



Session 2.5

Specific animal models and refinement

Poster

Refining rodent research at Johns Hopkins University by moving toward specific pathogen free status

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In November of 2003, Johns Hopkins University made a commitment to protect science through animal welfare refinements at the institution. An integral aspect of the plan was to eliminate all specific pathogens known to confound research data from our rodent colonies. January 2005 was set as a target deadline to eliminate all conventional or diseased rodent colonies from our Baltimore Medical Campus. Our goal was to convert all rodent colonies to specific pathogen free status with minimal scientific interference and expense. We utilised a number of strategies including revamping our sentinel program and importation quarantine services, decontaminating laboratory space and equipment, developing re-derivation and cross-fostering services, renovating existing facilities, abandoning outdated facilities, utilising ventilated and non-ventilated micro isolator housing, minimising the number of animals housed

outside of central facilities, mandating the use of aseptic procedures, and expanding the training of animal care staff, principle investigators and their staff. Although we did not meet the anticipated deadline for the complete conversion in animal health, we have currently eliminated approximately 70% of our conventional rodent populations. The creation of our cross-fostering program and the use of high risk return housing have greatly contributed to our success. We have faced a number of challenges such as; equipment shortages, unique investigator equipment needs, limited procedure space, disease outbreaks, developing traffic patterns, and investigator reluctance. The sheer magnitude of this project was a major factor in extending our deadline. We are now aggressively pursuing our goal and plan to achieve 100% success by January 2006.



Lecture

An *in vitro* flow adaptation chamber replaces animals in an Ischaemia/Reperfusion model to study oxidant generation

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Ischaemia/Reperfusion (I/R) is the cessation of blood supply to an organ for a temporary period followed by restoration of flow and is the cause of complications following surgery, thrombosis or organ transplantation. I/R injury is caused by the reactive oxygen species (ROS) generated by the vascular endothelium. Besides injury, ROS generated by the vasculature are also involved in endothelial cell signalling. Studies of endothelial responses have till date involved an extensive and indiscriminate use of animals. Here, we propose to drastically reduce the use of animals by an artificial capillary system (FiberCell-CellMax) that mimics endothelial cells in the vasculature. Cells isolated from a few mice and rats, expanded into larger cultures were subjected to *in vitro* I/R conditions. Earlier *in situ/in vivo* studies from our lab using rat and mouse lungs

have shown that the pulmonary endothelium was the predominant source of ROS generation with ischaemia. We also found that the cessation of flow triggers the closure of an endothelial K⁺ channel (KATP) resulting in ROS generation and all the other events that cause I/R injury.

This project investigated the endothelial ROS generation with a variety of KATP channel openers to evaluate their potential therapeutic applications. The ROS levels as monitored by H₂DCF-DA fluorescence showed a sharp increase with ischaemia and was significantly reduced by the presence of K⁺ channel openers such as cromakalim and lemakalim. These results indicate that the KATP channel was an important component of the ROS generation in the endothelium with ischaemia.

Lecture

Genetically modified interactions: Does the genetic modification of animals modify human-animal interactions?

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The interaction of animal care personnel with the animals in their charge can be influenced by a number of factors. It has been demonstrated, particularly in the farm animal welfare literature, that the interaction of animal care personnel with animals can have a strong effect on the animals' behaviour, productivity and wellness. Among species commonly used in biomedical research, it has been reported that mice are the least preferred species in terms of human-animal interactions in animal care facilities. In reviewing the literature and observing animal care personnel interacting with mice, it appears that the following factors may influence the manner in which mice are perceived:

Their small size, their particular behavioural characteristics, and husbandry constraints (such as housing in ventilated racks in barrier facilities). In addition, we are interested in whether animal care personnel perceive genetically modified mice differently than non-genetically modified mice, and whether this in turn has an effect on their interaction with the animals. The ability to carefully observe an animal's behaviour is key in assessing the animal's wellness, and in establishing appropriate study endpoints in order to minimise pain and distress. The difficulties in assessing mouse behaviour, in particular the behaviour of genetically modified mice, will be discussed.



