Reducing suffering through refinement of procedures: Report of the 2003 RSPCA/UFAW rodent welfare group meeting

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Summary

The RSPCA/UFAW Rodent Welfare Group holds a one-day meeting every autumn so that its members can discuss current welfare research and exchange views on rodent welfare issues. A key aim of the Group is to encourage people to think about the whole lifetime experience of laboratory rodents, ensuring that every potential impact on their wellbeing has been reviewed and refined.

The 2003 meeting was a case in point, since it covered a broad range of issues including refining experimental procedures*, reducing the number of animals used, assessing wellbeing, and reducing suffering in veterinary procedures. Speakers described protocols for refining salmonellosis studies in mice; improving pregnancy detection to reduce wastage in mice; refining repeat blood sampling in rats and mice; reviewing tooth trimming in genetically modified (GM) rodents; and the use of chromodacryorrhea objectively and non-invasively to assess stress in rats. Other presentations provided an update of progress made by the UK GM Mouse Welfare Assessment Working Group and set out recommendations for refining telemetry procedures (and husbandry) for rats and mice.

Refining infection studies in mice

George Grant, Rowett Research Institute

A number of major research programmes at the Rowett study the roles of gut bacteria in acute and chronic intestinal diseases of humans and other animals. This includes looking at the immune and inflammatory responses that occur in response to infection and the potential of commensal gut bacteria to modify these responses, with the aim of trying to enhance the host’s natural resistance to infection. Salmonellosis was chosen as the model of acute infection. While this was ideal for in vitro studies, the adverse effects in vivo presented us with significant ethical and welfare problems that had to be addressed.

The most widely used model of salmonellosis is based on susceptible mouse strains such as BALB/c or C57BL/6 (these are denoted by ity, i.e. infection by Salmonella typhimurium susceptible). After oral exposure to Salmonella typhimurium or S. enteritidis (≤10⁵ Colony Forming Units, CFU), these mice develop and rapidly succumb to severe systemic disease. This is used as a model of typhoid-like illness, but can lead to high levels of suffering and mortality and often requires large numbers of animals. Also, systemic infection is atypical for Salmonella typhimurium bacterium in most domestic animals and humans, in whom it causes gastroenteritis and destruction of intestinal tissue. There is thus a need for an alternative model that will cause less suffering, use fewer animals and produce more valid results.

* All experimental procedures described within this report were carried out under the authority of the Animals (Scientific Procedures) Act 1986.
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An alternative model of salmonellosis was developed, based on the C3H/HeN ity (infection by Salmonella typhimurium resistant) mouse. This strain is partially resistant to S. enteritidis, but does develop a persistent infection when given high doses (10^7-10^8 CFU). Initial attempts at dosing in jelly failed because the mice did not like it, but we have had greater success with chocolate drops. However, unlike in ity mice, there is no uncontrolled proliferation of pathogen in systemic tissues. Using a resistant strain has enabled the endpoint to be significantly refined and most mice maintain their body weight or grow over a 12-day experimental period, provided that they have attained a starting body mass of at least 19g. The mice are pair-housed in metabolism cages with a tunnel as a refuge; this does not interfere with urine and faeces collection because the tunnel has holes in and the mice keep their resting area clean.

Salmonellosis in the C3H/HeN mouse exhibits both gastrointestinal and systemic elements of infection, without high lethality. This has enabled detailed investigation of innate and adaptive intestinal and systemic immune responses to infection, with moderate numbers of experimental animals. It thus provides a good alternative to existing widely used models for study of most aspects of pathogenesis. In particular, the severity of the infection for the animal and the numbers required are greatly reduced. It may not be possible to replace all current uses of ity infection models with resistant strains, but anyone who wishes to use susceptible strains should have to justify not using the refined model.

