage top vertically along the wall.
Stereotype	Consecutively and repeatedly climbing, digging and jumping in the same position (for more than 30 seconds).
Others	Except the behaviours described above.

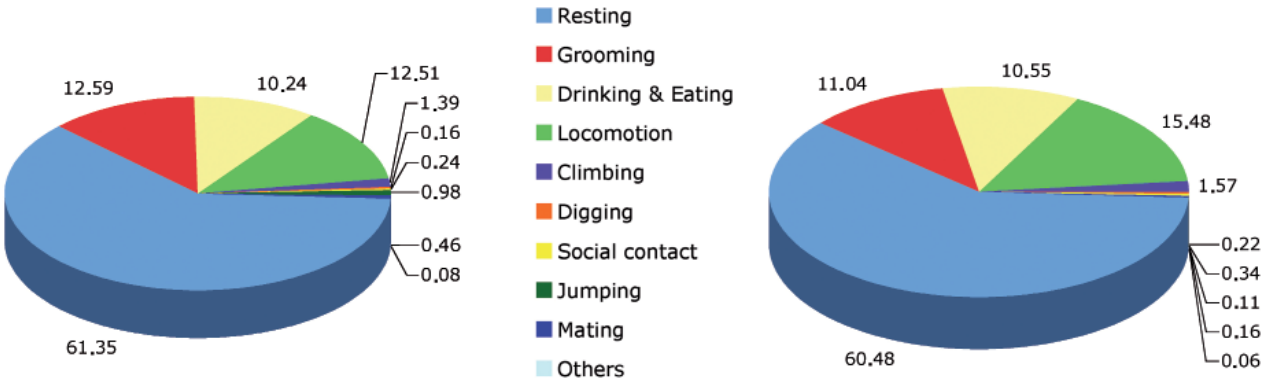

Figure 2. The time budget (%) of different behaviours (left: NE group, right: E group)

Experimental design

Following 4 weeks of adaptation at 10 weeks of age the animals were marked and randomly separated to the two rack systems described above. Breeding pairs were kept together (one pair/cage) during the entire experimental period from November to February.

After mice had been regrouped to one breeding pair per cage, breeding pairs were randomly recorded by video camera (Panasonic, AG-TL550E) until 36 weeks of age (each cage was recorded for 3x24 hours). Nest quality was graded every second day between 10:00 and 12:00. Breeding performance was recorded until 40 weeks of age, including litter size, number of pups weaned and body weight at weaning. Behaviours were defined and analysed following data collection. Time budgets were then constructed using the definitions given in Table 1. A mean value per cage was calculated.

Statistics

Data were analysed by StatView 5.0 software (SAS Institute Inc., Cary NC, USA, 1998). The normal distributed data were compared using a two-factorial analysis of variance with the factors 'rack system' and 'housing' to analyse the effects of the rack systems, the housing and the rack systems x housing interaction, with a significance level of 5%. Non-parametric tests, Mann-Whitney test, were performed for abnormally distributed data.

Two females, one from each housing type, died during the experiment. Therefore, the data were not included in the statistical analysis. The average duration of all behaviours of the two mice in the same cage was used for statistical purposes (n is equal to the cage number). The data of stereotypic behaviour were separated for three phases for detailed information.

Results

The effects of housing condition on home cage behaviours

In comparison to animals in the NE groups enriched animals spent more time on locomotion, climbing,

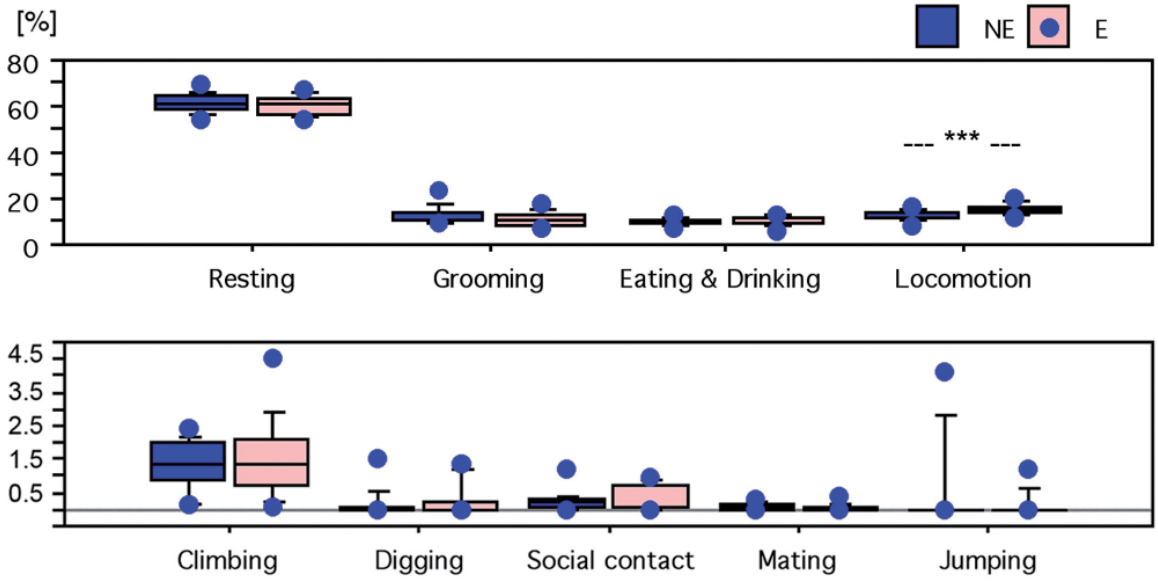

Figure 3. Relative duration (%) of home cage behaviours (n=15, ***: p< 0.001)

digging, social interaction, while non-enriched mice groomed, jumped and mated more often than the enriched group (Figure 2). Significant housing differences between the NE and E groups were found only in locomotive behaviour ($F_{1.26}$=18.152, p=0.0002, Figure 3).

The effects of rack system on behaviours

Animals kept in both rack systems showed similar relative duration (%), except resting, climbing and locomotion behaviour (Figure 4). Animals kept in open racks slept more (63.54±3.38%) than animals in ventilated cabinets (58.33±3.21%), this being observed for both housing conditions ($F_{1.26}$=8.659, p=0.0068). Mice kept in ventilated cabinets climbed more and showed more locomotion than those in open racks ($F_{1.26}$=9.054, p=0.0058 for climbing; $F_{1.26}$=6.316, p=0.0185 for locomotion). No rack system and housing interaction was found.

The effects of housing condition on stereotypic behaviour

Stereotypic behaviours, including stereotypic jumping, climbing and digging, were found more often in the non-enriched groups (5 cages in NE and 2 cages in E group) at the beginning of the experiment, the frequency of stereotyping observed decreasing with time (Figure 5). In the 3rd experimental period only one cage from each group showed any stereotypic behaviour. On average the duration of stereotypic behaviour was 1.64% in NE groups and 0.19% in E groups during the entire experiment, but no statistical difference between the groups could be detected.

Nest quality and breeding performance

Nest quality of E groups was better than that of NE groups (median: scale 2.000 for NE groups and scale 4.000 for E groups), this leading to a significant housing difference (Mann-Whitney test, p<0.0001).

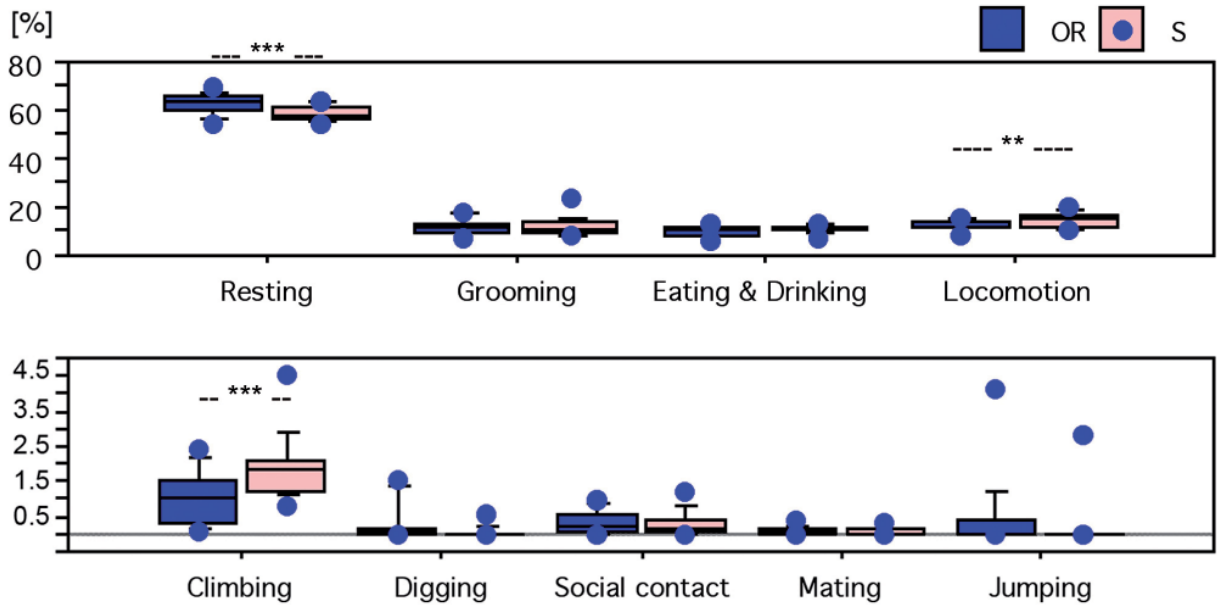

Figure 4. The relative duration (%) of different behaviours in different rack systems (OR: open rack, S: ventilated cabinet; ***: p<0.001, **: p< 0.05; n=15)

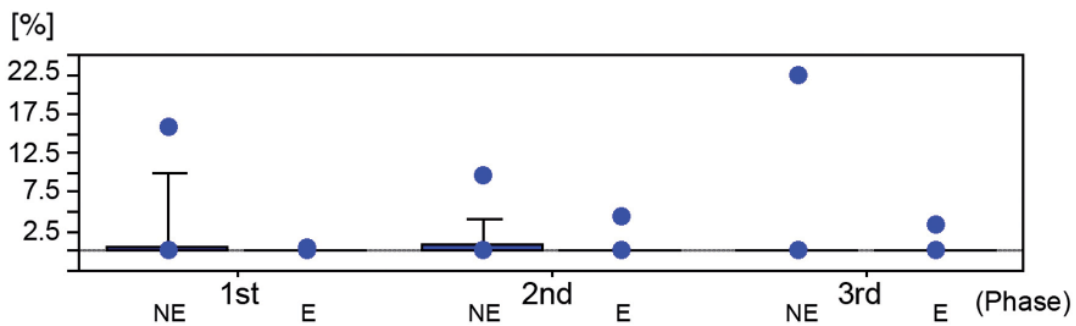

Figure 5. Relative duration (%) of stereotypic behaviour during three different phases (n=15)

The breeding performance of both housing groups was similar; no housing effect was found (data are not shown).

Discussion

Early studies have shown that providing nest material can lead to a better breeding performance in rats[8]. This was not supported in mice by previous studies[5,9] and the present study. Tsai *et al.*[5] reported that enriched housing caused a delay in the age of dam at first weaning in all rack systems (open rack, ventilated cabinet and IVC rack). The present study showed that enriched animals spent less time on mating behaviour and more time on locomotion/digging/climbing/social contact (Figure 2) in comparison to the NE group. The results indicate that playing and investigating the environment were more attractive for the animals kept in E cages. This may be the reason why enriched housing did not improve the breeding index.

An interesting finding is the difference between rack systems. The animals in open racks slept significantly longer than those in ventilated cabinets and showed significantly less climbing and locomotion behaviour. For this finding one possible explanation is that mice in open racks were not disturbed by vibrations compared with mice in ventilated cabinets. We could not found other reasonable explanations. To understand this phenomenon further studies are necessary.

The stereotypes may serve as general "coping mechanisms" (e.g. reviewed in Mason & Latham[10]). The present study found that the frequency of stereotypic behaviours decreased with time. Thus, it can be assumed that breeding mice need some time to adapt to their environment and enriched housing provided for the experiment may help to decrease this time needed for adaptation. Nevison *et al.*[11] reported that enriched housing (nest material and tunnel) can increase and decrease stereotypes in non-breeding mice; the results however varied according to the strain. The enriched housing significantly increased the stereotypic behaviour of DBA/2 mice. This indicates that nest material and a tunnel are not suitable for non-breeding DBA/2 mice.

According to the presented data, it can be assumed that enriched housing used in this study may reduce the stereotypic behaviour of breeding DBA/2 mice, at least in helping them cope with environmental or regrouping stress. Furthermore, the effects of enrichment could be different, these varying due to the enrichment given and the animals used in a study.

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Effects of IVC housing on laboratory mice welfare

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Abstract

The use of individually ventilated cages (IVC) has increased in recent years, as they provide better protection for animals and staff. However some studies have shown that they may have a negative impact on the physiology and welfare of animals (e.g. decrease in fertility, increase in anxiety); in addition, mice have been shown to prefer non-ventilated cages. One of the possible causes could be stress produced by ventilation; consequently, we studied the effect of the frequency of air changes per hour (ACH; 60, 40, 80 ACH in successive fortnights) on animal welfare (body weight, food and water intake, urine corticosterone, behaviour) and on cage environment (NH_3, CO_2, humidity, temperature) in CD1 and C57 female mice (bedding was corncob). Simultaneously, we investigated whether the use of environmental enrichment improved animal welfare in IVC.

Changes in ACH did not produce any significant changes in the welfare variables assessed. No differences were observed either between enriched and non-enriched groups. Temperature and humidity in cages depended on room values and not on ACH. Ammonia levels remained undetectable (<5ppm) and CO_2 was below the expected levels, with the exception of half of the CD1 cages at the end of the 40 ACH period.

Considering the results obtained, we can conclude that mouse welfare is not affected by different rates of ventilation (between 40 and 80 ACH), even if cages are changed once every two weeks. Lower values than recommended are still sufficient when the bedding used is corncob, which is extremely absorbent.

Keywords: animal welfare, individual ventilated cage, rack, air changes.

The use of individually ventilated cages (IVC) has increased in recent years as they provide better protection for animals and staff[1]. Several authors have studied the microenvironment in different IVC systems with different ventilation rates and cage cleaning regimens[1, 2, 3]. In general, all IVC systems were able to maintain microenvironment variables (CO_2, NH_3, temperature and humidity) within the established limits, even when ventilation rate was low (30-60 air changes per hour depending on animal density) [2], cage change frequency was lowered to once every two or three weeks[1], or even when there was a power failure for an hour[3]. The use of IVC systems showed additional advantages, such as the lowering of cage cleaning frequency, which also reduced pup mortality[1]. However, some systems presented drawbacks, such as a difficulty to maintain a negative pressure[3] and different effects on animal performance and well-being[3, 4, 5]. For example, fertility could be affected[4], and anxiety could be increased[5]. In addition, it has been shown that mice prefer non-ventilated cages[6]. Therefore, one of the possible causes of the different effects on animal performance and well-being could be the stress produced by the regimen of ventilation inside the cages. Consequently, the aim of this study was to investigate the effect of the frequency of air changes per hour on animal welfare and on cage environment in two commonly used strains of laboratory mice, in the IVC system in use in our centre. Simultaneously, we studied whether the use of environmental enrichment improved animal welfare in this type of IVC.

Materials and Methods

Animals and husbandry

Adult SPF female Hsd: ICR (CD-1) and C57/BL6JOlaHsd mice (25.1 g and 6-7 weeks of age, and 18.8 g and 8-9 weeks of age respectively, Harlan Laboratories Models, Europe) were used in the present study. Animals were housed in a rack with individually-ventilated cages (Tecniplast, Sealsafe plus, Italy). Cages were GM500, had a floor surface of 500 cm^2, the air inlet placed in the

upper area of the cage, and the outlet on the cage top. Each cage was filled with 1100 cm^3 of corncob bedding before autoclaving. Temperature and humidity were controlled and in accordance with national legislation. Animals were maintained in a 12h light/12h dark cycle, and light intensity was around 350-400 lux in the centre of the room. Food (Harlan Tecklad, Spain) and ultra filtrated water were available *ad libitum*. After delivery of the animals, a recovery period of five days was allowed.

Animal experiments were approved by the local IACUC and animal welfare standards were regulated in compliance with EU regulations and GlaxoSmithKline's policy on *Care, Welfare and Treatment of Animals in GSK*.

Experimental procedure

Ventilation regimens studied were 60, 40, and 80 Air Changes per Hour (ACH), in this order (Table 1). Animals were randomized and assigned to groups of 5. Ten cages of CD1 mice and ten of C57/BL6 mice were kept in the IVC with corncob bedding for two consecutive weeks per ventilation regimen without cage change, since this bedding has been shown to have excellent ammonia inhibiting characteristics [7]. Half the mice had nesting material (2 paper tissues) as environmental enrichment (+T groups).

Parameters studied related to animal welfare were body weight, food and water intake, urine corticosterone and behaviour. In addition, cage environment was checked measuring levels of NH_3, CO_2, relative humidity and temperature.

Methods

Behavioural assessment: Behaviour was observed every day in the morning (around 10:00) and nests were observed from 11:00 to 13:00. We observed whether animals displayed any undesirable behaviour (stereotypes, excessive aggression, etc) and where nests were built (or where animals slept). Stereotypic behaviour was defined as behaviours that are inappropriate, repetitive and unvarying in either goal or motor pattern[8]. Aggressive behaviour consisted on animals that bit, chased, or mounted others.

The weight of animals and their food intake were monitored at the end of each week. The water intake was monitored twice a week.

Corticosterone in urine: Urine was collected at the end of each week between 10:00 and 14:00; order of collection was thus randomized and time noted down in order to control for time of collection. Urine was obtained by handling the animals and rubbing their bladder over a Petri dish, placed in a vial and kept at -70ºC until assessed for corticosterone (Corticosterone EIA, Immunodiagnostic Systems Ltd) and creatinine (to control for urine concentration, a biochemical analyzer Synchron CX5, Beckman).

CO_2 and NH_3 were measured with colorimetric tubes on the last day of the week (Carbon Dioxide 100/a, Ammonia 5/a, Dräger).

Temperature and humidity: average temperature and average relative humidity inside the cages were daily measured by the rack. These data were compared with the data obtained for the room by the automatic control room system.

Statistics

A repeated measures analysis of variance was used to compare data (two-way ANOVA). Multiple comparisons of groups were performed by Bonferroni post-tests. GraphPad Prism Version 4.00 for Windows (GraphPad Software, San Diego California USA) was used for statistical analysis. P values of ≤ 0.05 were considered statistically significant.

Results

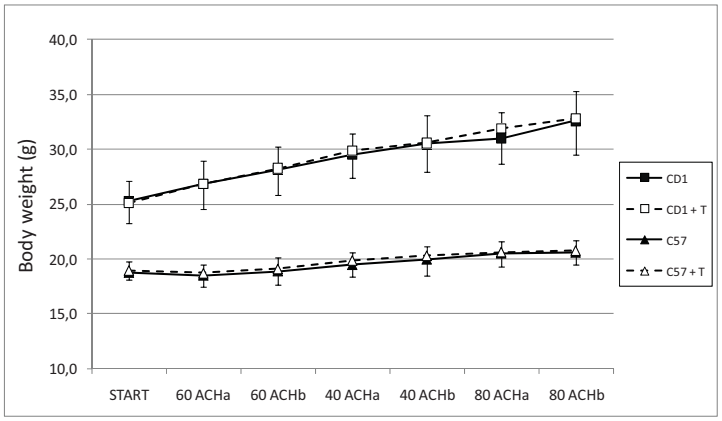
All groups gained weight normally week by week, according to our own experience with both strains and Harlan's data[9]. The only exception was the strain C57 in the first week of the experiment (Fig. 1). There were no differences between enriched and non-enriched groups.

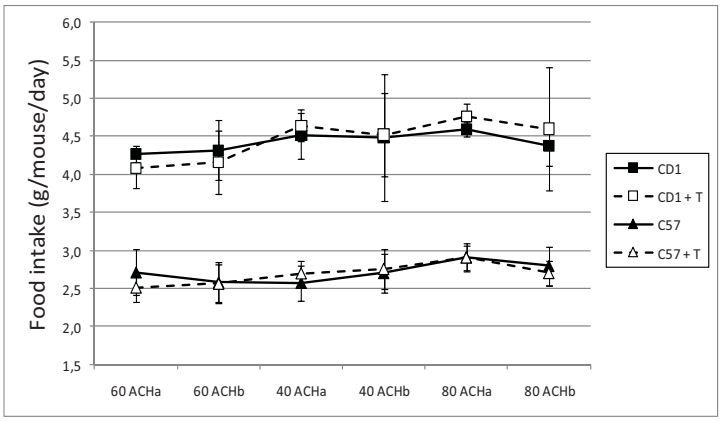
Food intake increased over time for CD1 mice (2-way ANOVA, p = 0.0018). Water intake remained stable throughout the experiment (Fig. 1). There were no significant differences between enriched and non-enriched groups.

Coefficient of urine corticosterone/creatinine (CCC) values were higher in the C57 strain than in the CD1 strain. CCC did not change for different ACH regimens in CD1 groups without enrichment. A significant increment in this coefficient was observed at the end of the first week with 80ACH for CD1+T groups, compared with other weeks, with the exception of week 40ACHb (Fig. 2). There were no significant differences between enriched and non-enriched groups of the same strain, although C57+T mice showed lower levels than non-enriched C57

Table 1. Experiment schedule.

WEEK	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
NAME	60 ACH a	60 ACH b	40 ACH a	40 ACH b	80 ACH a	80 ACH b
ACH	60	60	40	40	80	80
Cage change	no	yes	no	yes	no	yes





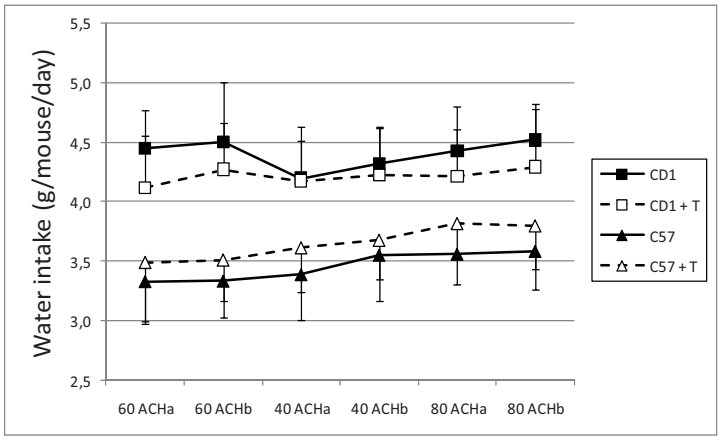


Figure 1. Mean & SD of: body weight (g) per group and week (top graph); food intake (g per mouse and day; middle graph); water intake (g per mouse and day; to facilitate visualization, only upper error bars are shown for CD1 strain, and only lower error bars are shown for C57 strain; bottom graph).

groups in the first week of the experiment (60 ACHa; Repeated measures Two-way ANOVA, and Bonferroni post-tests, $p < 0.01$).

There were no differences in behaviour between weeks or groups. Animals were very calm most of the time, although barbering was observed, mainly in C57, and also one stereotypic CD1 animal was found in the non-enriched group. Non-enriched groups' nests were smaller than enriched ones and away from open areas (sides of the rack). In all groups most nests were placed at the end of the cage (at least 70%), and ventilation did not affect nest placement.

Temperature values in the IVC were very stable (always 22°C) with similar values in the IVC and in the room (average room values ranged from 20.66 to 20.9°C) . Relative humidity varied with room values and not with changes in ACH. Average IVC humidity ranged from 43.4 to 47.3%, and average room humidity ranged from 35.8 to 48.6%. NH_3 values inside the cages were always undetectable (<5ppm). On average, CO_2 levels observed were always double in CD1 than in C57. There was a significant increase in levels for CD1 in 40 ACHb week, compared with other weeks. Levels also increased for CD1+T groups, but this was only statistically significant when compared with week 80 ACHb. Levels of CO_2 were significantly lower for non-enriched C57 at the end of the second week with 80ACH (Fig. 3).

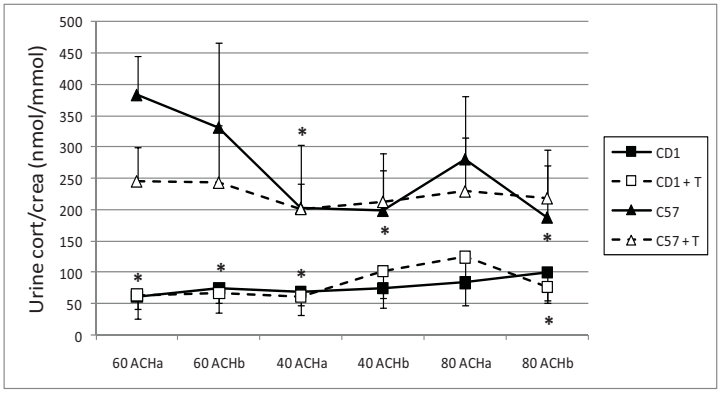


Figure 2. Mean & SD of urine corticosterone/creatinine levels (nmol/mmol). To facilitate visualization, only lower error bars are shown for CD1 strain, and only upper error bars are shown for C57 strain. CD1+T: * indicates p<0.001 when compared to week 80ACHa; C57: * indicates p <0.001 with respect to week 60ACHa, and p<0.05 with respect to 60ACHb.

Discussion

All animals ate, drank and grew well throughout the experiment independently of the regimen of ventilation of the cage. The only exception was the first week for the C57 strain, in which they lost weight; possibly being a more anxious strain[10], they needed a longer period of adaptation to the rack than the CD1 strain.

Coefficient of urine corticosterone/creatinine (CCC) values were higher in the C57 strain than in the CD1 strain, and higher for both in the first week of 80ACH. This increase could be due to the stress caused by doubling the ACH, although animals seemed to adapt to it, since values decreased again in the following week at the same ACH. CCC was also higher in the first week for the non-enriched C57 group. This could be due to a longer adaptation time to the rack or to the experimental procedures than that of C57 mice with enrichment, given that enrichment has been shown to reduce stress levels[11].

In general, animals were very calm and we did not observe abnormal behaviours or aggression. Barbering appeared in many C57 strain groups, but that was to be expected as this behaviour is very common in the strain[12]. Only one animal of the CD1 strain without enrichment showed undesirable behaviour (stereotypy), which is a frequency too low to be taken into account. Nests were very similar between weeks and strains, and the only observed difference was a bigger size of nests for enriched strains, most probably caused for the presence of nesting material.

Environmental variables inside the cages were very similar between weeks. The only undesirable finding was an increase in CO_2 levels in the second week with 40 ACH. This regimen of ventilation is probably too low to allow for two weeks without cage change and thus should only be used when cages are changed weekly. Ammonia inside the cages was always undetectable (<5ppm), probably due to the ventilated system and the good characteristics of the bedding[7].

To summarize, with regards to the results obtained, we can conclude that mouse welfare was not affected by different rates of ventilation **(**between 40 and 80 ACH**)**, even if cages are changed once every two weeks. Lower values than recommended by the manufacturers are still sufficient when the bedding used is corncob. The only undesirable finding was the increase in CO_2 values at the end of the second week with 40 ACH. Therefore, this ventilation regimen is not recommended unless cage change is increased to once a week.