Reducing wastage in mice by pregnancy detection

Ron Raymond, Cancer Research UK

Developing a more reliable technique for detecting pregnancy in mice can make a significant contribution to reducing overbreeding and wastage, since non-pregnant females can be mated again and animal supply can be more effectively matched to demand. Usually, visual observation of a vaginal “plug” is taken to be evidence that a female mouse has mated, and it is then assumed that she is pregnant. Alternatively, pregnancy can be determined by palpating the abdomen of a mated female at a later stage of gestation, e.g. 7 to 10 days. However, transabdominal palpation is not a precise technique at this stage; for example, gut contents can be mistaken for embryos. Although pregnancy is identified more easily by palpation at later stages, animals’ lives could have been wasted in the intervening period if females are found not to be pregnant. Besides the advantages with respect to wasting fewer animals and managing the colony, accurate confirmation of pregnancy at any stage of gestation would also benefit the management of ongoing embryology studies.

We therefore decided to investigate the feasibility of using ultrasound to diagnose pregnancy in the mouse. A project was initiated with two key aims; first, to characterise features for accurate pregnancy diagnosis; and second, to evaluate and establish a protocol for the use of diagnostic ultrasound as a routine technique in mouse pregnancy detection.

As there are concerns about the impact of ultrasound on laboratory rodents, we checked whether the scanner would be stressful to the mice. However, mice can detect ultrasound up to 100 kHz but the frequency of medical ultrasound is much higher at 1 to 20 MHz (1,000 to 20,000 kHz) and so the scanner is inaudible to the animals. Another issue that must be taken into consideration is the potential effect of exposure to ultrasound on developing embryos. Although there is no mention of detrimental effects caused by short-term exposure to ultrasound in the literature, we are monitoring the development of the embryos and juvenile mice carefully so that we can detect any adverse effects.

Method: Successive ultrasound examinations are being performed in “plugged” and “non plugged” females of various strains bred in-house. Before scanning, the abdominal coat is shaved, which can be done 2 to 3 days previously; the skin is cleaned with alcohol and scanning gel is applied. Animals are manually restrained and we have not found it necessary to anaesthetise or sedate them. However, the whole scanning process

Figure 1. Ultrasound image taken at PC12.5

Legend: We use the position just cranial to the inguinal mammary glands as an external anatomic reference for the transducer positioning. The uterine transverse section imaging and diameter measurement is performed cranial to the uterine bifurcation using the cross-section view of the 7th lumbar vertebra (solid arrow) and sacrum as an internal anatomic reference. Two gestational sacs are visible (dashed arrows).
obviously has the potential to cause distress and so careful, empathetic handling, positioning, clipping and cleaning are essential. It has been noted that performing these procedures with care also improves the quality of the overall imaging. We are still working to establish the most appropriate protocol for preparation for scanning.

Ultrasound imaging is performed on the entire abdomen with the mice manually restrained in the dorsal recumbency position. Females are scanned every day from post-coitum (PC) day one-and-a-half (PC1.5) until PC18.5 or 19.5 and also one day after they have littered. The main anatomic structure to be imaged and measured is the uterus (Fig. 1).

Results: Any detected features of pregnancy are measured and recorded. It usually takes 1 to 3 minutes to detect whether or not a mouse is pregnant, depending on the age of the embryo; for example, a 10 to 12 day embryo can often be detected in around a minute. The target period for initial investigation of uterine changes in diameter and echogenicity is PC4.5 to 5.5, because fertilised eggs are implanted into the uterus at PC4.5 and maternal decidual tissue growth one day later causes extensive uterine swelling. At PC7.5 there is an advanced decidual reaction in the uterus and the initiation of allantotis formation, so the study is also focusing on the changes that occur in the uterus during this period. At PC9 and beyond, pregnancy detection is 100%.

Conclusion: The scanning machine cost around £15,000 and training is necessary both in the use of the scanner and the interpretation of the scans. However, competence can be quickly achieved and the scanner has already enabled us significantly to reduce wastage. These is especially important with respect to transgenic mice where individuals are extremely valuable, and may be irreplaceable, yet are produced in very small numbers. The financial and ethical benefits of early pregnancy detection and minimising wastage of transgenic mice cannot be overstated. We now aim to explore other possibilities for using the scanner to refine procedures and reduce animal use.