On the other hand, we did not observe any relevant differences between enriched and non-enriched groups, with the exception of a lower ratio of CCC for the first two weeks for the C57 strain with tissues. This supports the idea that nesting material can

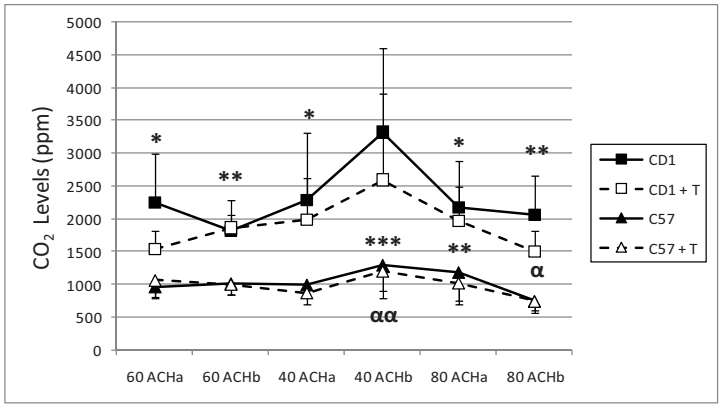


Figure 3. Mean & SD of CO_2 concentration (ppm) inside the cages per group and week. To facilitate visualization, only upper error bars are shown for CD1 strain, and only lower error bars are shown for C57 strain. CD1 strain: * indicates significant differences with week 40ACHb, α is used for CD1+T; C57 strain: * indicates significant differences with respect to week 80ACHb, α is used for C57+T (*/α = p<0.05; **/**αα** = p<0.01, *** = p<0.001).

reduce stress levels, thus improving animal welfare[11]. Additionally, not finding differences between enriched and non-enriched groups in many parameters that may be important for research (e.g. body weight, food intake), shows that enrichment might not interfere with experimental results in the conditions of this study.

Acknowledgements

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The effects of environmental enrichment on behaviour in the rat

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Abstract

Environmental enrichment (EE) has become a very important factor to consider in improving the well-being of laboratory animals. However, to date there has been limited investigation of EE on behavioural parameters. Thus, this study investigated the effects of EE and isolation housing on male Sprague-Dawley (SD) rats using a range of commonly employed behavioural tests. Three housing conditions were used, namely IC (isolation), SC (4/cage in standard cages 42 x 25.5 x 20cm), and EE who were housed (4/cage in 54 x 38 x 19cm cages) enriched with a range of toys/ objects. After 4 weeks of this housing the following tests were sequentially conducted: anxiety behaviours in the Elevated Plus Maze (EPM), immobility time in the Forced Swim Test (FST), locomotor activity in the homecage and latency to the escape platform in the Morris Water Maze (MWM). Data was collected using automated, semi-automated or manual scoring methods. As data was normally distributed, statistical analysis was carried out using one-way ANOVA. Following the 9-week period, IC rats gained significantly more weight than the SC and EE groups ($p < 0.05$). The only significant behavioural change occurred in the EPM, where EE and SC rats spent more time on the open arms and made more entries to the open arms ($p < 0.05$ vs. SC), suggesting that they were less anxious than the IC group. To conclude, the results suggest that EE, when compared to standard group-housed conditions, is not associated with any changes in commonly employed behavioural tests.

Keywords: environmental enrichment, social isolation, forced swimming test, elevated plus maze, Morris water maze

Environmental enrichment (EE) is an area of animal care and management which has received considerable attention in recent years. EE involves exposing laboratory animals to a physical and/or social stimulation that is greater than that they would ordinarily receive under standard housing conditions[1]. EE can be divided into two broad types, namely physical or social enrichment. Physical enrichment relates to structural modifications and/or the inclusion of novel objects in the cage with which the animals can interact and which provide them with exercise and multisensory stimulation. In the case of rats, these include bedding which is enhanced with natural materials, and the provision of plastic tunnels, wooden objects to gnaw, ropes, swings, running wheels, balls, ramps, ladders and other appropriately sized animal toys. Social enrichment on the other hand refers to housing social animals in groups wherever possible as rats are social animals. In reality, a combination of both physical and social enrichment approaches are most commonly employed.

The relevance of an enriched environment on learning was highlighted in the 1940's by Donald Hebb who noted that laboratory rats given freedom to roam in his home as pets had superior problem-solving and learning abilities than rats housed in standard laboratory conditions[2]. In the past 60 years, public pressure has led to increasing legislation governing the housing and care of animals in laboratories, farms and zoos. In 2007, the EU Commission issued guidelines for the Accommodation and Care of Animals used for Scientific and other Purposes, which included a section entitled "Housing, enrichment and care". As well as recommending the provision of social enrichment for rodents wherever possible, the guidelines also encouraged that appropriate physical enrichment should be made available, such as nesting materials, refuges, tubes, boxes and climbing racks (Commission Recommendation 2007/526/EC).

Research into the impact of housing conditions on rat brain development and behaviour generally addresses the effects of social isolation or impoverished conditions (IC) and of EE as compared to standard group-housing conditions (SC). Differential experience such as housing environment induces neurochemical changes in the cortex of the brain,

along with increases in cortical weight and thickness, as well as effects on plasticity including dendritic size and branching[1]. IC rearing produces lasting alterations in synaptic plasticity such as decreased dendritic branching[3] and reduced cortical thickness[4]. IC also influences neurochemistry as increased glutamatergic expression in the hippocampus[5] and enhanced dopaminergic activity in the nucleus accumbens and striatum[6] have been shown. The effect of IC housing on behaviour has led to the description of a "social isolation syndrome"[7] consisting of locomotor hyperactivity in a novel environment [8], increased anxiety in the elevated plus maze (EPM)[9], increased startle reactivity and reduced pre-pulse inhibition[10, 11] and impaired performance in tests of learning and memory[4, 12] when compared to SC animals. As behavioural indices in the EPM and open field tests (OFT) would suggest, IC rats show increased reactivity to stress and hypothalamic-pituitary-adrenal (HPA) axis hyperfunction compared to SC[13]. Such neurochemical and behavioural adaptations have led to the use of early social isolation as an adverse event and thus as models of human depression and schizophrenia[4].

As a lack of environmental stimulation affects neurochemistry, neural plasticity and behaviour so too does the additional stimulation provided by EE housing. Increased serotonergic activity in the PFC and hippocampus[14], increased hippocampal noradrenergic[15, 16] and glutamatergic transmission[17] as well as altered dopamine receptor density and transporter levels in the PFC[18] of EE rats compared to IC and SC controls are evidence of the capacity of EE housing to influence plasticity in the brain. In addition, neurotrophic factors such as brain-derived neurotrophic factor (BDNF) are involved in neuroplasticity for learning and memory[19] and compared to IC controls, EE rats have higher levels of BDNF in the hippocampus, basal forebrain, hindbrain and cerebral cortex[20]. EE animals appear to be less emotionally reactive than IC and SC controls[21], they display greater habituation and less locomotor activity in the OFT compared to controls[22] and greater open arm entries and time spent on the open arms of the EPM compared to IC and SC controls[16, 23]. EE rats also display improved learning and memory[12, 24] compared to pair-housed controls. An ever-growing body of evidence illustrates the potential benefits of EE following brain injury and CNS disease[25, 26].

Considering the potential effects of differential housing conditions on the rat brain and behaviour, housing environment ought to be taken into account in animal models of disease and treatment development. It can be argued that the aberrant effects of IC rearing may result in poor animal models[27] which may thus be of limited validity to the human conditions being modelled. On the other hand, as EE implementation becomes commonplace, its effects on the outcome on neurochemical and behavioural endpoints must also be considered. To date, few studies have investigated the effects of impoverishment and enrichment on baseline behaviours as compared to group-housed controls; many IC studies focus on profiling and treating symptoms of schizophrenia while many EE studies investigate its effect on brain injury and only employ SC as a comparative control. The purpose of the present study was to investigate the effects of housing rats in EE and IC compared to SC controls using a number of commonly-employed behavioural tests (namely locomotor activity in the homecage, anxiety-like behaviours in the EPM, symptoms of coping and despair in the forced swim test (FST) and learning and memory in the Morris water maze (MWM)). This was conducted with the aim of profiling baseline behavioural responses of animals housed in EE and IC, which may be regarded as at opposite ends of the spectrum, as compared to SC which can be considered as the central point.

Materials and Methods

Animals and housing conditions

Forty-eight male Sprague Dawley (SD) rats, aged 6 weeks old (150-170g) were supplied by Charles River (UK), animals were certified as "Conventional" by suppliers (non-specific pathogen free). All rats were housed in groups of 4 for four days acclimatisation, then randomly assigned to one of three housing conditions (n=16); isolation (IC), standard group-housed (SC, 4/cage) or housed with environmental enrichment (EE, 4/ cage).

The IC and SC animals were housed in standard-sized cages (42 x 25.5 x 19cm) with a layer of bedding material (wood shavings, Lillico Ltd., UK) while the EE animals were housed in larger cages (54 x 38 x 18cm) with the same bedding material and additional commercially available nesting material ("Safe 'n' Sound", Pennine, UK). The EE cages were equipped with various plastic and wooden toys such as plastic tunnels and coloured shelters, wooden tunnels and wooden chew blocks, as well as woven grass tunnels and cardboard boxes for shelters/hideaways (see figure 1 below). All animals were maintained in their respective housing conditions for four weeks before behavioural testing began and throughout the test period (total 9 weeks).

Animals were kept in a colony room on a 12-hour artificial light/dark cycle (08.00-20.00 h); the light intensity in the rats' cages was < 10 lux. The temperature was maintained at 19-23°C and humidity of 40-70%. Cage bedding was changed once weekly for the IC group and three times weekly for the SC

Figure 1. Enrichment cages with plastic and wooden commercially-available pet toys, shelters and tunnels

and EE groups. Animals had continuous access to rat chow (Harlan Ltd., UK) and tap water, which were replenished regularly. All animals were subject to health screens prior to their arrival by the suppliers (Charles River), after their arrival rats were weighed weekly and all remained healthy throughout the experiment. All procedures were approved by the Animal Care Research Ethics Committee and carried out under the guidelines of the Animal Welfare Committee, National University of Ireland, Galway and in accordance with the European Communities Council Directive of 24th November 1986 (86/ 609/ EEC).

Experimental Procedure:

Testing was carried out across three weeks following the four-week differential housing period, as illustrated in figure 2. Testing was carried out during the light phase, between 09:00 and 16:00 h and each test was separated by one week. Different rats were used in the FST, EPM and homecage monitoring tasks, while a subset (n=8) were randomly chosen for the MWM, thus these animals were exposed to two behavioural tests.

Forced Swim Test

The FST is a test of behavioural despair used to screen antidepressant drugs. During the test, rats were placed in glass cylinders, filled with water (23–25 °C) to a depth of 25 cm. The FST procedure consisted of a 15 minute pretest followed 24 hours later by a 5 minute test. The test was recorded onto DVD and later scored manually. The parameters of interest were time spent climbing and immobile (i.e. making minimum movements except those necessary to keep one's head above water). Increased activity and climbing are regarded as active coping in the FST whereas immobility reflects despair.

Elevated Plus Maze

The EPM consisted of a 4-arm maze (each arm is 40cm length x 12cm width) elevated ~50cm from the floor. The 4 arms intersect and form the shape of a plus (+). Two of the arms are open and two are enclosed, the closed arms are surrounded by walls ~30cm high and are illuminated at lux 36, the open arms are lit by two bulbs giving a lux of 90 on the open arms and centre section of the arena. The rat was placed into the centre of the maze facing an open arm and allowed to freely explore for 5 minutes, a camera located approx 1.3m above the maze recorded behaviour to DVD for later manual scoring. Between trials the maze was cleaned down and the rat returned to its homecage. The height and openness of the open arms are considered to be anxiety-provoking and thus avoided. The parameters of interest were entries to and time spent in the open/ closed arms of the maze.

Locomotor activity

Any effect of housing on locomotor activity was investigated in a homecage monitoring apparatus (HCMA). The HCMA consists of a mobile, steel structure measuring 163 cm in height, 127 cm in length and 52 cm in width which contains four adjacent wooden chambers (44 x 29 x 46 cm) each of which can accommodate a rat cage. An adjustable surveillance camera was positioned (~62 cm) above the base of each chamber, and this was connected to a DVD recorder and TV monitor which displayed the output. Rats were habituated in individual homecages with

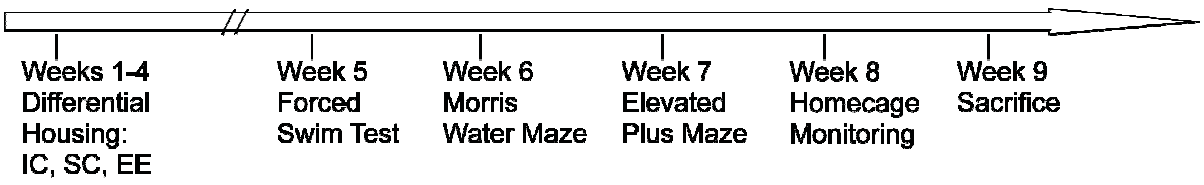

Figure 2. Timeline illustrating the experimental procedure employed for IC (isolated conditions), SC (standard group conditions) and EE (environmentally enriched) rats.

dark bedding and dark plates (to minimise interference for ethovision tracking) for three hours prior to testing. The homecages were placed in the monitoring apparatus and their activity was recorded for one hour. Ethovision® was used to record locomotor activity, i.e. distance moved (cm) and velocity (cm/sec).

Morris Water Maze

The MWM is a test of learning and memory consisting of a large circular tank (~ 1m diameter) filled with water to a depth of 69cm. The MWM test consisted of a four-day acquisition phase followed by a probe trial on day 5. During the acquisition phase a white escape platform (10cm diameter) was submerged in the southwest (SW) quadrant of the tank. The water was made opaque by adding white poster paint. Rats were gently placed in the pool, facing the wall, at one of four pre-determined release points (Southeast, Northwest, North, and East). Each rat was given 2 minutes to find the platform, if it failed to reach the platform in this time it was gently guided to the platform and remained there for 10 seconds before being removed, dried and placed into a recovery cage. The acquisition phase consisted of four trials per day for four days. On day 5, the probe trial was carried out; the platform was removed from the maze and the rat was placed in the pool at a novel starting point (North East), the trial consisted of 2 minutes free swimming before the rat was removed from the pool. Here the animal should have learned to go directly to the SW quadrant. Large geometric shapes (oval, rectangle, vertical lines) were placed at three locations around the maze to provide extramaze visual cues which remained constant throughout. The water temperature was maintained at 26°C (± 2).

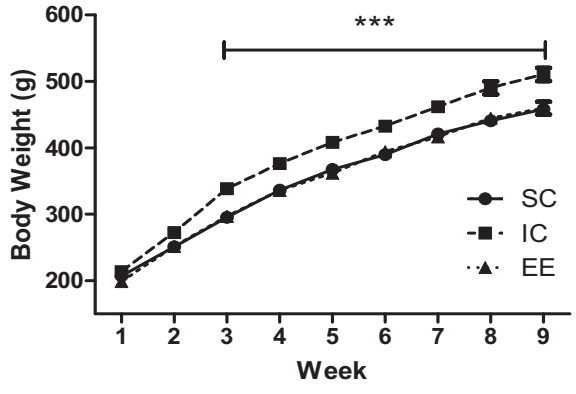

Figure 3. The effects of housing conditions on body weight.

The effects of housing in isolation (IC), group (SC) and enriched (EE) conditions on body weights over the 9-week experimental period. Data are expressed as mean ± SEM, 2-way repeated measures ANOVA *** $p <$ 0.001 IC compared to SC, n = 16.

Statistical Analysis

As data were normally distributed, two-way repeated measures ANOVAs were used to analyse any effects of differential housing on body weight gain and any main effects of housing or time on latency to find the platform in the MWM. One-way ANOVAs and Student-Newman Keuls post hoc tests were also used to analyse behaviour in the FST, EPM and locomotor activity. In all analyses the alpha level $p < 0.05$ was used.

Results

Body weight

There were significant main effects of housing condition [F (2, 42) = 109.7, $p < 0.0001$] and week [F (8, 35) = 642.0, $p < 0.001$] on body weights across the experimental period as IC rats were heavier than SC controls on weeks 3-9 (figure 3). There was no interaction effect.

FST

There were no effects of IC or EE housing on climbing and immobility behaviours in the FST (climbing [F (2, 15) = 0.44, $p = 0.655$], immobile [F (2, 15) = 0.38, $p =$ 0.689]) (figure 4).

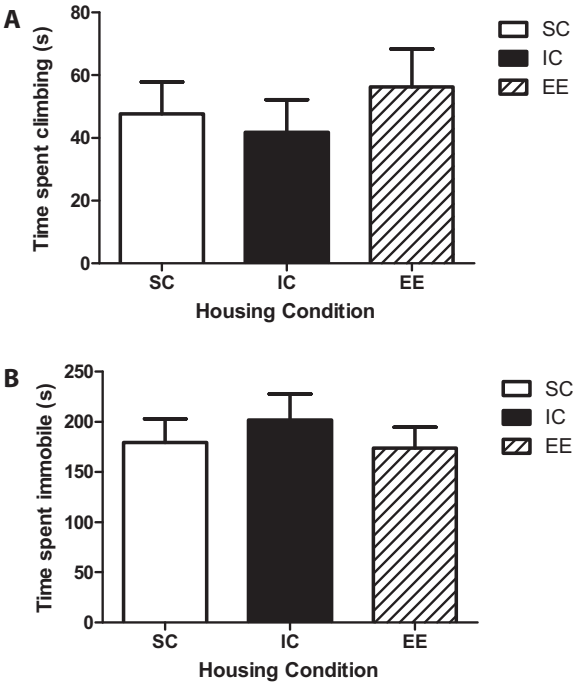

Figure 4. The effects of housing conditions on behaviour in the forced swim test.

The effects of housing in isolation (IC), group (SC) and enriched (EE) conditions on (A) time spent climbing and (B) time spent immobile in the forced swim test Data are expressed as mean + SEM, n = 6.

EPM

A significant housing effect on percent time spent in the open arms of the EPM [F (2, 15) = 7.01, p = 0.007] showed IC rats spent significantly less time in the open arms compared to EE and to SC controls ($p < 0.05$). This suggests that SC and EE rats are less anxious than those in the IC group. There was no significant difference between the groups in percent open arm entries [F (2, 15) = 2.85, p = 0.090] (figure 5).

Locomotor activity

There was no significant difference in distance moved or velocity of movement across IC, SC and EE conditions (distance moved [F (2, 9) = 1.36, p = 0.304], velocity [F (2, 9) = 1.37, p = 0.302]) (figure 6).

Morris Water Maze

There was a significant effect of time on latency to locate the platform in the MWM as all rats learned its location over the four days, however there was no significant main effect of housing [F (2, 21) = 2.39, p = 0.117], nor was there a housing x time interaction (figure 7). In addition there was no effect of housing on time spent in the target quadrant during the probe trial.

Discussion

In the present study, the effects of IC and EE conditions on a number of behavioural tests were investigated. There were significant effects of housing on body weight as IC rats were heavier than SC and EE groups at the end of the experiment. IC rats also spent

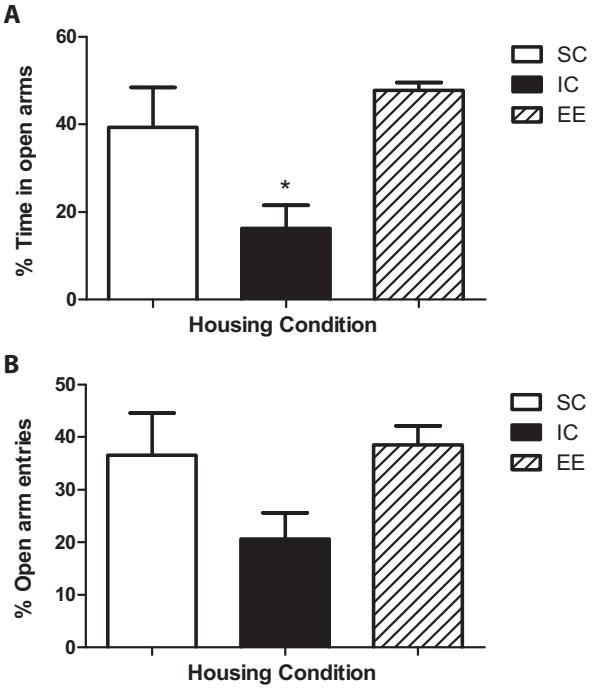

Figure 5. The effects of housing conditions on behaviour in the elevated plus maze.
The effects of housing in isolation (IC), group (SC) and enriched (EE) conditions on (A) percent time spent in the open arms and (B) percent open arm entries in the EPM. Data are expressed as mean + SEM, one-way ANOVA * $p < 0.05$ compared to SC, n = 6.

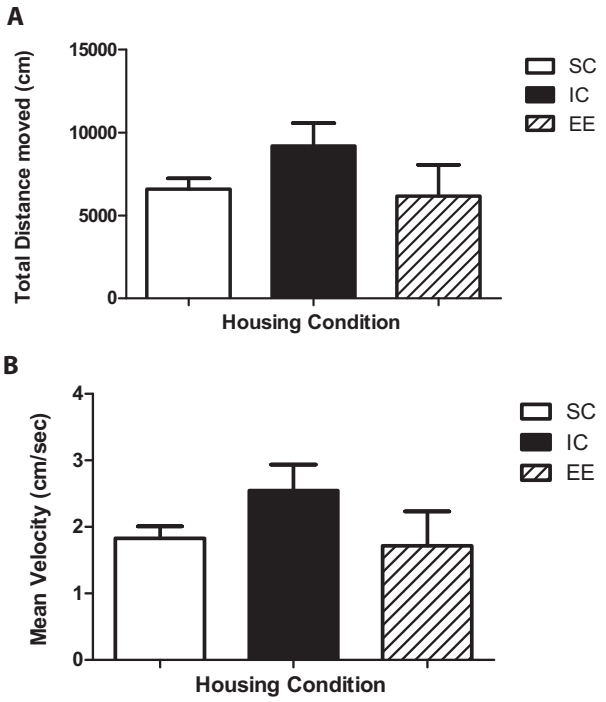

Figure 6. The effects of housing conditions on locomotor activity.
The effects of housing in isolation (IC), group (SC) and enriched (EE) conditions on (A) distance moved and (B) velocity in the HCMA for one hour. Data are expressed as mean + SEM, n = 4.

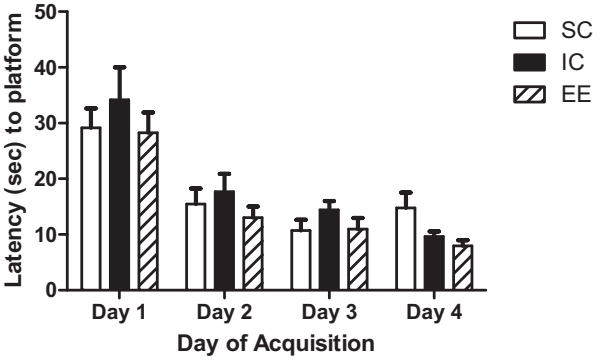

Figure 7. The effects of housing conditions on latency to locate the escape platform in the Morris water maze.
The effects of housing in isolation (IC), group (SC) and enriched (EE) conditions on latency to the platform in the MWM. Data are expressed as mean + SEM, n = 8.

significantly less time in the open arms of the elevated plus maze compared to SC controls. Differential housing had no effect on locomotor activity in the homecage monitoring apparatus, immobility in the forced swim test or escape latencies in the Morris water maze.

The data showed that IC rats gain more weight than those housed in social groups, this is consistent with previous studies[4, 28]. This may be due to differences such as competition for food, increased activity, decreased food consumption and factors such as stress and altered metabolism as a result of housing environment[29].

There was no effect of housing condition on activity in the forced swim test. A previous study demonstrated that 12 weeks EE housing for male SD rats produced the highest swimming and lowest immobility times compared to IC and SC controls[14]. The study carried out by Brenes and colleagues employed a longer period of EE housing and the animals were younger than those in the present study (30 days old compared to 44 days old) which may account for the conflicting results. In addition, Brenes used a larger cage (120cm x 70 cm x 100cm) to house all 15 EE animals together; compared to the EE condition in the present experiment (4/cage, 54 x 38 x 18cm), which could potentially alter the effect of EE on behaviour. Though the Sprague Dawley strain were used in the present study, stock may be an important variable when considering the forced swim test as Wistar rats showed no difference in immobility, swimming or struggling durations between those housed in IC or SC conditions[30], or between SC or EE groups[31].The finding that IC rats spent significantly less time on the open arms of the elevated plus maze compared to EE and SC is consistent with previous studies and suggests the IC rats have greater anxiety and less motivation to explore than those housed in social groups[16]. In the present study EE did not have an effect on elevated plus maze activity when compared to SC controls, this is in conflict with others who have found EE rats make greater percentage open arm entries than SC[32]. As the same rat strain and an 8-week EE protocol was employed by Pena and colleagues it may again be that housing in groups of 10-11 affected the EE experience in that study. On the other hand, in another study carried out by Brenes and others there was no effect of housing on percentage time on the open or closed arms in the EPM[15]. Rats housed in groups of 12 with EE in fact made a greater number of entries to the closed arms compared to IC and SC, which the authors suggest may be due to anxiogenic effects of lighting used, tail-marking and/or strain effects[15].

There was no effect of housing on locomotor activity in the homecage monitoring apparatus. Monitoring locomotor activity is usually carried out using a novel open field arena. In such cases IC animals have been more active compared to SC[10] and EE[10, 14]. The SC and EE rats in the present experiment were singly housed for monitoring in the homecage monitoring apparatus; as they were accustomed to social housing this may not provide an accurate depiction of homecage locomotor activity. In future it would be more suitable to monitor homecage activity in their regular groups or alternatively in the open field arena. Hellemans and colleagues attempted to reverse the hyperactivity effect of social isolation by housing animals in IC for 45 days and then EE for 45 days, however the hyperactivity persisted in spite of EE exposure. Interestingly, rats reared in EE for 45 days and subsequently housed in IC showed a partial reversal of this environmental neophobia[4]. This would suggest that the effects of IC rearing on behaviour depend on the developmental stage at which the rat is in IC.

In the Morris water maze differential housing had no effect on latency to locate the escape platform. Many EE studies employ the Morris water maze as a test of spatial memory and learning following models of brain/spinal cord injury. Rats housed in EE conditions have shown shorter latencies to reach the platform during acquisition and spent more time in the target quadrant in the probe trial compared to SC controls[24]. In one study IC controls took 6 days to reach the platform in minimum time (7-10 seconds), whereas the EE group reached this criterion on day 3 of training[12]. On the other hand, a study investigating the effects of prenatal lead exposure found that non-lead-exposed controls housed in EE or in pairs did not differ significantly in maze performance but EE lowered escape latencies of those in the lead-exposed condition[33]. Similarly EE housing improved the performance of rats exposed to hypoxic-ischemia compared to SC controls but EE had no effect on sham animals' escape latencies compared to the sham SC group[34].

IC housing for male SD rats over 9 weeks resulted in greater body weights compared to EE and SC groups and evidence of greater anxiety on the EPM than those housed in social groups. The absence of housing-induced differences in the forced swim test, Morris water maze and on locomotor activity may be due to methodological issues such as the EE protocol and the test apparatus used; in future a more complex EE protocol may be employed and the open field test would be useful to monitor locomotor and exploratory behaviour. Moreover, the sample sizes employed in this study (n=4-8) may be insufficient to detect subtle differences in behaviour as assessed by these tests. Though inconsistencies exist regarding the effects of EE, it is generally accepted that social housing and EE materials are important to animal welfare[35] and

that IC causes aberrant patterns of development [7]. However the implementation of EE is subject to financial and caregiver's time constraints[36], as well as uncertainties surrounding the possible unintentional effects of EE on the validity of research findings[37]. Questions related to EE to be addressed include: Any negative consequences of EE? Do different strains react differently to EE? Is there a critical period during which EE is best introduced? Considering the divergent types and conditions of EE, and the different durations of exposure, can an ideal EE protocol capable of replication be designed? Adequately controlled experiments will continue to contribute to a greater knowledge of the effects housing conditions such as EE and IC have on rat behaviour and neurodevelopment ensuring optimum conditions for the animals and for research.

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Body weight, feed intake and serum lipids of rats offered hazelnuts as environmental enrichment

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Abstract
Novel food items, such as hazelnuts, are attractive as environmental enrichment, since they form a substrate for both foraging and gnawing behaviour. Hazelnuts are particularly rich in unsaturated fat. A hazelnut, weighing 2 g, contains 820 mg crude fat, contributing with 15% of the total intake of dietary fat when feeding with a typical rodent diet. We examined body weight, feed intake and serum lipids in male and female Sprague Dawley rats, offered one hazelnut weekly each, for 10 weeks. Control animals were not offered hazelnuts. The animals were four weeks old at the start of the trial, and were fed *ad libitum* with a standard rodent diet containing 26.6% crude protein, 5.6% crude fat and 8.4% crude fibre. Body weight and feed intake was measured weekly, and at the start of the trial, after five weeks and after 10 weeks, blood samples were taken from the tail vein for analyses of serum cholesterol, triglycerides and free fatty acids. We found no differences in body weight and feed intake between animals receiving hazelnuts and control animals. Males reached a body weight of 437 $\pm$ 24 g and females 272 $\pm$ 17 g. No differences in serum cholesterol and free fatty acids were observed. Triglycerides levels were slightly elevated in females receiving hazelnuts, although not significantly. On the basis of this, we concluded that hazelnuts are an acceptable form of environmental enrichment, which does not affect growth and levels of serum cholesterol, triglycerides and free fatty acids, when offered once weekly.

Keywords: environmental enrichment, hazelnuts, cholesterol, serum lipids, rats

Nutritional enrichment has the potential of being an attractive supplement to social, behavioural and structural enrichment strategies, although novel food items may contribute to a greater variability in animal studies. On the other hand enrichment strategies should increase the frequency and diversity of positive natural behaviours, decrease the occurrence of abnormal behaviours, maximize the utilization of the environment and increase the animal's ability to cope with the challenges of captivity[1]. Novel food items, such as hazelnuts, are attractive as environmental enrichment, since they form a substrate for both foraging and gnawing behaviour. Potential interference with dietary intake may result in changed body weight, feed intake and serum lipids. Hazelnuts are particularly rich in polyunsaturated fat. A hazelnut, weighing 2 g, contains in average 850 mg crude fat[2], contributing with 15% of the total intake of dietary fat when feeding with a typical rodent diet (Table 1). In order to examine if hazelnuts were acceptable as an additional form of enrichment, we performed a study in which rats were offered one hazelnut per animal weekly for ten weeks. Body weight, food intake and serum cholesterol, triglycerides and free fatty acids were taken as parameters.

Materials and Methods

Forty eight newly weaned, 4 weeks-old Sprague Dawley rats (Taconic, DK-8680 Ry, Denmark) were fed *ad libitum* with a standard rodent diet containing 26.6% crude protein, 5.6% crude fat and 8.4% crude fibre (Altromin 1324, Lage, D-32791 Germany), with free access to drinking water. The animals were housed in groups of three of similar sex, in Macrolon type IV cages with aspen bedding material (Tapvei, Kortteinen, FI-73620 Finland), Enviro-dri bedding material (Shepherd Speciality Papers, Watertown, TN 37184, USA) and a red polycarbonate shelter, at 21-22°C and a relative humidity of 55-60%. As standard enrichment, one aspen biting block (Tapvei, Kortteinen, FI-73620 Finland) was given once weekly to each cage. The animals were randomly divided in groups of twelve animals, and one male and female group was offered

Table 1. Estimated contribution of one hazelnut to weekly nutrient intake of Sprague Dawley rat

	Nutrient value per hazelnut[1]	Weekly nutrient intake of Altromin 1324[2]	% of weekly intake
Energy	37 kJ	1666 kJ	2.2
Protein	210 mg	26.6 g	0.79
Fat	850 mg	5.6 g	15.2
Carbohydrates	235 mg	749 g	0.03
Fibre	140 mg	8.4 g	1.67

[1]USDA National Nutrient Database for Standard Reference, 22nd release, 2009
[2]Based on a daily feed intake of 20 g of Altromin 1324, catalogue values provided by the producer

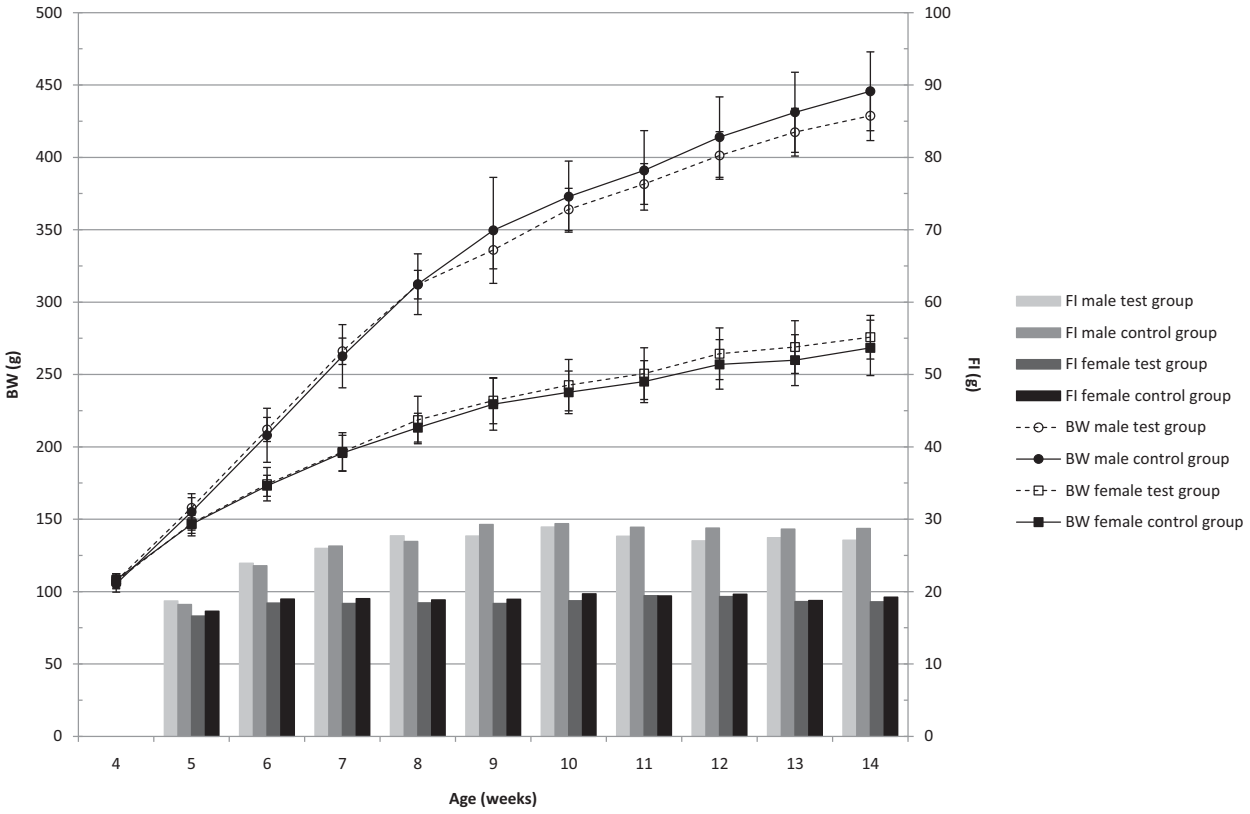

Figure 1. Average daily feed intake (FI), excluding hazelnut consumption, and body weight (BW) of Sprague Dawley rats offered one hazelnut weekly.

one hazelnut weekly per animal during daytime for ten weeks. The remaining animals did not receive hazelnuts, and acted as controls. The hazelnuts were soaked in 70% ethanol for 12 hours, where after they were dried in an incubator at 60°C for one hour, before they were offered to the animals. Only hazelnuts with intact shells were offered to the animals, in order to secure that the kernels had not been in contact with ethanol. Body weight and food intake were measured once a week during the morning. When hazelnuts were offered, the animals were observed and timed. At the beginning of the experiment (t=0 weeks), and after five and ten weeks (t=5 and 10 weeks), blood samples were taken from the tail vein during the morning, one day after hazelnuts were offered. The samples were centrifuged, and the serum was analysed colorimetrically for cholesterol, triglycerides and free fatty acids (Cobas Mira, Roche Diagnostics, CH-4070 Basel, Switzerland with ABX Pentra reagents, Horiba Medical, F-34184 Montpellier, France). Group means of body weight, feed intake and serum lipids were compared using the t-test (Microsoft Excel, Redmond, WA 98052, USA). The study was licensed by the Danish Animal Experiments Inspectorate (license nr. 2010/561-1900).

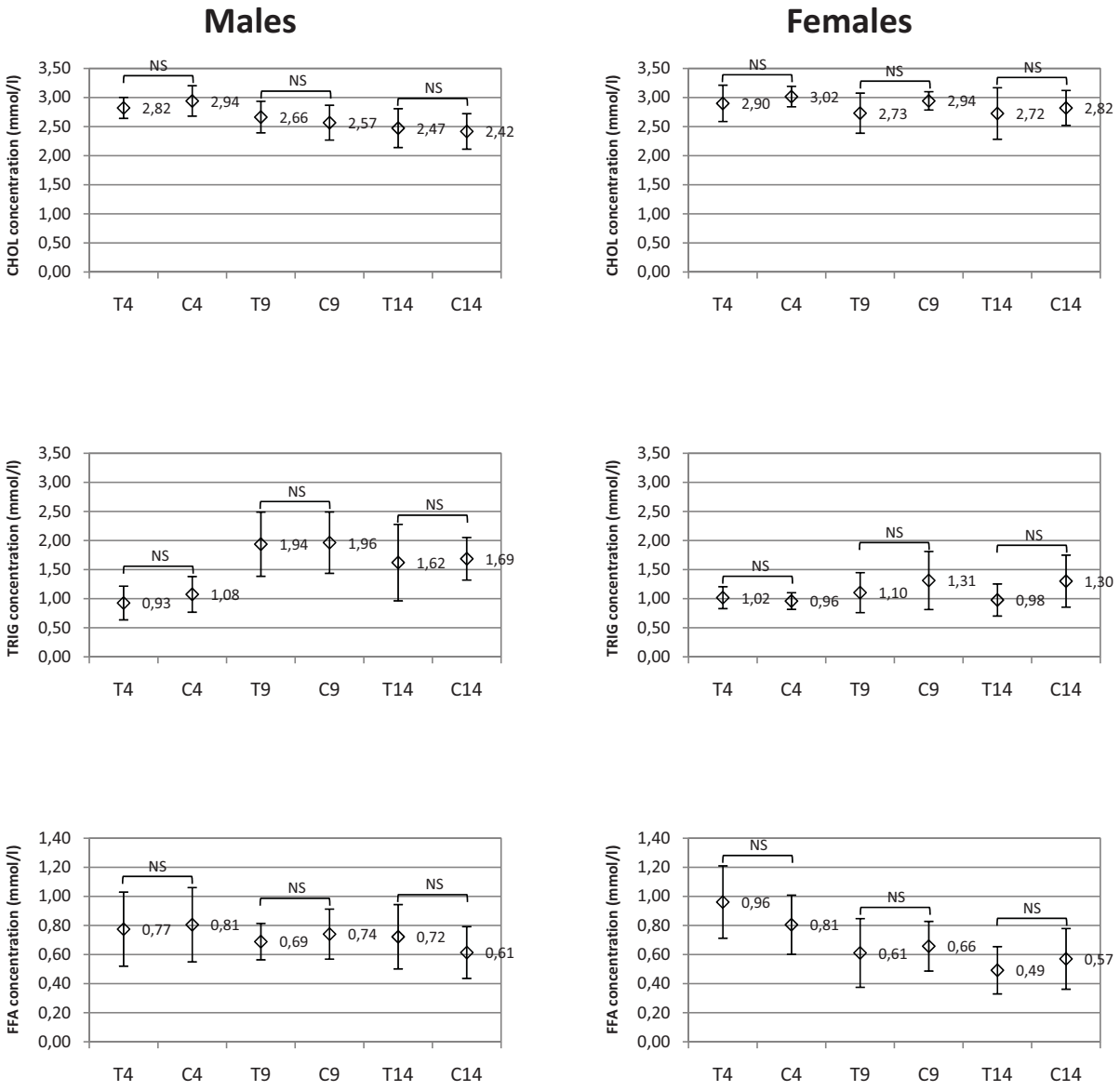

Figure 2. Serum cholesterol (CHOL), triglycerides (TRIG) and free fatty acids (FFA) concentrations of male and female Sprague Dawley rats offered one hazelnut weekly (mean ± SD). On the horizontal axis, T=test group and C=control group, whereas the number represents the age in weeks. NS= not significant.

Results

At the start of the experiment, at an age of 4 weeks, males weighed 106 ± 5 g and females 108 ± 5 g (pooled group means ± SD). During the first two weeks, no hazelnuts were consumed, but in the third week, at an age of 7 weeks, eight males and four females were able to open the shell and consume one hazelnut each. All animals were able to open shells and consume the hazelnuts at an age of 8 weeks, at which age males had a body weight of 312 ± 16 g and females of 216 ± 14 g (pooled group means ± SD). The hazelnuts were consumed readily, within 10 minutes after being offered. No competition was observed, as all animals were occupied. We found no differences in body weight development between animals receiving hazelnuts and control animals. Males reached a body weight of 437 ± 24 g and females 272 ± 17 g (pooled group means ± SD) at an age of 14 weeks. No differences in body weight and food intake were observed (Figure 1). No differences in serum cholesterol and free fatty acids were observed at t=0, 5 and 10 weeks. Triglycerides levels were slightly elevated in females receiving hazelnuts (0.98 ± 0.43 vs. 1.31 ± 0.26 mmol/l), at the end of the experiment (t=10 weeks), but this was not significant (Figure 2).

Discussion

First from an age of 8 weeks, all rats were able to open shells and consume the hazelnuts. At this age, males nearly had tripled (2.9 times) and females had doubled their body weight since the start of the experiment. Body weight and feed intake in animals receiving hazelnuts was similar to that of control animals, and typical for Sprague Dawley rats[3]. From other studies we know that structural environmental enrichment has no influence on body weight and feed intake[4], but it may be expected that nutritional enrichment would have an influence on these parameters. This was not the case in our study. No differences in serum cholesterol, triglycerides and free fatty acids were observed, even though the animals offered hazelnuts received 15% more crude fat than control animals. When feeding rats on a diet containing 14% additional saturated fat (palm oil, coconut oil) significantly increased levels of triglycerides, but no increased cholesterol levels, can be observed after 6 weeks of feeding[5]. The main source of fat in hazelnuts is monounsaturated and polyunsaturated and only 8% is saturated fat. It is well established that, because of this, nuts have a lowering effect on LDL-cholesterol[6 7], but nuts may regulate body weight in humans by suppressing appetite and fat absorption[6]. We did not see a lowering effect or a change in body weight, when offering one nut per animal weekly. Levels of serum cholesterol, triglycerides and free fatty acids were within normal range[8]. On the basis of this, we concluded that hazelnuts are an acceptable form of environmental enrichment, which does not affect body weight, feed intake and levels of serum cholesterol, triglycerides and free fatty acids, when offered once weekly.

The question if nutritional enrichment with one hazelnut per animal weekly contributes to an increased welfare of laboratory rats cannot be answered isolated, since nutritional enrichment with hazelnuts in this study was part of an enrichment programme including aspen biting blocks, paper strip nesting material and a polycarbonate nesting boxes. Variation of environmental enrichment may be an important factor, since enrichment is associated with habituation[9]. As such we consider nutritional enrichment to contribute to variation in enrichment programmes. The current study raises questions for further study, both with regard to the effects of an increased frequency of offering hazelnuts on physiological parameters as well the effects of nutritional enrichment on animal welfare.

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An effective and animal-saving strategy to control pinworm (*Syphacia obvelata*) infestation in a conventional mouse breeding unit.

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We present a procedure for the early detection and control of pinworm (*Syphacia obvelata*) infestation in a conventional mouse breeding unit. We screened 1541 old mice, retired from breeding, with monthly frequency, from genetically modified and inbred strains over a period of three years. The mice were tested evaluating their intestinal content by the plate method. As soon as the first positivity to *S. obvelata* was detected, a topical ivermectin treatment was applied. This cost-effective and efficacious procedure strongly reduced the prevalence of *S. obvelata* for long periods. According to the 3Rs principle, our approach replaces the common use of sentinels and reduces the number of animals sacrificed for monitoring.

Keywords: *Syphacia obvelata*, oxyuriasis, health monitoring, conventional colony, mice

Introduction

The mouse pinworm *Syphacia obvelata* is a common endoparasite that infests both conventional and SPF rodent colonies[1], and the infestation is maintained through ingestion of embryonated eggs. These can survive for long periods at standard conditions in animal facilities and can be easily aerosolized, resulting in widespread environmental contamination. The indirect transmission of these intestinal nematodes through contaminated food, water and bedding results in continual re-exposure to the parasite, making its eradication difficult. The importance of maintaining experimental mice under pinworm-free conditions has became recently evident: oxyuriasis strongly affects physiological functions, modulates the immune system and therefore biases the results of research[2,3]. Containment of infestation is possible with ivermectin, which proves highly effective and has a wide margin of safety. Herein, we present an efficacious and animal-saving strategy to control the prevalence of *S. obvelata* in a conventional mouse breeding unit (about 600 cages over 600 m^2). During a three-year period, we monthly screened for pinworm presence by the plate method in all the retired breeders and promptly administered ivermectin in case of a positive diagnosis. Our procedure, if routinely adopted, assures early detection of infestation and prevents widespread outbreak of pinworm for long periods of time. Finally, as shown in our study, use of retired breeders, instead of sentinels and/or randomly chosen mice from colonies, fully agrees with the 3Rs principle since it avoids unnecessary sacrifices.

Materials and methods

Animals

A total of 1541 retired breeders of both sexes, 6 to 12 months old from both genetically modified and inbred strains (BALB/c, C57BL/6, B6SJL/F1, FVB, SJL), were tested during a period of three years (July 2006 - July 2009). All animals were maintained at the animal facility of the University of Trieste under conventional conditions.

Husbandry conditions

All mice came from colonies kept in three rooms (A, B and C) specifically dedicated to reproduction. The mean number (min-max) of animals per month in the three rooms was, respectively, 1034.76 (790-1217), 414.73 (230-699) and 688.08 (345-1110). During the study, animals of different ages (from newborn to 24-month-old) were present in the three rooms. The rooms were kept at a temperature of 22±2°C, with a relative humidity of 40-60%, with 10-15 changes of air per hour and a light/dark cycle of 12/12 hours (lights on at 06:00). Changing of cages, feeding and cleaning were carried out by a single animal technician in rooms

A and B, while another person was responsible for room C. Room access was restricted to facility staff, who wore personal protective equipment (PPE) including face mask, latex gloves, lab coat and shoe covers, which were taken off when leaving the room. Bedding (IRS lignocel®) and food were changed once a week, whilst all empty cages were sanitized by overnight immersion in sodium hypochlorite (225 ppm). Mice were fed *ad libitum* with a pelleted diet (Global Diet 2018, Harlan Teklad, Harlan Lab., Udine, Italy) and drank tap water from bottles. Mice were housed in polycarbonate shoe-box cages (Tecniplast®) with (room B) or without (rooms A and C) filter tops (mod. 1264C; 267x207x140 mm; mean number of adult mice inside: 4; mod. 1284L; 365x207x140 mm; mean number of adult mice inside: 6; mod. 1290D; 425X266x155 mm; mean number of adult mice inside: 9). Room A contained 120 cages 1264C and 72 cages 1290D. Room B contained 60 cages 1264C, 30 cages 1284L and 50 cages 1290D. Room C contained 130 cages 1264C and 48 cages 1290D. With regard to animal health, infections with mouse hepatitis virus (MHV), mouse norovirus (MNV) and *Helicobacter* spp were present in all rooms. Animal care and management were in accordance with the EU guidelines (86/609/CE) and current Italian law (decree 116/92). Since only culled animals were used and no experimental treatments have been applied, additional ethical approval was not required.

Screening procedures

For the screening of *S. obvelata*, we analyzed all retired breeders that were in any case destined to culling. The analysis was carried out monthly over a period of 36 months, at the moment animals were retired from the colony. The plate method was used to diagnose *S. obvelata*. Mice were sacrificed by cervical dislocation and the caecum and the proximal colon were removed. A section of caecum-colon was placed at the centre of a Petri dish and cut lengthwise. A volume of preheated saline (40°C) of 2.5 ml and 0.3 ml, respectively was poured, at approximately 6 points, into sectioned intestine and periphery of the capsule, spreading the faeces, firstly at the centre and then at the edge of the dish. This operation was performed using a stereomicroscope. The study did not include counting the number of worms; the presence or absence of parasites in the sample was merely marked as either positive or negative. Data were expressed as prevalence P (the percentage of positive animals per screened animals).

Ivermectin treatment

Mice were treated with a solution of topically applied ivermectin[4]. In particular, one part of ivermectin (1% in glycerol; VIRBAMEC, VIRBAC S.A., France) was mixed with 10 parts of tap water in a 0.6 l spray-bottle. Each squirt was measured to deliver approximately 0.5 ml of solution or 0.5 mg ivermectin. Once a week, for three consecutive weeks, the solution was sprayed over the cages, during routine cage change, to disperse about 1 ml and 2 ml, respectively, for small (4-6 animals) and medium (10 animals) sized cages. The anthelmintic treatment was applied as soon as the first positivity to *S. obvelata* was detected in the single room; it was carried out during routine cage change, after shifting all animals, pups excluded, from dirty to clean cages.

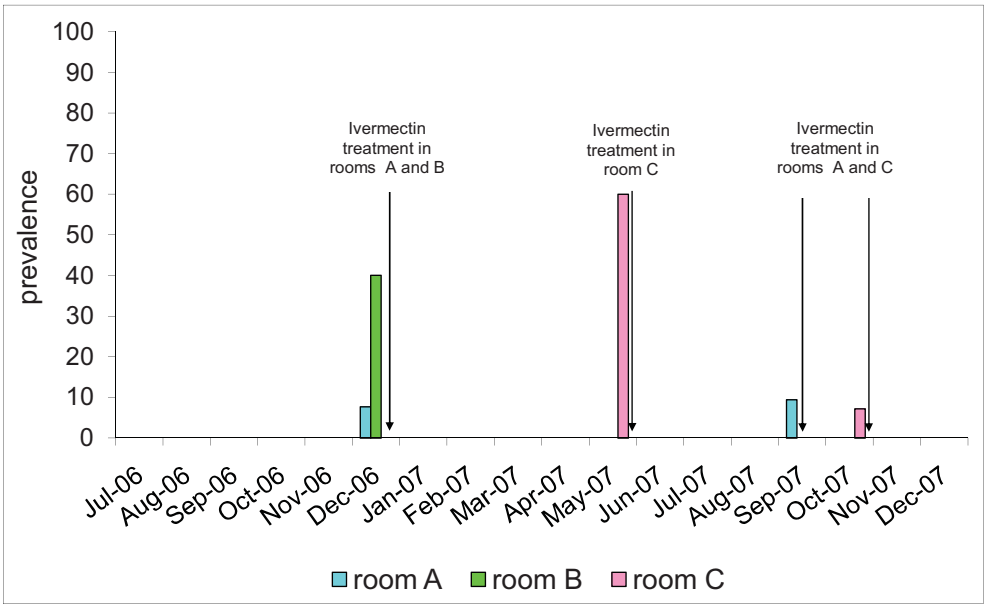

Figure 1. Trend of *S. obvelata* prevalence (P) in the case ivermectin was administered at the first detected positivity. Results are expressed as the percentage of positive animals per number of screened animals. Period from July 2006 to December 2007.

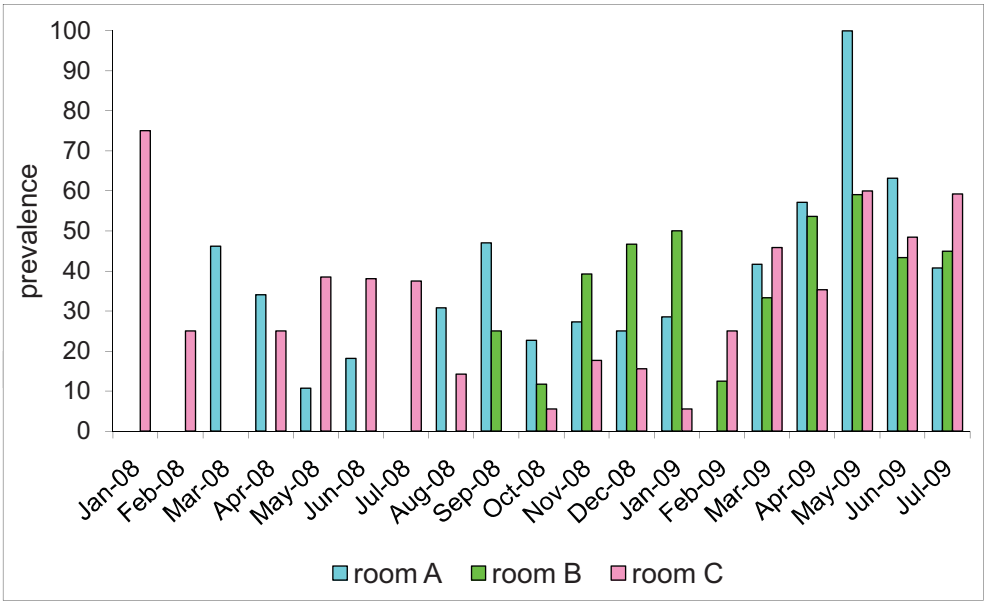

Figure 2. Trend of *S. obvelata* prevalence (P) after ivermectin treatment was discontinued. Results are expressed as in Figure 1. Period from January 2008 to July 2009.

Pups were added only after spraying to avoid topical application of ivermectin. Since the mist of solution was distributed to animals, bedding and cage walls, it follows that mice were not exposed to the full amount of the sprayed ivermectin, thus avoiding the risk of overdose. After January 2008, no further treatment was performed because of the presence of mutant strains sensitive to ivermectin, and we discarded the hypothesis to partially treat mice.

Statistical analysis

We used Fisher's exact test (P<0.01%) to compare the total number of infested mice detected in each room under study during the following periods: from July 2006 to December 2007 (with topical 1% ivermectin treatment) and from January 2008 to July 2009 (without ivermectin treatment)[5].