Repeat blood sampling from the saphenous vein in rodents
Gareth Hughes, Pfizer Global Research and Development, Sandwich

There are a number of techniques for blood sampling from mice, but many present practical, welfare or ethical problems. It is especially important to continually work to refine blood sampling, as it is such a common and frequent procedure. For example, at Pfizer we routinely conduct pharmacokinetic studies on rodents where repeat time point blood samples are required. We have routinely used one mouse per bleed time point to obtain a terminal sample on pharmacokinetic mouse studies, as this was necessary to acquire a sufficient volume for analysis. Now that more sensitive analysis equipment enables quantitative analysis to be performed on much smaller blood samples (e.g., 10μl in modern mass spectrometers), a broader range of techniques for blood sampling can be investigated.

Saphenous blood sampling was suggested after reviewing a method developed and published by the Laboratory Animal Centre of the Norwegian Institute of Public Health. Reasons for choosing this site were:

- this is a humane and practical alternative to methods that require anaesthesia
- repeat samples can be taken from the same animal without always having to re-puncture the vein
- the number of animals used is reduced
- inter-animal variation within a study is reduced
- it is a relatively straightforward and transient procedure
- no special equipment is required
- it can be a single operator procedure.

The following description of the protocol is a short summary to illustrate the basic principles of the technique – please read the full reference and consult your attending veterinarian (and check that it is permitted under your project licence, in the UK) before attempting this method. Also note that the BVA(AWF)/FRAME®/RSPCA/UFAW Joint Working Group on Refinement (JWGR) report on the removal of blood from laboratory mammals and birds does not recommend the use of the saphenous vein for rodents or lagomorphs. This is because the report was produced at a time when this site was not widely used.

Briefly, the rear leg of the mouse is carefully shaved (which can be done the day before) and the animal is placed in a thermostatically controlled chamber at 37°C for 10 minutes to aid peripheral vasodilation. The mouse is then restrained in an adapted tube so that the limb can be extended to raise the saphenous vein, which runs along the dorsal surface of the rear limb. Petroleum jelly is applied to the shaved area before venipuncture, which helps to prevent the blood from spreading and reduce the risk of clotting. The vein is punctured with a 23G hypodermic needle and the sample collected with an adapted syringe connected to a microvette tube. This creates a vacuum that draws

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* This is done using a 10 MHz linear-array transducer (SonoSite® Inc., Bothell, USA).

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* British Veterinary Association Animal Welfare Foundation, Fund for the Replacement of Animals in Medical Experiments.
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the blood sample into the tube. After collection, applying pressure to the puncture site with a cotton bud stems the blood flow.

Repeated blood samples can be obtained by either gently removing the scab or clot from the puncture site, or by re-puncturing the vein. Re-opening the original venipuncture site should be viewed as an option to reduce discomfort where possible, but its appropriateness should be judged on a case-by-case basis. It is essential to handle and restrain animals gently when doing this and not to persist in trying to remove a scab or clot if this would cause more discomfort than re-puncturing.

We have successfully used the saphenous vein site to obtain multiple blood samples of 50-75μl per mouse on each occasion throughout a study. For example, on one occasion we took four 50μl samples from each mouse in a 24 hour period, thereby reducing the number of animals used by 75%. This is a dramatic reduction in the number of animals required to complete a study and saphenous vein sampling also reduces the number of times a blood vessel needs to be punctured in comparison with other methods of blood sampling. Saphenous vein blood sampling has now been used successfully on several studies and we have now progressed to using this technique on rats.

Nice smile but bad news:


tooth trimming in mice

David Morton, University of Birmingham

Incisor trimming is undoubtedly stressful for rodents and can be painful if the pulp cavity is affected through heat or exposed through splintering the base of the tooth. Pain may also be caused by pressure at the level of the root periodontal ligament. As with all other potentially painful or distressing interventions, tooth trimming needs to be refined and only carried out when there is a demonstrable need to do so.

This study came about as a result of a transgenic line of mice that had a skeletal deformity leading to overgrown lower incisors, which were frequently trimmed. The upper incisors grew slowly and inwards towards the hard palate and had an altered consistency but were also a potential problem. The frequency of trimming increased to 3 times weekly but the animals did not seem to be thriving, which could have been due to the genetic defect or to the frequency of tooth trimming. We were concerned about exceeding the severity limit of the procedure and decided to evaluate the tooth trimming protocol to see whether we could reduce the impact on the animals.