Results

Parasitological screening was started after an initial ivermectin treatment performed in June 2006, and was continued for the following three years, during which time a total of 608, 457 and 476 mice were analyzed, respectively, in rooms A, B and C, with a mean (min-max) of 16.89 (3-47), 14.28 (1-49) and 14.00 (1-54) per month. In the first five months of the screening, no positivity to the parasite was detected in rooms A and B, while detection was delayed up to the tenth month in room C (Fig. 1). When *S. obvelata* was detected for the first time, although in different moments, the prevalence P was 8%, 40% and 60%, respectively, in rooms A, B and C (Table 1). It was then decided to treat all animals in the positive room with ivermectin, which led to lack of detection of the parasite for 8, 20 and 4 months, respectively, in rooms A, B and C. When *S. obvelata* was detected again in rooms A and C, the P was 9% and 7%; the reinfestation led to a second treatment with ivermectin in these two rooms. In room B, only one treatment had been performed in December 2006, since during the following 20 months mice were negative for the pinworm. Figure 1 shows that during the above-mentioned period, in which ivermectin had been promptly administered at the first detected positivity, the *S. obvelata* prevalence remained close to zero in the most of samples (88.8%). Although no further treatment was performed after January 2008, we kept monitoring the parasite infestation which showed a fluctuating but progressive increase. Figure 2 shows that during this period the P was equal to zero only in 25% of samples and progressively increased, reaching 100% in one case; the first positivity was detected in January for room C, in March for room A and in September for room B (Table 1). Statistical results for the detection of infestation in each room are summarized in Table 2. We compared the first period from July 2006 to December 2007 (with topical ivermectin treatment after any detected positivity) and the last period from January 2008 and July 2009 (without ivermectin therapy). In all three rooms the prevalence observed during the first period was significantly (P<0.01%) lower than that in the second period.

Discussion

We herein present a simple procedure for the early detection of pinworm infestation in a conventional

Table 1. Oxiurids detected from intestinal tract of different mice from rooms A, B and C. "nd" indicates data not detected, "P" indicates prevalence

		room A			room B			room C		
		No. of mice	positive mice/ screened mice	P	No. of mice	positive mice/ screened mice	P	No. of mice	positive mice/ screened mice	P
period with treatment	Jul-06	1158	0/5	0	382	0/16	0	477	0/8	0
	Aug-06	1043	0/15	0	423	0/9	0	472	0/1	0
	Sep-06	1217	0/14	0	359	nd	nd	409	nd	nd
	Oct-06	1208	0/3	0	396	0/6	0	345	nd	nd
	Nov-06	1088	0/9	0	354	0/3	0	450	nd	nd
	Dec-06	1174	1/13	8	370	4/10	40	380	0/8	0
	Jan-07	976	0/6	0	476	nd	nd	410	0/4	0
	Feb-07	790	0/18	0	243	0/4	0	397	0/9	0
	Mar-07	828	0/14	0	230	nd	nd	464	0/11	0
	Apr-07	840	0/9	0	236	nd	nd	458	0/11	0
	May-07	936	0/15	0	261	nd	nd	639	3/5	60
	Jun-07	913	nd	nd	314	0/3	0	699	0/3	0
	Jul-07	958	0/3	0	399	0/5	0	747	0/3	0
	Aug-07	865	0/17	0	398	0/1	0	749	0/4	0
	Sep-07	987	3/32	9	293	0/9	0	816	0/16	0
	Oct-07	1010	0/22	0	343	0/7	0	878	1/14	7
	Nov-07	996	0/25	0	305	0/14	0	1012	0/6	0
	Dec-07	834	0/12	0	284	0/3	0	1110	0/21	0
period without treatment	Jan-08	886	0/20	0	342	0/7	0	956	3/4	75
	Feb-08	976	0/32	0	375	0/8	0	858	5/20	25
	Mar-08	1025	6/13	46	413	0/11	0	823	0/12	0
	Apr-08	1059	16/47	34	354	0/11	0	757	2/8	25
	May-08	1091	3/28	11	344	0/17	0	733	5/13	38
	Jun-08	1034	4/22	18	288	0/7	0	797	8/21	38
	Jul-08	1138	0/8	0	452	0/8	0	767	3/8	38
	Aug-08	1157	4/13	31	412	0/19	0	697	3/21	14
	Sep-08	1164	8/17	47	415	5/20	25	720	0/10	0
	Oct-08	1095	5/22	23	463	2/17	12	700	1/18	6
	Nov-08	1120	6/22	27	579	11/28	39	740	3/17	18
	Dec-08	1088	2/8	25	536	7/15	47	688	5/32	16
	Jan-09	1071	4/14	29	594	12/24	50	800	1/18	6
	Feb-09	1101	0/10	0	567	3/24	13	828	3/12	25
	Mar-09	1107	15/36	42	588	3/9	33	746	11/24	46
	Apr-09	1064	8/14	57	627	22/41	54	699	6/17	35
	May-09	1110	4/4	100	613	13/22	59	733	6/10	60
	Jun-09	1105	12/19	63	699	13/30	43	767	16/33	48
	Jul-09	1074	11/27	41	618	22/49	45	738	32/54	59

Table 2. Total prevalence of pinworm infestation in the three rooms under study. *$P<0.01\%$, Fisher's exact test, "P" indicates prevalence

	positive mice/ screened mice (P) with treatment	positive mice/ screened mice (P) without treatment
room A	4/232 (1.7%)*	108/356 (28.7%)*
room B	4/90 (4.4%)*	113/360 (31.0%)*
room C	7/128 (3.1%)*	110/348 (32.1%)*

animal facility dedicated to the breeding of genetically modified murine lines. In mice, the positivity to *S. obvelata* peaks at 3-4 weeks of age[6,7] and decreases as animals become older[8]. However, our study demonstrates that older mice also (6 -12 months of age) are still useful for diagnostic surveys. Routine tests consist in the detection either of eggs (by faecal floatation, anal swab or tape test in the perianal region), or of the parasite in its larval/adult stage in the intestine (necroscopy of the mouse with analysis of the faecal content of caecum and colon). The test on living animals is affordable and does not compromise their health, however it is the least sensitive. The so-called "plate method" allows the direct detection of the parasite in the large intestine and proves therefore the most reliable[2,9-11]: Therefore it was chosen for the present study, considering that the mice were destined to culling. The programmed application of the plate method to all the retired breeders in our facility allowed the analysis of a large number of mice. This was possible because our facility is entirely devoted to the maintenance of genetically modified murine lines (the number of lines was 35 in 2007, 40 in 2008 and 57 in 2009), with a breeding plan that schedules the substitution of old breeders with younger ones at predetermined time points (every 2-3 months for each line). In this way, although each line is maintained with a limited number of animals (4-5 cages containing 3-4 breeders each), a constant survey is carried out, using a substantial number of mice from each single room, which can be considered an isolated microbiological unit. Some old breeders are randomly retired from each room on a monthly basis, thus providing an instant photograph of the situation and a constant update of the progression of the infestation both during anthelmintic treatment and after treatment was discontinued. The data comprise a relevant number of animals, hardly affordable with a sentinel program.

Ivermectin was chosen for treatment, both for its efficacy against *S. obvelata* and its wide safety margin in vertebrates[4,12]. Ivermectin is an agonist of the GABA (γ-amino-butyric acid) receptor, and its efficacy and safety derive from the different distribution of these receptors in mammals (mainly in the central nervous system) versus nematodes and arthropods (peripheral nervous system and neuromuscular junction). Various methods of administration have been described[2]; we chose the topical method because of its simplicity of application and the possibility of mass treatment, when compared with oral administration in the drinking water or subcutaneous injections. Spray application of ivermectin is safe with no risk of overdose because animals do not take in the entire sprayed dose. Administration of ivermectin entails deleterious effects in immature young mice and genetically modified animals with compromised detoxifying complexes (e.g. mdr1a and mdr1b knockout mice)[2]. The risk of neurotoxicity in newborns was reduced by administering the drug to adults in the clean cage before transferring pups[9], while in the case of sensitive lines, it is suggested to use other compounds (e.g. fenbendazole).

Considering the conventional conditions of our facility, we aimed at containing pinworm infection, with no ambition to eradicate it. As shown from data obtained in room B, the use of filter tops represented a key role in the containment of pinworms for the long period observed (20 months). In this regard, the efficacy of filter tops in bio-containment has been previously documented: one study reported that filter tops could exclude pinworms in 17 of 18 cages for a period of eight weeks, whilst only 4 of 16 unprotected cages were free of the parasite during the same period[13]. We have no explanation for the different duration of effect of the two successive treatments in the same room, considering that the procedures were identical in the two periods. However, it must be noted that lack of analyzed mice from September to November 2006 in room C and from March to May 2007 in room B does not rule out the possibility that a comparable infection was present but undetected.

In conclusion, the procedure herein described allows the analysis of a single, homogeneous microbiological unit using a large number of sample animals on a monthly basis, offering a more detailed and less expensive survey compared to a monitoring program with sentinels. Such a constant survey, combined with a prompt anthelmintic treatment and simple static protection measures (i.e. top filters), allows efficient containment of pinworm infestation for at least six months. Lastly, the choice to examine retired breeders circumvents the use of healthy sentinel animals, sparing unneeded sacrifices and

allowing an effective enforcement of the ethical 3Rs principle – reduction and replacement - in laboratory animal use.

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Welfare of animals and employees in connection with slaughter or euthanasia of animals

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Abstract

The aim of the current project is to study the relationships between investments in human resources and animal care, employee subjective well-being (SWB) and animal welfare (AW), and the organisational efficiency at different workplaces where animals are handled and routinely killed. A three-year interdisciplinary collaboration between veterinary medicine, social science and humanities was initiated in 2009. SWB refers to a person's evaluation of his/her life. Work place and job satisfaction are assumed to influence SWB. The most general definition of AW states that AW occurs when the animal is in harmony with its environment. One important environmental factor for animals is human handling. Content abattoir or laboratory employees are more likely to interact with fellow workers and animals in a friendly way, and non-stressed animals are easier to handle and less likely to cause irritation and stress among the staff. Hence, we hypothesise that there is a mutual dependency between employee SWB and AW at these workplaces, resulting in a virtuous or vicious circle. Employees and animals at abattoirs and animal laboratories in Sweden will be studied. Both abattoirs and laboratories use animals in their production and kill them on a routine basis. However, they differ in e.g. gender distribution, level of education and physical and mental work load. Data will be collected through questionnaires, interviews, analyses of strategic company documents and observations of humans and animals in interaction. The first results are expected in 2011.

Keywords: welfare, employee, animal, slaughter, laboratory

Research has shown that stockperson behaviour and attitudes effects animal welfare.[1] There are also studies showing positive effects of human-animal interaction on human well-being, and animals can be used in treatment of physically and psychologically ill people, for example in riding therapy.[2]

Handling of laboratory animals or slaughter animals in connection with euthanasia or slaughter can cause pain, distress and decreased animal welfare (AW). Pain and distress can greatly affect results from experiments through the release of neurotransmitters and hormones.[3] Chronic stress increases the level of cortisol in the blood which e.g. has an inhibiting effect on the immune system and the growth of young animals. Acute stress, such as pain, causes a number of physiological responses of which many are related to increased levels of the stress hormone adrenaline.[4] Stress before slaughter can cause reduction of meat quality and condemnation of carcasses.[5] Handling and killing of large numbers of animals demands highly skilled staff to secure good AW, which is closely connected to management and organisational culture. Security and care for both employees and animals emanates from the behaviour of managers at all levels and is institutionalised through organisational structure. Tradition-bound reasoning, insufficient or

inefficient information and conflicts between different organisational levels are likely to pose threats to AW.

Objectives

The objective of the project is to describe relationships between resources invested in human resources (HR) and animal care, actual levels of employee subjective well-being (SWB) and animal welfare (AW), and the company efficiency (E) at industrial workplaces where animals are handled and routinely killed. The following hypotheses are put forward:

1. There is a positive association between actual employee SWB and AW, resulting in positive reinforcement (Figure 1). However, under some circumstances, investments in AW can cause a reduction of SWB.
2. Company investments in HR and in AW have a synergistic effect on E (Figure 2).
3. The relationship between SWB, AW and E varies between different work environments, depending on e.g. workplace status, type of work and gender skewness among employees.

The results from this project are expected to contribute to a better well-being for staff and animals at abattoirs and animal labs and also on farms. The results might be used in other areas where individuals are dependent on the benevolence of care givers such as in health care and old peoples' homes. In that way, the project may contribute to economically and socially sustainable development in society.

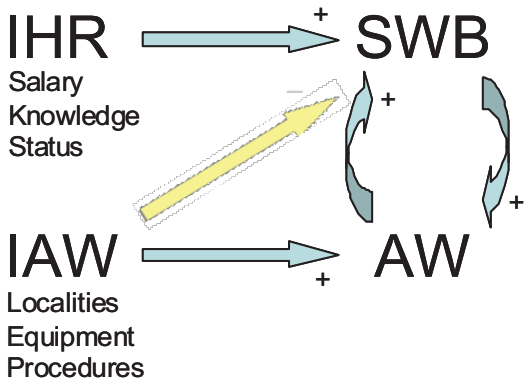

Figure 1. Hypothesised relationship between company investments in human resources (IHR), investments in animal welfare (IAW), actual levels of employee subjective well-being (SWB) and of animal welfare (AW) at workplaces where animals are killed.

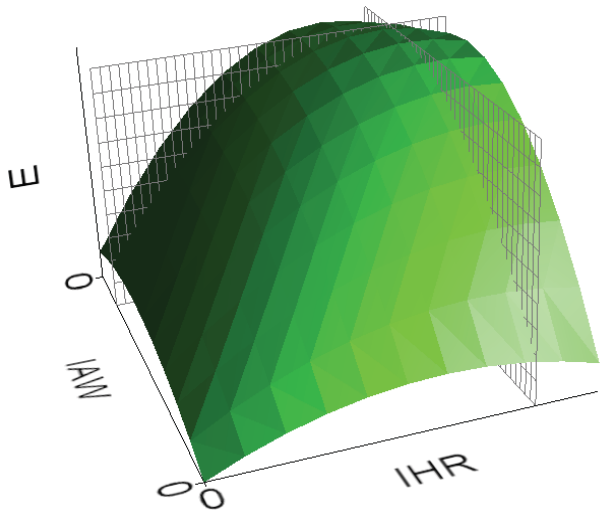

Figure 2. Hypothesised relationship between company investments in human resources (IHR), investments in animal welfare (IAW) and organisation efficiency (E). The marked point of intersection indicates the point of maximum efficiency.

Theoretical background

Animal food production matters to everybody. It influences the environment, the food that people eat and the ethical values that guide the relationship between humans and animals. Including the processing industry and subcontractors, the food sector is one of the largest industrial branches in Sweden. At present, there are 66 Swedish abattoirs for cattle, sheep or pigs, and 23 for poultry.[6,7] In 2009, 460,000 cattle, 255,000 sheep, 2,970,000 pigs, and 75,000,000 chickens were slaughtered in Sweden.[8,9]

Abattoirs can be compared with other workplaces where animals are routinely killed after close contact with staff, for example animal laboratories. According to the European definition of experimental animals 501,000 experimental animals, of which 216,000 mice and 55,000 rats, were used in Sweden in 2008.[10] There are obvious differences between abattoirs and animal laboratories. In the former, the majority of the employees are male, the level of education is low, there are high demands of efficiency along the production line, the work is physically heavy, risky, dirty and noisy, and the work status is low. In animal laboratories on the other hand, females predominate among the staff, there is no conveyor line, the work is more academic, physically lighter, usually clean and quiet, and the work status is higher. In these and probably other respects related to the purpose, organisation, and management of respective companies, the two branches are each other's opposites.

Subjective well-being (SWB) refers to people's overall satisfaction with life. It contains a cognitive (thought-related) judgement of satisfaction and an emotional (mood-related) component of feeling well.[11] There is a distinction between subjective well-being and well-being at work, though both terms are components of mental health. There are also spill-over effects from job-satisfaction to SWB, and the other way around; if you enjoy life in general, it's more probable that you are also happy at work.

Animal welfare (AW) has been defined scientifically in various ways.[12,13,14,15] The concept AW is mainly based from the animal's point of view and is concerned with all aspects of its genotype, behaviour, environment and interaction with its environment. Although it is generally recognised that feelings are very important, there is still some controversy as to whether AW should be defined ultimately in terms of the animal's emotions and feelings only or in terms of biological functioning and state with regard to attempts to cope.[16]

There is substantial knowledge that workplace factors influence employee SWB.[17,18,19] Work-place factors related to motivation can be *intrinsic* (e.g. employee influence, the satisfaction of doing a good job, importance and variation of tasks, and a moderate level of difficulty) or *extrinsic* (e.g. level of education and knowledge, salary, status, and physical and psychosocial work environment), of which the former are more strongly correlated to job satisfaction and happiness.[18] The work environment affects work satisfaction, sick leave and turnover of staff.

There is also good scientific evidence that AW is affected by the way the animals are treated and handled by humans, whose level of knowledge, attitudes and personalities are important influential factors.[20,21,22,1] Handling studies show that fearfulness of humans can cause a chronic stress response leading to decreased growth and productivity of farm animals.[20] Research shows that there are many positive effects of close interaction between pets and their owners.[2] In Animal-Assisted Activity and Animal-Assisted Therapy, animals are used in the care and development of people.[23,24] In such cases, animals are expected to enrich the life of humans and to promote e.g. rehabilitation. In line with previous research we propose that a healthy, satisfied and happy abattoir or laboratory employee is more likely to interact with his or her environment in a friendly way, and to treat fellow workers and animals well. At the same time, a healthy, non-stressed and well-treated animal is easier to handle, and less likely to cause irritation and stress among the staff.

Hence, we hypothesise that there is a mutual dependency between employee SWB and AW at these workplaces, i.e. reinforcement resulting in either a virtuous or a vicious circle. On the other hand, one-sided company *investments to secure* AW might cause extra work load or envy and thus reduced employee welfare, which in turn could *cause negative attitudes* towards the animals (Figure 1). Investments in well-being among both staff and animals should promote work efficiency and save money for the company, regardless of branch (Figure 2). For example, it can be expected that the company's economic efficiency increases with investments in both employee SWB and AW, up to a maximum beyond which it can be expected to drop. Such investments are also expected to act synergistically, i.e. the increase in net return is greater if both types of investments are made simultaneously. As Figure 2 predicts, the effect of altering investments in either employee SWB or AW is greatest at maximum net return (indicated by the large slopes of the curves), indicating an incentive for maintaining both at a high level. The effect could be superior work environment, higher efficiency in the work process with fewer losses (e.g. condemnations at slaughter), improved product quality and improved consumer confidence (e.g. reduced pressure from animal activists on the employees). The described relationships might vary between different branches and types of workplaces.

Economic efficiency in general denotes how well a system uses the ingoing resources to produce an output. The overall measure on organisational performance is the financial outcome. Efficiency is not, though, a plain quantitative concept. It is usually assumed that there is a positive relation between investment in employee well-being and long term efficiency of the organisation.[25] Short-term efficiency, however, can often be reached without any such concern. Machine-like organisations, where employees are more or less regarded as parts of a mechanistic system, can be run effectively as long as people act as parts of the machine. When they, however, start to reflect, address problems, or take action, the machine turns into human co-operation and relations. In the former case, employees take a docile role, not committed and non-responsible. In the latter case, they use and develop their capabilities and take responsibility. Most of us, usually, act somewhere in between these extremes.[26,27]

Material and Methods

We established co-operation with two abattoirs and two animal laboratories in 2010. Collection of data, which will be a combination of quantitative and qualitative data, will start in spring 2011 . Data on subjective well-being will be collected through questionnaires and interviews of employees. There are e.g. questions on a persons feeling of joy and

sadness during the last month and about their general satisfaction in life. Animal behaviour will be observed at and around the time of killing to assess AW. At the same time, interactions between humans and animals will be observed by another observer. The behaviour of the observed animal could then be correlated to the interaction that has taken place. There will also be an ethnographic description from each abattoir and laboratory focusing on the interaction between the animals and the staff to complement the collected quantitative data. Company documents and statistics on e.g. staff turnover, sick leave, investments in HR, leadership training and investments in animal care will be collected. Quantitative data from the questionnaire and the observations will mainly be analysed and presented descriptively, and qualitative data from the interview, the observations and the ethnographic description will be analysed by content analysis. Results from abattoirs and animal laboratories will be compared. The use of animals and humans in the project was approved by the regional animal ethics committee and the regional ethical review board, respectively, both situated in Gothenburg, Sweden. The first results are expected in 2011.

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Workshop: Beyond housing … adding value with the 3Rs

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Keywords: primates, breeders, social housing, training

The aim of the workshop was to examine factors that impact on the welfare and scientific integrity of regulatory toxicology studies in primates.

Working with breeders

The first part of the session was presented by Jörg Luft, who discussed working with breeders. He discussed public perception about the way in which primates are housed and treated in laboratories. He looked at the lifecycle of a laboratory primate at the breeding establishment and then went on to discuss the ways in which, working within a toxicology laboratory, we might work alongside breeders. Areas of importance are health of the animals, conditions to ensure a suitable, enriched environment, and also opportunities for social interaction with conspecifics, and, importantly, with care staff.

Jörg pointed out that traditionally there has been an emphasis on health of the animals, which, though important, has led to compromise in other areas. He discussed the types of enclosures that may be used at breeding establishments, suggesting that improvements may be made by providing more places for the animals to sit. These are especially useful if they are high up to allow the animals to feel safe, but also close to the front of the pen, so that care staff may be approached and opportunities for positive interaction with staff are maximised. Ideally such a perch should allow the animals to sit comfortably, bearing weight on the ischial callosities, and be made from wood. Enrichments within the enclosures, such as swings, are important. In addition, a floor type which allows foraging is needed to allow natural behaviour. Interaction with care staff, with the use of appropriate PPE, can still be maximised to socialise the animals to future handling. However, many of these developments potentially compromise hygiene and a careful balance must be struck.

When animals are due to be shipped from the breeders, a quarantine period may be required. Although this often requires isolation of the animals from conspecifics, interaction with care staff, with the use of appropriate PPE, can still be maximised. Pair housing during quarantine periods should be considered.

In conclusion, Jörg emphasised the importance of keeping lines of communication open between breeders of primates and end users, so that suggested changes at the breeding facility may be discussed and the implications of these for health, but also for welfare of the animals might be considered in detail.

Refinement of social housing

The next part of the session was presented by Wolfgang Müller, and looked at refinement of social housing. Wolfgang discussed the importance of provision of sufficient space, in line with the ETS 123 requirements, and then went on to discuss development of social housing at Covance's facility in Münster, Germany. Here a system of multifunctional social housing was developed, passing through several generations of prototypes. Selection of compatible cage mates to occupy this housing requires careful consideration. For each animal, the history, origin, prior experience and previous grouping is taken into account. Animals are then observed once they have arrived at the facility, and snapshot dominance matrix ethograms are made. This involves scoring the affiliative and the dominant/subordinate interactions of the animals. Once scored, animals are grouped by scores to ensure the correct balance in the group with suitable dominant and subordinate animals. To allow this type of scoring to take place, staff must be trained in the normal behavioural repertoire of the animals and its interpretation. In addition, animals are trained to interact positively with care staff, using treats as positive reinforcement. The three described elements – optimized cage type, training of staff and animals and the systematic approach to assign compatible cage mates – form the basis (or pillars) of the "Three Pillar Housing Concept" for NHPs at the Münster facilities.

After this section of the workshop, there was a lively discussion around understanding interactions of primates, and the usefulness of a behavioural scoring system where only a brief behavioural snapshot was taken.

Training of the primates

Angela Bodey discussed various aspects of training of the animals, beginning with the thesis that if the animals enjoy good welfare then the quality of scientific data produced will be better, allowing more robust interpretation. Angela discussed a number of training initiatives used at Covance Harrogate. The first considered an enhanced socialisation programme, where increased time for socialisation and increased use of treat feeding by care staff led to recordable reductions in blood pressure compared with animals given a standard socialisation programme.

Angela then discussed restraint of animals, in this context for the acquisition of ECGs, sharing data which showed that development of a tube for restraint of the animals led to better quality data allowing more detailed interpretation. In addition, it was shown that heart rates were lower when tube restraint was used rather than manual restraint, assuming appropriate habituation to the restraint procedure. Habituation of the animals to wearing jackets was illustrated. This allowed the use of jacketed external telemetry for ECG recording, yielding even better quality data, with recordings taken from the animals whilst group housed in their home pens.

This section of the workshop finished with comments about the use of positive reinforcement training programmes, especially for animals expected to spend considerable time in the facility, not only to improve their co-operation with various procedures, but also to improve their welfare, viewing a training programme as a form of enrichment.

There was then further discussion between participants, around the use of tubes for restraint, and the welfare implications of this, as well as the importance of increased awareness and communication between all stakeholders in order to achieve the goal of better welfare and therefore better science.

Procedures on Animals

Perioperative and surgical procedures which minimize pain and distress in swine: A review

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Abstract
Swine are commonly used as a general surgical model. Included in the major surgical models are those of the cardiovascular system, digestive system, urogenital system, organ transplantation, wound healing, and bioengineering studies. Swine are a preclinical model for the implantation of devices and biomaterials in many organs and systems. Implanted devices include stents, grafts, tissue engineered structures, mechanical devices and artificial organs. With their increased international use veterinarians and research staff need to be involved in providing for the needs of the animals in the least stressful manner. This includes the use of proper housing, socialization, selection of anesthetic protocols, preemptive analgesia and proper perioperative monitoring. Surgical procedures using surgical techniques which are less invasive than open techniques are increasingly being used in research and practice. One of the newest developments in these types of procedures is natural orifice transluminal endoscopic surgery (NOTES). This manuscript reviews the principles and practices of these various techniques.

Keywords: swine, miniature swine, surgery, perioperative care, NOTES surgery

Both domestic and miniature swine are used commonly in invasive surgical procedures requiring intense perioperative care protocols to minimize their pain and distress.[1-3] Swine are social animals and gain from having non-stressful social interactions both with other swine as well as humans. Inherent in their care is the use of preemptive analgesia for surgical procedures as well as surgical techniques which minimize the incision size and postoperative wound healing timeline. New techniques in surgery are being developed and tested in swine which also accomplish this, such as natural orifice transluminal endoscopic surgery (NOTES).[4-6] A review of techniques of minimizing pain and distress and an introduction to NOTES surgical techniques are described in this manuscript.

Preoperative and Anesthetic Considerations[1-3]

It is best to house animals for a seven day acclimation period prior to using them for survival surgical procedures. This allows animals to recover from shipping stress, accommodate to dietary changes and acclimate to personnel and husbandry methods. Interaction with other swine can be accomplished without housing them in groups. Having swine in the same room with visual and olfactory contact provides a form of interaction. If the pens are designed so that swine have nasal contact with other members of their species then this also provides for this intraspecies interaction. During this time personnel should handle the animals and provide them with food treats to allow non-stressful interactions to develop which will facilitate postoperative care.

Swine, even miniature breeds, are powerful animals and should be controlled using non-stressful techniques rather than being manhandled. Use of restraint slings is recommended (Figure 1). Swine will always move away from an object that is pointed at their snouts so "hog panels" can be used to direct animals without manual restraint.