In particular, we investigated whether it would be better for an anaesthetic to be used or whether this would be even more disruptive; alternatively, could the teeth be just left to grow, or trimmed less frequently? We also needed to consider whether females could safely be mated and allowed to give birth, care for pups and so on. We used daily observation of appearance, posture and behaviour, and body weight as markers of welfare.

Growth rate curves were equally affected whether or not an anaesthetic was given for tooth trimming. However, when we changed the frequency of the trimming procedure to every 3 weeks or so, body weight gain did not dip even though the incisors grew well beyond the nose. The level of the nose is well away from the pulp cavity and teeth were subsequently trimmed to this level. Initially, the teeth were carefully trimmed with either a miniature rotating dental saw or scissors, but we discovered that the tooth consistency was softer than normal so that we were able to trim the teeth with skin clip/staple removers with a sharp, anvil cutting edge. These were easier to use and enabled a faster, more accurate trim. One mouse subsequently nearly doubled her body weight in just a few weeks and has successfully reared a litter despite her epignathic appearance, which is reminiscent of an upturned walrus! Reducing trimming frequency in this instance was critical for good welfare and weight gain, especially in this line of genetically modified mice. These observations may have implications for trimming the incisor teeth of other rodents and lagomorphs.

Non-invasively assessing disturbance and stress in laboratory rats by scoring chromodacryorrhoea

Georgia Mason, David Wilson & Charlotte Hampton, University of Oxford and Hanno Würbel, University of Giessen, Germany

Interpreting behaviour and assessing wellbeing can be difficult in rodents, and so there is a pressing need for more effective and objective ways of detecting suffering so that procedures, husbandry and endpoints can be refined. This study, which aimed to see whether chromodacryorrhoea* could be scored in a quantitative way, came about after we noticed that several rats in our unit had small (~ 1 mm diameter), transient spots of dark red secretion around their nostrils after their light cycle had been accidentally disrupted. We researched whether chromodacryorrhoea had been

* In rats, glands next to the eyes secrete red porphyrins and other compounds. High levels of secretion lead to ‘chromodacryorrhoea’ (red or ‘bloody’ tears), which is widely recognised as a sign of stress and/or respiratory disease.
evaluated as an indicator of suffering, and found that only one paper had assessed its relationship with experimental stressors such as restraint, almost all studies scored it in a qualitative, ‘all or none’ way, and all focused on severe cases only. However, mild chromodacryorrhea occurs after less serious disturbances, which suggests that it could provide a useful tool for assessing rat wellbeing and incipient discomfort or distress. The resulting study has been published in more detail elsewhere; please see this reference for full details of the protocol.

We developed a scoring system for recording chromodacryorrhea (Table 1), and investigated whether the very low-level, transient secretions of normal, healthy rats correlated with low to moderate levels of stress or disturbance. Rather than experimentally exposing our subjects (24 young adult Lister Hooded, both sexes, housed in single-sex pairs or triplets in 11 cages) to stressors, we made opportunistic use of likely sources of low-level stress within the unit. These were as follows: 1) building maintenance work, which took several hours and involved several potential stressors; and 2) visits by unfamiliar humans, and the other sources of mild disturbance normal in an animal unit.

Initially, we scored the rats according to the system in Table 1 one hour after the disturbance and the results for building maintenance work were as shown in Fig. 2. This showed that chromodacryorrhea could be quantified and that it significantly increased with disturbance, although it was always subtle and transient. However, the study was not ideal as the treatment scores were obtained in the afternoon, whereas most baseline scores were taken in the morning. We therefore set up a second experiment to compare ‘disturbed’ days with undisturbed days, in which rats were always scored in the morning and any unusual events, e.g. visitors, outside noises or fights between rats, were recorded. The mean scores for disturbed animals were lower than those in Fig. 2 (see full reference for details), but chromodacryorrhea still increased significantly above control levels on days when there was mild disturbance. Throughout all of the studies, individual rats scored consistently and there was also significant inter-observer reliability between independent scorers.