Swine cannot bend to chew or scratch at their incisions; consequently, they will rub an area that is irritated. These behavioral traits need to be taken into consideration during the preoperative planning sessions. Caging needs to be designed which provides the animals with a comfortable and dry environment free of objects against which they can rub. They also require enrichment toys that they can manipulate to satisfy their "rooting" behavior. Swine develop a dunging pattern and will defecate at an area away from their food dish. Water should be supplied with an automatic watering system. Food dishes should

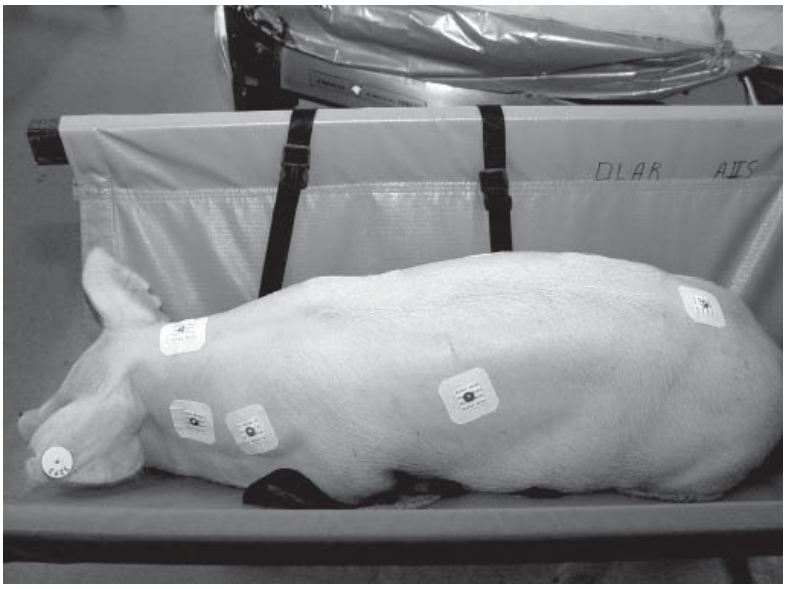

Figure 1. The pig is being restrained in a humane sling and has adhesive strips attached for recording an electrocardiogram.

be tightly attached to the side of the cage and the latches on the cage should be secure. The best type of flooring to use is elevated fiberglass slats with moderate grit.

Swine can be routinely fasted for 12 hours prior to surgery, but they should not have water restriction unless the procedure is a gastrotomy. It requires 2-3 days of fasting to empty the spiral colon but oral purgatives may be used to accelerate the process. If swine are housed on contact bedding such as hay or wood chips, they will eat it when fasted and may become impacted. It is not a good practice to house swine together in the postsurgical period because they are cannibalistic and the dominant pig may attack the wounds of a subservient pig. Wounding each other while developing the social domination order in group housed pigs also provides a complication if they undergo surgery with an infected wound on some part of their body.

Preanesthetic administration of tranquilizers and anticholinergics is helpful in sedating swine. Intramuscular injections are painful in swine due to their dense muscular tissue. Subcutaneous injections into the side of the neck using butterfly catheters are the preferred method of giving injections. Without restraining the animal the catheter needle is quickly inserted into the neck behind the ear. The end of the catheter connected to the syringe is then picked up and the injection given. Acepromazine and diazepam are non-painful and can be given preliminarily 5 minutes before giving an induction dose of an agent such as ketamine to minimize distress in the animals. Atropine may be used to help dry oral and respiratory secretions in order to facilitate intubation.

Anesthesia and Analgesia Techniques and Agents

No single anesthetic or analgesic protocol can be used for all surgical procedures. The physiologic effects of the agents have to be considered in order to avoid complicating effects on the research. If the effects of the anesthetic do not matter, then general anesthesia with the inhalant agents isoflurane or sevoflurane should be the default. Preemptive analgesia should be used for all survival surgical procedures. This consists of administration of a systemic NSAID and/or opioid analgesic prior to making the skin incision with consideration of other adjunct techniques. Local anesthetics block nociception and can be used either as an infiltration of the skin incision or as a regional block. Epidural morphine is effective for analgesia of the abdomen and hindquarters. The recovery period will be shortened by using these preemptive techniques.[1]

Aseptic Preparation, Intraoperative Monitoring and Care[1-3]

While under general anesthesia for major surgical procedures all animals should have their temperature, ECG and respiratory parameters monitored and recorded as a minimum. Noninvasive blood pressure monitoring, pulse oximetry, end-tidal CO_2, and blood gas determinations are useful and may be essential for some procedures.

Homeostasis needs to be controlled by maintenance of core temperature and fluid support. A flow rate of 5-15 ml/kg of Lactated Ringer's solution or other isotonic solution will provide fluid support during general anesthesia. Core temperature can

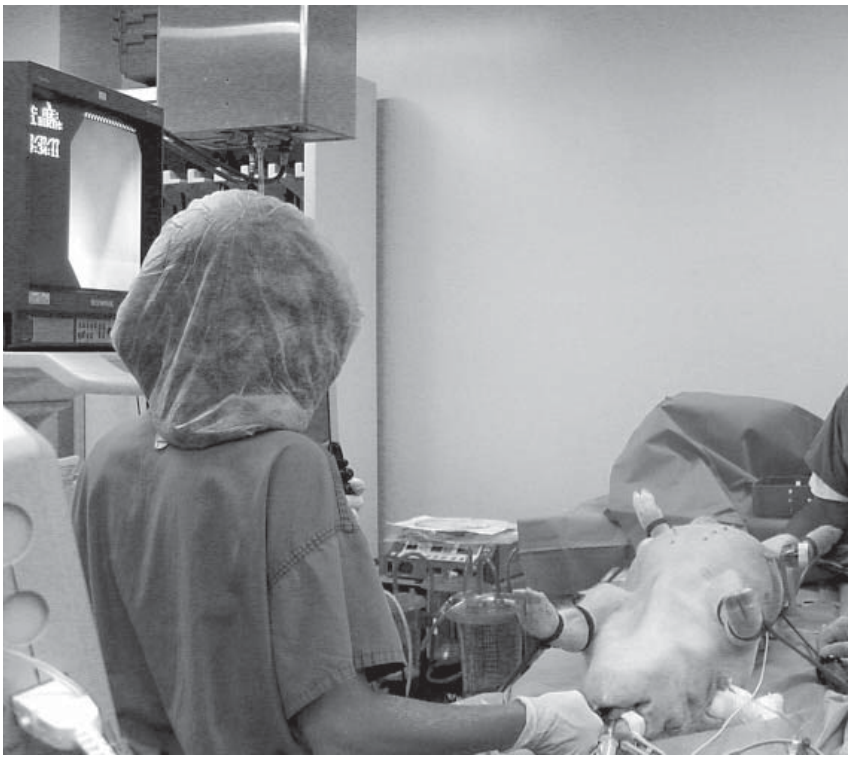

Figure 2. A transgastric NOTES procedure is being performed on the pig.

be maintained using circulating water or forced air blankets.

Strict aseptic technique should be utilized for all surgical procedures. The initial skin preparation should be done in a room separate from the operating room. Skin preparation usually consists of alternating scrubs with a bactericidal soap and alcohol. A final sterile prep should be performed in the operating room prior to surgical draping of the animal. Application of iodine-impregnated sticky drapes prevent any contamination of the wound from the skin as well as the use of wound protectors for the edge of the skin. Animal prep, surgeon prep and the operating room need to be separate rooms to prevent cross contamination. Other areas that are required are an area to recover animals from anesthesia, instrument cleaning and storage of sterile supplies.

Postoperative Care[1-3]

Animals need to be continuously monitored until they recover from anesthesia and have regained a righting reflex. During this time heart rate, rectal temperature and respiratory rate should be monitored as a minimum. It is also helpful to monitor ECG and pulse oximetry. For complex cases the blood pressure should also be monitored.

Assuming preemptive analgesia was used as described above, most cases will require analgesics for only a few days. Animals should be monitored for incisional pain and inflammation and normal physiologic function. Parameters that should be considered include appetite, bowel movement, urination, normal posture, normal behavior, rectal temperature, heart rate and respiration. As a minimum animals should be monitored until sutures are removed or the surgical wound is healed which generally is approximately two weeks. If a diseased state was created, such as a heart failure or transplant model, then the animals may have to be monitored daily for extended periods of time. Some protocols may require monitoring of blood chemistry values or other measures of physiologic function.

Natural Orifice Transluminal Endoscopic Surgery (NOTES)[4-6]

NOTES surgical techniques have been developed as a result in advancement of laparoscopic and endoscopic surgical techniques. The basic technique involves passing a modified endoscopic tube through a natural orifice into the abdominal and thoracic cavities in order to perform a procedure. Natural orifices which have been used are the mouth, vagina and rectum (Figure 2). For example, a cholecystectomy can be performed by passing the tube through the mouth, down the esophagus and into the stomach. A perforation is made in the gastric wall, dilated with a balloon catheter and the endoscopic surgical device passed into the abdominal cavity. Endoscopic devices typically have a fiber optic camera and various grasping and cutting devices attached, as well as suction. The surgical procedure is performed by visualizing the field on a TV screen, in the same manner as for laparoscopic surgery (Figure 3).

Following the procedure any surgical incision is closed using a snare and clip technique (Figure 4). For example in the stomach the gastric wall would be inverted into the lumen and closed with the snare. The same procedure could be performed by either perforation of the vaginal wall or colonic wall. The structures which are penetrated can be pre-flushed with an antibiotic and saline solution. Likewise, the abdominal cavity can be flushed and drained if contamination is felt to be an issue.

Swine have been used almost exclusively as the research and training model for these procedures.

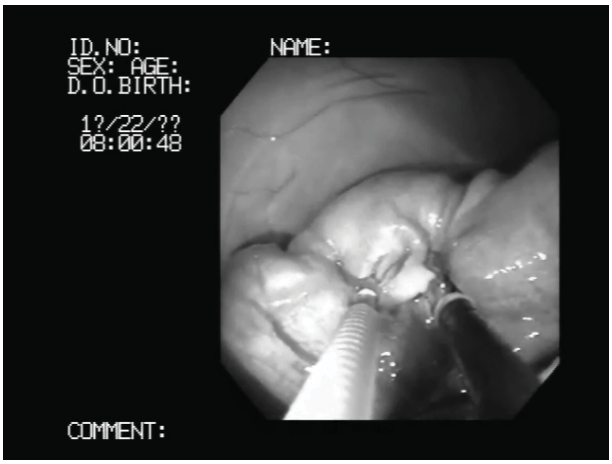

Figure 3. A colonic biopsy is being performed using a transgastric NOTES approach.

Some procedures have begun to be performed in humans as well. Procedures which have been studied and applied include cholecystectomy, ovariectomy and intraabdominal biopsy. Other procedures are under development including intrathoracic procedures.

Advantages to this technique are that it does not involve an open surgical procedure and penetration of the natural orifices is painless and may be able to be performed on an outpatient basis. The Natural Orifice Surgery Consortium for Assessment and Research Committee (NOSCAR) has set standards for the Society of American Gastrointestinal Endoscopic Surgeons (SAGES).[4] They list the following theoretical advantages to NOTES: avoidance of wound infections, less adhesions, decreased ileus, rapid recovery, less pain, avoidance of hernias, more cosmetic surgery, and less invasiveness. These advantages have been confirmed in some experimental studies in porcine models.[4-5] This may be considered a form of refinement since it reduces healing time and the invasiveness of the surgical procedure.

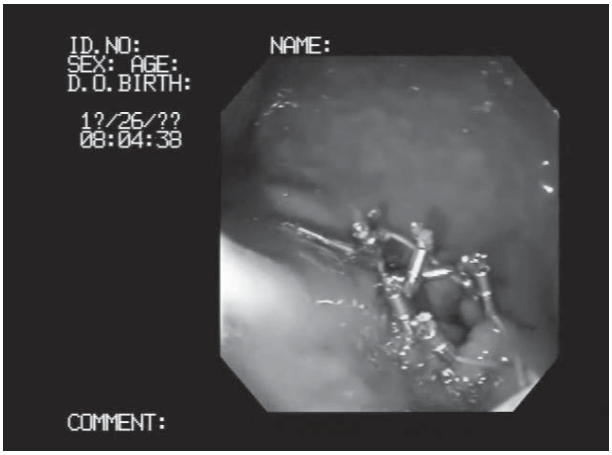

Figure 4. Closure of a NOTES wound using titanium snare clips.

There are procedural related issues to be addressed. There is a need to develop better endoscopic instruments, closure techniques and to address retraction/visualization issues. It is likely that NOTES will become a new revolution in surgery in the same manner as laparoscopic surgery evolved in the 1980's and 1990's. And just as laparoscopic surgery in companion and research animals is now commonplace it is likely that NOTES will also be developed in the same manner.

Discussion and Recommendations

Swine have replaced dogs and other species internationally as the general surgical model for research and training in biomedical research. There are certain critical factors that should always be considered when designing a surgical protocol for them. The anesthetic and analgesic regimen should be based upon the physiologic effects of the agents selected and their potential compatibility with the goals of the project. For survival surgical procedures swine should be acclimated to the facility and socialized with the personnel involved in the project. Preemptive analgesia should be performed as a routine. Housing should be designed to be non stressful for the animals. Surgeons should be properly trained in performing surgical techniques in this species. With a team approach of veterinarians, technical staff and researchers it is possible to conduct major experimental surgical protocols in swine with minimal complications.

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Voluntary ingestion of buprenorphine as a refined analgesic strategy

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Abstract

Buprenorphine is a widely used analgesic for laboratory rodents. Administration of the drug in an attractive food item for voluntary ingestion is a desirable way to administer the drug non-invasively. The method refines the standard analgesic procedure and has the potential to improve the welfare of laboratory mice and rats. However, many aspects of the voluntary ingestion method still need to be investigated. This paper examines the concept of voluntary ingestion of analgesia and reports new findings about voluntary ingestion of buprenorphine in mice.

Keywords: analgesia, voluntary ingestion, mice, refinement,

Introduction

It is a moral mandate to eliminate avoidable pain and stress from animal experimentation. In addition to being unpleasant and potentially distressing for the animal, pain and stress can slow recovery after surgery, interfere with the normal physiology and reduce the animals "self-maintenance" behaviour.[1-3] Prolonged pain may slow healing, cause organ dysfunction, impair the immune system and may result in detrimental endocrine and metabolic changes.[4] Beside the adverse consequences for the suffering animal, pain and stress increase the variation within and between animals and constitutes an unwanted source of experimental error. Appropriate pain treatment should therefore always be given animals subjected to invasive procedures. However, concerns have been raised that analgesics may interfere with the animal model.[5;6] Further research is needed in order to evaluate the influence of analgesia on various models, but letting animals suffer from post-operative pain is a serious welfare concern.[7]

Buprenorphine is a highly potent opioid and is widely used as an analgesic in laboratory rodents subjected to mild to moderate surgical procedures. The recommended route of subcutaneous injection requires dosing every 8-12 hours with doses of 0.05-0.1 mg/kg bodyweight [bw] in mice and rats. These doses have been effective in reducing pain perception in several clinical studies and analgesiometric tests.[8] However, the repeated dosing may stress the animals and result in fluctuating serum concentrations if not injected at correct intervals. The frequent dosing may further disrupt the animal's diurnal rhythm, and frequent stressful experiences may affect the experimental outcome.[9;10] Furthermore, small animals subjected to injections, often display symptoms of distress. Alternative non-invasive routes of administrations may thus refine the standard analgesic procedures and should be welcomed.[7]

Efficiency of orally administered buprenorphine

Oral dosing of buprenorphine has resulted in high and longer lasting serum concentrations than subcutaneous dosing in mice.[11] However, oral dosing by gavage requires restraint of the animal. The potential stress of this procedure can be eliminated by allowing the animal to voluntarily consume the drug, a method which has gained some acceptance as an analgesic regimen in rats.[12-14] It has recently been demonstrated that it is possible to reduce the adverse corticosterone induction associated with invasive procedures in laboratory rats, using a self-administration system offering operated rats Nutella® (a nut-based paste for human consumption) with buprenorphine.[12] Voluntary ingestion of buprenorphine in rats has, however, had different degrees of success. Doses 100 times higher than those recommended for subcutaneous injection have been claimed necessary to induce serum concentrations of buprenorphine providing effective analgesia in analgesiometric tests.[15] A study by *Thompson et al* concluded that only the recommended dose of 0.05 mg/kg bw buprenorphine given by

subcutaneous injection is successful in increasing the latency time in the hot water tail flick test.[16] Furthermore, a study using fruit flavoured gel as the food item, demonstrated that oral administration of buprenorphine in concentrations inducing appreciable analgesia resulted in unpalatable mixtures not voluntarily consumed by the rats.[17] However, a recent study by Leach *et al* demonstrated that buprenorphine mixed in jello or syrup was effectively ingested by all the rats in the study, but the analgesic effect measured by thermal antinociceptive thresholds were of shorter duration that after subcutaneous dosing.[18] By contrast, Goldkuhl and co-workers found that oral doses of 0.4 mg/kg bw reduced the post-surgical level of circulating corticosterone in rats subjected to permanent catheterization.[19] In agreement with this, oral doses of 0.3 mg/kg bw have been demonstrated to be efficacious in inhibiting post-surgical body weight loss and reduced food and water intake.[13] The discrepancies may be due to differences in study design, pain assessment methods and strains tested. Therefore, comparing data obtained from analgesiometric tests with studies investigating clinical post-operative pain may not be meaningful.[20;21] Standard pharmacokinetic indices of buprenorphine suggest that oral dosage should be 10 times the parenteral dose to compensate for the difference in bioavailability[22], which agrees well with the findings of Goldkuhl and co-workers[19] and Flecknell and Roughan[13].

Voluntary consumption of buprenorphine

One of the earliest studies describing self administration of an analgesic was reported in 1980. Colpaert and co-workers demonstrated that rats injected with *Mycobacterium butyricum* developed chronic arthritis and drank significantly more suprofen solution compared to control rats.[23] Later, Debb and colleagues provided rats buprenorphine mixed in drinking water and investigated the effects in analgesiometric tests.[24] This resulted in increased reaction time in tail immersion test. Mixing analgesics in drinking water has, however, been debated since the drinking behaviour is often negatively affected by adverse taste or smell of the solution.[25] Furthermore, the animals have to consume a sufficient amount of the solution to provide an efficacious dose of the analgesia and each animal's water intake should be monitored in order to estimate the concentration of the analgesic in the circulation. Finally, since rats consume more than 90% of their daily water intake during the dark phase of a light/dark cycle, analgesic dosing during daytime may be insufficient.[26] However, some studies document that the voluntary drinking method can be successful and allows the animals to self-administer the analgesia on demand.[27]

Another way of administering the analgesic is mixed in a food item. Several studies have shown that administration of buprenorphine in a desirable food item for voluntary ingestion is an attractive way to administer the drug non-invasively.[5;28;29] Chocolate, jello (berry, orange, lime and strawberry flavors), syrup, marmalade and the commercial nut paste Nutella® are some of the food items used.[5;14;19] We have recently investigated how readily female and male mice of two different strains consumed buprenorphine mixed in Nutella and potential effects of variation between genders and strains on ingestion time and subsequent serum concentrations of buprenorphine.[30] In this study, we tested 1.0 mg/kg bw and 3.0 mg/kg bw buprenorphine (Temgesic, Schering-Plough Europe, Brussels, Belgium) mixed in 10 g/kg bw Nutella (Ferrero, Pino Torinese, Italy). Nutella 10 g/kg bw was used as control. The study demonstrated that even high doses of buprenorphine in Nutella were successfully ingested by male and female BALB/c and C57BL/6 mice. This is in contrast with a previous study in mice, concluding that voluntary ingestion of buprenorphine (mixed in a pina colada-flavored treat) is not possible due to the adverse taste.[31] Furthermore, in contrast with a study where rats were offered buprenorphine mixed in a gel ("buprenorphine-jello"), there was no difference in the time the mice spent on ingesting the treatments compared to the Nutella control.[17] Even after treatment with the high dose (3.0 mg/kg bw; 60 times the subcutaneous dose of 0.05 mg/kg bw), we were not able to detect any difference compared to pure Nutella. This may be related to the high palatability of the Nutella. Furthermore, we found that female mice consumed the treatments significant faster than male mice. The reason for the sex difference in ingestion time may not be related to response to the novel food, but may reflect the females' preference for sweet food items, as demonstrated in several studies in rats.[32-35] Similar studies have, to our knowledge, not been performed in mice.

A significant reduction in the start time of ingestion (the time from providing the buprenorphine-Nutella mix to the time when the animal began eating it) was seen after repeated exposure in both BALB/c and C56BL/6 mice.[30] Likely, this is caused by the neophobic nature of mice.[36] Neophobia ("fear of the new") is commonly observed in both mice and rats and it is an important part of these animals' survival mechanism. This reaction to novel food items protect the animals from eating food that provokes or causes sickness.[37;38] Mice should therefore be given Nutella 1-2 times before an experiment in order to habituate to the novel food. The same have been reported in rats.[12] For both species, the animals' reactions to the novel food may depend on social status, strain, odour, previous exposure and experiences, age, sex

and exposure via conspecifics.[39-41] However, when comparing female and male mice of the two strains, we did not find any differences in start time of ingestion, despite male mice being considered less neophobic than female mice and superior in regards to localizing and recognizing new objects.[42-44]

When comparing serum buprenorphine levels 17 hours post administration of voluntary ingestion of 1 and 3 mg/kg bw buprenorphine mixed in Nutella, we observed a tendency towards C57BL/6 having higher levels of serum buprenorphine than BALB/c mice.[30] Strain differences in metabolism and effect of drugs are well recognized in mice.[45-48] Further studies are therefore needed to evaluate this potential difference at other time points and to assess the biological consequences of the analgesic treatment and dosing. To our knowledge, pharmacokinetic analyses of buprenorphine have not investigated the variation between different strains of mice. However, genetic variation in analgesic potency, tolerance and physiological effects of morphine has been reported[49-52] both due to behavioural and genetic differences[53;54]. Similar strain specific reactions have been reported in mice in relation to buprenorphine[55], but the actual serum concentrations of buprenorphine have not been investigated.

The differences in start time after repeated exposure and the sex differences in voluntary ingestion time in male and female BALB/c and C57BL/6 mice did not affect the subsequent serum concentrations of buprenorphine measured 17 hours post administration.[30] This is probably because these differences were too small to have any effect on the serum concentrations when measured several hours post administration. It is therefore possible to achieve measurable levels of circulating buprenorphine in mice 17 hours following presentation of the drug, regardless of the sex. This indicates that buprenorphine can be administered well in advance as a pre-emptive analgesic using the present voluntary ingestion scheme. However, further studies on this issue are needed to verify whether the observed serum concentrations of buprenorphine also have a significant biological effect in reducing post-surgical pain.

Concluding remarks

Several studies have shown that administration of buprenorphine in a desirable food item for voluntary ingestion is an attractive way to administer the drug non-invasively in rats. We have recently demonstrated that even high doses of buprenorphine in Nutella will successfully be ingested by two commonly used strains of mice. Voluntary ingestion of buprenorphine mixed in Nutella is thus an effective way of achieving high levels of circulating buprenorphine, since the palatability of the drug-Nutella mix is high and since oral ingestion results in long-lasting high serum concentration levels. The present voluntary ingestion method in mice is thus a new way of administering analgesia that refines the standard analgesic procedure and has the potential to improve the welfare of laboratory mice.

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Refining the experimental models of chondral and osteochondral injuries in the sheep knee: an improved tool for preclinical studies in regenerative medicine

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Abstract

The purpose of this study was to refine the sheep model of articular cartilage lesions, for testing the therapeutic potential of new approaches in regenerative medicine in the preclinical animal model. Twenty-four sheep divided into two groups (chondral lesion group and osteochondral lesion group) were used in this study. We developed minimally invasive surgical procedures (arthroscopy) for experimental lesion induction and cellular therapies implantation. We used as non-invasive diagnostic methods the ultrasound (US), the magnetic resonance imaging and radiography to characterize the lesion and to assess the progression of the applied articular treatments during the entire experiment. Reproducible experimental lesions were achieved by arthroscopy. Lesion and treatment evolutions were successfully performed by non-invasive diagnostic methods and US reveal to be a useful tool imaging the injured articular cartilage. We conclude that the application of the developed refined techniques promotes animal welfare, and increases research quality.

Keywords: chondral lesion, osteochondral lesion, arthroscopy, ultrasound, sheep knee, cellular therapies

Articular cartilage damage frequently results from injury or diseases such as rheumatoid arthritis or osteoarthritis. It has a significant social and economic impact on the aging population.

The articular cartilage response to injury depends on the severity and depth of the injury.[1,2] The avascular nature of articular cartilage means that pure cartilage injuries do not cause haemorrhage or fibrin-clot formation. The chondrocytes respond by proliferating and increasing the synthesis of matrix macromolecules near the injury location, but they cannot restore the surface.[1] On the other hand, a full-thickness lesion that penetrates the subchondral bone provides access to cells, blood supply and hematoma formation. It results in fibrocartilage formation, a repair tissue with different characteristics from hyaline articular cartilage.[3]

Regenerative medicine is an emerging field that seeks to repair or replace injured tissues and organs through natural or bioengineered means. Recent research on stromal mesenchymal stem cells (MSCs) has provided a new opportunity for bone and cartilage tissue engineering.[4] This is a promising alternative to overcome the limitations of the conventional approaches for cartilage repair, but it has to be validated in large animal models.

The ovine experimental model is widely used as a preclinical model in articular cartilage regeneration and repair.[5] Improving the 3Rs guidelines in this model is imperative, as published research in the area highlights the habitual use of invasive methodology (arthrotomy) for lesion induction and assay-product implantation.[6,7]

We present our experience concerning the generation and assessment of chondral and osteochondral lesions in the ovine knee for testing the therapeutic potential of new approaches in regenerative medicine. In order to refine our research we developed minimally invasive surgical procedures for experimental lesion induction and assay-product application. We used non-invasive diagnostic methods to characterize the lesion and to assess the progression

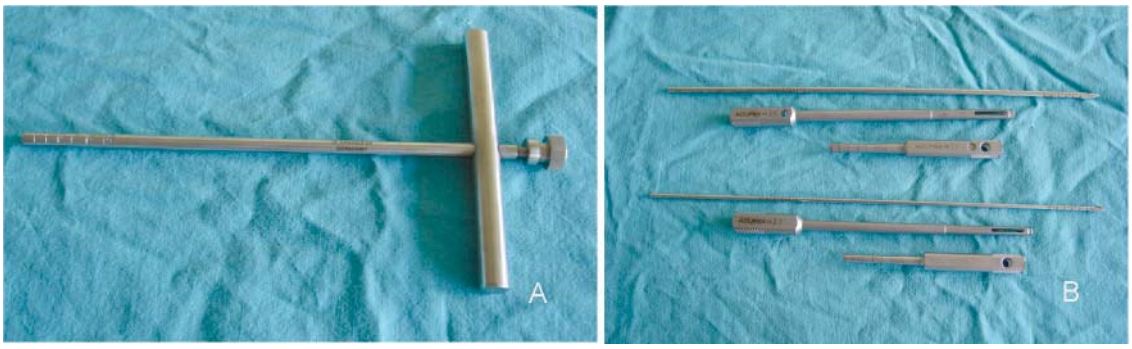

Figure 1. Surgical instruments used to induce the experimental lesions. **A-** Michelle throcar, used to perform the chondral lesion. **B-** Mosaycoplasty cannulas, used to perform the osteochondral lesion.

of the applied articular treatments during the entire experiment.

Materials and Methods

Animals: With the approval of the Animal Ethics Committee of the Autonomous University of Barcelona, 24 healthy 2 year old female sheep, Ripollesa breed, weighing 50 ± 6 kg were used in the study. The animals were divided in 2 groups, based on the kind of cartilage lesion that was experimentally induced: Group I (10 animals) for the study of chondral lesion and Group II (14 animals) for the study of osteochondral lesion.

All surgical procedures were developed in a preliminary study in sheep cadaver, in order to achieve accurate arthroscopic techniques. For this purpose 12 animals were euthanized at the end of other experimental procedures, with no previous experimental manipulation of knee articulation.