<table>
<thead>
<tr>
<th>Score</th>
<th>Description</th>
<th>Example of appearance of nose (diagrammatic)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No chromodacryorrhea</td>
<td><img src="image1" alt="Diagram" /></td>
</tr>
<tr>
<td>1</td>
<td>Very slight, inconspicuous chromodacryorrhea, e.g. one small spot &lt;1mm across (often just lateral to a nostril), only visible if rat pushes nose out between the cage bars.</td>
<td><img src="image2" alt="Diagram" /></td>
</tr>
<tr>
<td>2</td>
<td>Slight, rather inconspicuous chromodacryorrhea, e.g. one spot 1.1 mm across, or several spots &lt;1mm across, only visible if rat pushes nose out between the cage bars.</td>
<td><img src="image3" alt="Diagram" /></td>
</tr>
<tr>
<td>3</td>
<td>Noticeable chromodacryorrhea, e.g. one spot 2.3 mm across, or several spots 1.1 mm across, often around nostrils and surrounding fur.</td>
<td><img src="image4" alt="Diagram" /></td>
</tr>
<tr>
<td>4</td>
<td>Relatively severe chromodacryorrhea, e.g. several patches 2-3mm across or more, around nostrils and surrounding fur, and visible even in an estraul at the back of the cage. Rare secretions around eyes were also given this score.</td>
<td><img src="image5" alt="Diagram" /></td>
</tr>
</tbody>
</table>

Table 1. A scoring system for quantifying chromodacryorrhea.

Figure 2. Chromodacryorrhea scores for male (M) and female (F) rats one hour after manual work in animal house. n = number of cages; treatment effect: $F_{1,9} = 42.25$, p<0.0001

We concluded that:
- low level, relatively inconspicuous and transient forms of chromodacryorrhea in healthy rats can be quantitatively scored, with reasonable inter-observer reliability
- chromodacryorrhea seems to track environmental disturbances in a very sensitive way, appearing within minutes following the stressful event
- as the red stain seems to be groomed away following an acute stressor, its continual presence indicates high levels of chromodacryorrhea production and/or reduced levels of grooming, either of which suggest that animals are experiencing chronic stress
- scoring chromodacryorrhea could be a simple, practical and non-invasive way of sensitively assessing the impact on rats of housing, husbandry, or procedures for researchers, technicians and vets to use.
Cage-side assessment of GM mouse welfare
Laura Playle, Medical Research Council (MRC) Centre for Best Practice for Animals in Research (CBPAR)

In June 2001 the Biotechnology Working Group of the Animal Procedures Committee (APC) published a report assessing the adequacy of the present regulatory regime to deal with current and future scientific developments in biotechnology. This report made a series of recommendations regarding genetically modified (GM) organisms, some of which were specific to the welfare assessment of GM mice. Effective welfare assessment was highlighted in the report as essential because it is ethically important (and a legal requirement) to minimise laboratory animal suffering, which necessitates effective monitoring. Welfare assessment can also inform phenotype assessment, which will benefit the science.

In response to the APC Report, the main UK funders of biomedical research (MRC, Biotechnology and Biological Sciences Research Council, Wellcome Trust, Cancer Research UK) met to consider how to progress the recommendations. It was agreed that a GM mouse welfare assessment working group would be established, to be convened by CBPAR as part of its role as a focus for coordination and collaboration between organisations using animals in research. It was also decided to adopt the term “genetically altered” (GA), to encompass mice produced by transgenesis, mutagenesis and harmful spontaneous mutations.

The Group was set up by drawing members from a wide range of backgrounds including industry, academia, breeding establishments, animal welfare organisations and the Home Office. The Group’s remit is to review potential welfare issues, summarise current methods of assessment, recommend best practice for assessment and identify areas for future research.

The Group considers that the issues surrounding the welfare of GA mice are basically the same as for conventional strains; however, it is essential to ensure that an assessment system is in place that is robust enough to identify pain, disease or negative emotional states such as anxiety or fear should they occur. Phenotype and welfare assessment schemes vary between establishments, so the Group defined the characteristics of good practice in basic welfare assessment. A scheme should be practical and not overly complex, minimally invasive, generally applicable (not facility dependent), have appropriate controls and include appropriate record keeping. It should also be sufficiently flexible to allow the addition of more specific tests according to the predicted outcome. The Group is now building on these principles to make recommendations for welfare assessment at different life stages (e.g. neonatal and weaning) and colony-wide assessments. It is also considering in depth the concept of the “Mouse Passport” and what type and level of information this should include.