Anaesthesia: All surgeries were performed using aseptic techniques and under general anaesthesia. Animals were premedicated with an intramuscular injection of 0,01 mg/kg of buprenorphine (Buprex®, Schering-Plough, S.A.) and 0,2 mg/kg of midazolam (Dormicum®, Roche). After pre-oxygenation with a face mask, they were induced with 4 mg/kg intravenous propofol (Propofol®-Lipuro 1%, BBraun Melsungen AG). The animals had endotracheal intubation and were maintained on isoflurane 2% (Isoflo, Abbott laboratories Ltd) with 100% oxygen. Esophageal intubation was used to prevent ruminal bloat. A continuous infusion of Ringer lactate (Ringer lactate, BBraun Melsungen AG) was administered at 10 ml/kg/h during surgery. Intra-operative monitoring consisted of electrocardiography, pulse oximetry, non-invasive blood pressure and capnography (VetCare® multiparametric monitor, BBraun Germany).

Arthroscopy: The experimental lesion was made by using arthroscopic surgery, in both knees. With the articulation positioned in flexion, each joint was approached via two stab incisions of 3 mm: one lateral and another medial to the distal aspect of the patellar ligament. The lateral arthroscopic portal allowed the insertion of the arthroscope (and the optics) for the visualization of the medial condyle as well as the joint irrigation. During surgery, the joints were irrigated by saline solution (Grifols) of 3000 ml with the irrigation set with double silicone terminal (Fluxisol®, Grífols) at room temperature. A mechanical shaver was introduced by the medial arthroscopic portal, being used to remove the fat pad, allowing a clearer view. Once cleaned, the shaver was removed.

In the animals belonging to group I, a chondral lesion was performed. For this purpose, a Michelle punch (Insorvet SL) with a diameter of 5 mm (Figure 1 A) was inserted through the medial arthroscopic portal. The extremity of this instrument was pressed against the articular surface of the medial femoral condyle, applying a rotation movement in 3 contiguous areas (Figure 2A). This way, the indentations of 1mm of this cannula rubbed the cartilage eroding the articular cartilage, in a surface area of 60 mm^2, without reaching the subchondral bone.

In the animals belonging to group II, an osteochondral lesion was produced. The same arthroscopic approach was performed, with a lateral and a medial portal to the patellar ligament. A mosaicoplasty set (Smith and Nephew Inc, USA) composed by a donor and a receptor cannula (Figure 1 B) was used to perform the lesion and to place the colonized scaffold. For this purpose, the cylindrical hollow punch with an inner diameter of 2,7 mm (donor cannula) was placed through the medial arthroscopic portal. This instrument was used to perform a cylindrical osteochondral lesion of diameter 3,5 mm and 5 mm depth in the medial femoral condyle of each knee. The skin incisions were closed with 2 surgical staples.

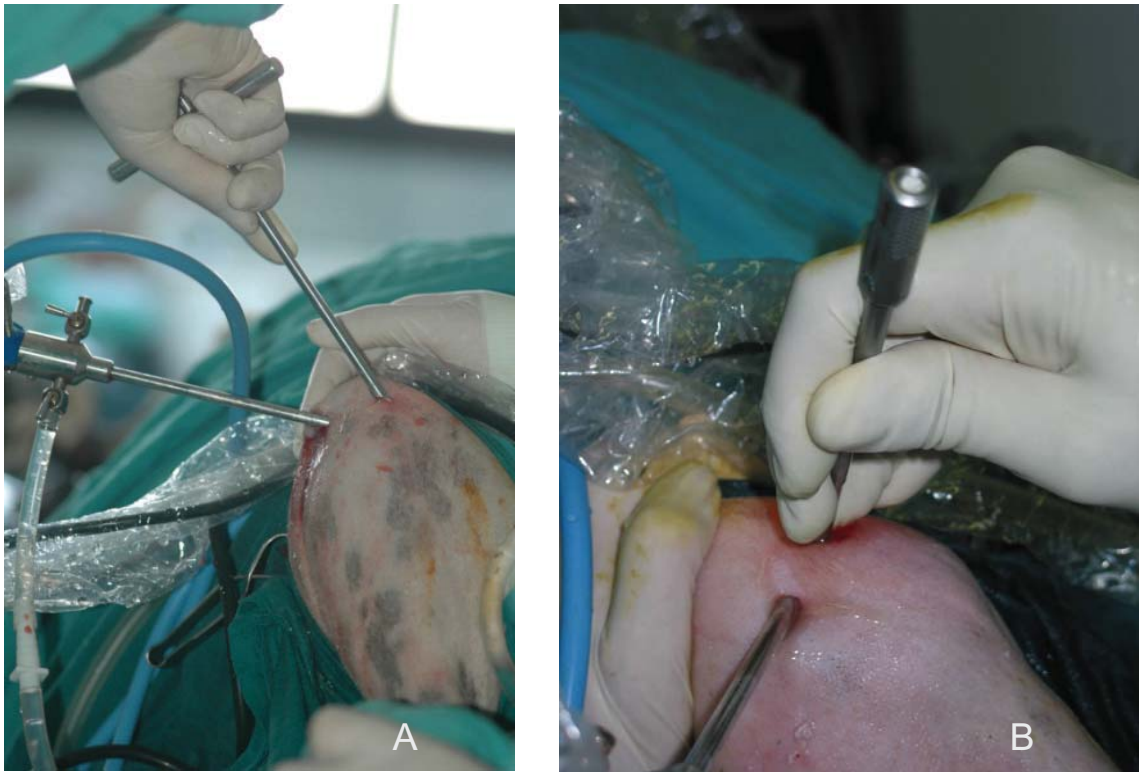

Figure 2. Arthroscopic surgery in the ovine experimental model. **A-**Arthroscopic portals in group I. **B-** Introduction of the scaffold through the mosaicoplasty cannula, in group II.

Post operative care: All animals received subcutaneously meloxicam (Metacam, Boehringer) 0,2 mg/kg for 10 days and transdermal fentanyl (Durogesic) 100 µg for post-operative pain relief. For the prophylaxis of peri-operative infection the animals received intravenously 22 mg/kg of cefazolin (Kurgan, Normon Laboratories) in the induction of anaesthesia and 15 mg/kg amoxicillin (Duphamox® L.A., Norbrook Laboratories) every 48 hours for 10 days.

Cellular therapies application: Group I: The selected approach to chondral lesions regeneration was the intra articular injection of MSC derived from bone marrow (bmMSC) suspended in platelet rich plasma or in saline solution, 30 days after arthroscopy for lesion production. Controls consisted in injection of the vehicle without bmMSC. The intra-articular application of the experimental products was made by ultrasound-guided injection, using aseptic technique and under general anaesthesia. In order to facilitate the procedure, a multiple infusion 3 way valve set with extension (Discofix® C-3, B.Braun Melsungen AG) was used connecting the 5ml syringe (Injekt®, B.Braun Melsungen AG) and the needle 18Gx 1 ½ ´´ (Sterican®, B.Braun Melsungen AG).

Group II: The approach to osteochondral lesions regeneration was made using a Polilactic-poliglicolic acid (PLGA) scaffold colonized with autologous MSC derived from adipose tissue, bone marrow or cartilage. Controls consisted in non-treated lesions and in non-colonized scaffolds implantation. The implantation of the colonized scaffolds was made by arthroscopic surgery, during the same surgery used to induce the osteochondral lesion. The scaffold of cylindrical shape and 4 mm diameter and 7 mm high was placed through the receptor cannula of mosaicoplasty (Figure 2 B), filling the lesion.

Imaging the knee: In both groups each knee was assessed by two radiographic projections: lateral and anteroposterior views. These projections were made to all animals before the experimental lesion was made and they were repeated at the end-point.

Ultrasound (US) diagnosis: This exam was performed to all animals belonging to group I. Examination of the knees was made using a 10 MHz transducer (Ultrasound apparatus Acuson®, Siemens). Basal images were taken before experimental manipulation took place and 7 days after lesion induction. The following examinations were made at the end-point as blind-examinations, without previous knowledge of which treatment was applied to the articulation. To visualize the structures, the articulation was positioned in flexion. The medial condyle was visualized from a medial and sagital portal, placing the transducer longitudinally and transversally.

Magnetic resonance imaging (MRI): MRI was performed on both knees of all animals before

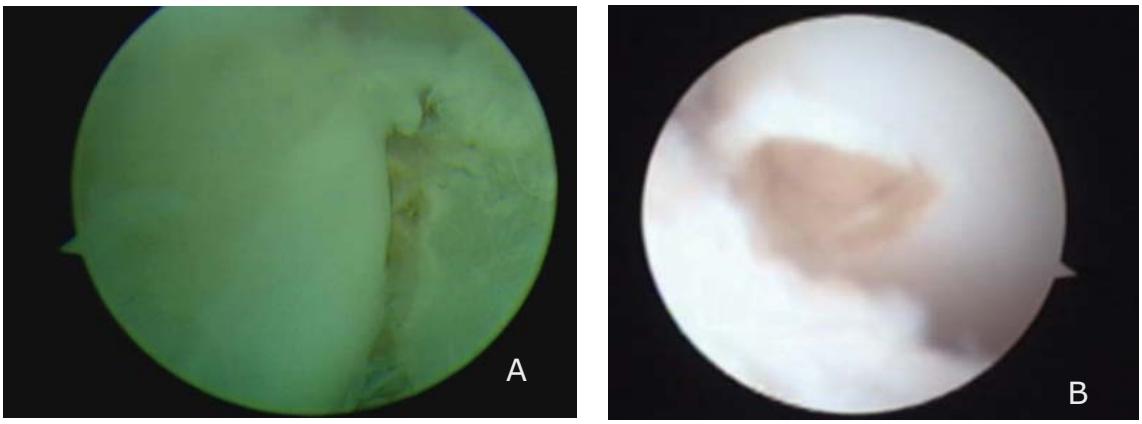

Figure 3. Arthroscopic images of the medial femoral condyle, after induction of the surgical lesions. **A-** Chondral lesion. **B-** Osteochondral lesion.

the lesion induction and at the end-point. In the animals of group I MRI was also performed 7 days after lesion induction. MRI was conducted using a 0,2 T unit with open permanent magnet (Vet-MR, Esaote S.p.a., Genoa, Italy). This exam was performed under general anaesthesia with the animal positioned in sternal recumbency with the leg extended and symmetrically placed inside a dual phased array coil. Sequences included axial, coronal and sagital in SE, FE and FSE in T1, T2 and STIR.

Macroscopic assessment: Half of the animals were sacrificed at 6 months and the rest 12 months after assay product implantation. The euthanasia was performed by administrating an intravenous injection of 80 mg/kg of thiopental (BBraun), after previous sedation with an intramuscular injection of 0,01 mg/kg of buprenorphine (Buprex®, Schering-Plough, S.A.) and 0,2 mg/kg of midazolam (Dormicum®, Roche). Full necropsy of animals was performed. Medial femoral condyles were excised for histological analysis.

Histological evaluation: After 10% buffered formalin fixation, the samples were decalcified (Decalcifier I®, Surgipath Canada Inc). Medial femoral condyles were sectioned at a thickness of 3 mm, perpendicular to the lesion area. Samples were embedded in paraffin and sectioned at a thickness of 4 µm using a microtome of rotation. Haematoxylin and eosin staining was performed. All sections were assessed according to International Cartilage Repair Society guidelines.[8]

Results and Discussion

Reproducible experimental lesions were achieved by arthroscopy (Figure 3). Animals were ambulatory immediately after anaesthesia recovery, and lameness was gone after 4 days post surgery. No surgical complications were observed.

Arthroscopy was an effective technique to implant the scaffolds and US was a safe imaging technique for guiding intra-articular injections, showing the proper needle positioning inside the joint cavity. This is especially important because a specific dose of cells was being tested, and the injection in peri-articular tissues would mean an altered dose.

The assessments of the lesion and treatment evolution were successfully performed by non-invasive diagnostic methods. Definitive results were obtained

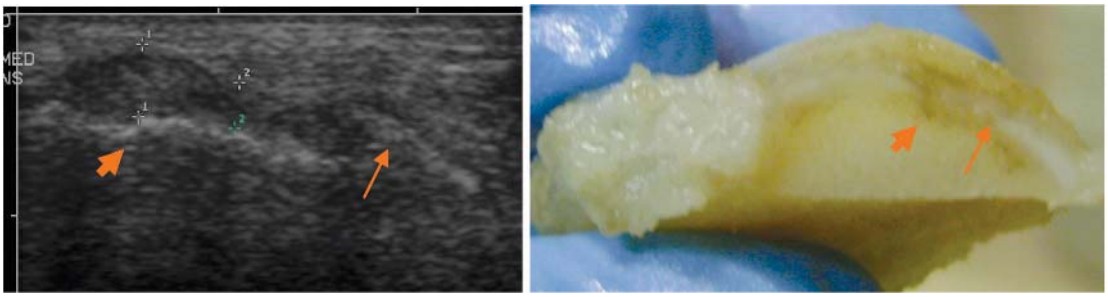

Figure 4. Ultrasound imaging of articular cartilage of femoral condyle, and comparison with macroscopic view. **A-**Ultrasound image of cartilage. **B-** Macroscopic aspect in transversal cut. The arrows sign the alteration of cartilage-bone interface.

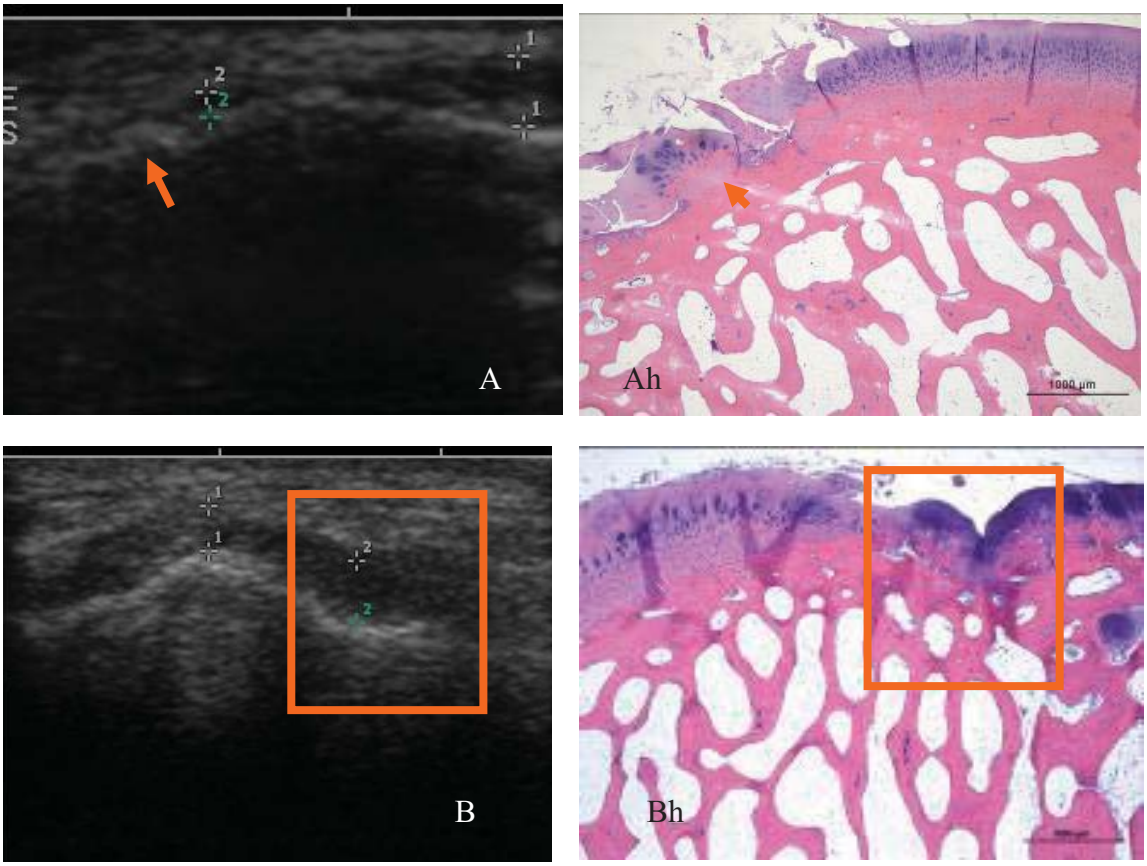

Figure 5. Ultrasound imaging of articular cartilage of femoral condyle, and comparison with biopsy histology. **A-** US image. **Ah-** Histological aspect of cartilage A. **B-**US image. **Bh-** Histological aspect of cartilage B.

by histology. Radiographic examination of the joints didn't show any alteration.

US of articular cartilage of medial femoral condyle revealed to be a useful and practical tool assessing the evolution of femoral condyle cartilage, suggesting some alterations observed after animal sacrifice (figure 4).

Human hyaline cartilage is described as a well-defined anechogenic or homogeneously hypoechogenic band between the chondrosynovial and osteochondral margins. [9,10] Normal articular cartilage of sheep has a similar aspect.[11] The lack of echoes is due to uniform transmission of sound wave in cartilage with high water content and densely packed and organized collagen.[12,13] The absence of echoes of the cartilage layer and the sharpness of the margins are its principal features in healthy subjects, corresponding the sharp margin to smooth surface of healthy cartilage.[9,10,14]

In our study, we found that at the end-point time the medial femoral condyle cartilage appeared in some animals with a variable degree of echogenicity and with some alterations in the sharpness of the margins. We saw that these US image alterations correspond to histological alterations: the US alteration in the sharpness of the osteochondral margin corresponded to alterations in the calcified cartilage layer in histology. Furthermore, alterations observed by US in the chondro-sinovial margin corresponded to superficial cartilage histological fibrillations (Figure 5).

The described earlier US features of osteoarthritis in human are loss of clarity of the cartilage band and loss of sharpness of the margins. The loss of sharpness of the interface is due to scattering of sound by a rough surface. The increased echogenecity may represent structural alteration such as fibrillation of cartilage and cleft formation. In the later stages, an asymmetric narrowing of the cartilaginous layer occurs.[10] However it is known that the loss of sharpness of the interface can make the placement of markers, for thickness measurement, difficult.[12,13] Because of this, clarity and sharpness of the cartilage images are considered the best predictors of cartilage alteration, correlating significantly with gross findings of the specimen.[12]

The main limitation of US is the inability of the beam to penetrate the bony cortex. Thus, US visualization of the articular cartilage is restricted by the acoustic windows, their width being determined by the anatomy of the joint under examination.[10] The bone alterations that can be detected by US are loss of continuity of the bone profile or an increased intensity of the posterior bone-cartilage interface, that may reflect subchondral bone sclerosis or loss of

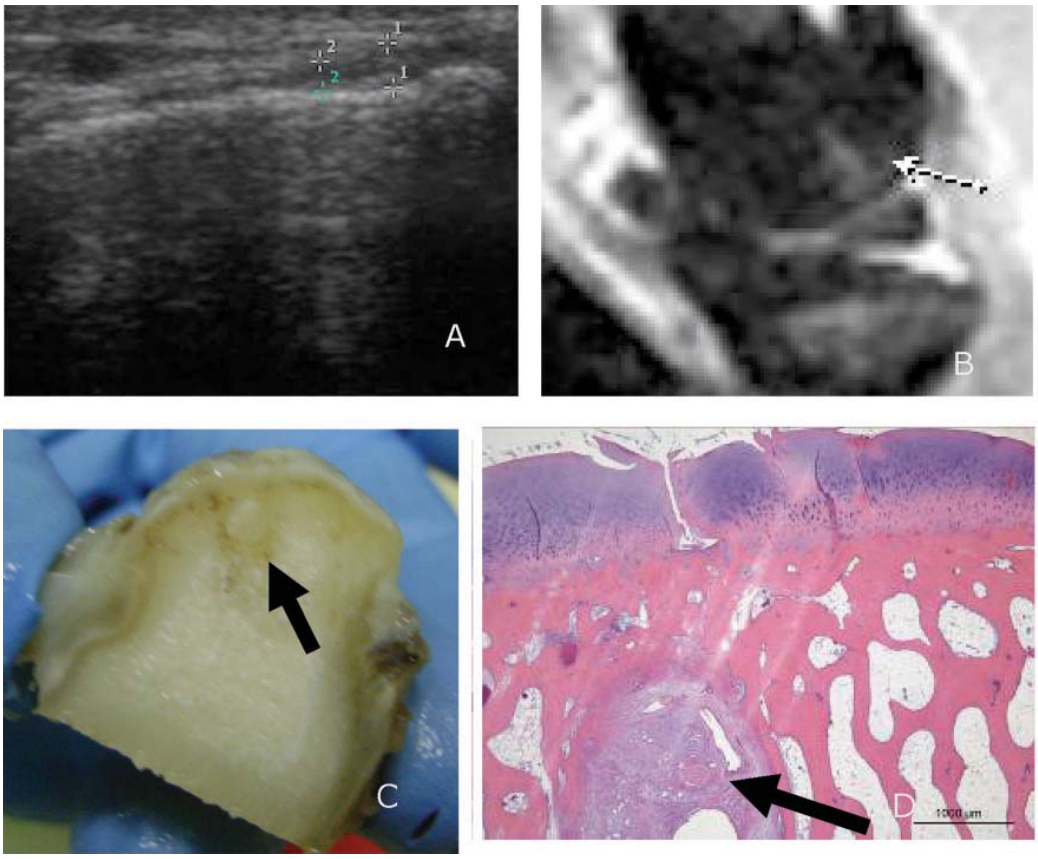

Figure 6. US and MRI images of the articulation, macroscopic and histologic aspect of the femoral condyle. **A-**US image of cartilage. **B-**MRI image of the knee. The subchondral bone has a zone with abnormal signal (arrow). **C-**Macroscopic aspect of the femoral condyle, in sagital cut. The arrow signals subchondral bone reaction. **D-** Histological image where subchondral bone alteration is present (arrow).

overlying cartilage.[13] Difficulty to measure cartilage thinning and the fact that US is an operator-dependent imaging technique are also important limitations, as the recorded US images largely display the subjective selection of findings observed by the individual performing the examination.[10] In order to minimize this limitation, we defined a standardized examination protocol, we took basal images and we performed blind examination of the articulations after assay-product implantation.

MRI was useful to detect subchondral alterations. The fact that we used a 0,2 T unit limited the ability to detect cartilage lesions that didn´t produce a reaction of the subchondral bone. However, by crossing information given by US examination of cartilage with MRI information of underlying subchondral bone, we were able to obtain useful qualitative information about the cartilage repair process (Figure 6).

Some studies, applied to human cartilage, correlate the US grading of osteoarthritic cartilage with histological grading.[9,14] Our preliminary results in the sheep model suggest that in this animal model, US can provide useful information for qualitative assessment of the cartilage repair process. This information can be important for monitoring the evolution of the cartilage regeneration response in large time periods experiments, in a non-invasive, non-painful and cost-effective methodology that can be performed on animals under a light sedation.

In our future work we will study, with a larger number of samples, the correlation of sheep articular cartilage US grading with histology, in order to establish a semi-quantitative in vivo US assessment of articular cartilage in this animal model.

Conclusions

The application of the developed refined techniques in the field of cartilage repair promotes animal welfare and increases research quality.

Arthroscopy was an appropriate surgical technique for chondral and osteochondral experimental lesion induction in the ovine knee.

US revealed to be a useful non-invasive technique in the assessment of the injured articular cartilage in the sheep knee, making possible the prediction of small alterations in the cartilage band. MRI was important to detect deeper subchondral bone alterations.

Refining the preclinical animal model facilitates the extrapolation of methodologies on assay-product application and diagnosis techniques in future clinic human trials.

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Validation of an automatic device for fasting mice

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Abstract

In Drug Discovery, it is sometimes necessary to fast animals to control the effects food may have on oral absorption of drugs; however, fasting periods are usually set too long. In a previous study, our team had shown that long fasting periods of 16 hours caused alterations in animal physiology and welfare. In this study, our aim was to validate a new automatic device to fast mice, which we named "automatic feeder", and which facilitated a reduction in the length of fasting. For this purpose, we compared 8-hour fasting using the feeder against two manual methods (a formula to calculate food intake until the start of fasting and manually withdrawing the food). Cages had a grid floor instead of bedding, to stop animals from ingesting their faeces. Three controls were included: a 16-hour fasting group (the standard length of fasting in most labs) and two non-fasting groups, one with bedding, with the purpose of distinguishing the stress caused by the grid from the stress caused by fasting. There were no differences between the three 8-hour groups. The 16-hour group did not show a significant reduction of stomach contents compared to 8-hour groups, while exhibiting an important reduction in welfare (increase in corticosterone, greater loss of body weight, loss of liver weight and depletion of liver glycogen contents, and a greater alteration of biochemical and haematological values). In conclusion, each of the three 8-hour methods is useful for fasting animals and represents an improvement in welfare with respect to longer fasting times. In addition, the automatic feeder offers advantages over the two manual methods, as it provides a more accurate period of fasting than the formula, and staff does not need to be present at night to withdraw food.

Keywords: animal welfare, automatic feeder, fasting, drug discovery, refinement.

In Drug Discovery, it is sometimes necessary to fast animals to control the effects food may have on oral absorption of drugs. However, fasting periods are usually set based on practical reasons and not on scientific reasons[1]; food is usually withdrawn in the evening, when staff leave, and the experiment is carried out the next morning, producing a long fasting period of approximately 16 hours[1, 2]. Consequently, the animals' physiology and welfare are altered [1, 3] and possibly the experimental results could also be altered. In a previous study[3], our team had shown that long fasting periods of 16 hours caused and alteration in animal physiology and welfare. Thus, in order to avoid long periods of fasting, we proposed the use of shorter periods of time, such as 8 hours instead of 16 hours. The main problem was the difficulty of convincing researchers of the need to come to the animal facility at night to withdraw the food for an experiment taking place the following morning. For that reason, we developed two methods to circumvent this inconvenience and ensure that fasting length was kept to a minimum. Firstly, we developed a formula to calculate food intake, so we could leave the amount of food needed until the set time for the start of fasting. Since food intake could vary and, as a result, also the time of the start of fasting, we aimed to improve the fasting technique and designed an automatic device, which we named "automatic feeder", and which prevented animals' access to food at the chosen time (both formula and feeder are explained below). The aim of this study was to verify the suitability of this new automatic feeder. We compared 8-hour fasting using the feeder against the two manual methods (the formula to calculate food intake until the start of fasting, and manually withdrawing the food).

Materials and Methods

Animals and husbandry

Thirty adult SPF female ICR (CD1) mice (30g, Harlan Laboratories Models, Europe) were used in the present study. After delivery of the animals, a recovery period

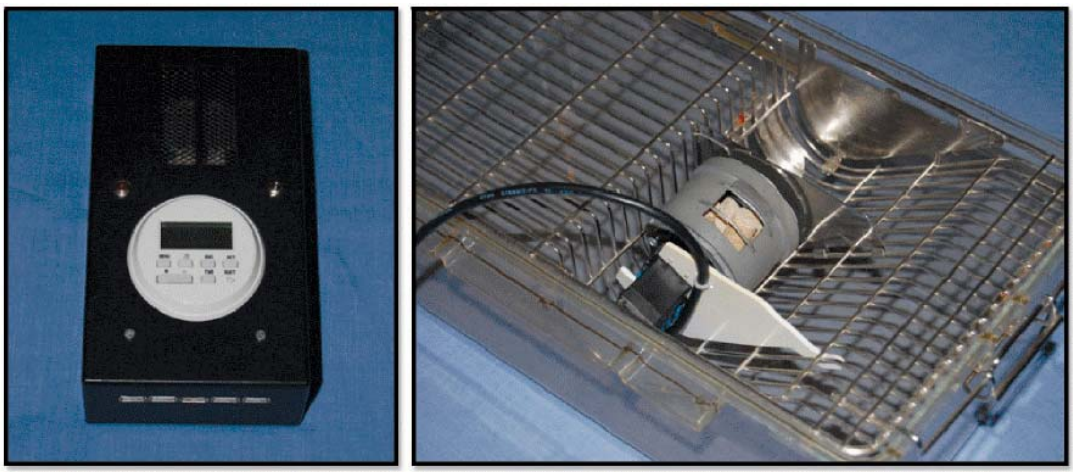


Figure 1. Automatic feeder. Timer (left) and feeder (right). The feeder turns up at the start of fasting so the animals cannot reach the food.

of five days was allowed. Animals were housed in standard polisulfone cages (Techniplast, Italy) with corncob bedding or grid floor in a temperature and humidity controlled room and maintained in a 12h light/12h dark cycle. Food (Harlan Tecklad, Spain) and ultrafiltrated water were available *ad libitum*.

Animal experiments were approved by the local IACUC and animal welfare standards were regulated in compliance with EU regulations and GlaxoSmithKline's policy on Care and Ethical Use of Animals in Research.

Experimental procedure

Animals were randomized and assigned to groups of 5, one per each experimental condition. Cages had a grid floor instead of bedding, to stop animals from ingesting their faeces. Three groups of animals were fasted for eight hours: 1) food was withdrawn manually 8 hours before euthanasia (8h), 2) 8 hours fasting with the use of an automatic feeder (8h+CA), and 3) 8 hours fasting by using the formula shown below (8h+F). Two control groups were added to the experiment: Control 1, non-fasted and corncob bedding; Control 2, non-fasted and grid floor, with the purpose of distinguishing the stress caused by the grid from the stress caused by fasting. In order to compare these results with the standard length of fasting in most labs, one more group was added: 16 h, 16 hours fasting starting at 18:00 the day before.

All animals were weighed at 18:00 the day before the experiment. At that time, food was withdrawn from the 16h group, the timer set for the 8h+CA group, and food calculated for the 8h+F group. Food was manually withdrawn at 2:00 for the 8h group. Animals were euthanised after the set hours of fasting, at 10:00 in the morning. The following data were taken: body weight; liver weight and glycogen contents; blood for biochemical and haematological analysis; weight of stomach contents; and observation of bowel contents.

Formula

In order to know the amount of food eaten by mice during the night and the day, we weighed the food at artificial dusk and dawn and calculated food intake per hour and gram of mouse at night and during the day. Our food intake results were slightly under those in the literature (3.8 g/30g female mouse/complete day, compared to 4-5 g[4]). To produce the formula, the average food consumption per hour and gram of mouse was used, separated by day and night:

*Food to leave = (day hours*0.0026 + night hours*0.008)*group weight*

In our case: To leave the food at 18:00 and to start fasting at 2:00 the next day (lights are off at 20:00).

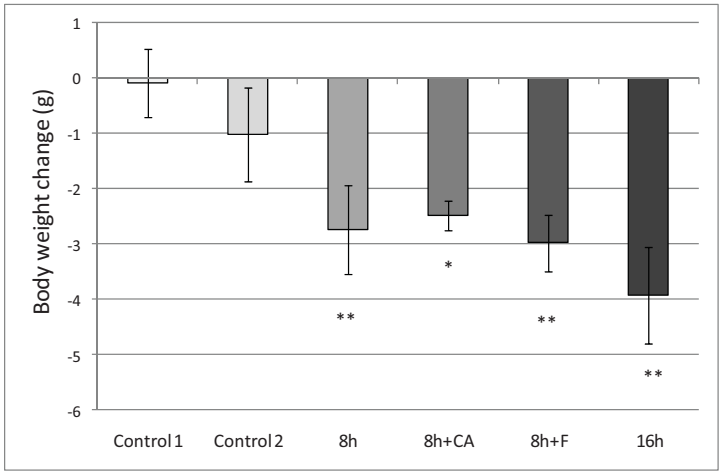
Food to leave = (2 day hours*0.0026 + 6 night hours*0.008)*(155.6g of mice) = 8.28g food.

Automatic feeder

The automatic feeder was composed of a cylinder, where food was placed, and a timer to select the start of fasting (Figure 1). The cylinder was made of stainless steel, and had a rectangular opening through which animals could reach the food. It was placed on the cage grid, where food would normally be. The timer controlled the cylinder, making it rotate on its axis, so the opening faced down when animals were allowed access to food, and up when fasting was required.

Statistics

A one-way analysis of variance, or on ranks if data were non-parametric, was used to compare data. Multiple comparisons of fasted groups versus the Control 2 group were performed by Dunnets´s or Dunn's Multiple Comparison Test. GraphPad Prism Version 4.00 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com) was used for statistical analysis. P values of ≤ 0.05 were considered statistically significant. All mean values given in the text and tables include the standard deviations of the means.



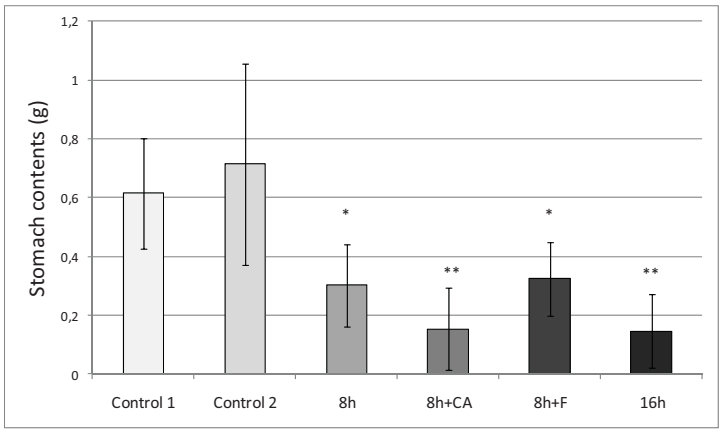
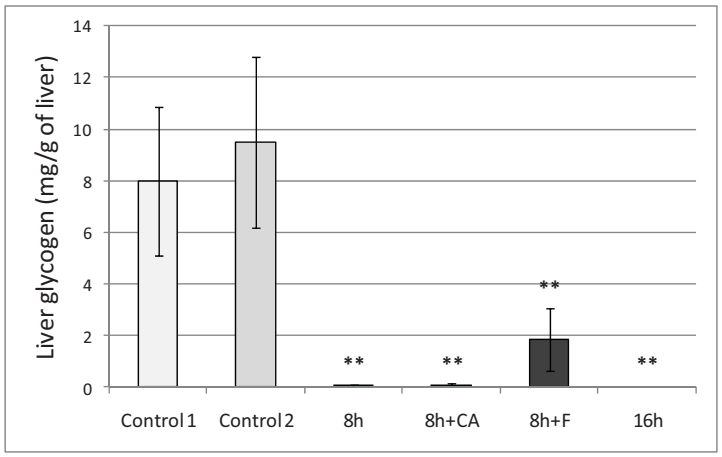


Figure 2. Up: body weight loss after fasting (g). Down: stomach contents (g).

Mean & SD. * p<0.5, ** p<0.01 when compared to Control 2 group (1-Way Anova and Dunnet's multiple comparison test).



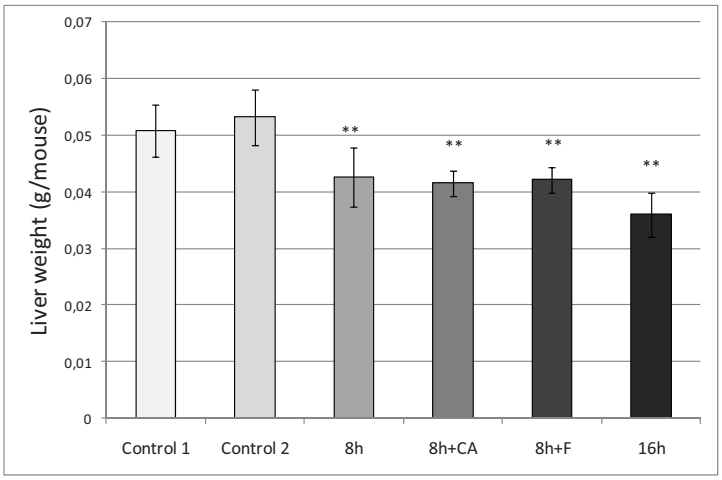


Figure 3. Up: liver glycogen (mg/g of liver). Down: liver weight (g/mouse).

Mean & SD., ** p<0.01 when compared to Control 2 group (1-Way Anova and Dunnet's multiple comparison test).

Table 1. Mean and *SD* of biochemical and haematological parameters that showed statistical differences with Control 2, or deviation from internal reference values (in parenthesis). * p<0.5, ** p<0.01 when compared to Control 2 group (1-Way Anova and Dunnet's multiple comparison test).

	TBIL	(mg/dl)	BUN$	(mg/dl)	PHOS	(mg/dl)	GLU	(mg/dl)
C1	0,30	*0,00*	14,20	*1,10*	8,68	*0,41*	189	*14*
C2	0,30	*0,00*	13,00	*2,24*	8,54	*0,72*	181	*22*
8h	0,28	*0,04*	15,20	*2,95*	9,54	*0,59*	141**	*22*
8h+CA	0,28	*0,04*	15,80	*0,84*	10,22*	*0,95*	(137)**	*17*
8h+F	0,30	*0,00*	14,80	*1,79*	9,20	*1,16*	140**	*13*
16h	(0,24)	*0,05*	21,00**	*4,18*	9,26	*0,60*	(85)**	*12*
	Na^+	**(mmol/L)**	**K^+**	**(mmol/L)**	**TP**	**(g/dl)**	**GLOB**	**(g/dl)**
C1	154,40	*1,14*	(8,48)	*0,04*	5,72	*0,16*	1,78	*0,11*
C2	155,60	*1,34*	(8,50)	*0,00*	5,56	*0,32*	1,62	*0,16*
8h	159,20**	*1,48*	(8,50)	*0,00*	5,82	*0,31*	1,98*	*0,08*
8h+CA	158,20	*2,28*	(8,50)	*0,00*	5,72	*0,13*	1,82	*0,08*
8h+F	156,60	*2,07*	(8,50)	*0,00*	5,90	*0,24*	1,86**	*0,09*
16h	161,40**	*0,89*	(8,50)	*0,00*	6,18*	*0,25*	2,00**	*0,17*
	AST$	**(IU/L)**	**CK**	**(IU/L)**	**RBC$**	**(10^6/µl)**	**Hb**	**(g/dl)**
C1	57,54	*5,64*	61,54	*38,11*	9,01	*0,17*	15,26	*0,47*
C2	54,89	*4,28*	76,17	*23,77*	9,18	*0,31*	15,48	*0,26*
8h	83,61*	*17,87*	150,69	*54,31*	9,00	*0,30*	15,48	*0,33*
8h+CA	81,86**	*5,28*	139,00	*50,89*	9,30	*0,08*	15,50	*0,35*
8h+F	75,97	*19,30*	132,56	*70,71*	9,53	*0,40*	16,28**	*0,45*
16h	82,77**	*13,75*	193,53*	*102,29*	(10,31)	*0,89*	16,74**	*0,40*
	Hct	**%**	**VCM**	**(fl)**	**CHCM$**	**(g/dl)**		
C1	45,90	*2,36*	51,00	*1,87*	33,28	*1,26*		
C2	46,26	*1,31*	50,60	*2,07*	33,50	*1,09*		
8h	43,54*	*0,67*	48,60	*1,82*	35,54	*0,57*		
8h+CA	44,04	*0,61*	47,40	*0,89*	35,22	*0,54*		
8h+F	46,88	*1,74*	49,40	*1,14*	34,76	*0,38*		
16h	46,86	*0,94*	46,75*	*4,03*	35,69**	*0,22*		

TBIL: Total Bilirrubin, BUN: Blood Ureic Nitrogen, PHOS: Inorganic Phosphorous, GLU: Glucose, TP: Total Protein, GLOB: Globulins, AST: Aspartate Aminotransferase, CK: Creatin-Kinase, RBC: Red Blood Cells, Hb: Hemoglobin , HCT: Hematocrit , VCM: Mean

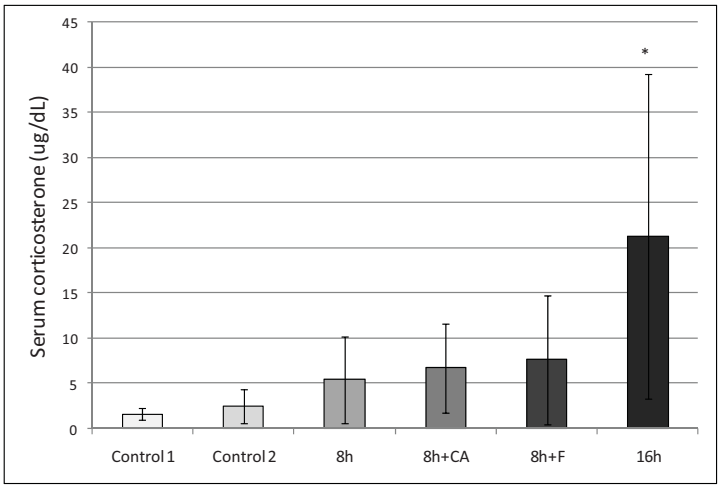


Figure 4. Serum corticosterone (ug/dL). Mean & SD. * p<0.5 when compared to Control 2 group (Kruskall-Wallis and Dunn's multiple comparison test).

Results

The automatic feeder turned off at the set start of fasting and thus prevented animals from reaching the food from then on. With the formula, there was still a small pellet available at the start of fasting (3g), and consequently that group started fasting later than the rest of the groups.

All fasting groups lost body weight, and showed a reduction in liver weight and in hepatic glycogen contents, when compared to group Control 2 (Figures 2-3). The reduction in hepatic glycogen contents was less pronounced in group 8h+F.

All fasting groups showed reduced stomach contents compared to Control 2 (Figure 2). This reduction was greater in the 8-hour fasting group with automatic feeder (8h+CA) and in the 16-hour fasting group. Moreover, there were no significant differences in stomach contents between the 8-hour fasting groups and the 16-hour fasting group. All fasting groups also showed an empty small intestine.

All fasting groups showed some changes in the biochemical and hematological analysis (Table 1), but these were especially noticeable in the 16-hour group. The most obvious changes were a decrease in glucose (decreased from 181±22 in C2, to 85±12 in the 16-hour group), and an increase in protein and ion concentration (including blood ureic nitrogen, Na^+, total protein, and globulins; see Table 1 for values).

Serum corticosterone concentration was significantly higher in the 16-hour group only (Fig.4). Finally, no significant differences were observed between Control group 1 and 2.

Discussion

Fasting caused a body weight loss, a decrease in liver weight and glycogen content, a decrease in stomach and small intestine contents, and an alteration of the biochemical and haematological values. For longer times of food deprivation (16 hours), fasting caused a greater decrease in body and liver weight, a complete depletion of hepatic glycogen contents, greater changes in the biochemical and haematological values, as well as a considerable increase in serum corticosterone. At the same time, this longer period of fasting did not produce a significant reduction in stomach contents when compared to any of the 8-hour fasting groups; on average, stomach contents for the 16-hour groups were very similar to the 8-hour fasting group with automatic feeder (8h+CA). This means that an 8-hour fasting period is enough time to find an empty stomach.

Group 8h+F showed a greater hepatic glycogen content than other 8-hour fasting groups, since there was food still available in the cage at the calculated start of fasting. In spite of this, all other parameters were pretty similar to the rest of the 8-hour fasting groups.

Although all fasting groups showed changes in the biochemical and hematological analysis, it was the 16-hour fasting group which showed greater alterations. The decrease in glucose was obviously due to fasting. The increase of AST and CK could be related to stress[5]. The increase in the rest of the biochemical and hematological variables seems related to dehydration, probably due to a reduction in water intake accompanying fasting.

Serum corticosterone concentration was significantly higher in the 16-hour group only, indicating an increase in stress levels in this group mainly.

There were no significant differences between Control group 1 and 2, showing that observed changes in the experiment were definitely due to fasting and not to the stress produced by the grid floor.

In conclusion, each of the three 8-hour methods is useful for fasting animals and represents an improvement in welfare with respect to longer fasting times. Additionally, the automatic feeder offers the advantages of providing a more accurate period of fasting than the formula, and not needing to withdraw food at night manually, thus improving science as well as being more practical.

Acknowledgements

The automatic feeder was originally conceived by people in LAS Spain department and designed and built by David Garijo. In addition, we would like to thank the Pharmacology team for their analysis of blood parameters and liver glycogen, and the rest of the LAS team for their continuous support.

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Stress response of mice under different volatile anaesthesia

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Abstract

Anaesthesia is often recommended to minimise discomfort caused by experimental procedure, e.g. blood sampling. However, it has been reported that anaesthesia with diethylether causes a pronounced endocrinal stress response in rats. Thus, it is important to determine whether anaesthesia itself will cause stress. The present study focused on the stress response of different anaesthetics (sevoflurane, isoflurane, ether and CO_2). Corticosterone concentration and Open Field Test were measured as indicators of stress response.

In total, 60 inbred BALB/cOlaHsd mice, obtained from Harlan Winkelmann (Borchen, Germany), were randomly allotted to six experimental groups, in groups of five. With the exception of the control group mice were anaesthetised with sevoflurane isoflurane, ether or CO_2. The control and sham group (not anaesthetised) were also transferred into the same box before blood sampling, but only the sham group received an airflow (same speed as under anaesthesia). Blood samples were collected twice, one week before the experiment and 15 minutes after the experimental procedure. The Open Field test was performed immediately after the second blood sampling.

Significant increased corticosterone levels were found for sham and different anaesthetic groups after the experimental procedure. Compared to the control group corticosterone concentration increased significantly after anaesthesia. In the Open Field test significant differences between groups were found for the total distance mainly due to the differences between groups under isoflurane and ether anaesthesia, while for the crossing frequency the main difference was found between the sham group and groups with isoflurane & CO_2.

Keywords: volatile anaesthesia, stress, mice, corticosterone, open field

In laboratory animals inhalation anaesthesia is often used for short-acting anaesthesia to minimise stress caused by experimental procedure e.g. blood sampling. A closed anaesthetic system is commonly used for the induction of inhalation anaesthesia[1]. However, it has been reported that some anaesthetics such as ether can cause endocrinal stress because it is highly irritating to the respiratory system[2]. The use of CO_2 as inhalation anaesthetics is also discussed very controversially. Various authors measured a variety of behavioural and physiological responses as indicators of pain and stress in studies with CO_2 such as hyperventilation or escape behaviour. Other authors prefer CO_2 as an anaesthetic as well as for euthanasia[3,4,5]. In a previous study we found that volatile anaesthesia (ether and CO_2) caused more stress than retro-bulbar blood sampling without anaesthesia[6]. Other possibilities for inhalation anaesthesia are sevoflurane and isoflurane. Both demonstrate a very rapid induction and recovery from anaesthesia and are non-irritant[7]. Nonetheless, there are no results for endocrinal stress during or after anaesthesia with these agents.

Thus, this study focuses on the stress response of different short-term inhaled anaesthetics (sevoflurane, isoflurane, ether and CO_2), which are performed for rapid routine procedures such as blood sampling. Corticosterone (CORT) concentration and the Open Field Test were measured as indicators of stress response.

Materials and Methods

Animals: In total, 60 female BALB/cO1aHsd mice, specific pathogen free according to the FELASA recommendation[8], about 6 weeks old, obtained from Harlan Laboratories, were marked by ear puncturing and randomly allotted to six experimental groups. The animals were distributed to 12 Makrolon type III cages, in groups of five. At the beginning of the study the mice had an average weight of 18.7 g.

Environment*:* All animals were maintained in a scantainer (Scanbur AS Denmark), with air exchange 10-16 times per hour. The room temperature was 22 ± 2°C and 50 ± 10% relative humidity, with a 12/12 hour

light/dark cycle (light on at 6:00) and at a light intensity of 50±10 Lux inside the scantainer.

Food and water: Tap water in drinking bottles and pelleted food containing 19.0% protein, 4.0% fat, 6% fibre and 7% ash (Altromin No. 1324, Altromin GmbH, Lage, Germany) were given ad libitum.

Bedding and nest material: As bedding, soft wood shavings were provided for each cage (Altromin Type 5, Altromin GmbH, Lage, Germany). Cage and bedding were changed once a week (always on Thursdays). For environmental improvement nest material (Nestlets, EBECO) was placed in the cages after cage changing.

Experimental groups*:* In total 60 animals were tested in 6 groups with 10 female mice aged 8 weeks. Four different volatile anaesthetics (ether, CO_2, isoflurane and sevoflurane) were examined. Besides the groups controls and sham, anaesthesia was performed.

Anaesthesia: All animals were placed in a Perspex box (15 cm x 13 cm x 28 cm, designed for anaesthesia). In this box anaesthesia was induced either with 8% sevoflurane vaporised with oxygen (5 l/min) or 4% isoflurane vaporised with oxygen (5l/min). For CO_2 anaesthesia, a special particularly developed lid[9] with a CO_2 inflow of 4.6 l/min was introduced into the box. The lid can provide a very even CO_2 distribution in the box and therefore can prevent severe distress or pain. Ether anaesthesia was initiated with 8 ml ether inside a gas-washing bottle, which was vaporised with 5 l/min oxygen. All animals were taken out of the box when breathing was clearly depressed. Sham and control animals were placed in the same box for 78 sec (mean time of induction for all anaesthetics) without anaesthesia, but the sham group received an air-inflow of 5 l/min. An overview is given in Table 1.

Corticosterone analysis*:* For determining the basal value of corticosterone 150 µl blood was taken one week after arrival by retro-bulbar venous puncture in a Natrium Heparin capillary.

A second blood sample (150 µl) using the same method during the experiment without anaesthesia was taken from each mouse 15 min after anaesthesia. Blood plasma was collected after centrifuging for 4 min (12000 rpm). The corticosterone concentration was analysed using a competitive corticosterone ELISA (IBL) and measured with a microtiter reader at 450nm.

Open Field Test: Following the blood collection the mice were introduced into the open field (60 cm x 60 cm) for 5 min. During the test the animals were videotaped by a camera (Ikegani). The video was analysed with Etho Vision (Noldus, version 3.1). The total distance and the frequency animals crossed between the outer and inner zones were recorded.

Experimental design

During 12 days of adaptation the animals were handled daily over 7 days. Each animal was put on the arm for at least 1 minute to minimise stress during the experiment. For measuring the basal corticosterone value blood was taken one week after arrival. At 8 weeks of age the experiment was performed and finished

All tests were performed within six days between 9:00-12:00 in the morning, 10 mice per day. Only one animal per cage was taken out each day to avoid additional stress by opening the cage a second time. The animal was taken out of the cage and transferred into a transport cage and taken to the experimental room. Immediately after arriving the animal was, depending on the test group, anaesthetised.

Following the anaesthesia the mice stayed in the transport cage for 15 min. From each mouse a blood sample was taken by retro-bulbar puncture without anaesthesia for corticosterone measurement. Finally the animals were introduced into the open field for 5 min and then were returned to their home cages.

Statistics

All data were analysed using StatView software (version 5.0, SAS Institute Inc., Cary, NC, USA, 1998). The data were analysed for normal distribution followed by ANOVA and a Scheffé -test with a significance level of 5%. Non-normal distributed data were analysed by non-parametric test (Kruskal-Wallis test or Mann-Whitney U test).

Table 1. Overview of doses and gas inflow

	Treatment	Doses	Oxygen inflow
Anaesthesia	Sevoflurane	8%	5 l/min
	Isoflurane	4%	5 l/min
	Ether	8 ml	5 l/min
	CO_2	---	4,6 l/min
Without Anaesthesia	Sham	---	5 l/min
	Control	---	---

Results

Blood corticosterone concentration

In comparison to the basal value corticosterone concentration rose significantly after anaesthesia (sevoflurane, isoflurane, CO_2, ether: $p<0.001$) as well as after sham procedure ($p=0.009$) (Figure 1). Even though the corticosterone concentration of control group increased, it did not reach a statistical difference.

Significant differences were found for the change in the corticosterone concentration ($F_{5,54}=11.954$, $p<0.0001$). The increased corticosterone levels of each group were: isoflurane (1816.2±386.8 nmol/l), sevoflurane (1527.2±397.9 nmol/l), ether (1444.3±405.0 nmol/l), CO_2 (1440.7±337.2 nmol/l), sham (773.5±535.1 nmol/l) and control (358.2±790.2 nmol/l). Compared to the control group corticosterone concentration increased significantly after anaesthesia ($p=0.0004$ for sevoflurane, $p<0.0001$ for isoflurane, $p=0.0012$ for ether and $p=0.0012$ for CO_2). There were no significant differences between groups anaesthetised by ether, CO_2, isoflurane and sevoflurane, nor between the sham and control groups (Figure 2).

Locomotor activity in the Open Field test

Significant differences were found for the Open Field test in locomotor activity ($F_{5,47}=3.528$, $p=0.0086$ for total distance; $F_{5,47}=2.868$, $p=0.0243$ for frequency). For the total distance the statistical difference is mainly due to the difference between groups under isoflurane and ether anaesthesia ($p=0.0581$), while the main difference was found between the sham group and groups of isoflurane & CO_2 for frequency (Table 2).

Discussion

The aim of this study was to compare four volatile anaesthetics (CO_2, ether, sevoflurane, isoflurane) commonly used for minor procedures, determining their impact on the animals after short exposure. The secretion of glucocorticoids is a classic endocrinal response to stress[10]. The results show that corticosterone concentration of all anaesthetised mice is higher than the basic value of non-stressed mice, this being independent of the anaesthetics. There is nearly no difference between the corticosterone levels following the different anaesthetics. Animals with sham anaesthesia also show a higher corticosterone level in

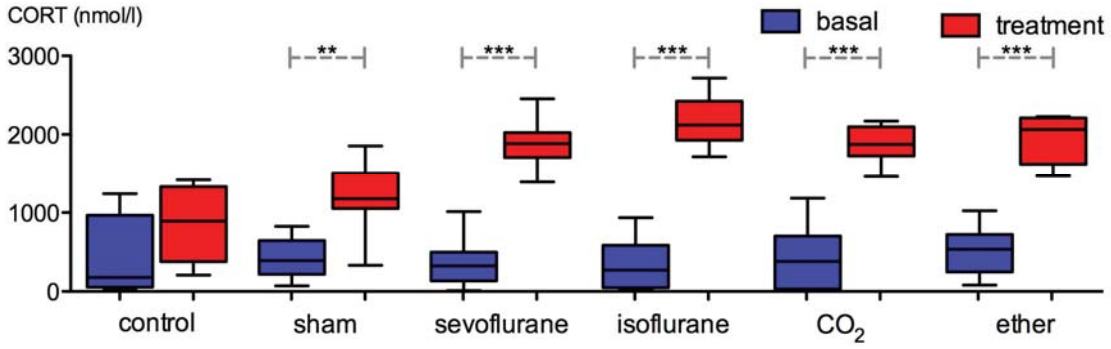

Figure 1. Box plot of basal CORT concentration and the concentration after experimental procedure (n=10, *** p<0.001; ** p<0.05).