The working group met 3 times in 2003 and its report will be produced later in 2004. It will be available on the CBPAR website when it is published; meanwhile visiting the site and clicking on “general resources”, “species” and then “rodents” will call up a list of resources to help improve the welfare of GA and conventional mice.

Refinements in telemetry procedures (and husbandry) for mice and rats: the new JWGR report
Penny Hawkins, RSPCA

Telemetry is widely viewed as benefiting science and animal welfare because it can reduce stress caused to animals (e.g. by restraint), enable reductions in animal numbers, and provide indicators of animal wellbeing to help implement humane endpoints. However, telemetry studies can have a significant impact on the wellbeing of individual animals and this must be taken into account and minimised. The JWGR has recently published two reports on refinements in telemetry, addressing procedures and husbandry. The reports identify three key sources of harm associated with telemetry: (i) surgical implantation or attachment procedures; (ii) the physical impact of the device on the animal after it has been implanted or fitted; and (iii) distress induced by housing animals individually (this may be done because they have been implanted with telemetry devices that all transmit at the same frequency).

In the case of rodents, the mass of the device can have a significant impact on the animal. The device mass that rodents, especially mice, are expected to tolerate is disproportionately large in comparison with other species. For example, mice are often implanted with transmitters that represent 20% of their body mass, and the JWGR believes that this is a serious welfare issue. In the short term, changes in body mass and behaviour following implantation in mice suggest that well-being is impaired for a week following surgery, not only due to surgery but also to the mass and volume of the device. The report provides guidance and makes a number of recommendations on selecting devices, perioperative care including pain management, and considering alternative technology such as passive transponders or totally non-invasive systems wherever possible.

An ideal telemetry system would allow animals to be
housed in stable, compatible groups. While implantable devices can facilitate this because there are no external components that other animals could interfere with, commercially available devices presently all transmit at the same frequency and so animals may be singly housed. This contradicts current thinking about group housing and, until devices that transmit on different frequencies are available, it is important to find alternatives that allow rodents to be at least pair housed. The report sets out possible solutions including the "buddy" system, where animals are pair housed but only one is implanted, or using devices that can be switched on and off in situ. It also provides guidance on selecting suitable rats and mice for telemetry studies, forming stable groups and regrouping animals after surgery.

More information about the content of both sections of the report is available online. This also includes guidance on legal issues relevant to telemetry; an example of good practice in writing up materials and methods sections of telemetry papers; and guidance notes for setting out project proposals involving telemetry or data logging.

**Conclusion**

We hope that this meeting report will help technicians, scientists, veterinarians and all those concerned with reviewing projects involving laboratory rodents to reduce suffers and improve welfare throughout the animals' lives. On the basis of the 2003 meeting, the Rodent Welfare Group would encourage the following:

- for salmonellosis studies in vivo, question any use of susceptible strains and give serious consideration to non-susceptible strains
- review the success rate of pregnancy detection and evaluate the potential to reduce wastage by using more effective techniques such as ultrasound
- review blood sampling protocols, using the JWGR report and taking developments in the sensitivity of analysis equipment into account; consider the saphenous vein technique
- regularly review veterinary and husbandry procedures. Do any have the potential to cause distress, e.g. tooth trimming in rodents and lagomorphs? If so, are they really necessary, could they be refined and could they be performed less frequently?
- review how discomfort, pain, suffering and distress are assessed and monitored, for both conventional and GM strains of rodent. Consider trialling new techniques including chromodacryorrhoea in rats. Check the CBPAR website for recommendations for GM mouse welfare assessment and for resources to improve the welfare of all rodents
- review telemetry procedures involving rodents; include reducing device impact, refining surgery and facilitating group housing.

**Acknowledgements**

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12. www.mousespecifics.com