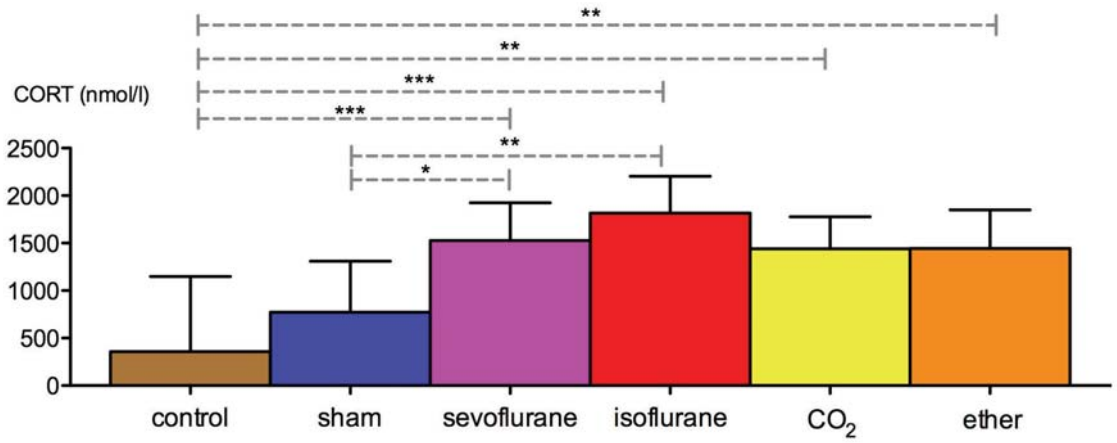

Figure 2. The increase of CORT concentration (Mean± SD) after experimental procedure (n=10, ***p<0.001; **p<0.05; *p<0.1

Table 2. Travel distance (cm/5 min) and frequency of crossing (mean ± SD) in the Open Field Test

Treatment	Total distance	Frequency
Sevoflurane	2033.5±445.2	15.8±10.7
Isoflurane	1556.1±598.5	10.8±9.9
CO_2	1612.4±948.6	10.0±10.0
Ether	2805.2±155.4	12.0±7.0
Sham	2503.4±227.2	23.2±8.0
Control	2322.2±498.1	17.5±7.3

comparison to the control group. This indicates that the gas inflow alone into the box may also raise the corticosterone concentration. Thus, the manipulation of animals and the anaesthesia itself are both stressors.

In a previous study[6] we showed that short-term volatile anaesthesia (ether and CO_2) caused more stress than retro-bulbar blood sampling without anaesthesia, but there was no difference between both anaesthesics. The present study also found no difference between the corticosterone levels following the different anaesthetics. This suggests that all the inhalation anaesthesia performed in the present study led to a similar level of stress, even though the reason causing stress may differ to ether (highly irritating to the respiratory system).

Quartermain et al.[11] showed that stressors reduce locomotor activity in the open field test. In the present study animals which had received ether anaesthesia travelled significantly more than animals which had received isoflurane anaesthesia. Comparable results were also found by a previous study[6], mice given ether anaesthesia travelling more than after CO_2 anaesthesia. During the experiment all animals, independent of treatment, showed a similar crossing frequency. The results indicate that the impact of short-term inhalation anaesthesia in the open field test may be mild after 15 min even though the residual anaesthesia could still play a role. Thus, it may not be ideal timing to use the open field test to determine the stress response of short-term inhaled anaesthesia.

Based on our data the corticosterone level rose significantly after short-term volatile anaesthesia and there were no significant differences between the four anaesthetics used. It can be concluded that all inhalation anaesthetics used in this study cause temporary stress in mice and that there is no relevant difference between the different anaesthetics. Therefore, it should be taken into consideration whether anaesthesia used for short experimental procedures such as blood sampling reduces stress or actually causes more stress than the experimental procedure itself.

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Application of inhalational anaesthesia to miniature pigs housed in isolators

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Abstract

Procedures with a miniature pig in an isolator often requires injectable anaesthetic for safety and prompt performance. The injection of an anaesthetic may, however, be problematic if the animal is not cooperative. The purpose of this study is to evaluate the effectiveness of inhalation anesthetic with a novel installation in the isolator. Four male miniature pigs were individually housed in large or small isolators for 12 weeks. When the experiment started, the two, older animals for the large isolator weighed 24.2±0.2 kg, and the two, younger ones for the small isolator weighed 14.7±1.0 kg. A modified ethogram was applied to evaluate the activity of the pigs after the initiation of sevoflurane administration. Sevoflurane was administered into the isolators with total amounts of 87 ml (large isolators) and 51 ml (smaller isolators). The amounts were divided to 5 or 10 installments which were administered every 5 minutes and the activity of the pigs was followed. In the large isolators, the activity levels of the animals were similar with the five and ten installments, respectively. In the small isolators, the administration of sevoflurane with five installments caused a more rapid effect than the administration of ten installments. In conclusion, sevoflurane is a successful chemical restraining agent when used for the animals housed in isolators. The optimal handling time for the animals is 20-30 minutes after initiation of the administering. If the animal must have a recumbent posture, an injectable anaesthetic would also be needed.

Keywords: inhalation anaesthetic, miniature pig, isolator

The miniature pig has been noted as a source of organs for xenotransplantation, because it is similar to human beings in its physiology, anatomy structure and organ size. For a successful xenoplantation, gnotobiotic miniature pigs are housed in isolators. The handling of pigs in isolators is not easy. Usually an injectable anaesthetic is used with these animals during procedures for both safety and prompt performance of examinations, diagnoses and therapies. The injection of an anaesthetic may, however, be problematic if the animal is not cooperative. Moreover, restraining of the pigs for the injection procedure may be stressful for the animals. The purpose of the present study was to evaluate the effectiveness of inhalation anaesthetic administered into the isolator in order to develop less stressful restraint methods.

Materials and Methods

This study was approved by the Institutional Animal Care and Use Committee, Asan Institute for Life Sciences, Asan Medical Center. Four male specific pathogen-free miniature pigs (mixed with Yucatan, Native pig, Pigmy pig, and Miniature pot belly) were purchased from PWG Genetics Korea, Ltd. The pigs were three, six, eight, and twelve months old and clinically healthy. They were individually housed in large or small isolators (approximately 1450 and 850 liters, respectively) for 12 weeks. When the experiment started, the two older animals in the large isolators weighed 24.2±0.2 kg (mean±SD) and the two younger ones in the small isolators 14.7±1.0 kg. The pigs were fed once daily with a commercial diet (Imperial 4®, SCF, Korea) and had free access to RO (Reverse Osmosis) water. The feeding was restricted to prevent obesity[1]. The daily amount of food was 590 g (larger pigs) and 400g (smaller pigs), at a feeding level of 60% of the *ad libitum* food intake[2].

Sevoflurane was chosen to be used because of its rapid induction and recovery and its inoffensive odour[3]. The concentrations and methods of administration to the large and small isolators are shown in Table 1. Sevoflurane liquid was delivered into the isolators through the infusion port with a

syringe. The delivered sevoflurane was fallen into the container in the isolators, with a small fan operating with battery for rapid vaporization (Figure 1). The total amount of sevoflurane was 87 ml for large and 51 ml for small isolators. This amount was divided into 5 or 10 installments, which were administered every 5 minutes. The volumes of the installments were 17.4 ml for 5 dosages and 8.7 ml for 10 dosages with the large isolator and 10.2 ml and 5.1 ml with the small isolator, respectively. The first two pigs were treated with the administration of 10 installments and the second two pigs with the 5 installments for 2 times once a month.

A modified ethogram[4] was applied to evaluate the activity of the pigs, and the scoring is shown in Table 2. The activity was evaluated with scores of twelve points every 5 minutes for 1 hour after the initiation of sevoflurane administration. The isolator was turned off during the experiments for guarantee of an effective concentration of sevoflurane gas. The concentration of CO_2 in the isolator during the experiments was approximately 1,000 to 10,000 ppm.

Results

The activities of the pigs during the sevoflurane administration are shown in Figure 2 and Table 3.

In the large isolators, 5 and 10 installments caused a similar pattern of behaviour. The standing up position was maintained from 25 minutes to 60 minutes with both installments, although there was the almost sitting posture at 40 minutes in the 5 installments as compared to the measurement in the 10 installments. When the activities of the pigs were calculated with scores of twelve points every 5 minutes for 1 hour, they were similar with the 5 and 10 installments (mean±SD: 1.25±0.0 and 1.25±0.11, respectively).

The activities in the small isolators, however, showed a tendency of decrease with the 5 installments during the period from 5 minutes to 45 minutes as compared to the 10 installments. The activities in the small isolators presented more rapid sedation when conducting 5 installments, compared with 10 installments. When the activities of the pigs were calculated with scores of twelve points every 5 minutes for 1 hour, the average value differed with the 5 and 10 installments (mean±SD: 1.25±0.0 and 0.80±0.18, respectively).

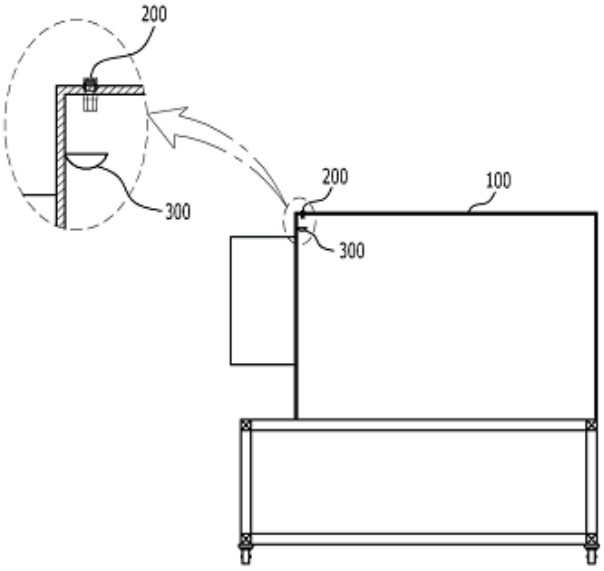

Figure 1. Scheme of the infusion device of inhalation anaesthetic installed within the isolator.
100: Isolator, 200: Infusion port, 300: Container for inhalation anaesthetic

Table 1. Concentrations of sevoflurane anaesthesia administered into the isolator for the gnotobiotic miniature pig. The total volume needed to get the concentration of 60 ppm was calculated. This volume was administered in 5 or 10 installments every 5 minutes.

Isolator	Capacity (L)	Volume of sevoflurane required per 1ppm (ml)	Total accumulated concentration (ppm)	Total administered volume (ml)	Two methods of administration (every 5 mins)
Large	1450	1.45	60	87	5 & 10 times installments
Small	850	0.85	60	51	

Table 2. Evaluation criteria for the activity of the miniature pigs.

Scoring system	+2	+1	0	-1	-2
Behavior	Active		sitting position	Inactive	
	walking rooting eating drinking	standing up		lying down agitatedly	lying down quietly

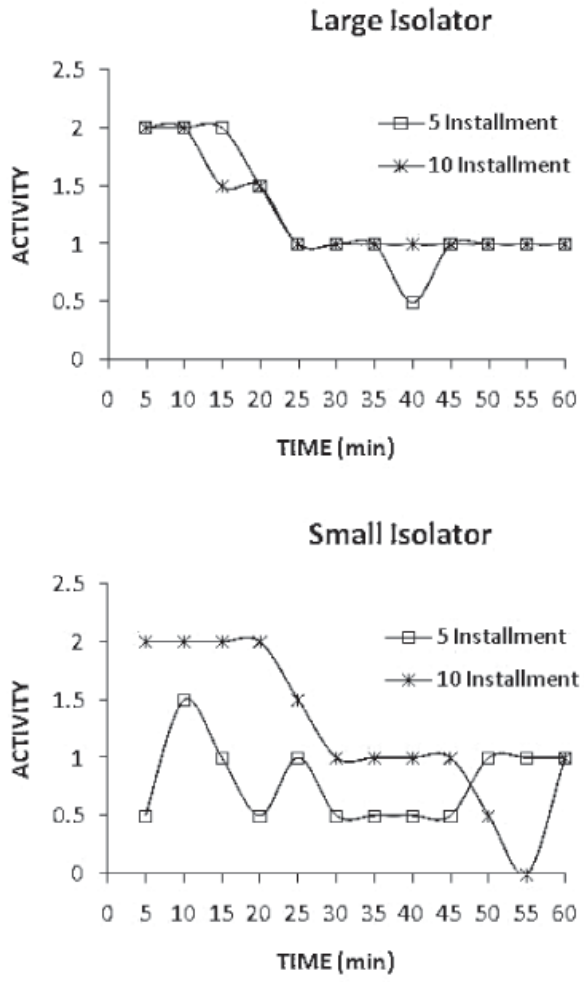

Figure 2. The activity of the pigs were evaluated every 5 min for 1 hour after the initiation of sevoflurane administration. The total amount of sevoflurane was 87 ml for large and 51 ml for small isolators, and divided into 5 or 10 installments, and then the dosages were administered every 5 minutes.

Table 3. Activity of the miniature pigs after sevoflurane administration. The data are presented as mean±SD, which were calculated with scores of twelve points every 5 minutes for 1 hour.

Installments	Large isolator	Small isolator
5	1.25±0.11	0.80±0.18
10	1.25±0.0	1.25±0.0

Conclusions

Sevoflurane is a successful chemical restraint agent to be used when minor procedures are performed to the animals housed in isolators. The optimal time to start procedures is 20-30 minutes after the initiation of administration. If a recumbent posture of the pig is wanted, an injectable anaesthetic would also be needed.

Acknowledgement

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A novel method of oral endotracheal intubation utilizing an endoscope for inhalation anaesthesia in rabbits

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Abstract

Endotracheal intubation in rabbits is technically difficult. This difficulty may prevent the use of an inhalational anaesthesia in rabbits. Considering the animal welfare, the inhalational anaesthesia is in general thought to be better than the use of injectable anesthetics, and its use would be a refinement. Hence, new techniques to enable the endotracheal intubation in rabbits are needed. We developed a novel method of oral endotracheal intubation utilizing an endoscope. Anaesthetized animals were placed in the spine position on a newly developed work stand which was tilted at a 30 degree angle during intubation. An endoscope equipped with a 150 mm long probe, 1.6 mm in diameter and 17000 pixel counts, was used. The endoscope was passed through a 3.5 mm endotracheal tube with the stylet which was passed through the oropharynx under a visual control using a monitor connected to a computer. Following visualization of the larynx, the endotracheal tube was gently advanced into the trachea on expiration. After confirming the tracheal cartilages, the endoscope was withdrawn from the endotracheal tube and the tube was left in the trachea. This endotracheal intubation method enabled us to conduct the safer anaesthesia in rabbits.

Keywords: endotracheal intubation, inhalant anesthetics, endoscope, stylet, animal welfare

Endotracheal intubation of rabbits is more difficult than in most other species, such as dogs, sheep, pigs, old-world primates and large birds[1, 2]. The larynx is difficult to visualize directly[2, 3], because of the anatomy of the rabbit which includes large incisors, a long and narrow cavity, a thick tongue, and the restricted mobility of the tempero-mandibular joint[3]. Numerous descriptions of techniques and devices for the endotracheal tube placement have been described, such as classic and modified 'blind' techniques, pediatric laryngoscope, otoscope, endoscope and laryngeal mask airway[3]. More recently, the modified 'blind' techniques, using nasotracheal intubation[4] or laryngeal tube[5], were reported. The 'blind' technique may, however, be problematic from point of view of the animal welfare: The laryngeal mucosa may be damaged very easily[3], but the mucosal damages cannot be recognized by an operator during the 'blind' technique. Hence, an ideal method of endotracheal intubation has not yet been established in rabbits, which may hinder the refinement of anaesthesia methods in rabbits. The aim of present study was to find a method of good visualization for larynx. Thus we used an endoscope, a newly developed metal hollow stylet, a workstand and an endotracheal tube for endotracheal intubation in rabbits.

Animals and anaesthesia

Female New Zealand white rabbits (n = 4, weight 2.5 to 3.0 kg) were used. These rabbits were negative for tested bacterial, viral, helminthes, protozoan, and arthropod pathogens. They were allowed *ad libitum* access to commercial rabbit diet and to water via a plastic bag with a stainless steel ball bearing sipper.

All procedures in this study were approved by the Institutional Animal Care and Use Committee. The animals did not fast prior to anaesthesia. Anaesthesia was induced with medetomidine (0.5 mg/kg intramuscularly; Nippon Zenyaku Kogyo Co., Ltd. Koriyama, Fukushima. To ensure the adequate level of anaesthesia, clinical signs were observed: lack of muscle and jaw tone, absent palpebral response, decreased respiratory and heart rate and loss of pedal reflexes. In the case of insufficient anaesthesia or between each trial of endotracheal intubation, oxygen-

volatilized sevoflurane (3% to 5%, Abbott Japan Co., Ltd. Tokyo) delivered via a vaporizer (Natsume Seisakusho, Tokyo) was administered–using a face mask.

Technique

An ultra-thin endoscope (TESALA™, AVS Co., Ltd. Tokyo, Japan) consisted of the camera unit, hand piece and flexible fibre probe (outer diameter, 1.6 mm; length, 150 mm; 17000 pixel counts). The endoscope was connected to a PC or monitor for visualization.

After the adequate anaesthetic depth was achieved, the rabbit was gently placed in the spine position on a newly developed work stand which curved at 30 degrees. The rabbit was facing the handler and operator. The mouth of the rabbit was fully opened by the handler using operation strings, which were connected to upper and lower incisors. The tongue of the rabbit was carefully held by operator's subordinated hand while operator's dominated hand induced an endoscopic probe, metal hallow stylet and endotracheal tube (Figure 1D; ESE unit) into the oral cavity. The ESE unit comprised the endotracheal tube (Figure 1A; inner diameter 3.5 mm; length 15 cm; Fuji Systems Co., Ltd. Tokyo), a newly developed aluminum hollow stylet (Figure 1B; inner diameter 2 mm; length 13 cm; tip of stylet curved approximately 30 degrees) and a flexible fibre probe (Figure 1C).

The operator induced the ESE unit into the oral cavity and slowly advanced it by following the hard palate to the back of the oropharynx and a pair of the tonsils (Figure 2A). While inserting the ESE unit, the operator was able to investigate the laryngeal regions thoroughly through the endoscopic views. The operator could observe a pale triangle of the epiglottis (Figures 2B and 2C) at the laryngeal region. When the ESE unit moved forward, the operator clearly recognized a pair of arytenoid cartilages (Figure 2D) which came into view. Then, the operator could advance the ESE unit into the glottis, and the pair of vocal cords (Figure 2E) became visible. After the vocal cords, the tracheal cartilages were observed (Figure 2F) and the endotracheal intubation was completed. All four rabbits were intubated orally within two minutes. No signs of laryngeal spasm or bleeding due to laryngeal damages were observed.

Discussion

Numerous reports on the rabbit endotracheal intubation have been published. The intubation methods can be categorized into two techniques, 'blind' intubation[2-4] and intubation using a laryngoscope[2] or rigid endoscope[6]. 'Blind' intubation has been reported to require very careful positioning where the rabbit was placed in the prone or supine position and the endotracheal tube was induced via oral or nasal routes.

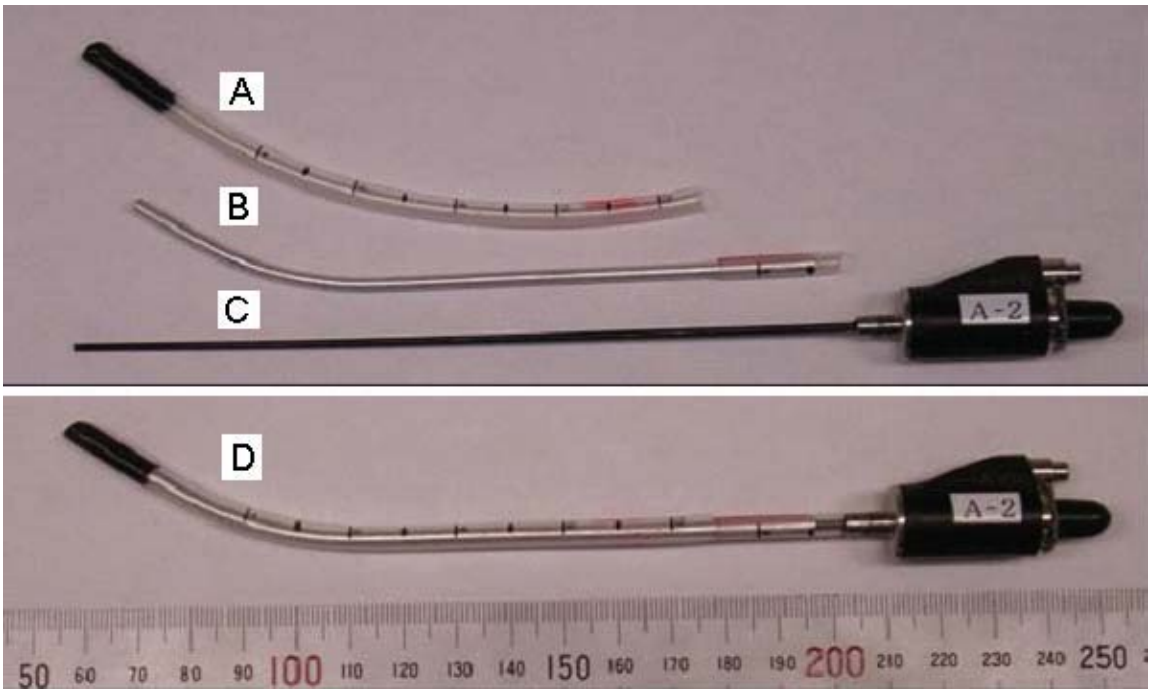

Figure 1. Components of ESE Unit: The ESE unit comprises of the endotracheal tube (A; inner diameter 3.5 mm; length 15 cm; Fuji Systems Co., Ltd. Tokyo) a newly developed aluminum hollow stylet (B; inner diameter 2 mm; length 13 cm; tip of stylet curved approximately 30 degrees) and a flexible fiber probes (C). The lower picture shows that three components are prepared for endotracheal intubation as ESE Unit (D).

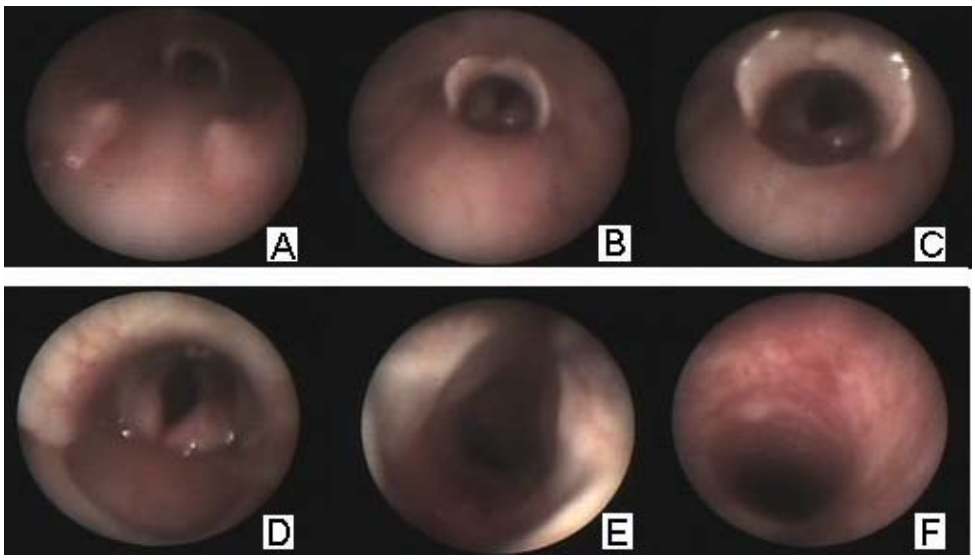

Figure 2. A serial views of laryngeal region: A pair of the tonsils (A), a pale triangle of the epiglottis (B and C), a pair of the arytenoid cartilages (D), the vocal cords (E) and the tracheal cartilages (F) were observed.

Inglis and others[3, 7] reported that the mouth, larynx and trachea had to be brought into linear alignment for successful intubation. In an outstretched prone position, the rabbit's head was tipped and the neck was extended until the head was upright at a right angle to the rest of its body[8]. If intubated in the spine position, the neck was hyperextended by fashioning a holder from styrofoam[9].

For using 'blind' intubation, the confirmation of the endotracheal tube placement was based on observing condensation of breath on a cold surface, or movement of a piece of tissue paper or a fur from the animal placed at the end of the tube[2], but the direct confirmation of the endotracheal intubation was not possible. Moreover, 'blind' intubation may damage the laryngeal mucosa easily but the damages may not be recognized.

The endotracheal intubation methods with good visualization have also been reported. For visualizing the larynx, the rabbit was positioned on its back and the otoscope or laryngoscope[2,3] or a rigid endoscope[6] was introduced into the mouth to ease placement of the endotracheal tube in the trachea. These methods could also be used with a combination of stylet and tube and they are suitable for larger rabbits (>3kg)[7]. They may, however, be inappropriate for smaller rabbits.

Other methods using an airway device[10], a laryngeal mask airway[11], or a laryngeal tube[5] have also been reported. The airway device and laryngeal mask airway were designed to surround and seal the larynx and were inserted blindly. According to the writers, these devices were easier to place than an endotracheal tube. The laryngeal mask airway, however, emitted greater waste anaesthetic gas, especially when staff had limited experience[11]. The laryngeal tube was reported to be inserted blindly and without difficulty as an airway management device in rabbits <5 kg. These devices might damage the mucosal epithelium around larynx, and operators are not able to observe bleeding or damage of mucosa with the blind method. Therefore, it was concluded that there were no appropriate endotracheal intubation methods for rabbits[5].

Before this study, we tried to apply a laryngoscope to insert the laryngeal tube. However, we could not observe the laryngeal region in some rabbits due to limited mouth opening. For the purpose of good and constant visualization of the laryngeal region, we found that the endoscope with a thin flexible probe enabled insertion of an endotracheal tube. However, we were not able to advance the endoscope due to the flexibility on the tip of the endoscope. Because of the anatomical characteristics of rabbit, the straight laryngoscope of the endoscope was presumed not to assure a constant good visualization of the laryngeal region in particular glottis. Indeed, a flexible endoscopic probe, used as a guide-wire, has been used in rabbits[12].

As a result, we developed a hard hollow stylet, which was inserted into the endotracheal tube. Consequently, we applied an aluminum hollow stylet with a 30 degree bent at the tip. The stylet was thin enough to be used within an endotracheal tube, and by this way we could also use a flexible probe of the endoscope. During the endotracheal intubation, the laryngeal regions and the area within trachea could be observed clearly through the endoscopic views.

The results of this study suggest that endotracheal intubation using an endoscope can be used as a clinically preferred method in the rabbit.

In the future, using an alternative method with a laryngoscope along with our method, a comparison study will be conducted to reveal the usefulness and learning speed for the beginner of endotracheal intubation in the rabbits.

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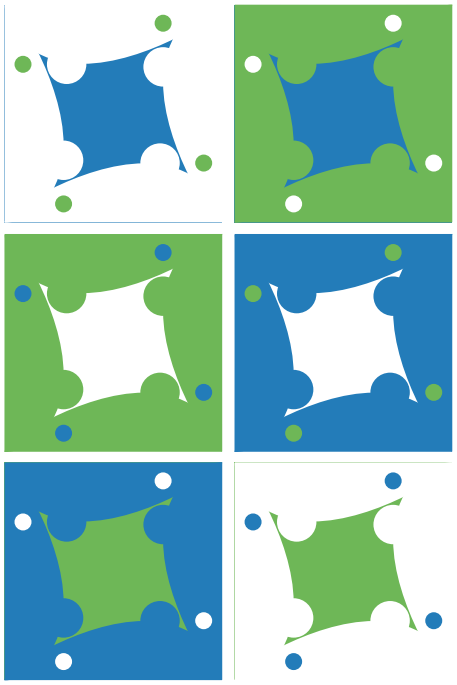

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