Lecture

Reduction of captivity stress on single housed pigeons through an enriched environment program

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Repetitive stereotypical behaviours have developed in pigeons that have been chronically housed in our vivarium for a number of years. We were not sure if these stereotypes were due to boredom and frustration or distress experienced from the barren, single-housed environment in which the birds live. Under current conditions, there is no opportunity for the pigeons to engage in species-specific behaviour such as contact with conspecifics, foraging or locomotion. We tested the bird's faecal corticosterone levels under three distinct conditions.

Eighteen white carneaux pigeons were chosen and stereotypical behaviours were recorded and monitored for a period of four weeks. Next, birds were put into a crowded condition, two in a

single home cage. In the last condition, 3 sets of 6 birds were group housed in a 5"x8" flight cage. The cage included various enrichment items. Faecal samples were collected in all three situations and are in the process of being assayed. The expected results are that faecal corticosterone levels will be high in the control group, higher in the crowded condition and lower in the flight cage.

Expectations are based on the evidence that corticosterone is a clear indicator of stress in birds (Harvey et al., 1980; Harvey et al., 1984) which unfailingly increases after a range of stressful events such as crowding, handling and captivity (Siegel, 1980).

Poster

Introduction and use of the saphenous vein blood-sampling technique in the mouse

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Introduction: The conventional method within safety assessment for obtaining multiple time-point blood samples in mouse studies has involved euthanising an animal for each sample. By adopting the saphenous vein blood-sampling technique makes it possible to obtain between one and three additional samples from each mouse, considerably reducing the number of animals required to achieve the objective of these studies.

Method: Thermostatically controlled warming cabinets are used to increase the body temperature of the mice prior to sampling, the mouse then being placed into a restraining device and the leg to be sampled is held so that a slight pressure is applied to the saphenous vein. The vessel is then punctured and the blood collected into an appropriate sample tube.

Results: Since the introduction of this technique within safety assessment, it has been used routinely for pharmacoki-

netic and toxicology studies, with an approximate 50% reduction in the number of animals required.

Discussion: This technique does not appear to cause the animals undue stress, although slight bruising and swelling around the sampling site is occasionally recorded.

200 µl of blood is the maximum practical volume obtainable at each time-point, making it a prerequisite that the required experimental analysis can be accomplished with this relatively small volume.

Nevertheless, saphenous vein blood-sampling has proved itself to be an extremely positive welfare initiative, capable of producing scientifically valid results, whilst substantially reducing the overall numbers of mice.

Poster

3R compliant biomaterial testing: HET-CAM evaluation of biomaterials gives comparable results to *in vivo* models regarding biocompatibility patterns

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The implantation of new biomedical devices into living animals without previous biocompatibility evaluation is possible based on current legislation. The HET-CAM test offers a 3R compliant partially immunodeficient model allowing the simulation of transplantation experiments prior to animal testing. To compare these data with data acquired by animal experimentation, a meniscus regeneration experiment was repeated using the HET-CAM assay.

The meniscus implant was a cut into 5x5 mm pieces and applied onto the CAM. Samples were incubated for 3 days followed by blood sampling, digital documentation and histological evaluation. The meniscus devices were tested in a sheep model by the Dept. of Orthopaedic Surgery and data were supplied for comparison.

Analysis of the sheep knee joint showed good integration and vascularisation of the implant. HET-CAM analysis revealed a firm

attachment of the samples and a high vascularisation. A synovial hypertrophy observed in the sheep was visible as hypertrophy of the CAM reticular connective tissue. Both models showed an inflammatory response and a foreign body tissue reaction. Analysis of synovial smears and blood withdrawn from the CAM indicated a lymphocytosis.

Fertilised, 10 days incubated chicken eggs can show a tissue response similar to an animal model. Biocompatibility and -incompatibility can be tested prior to *in vivo* implantation. The routine application of the HET-CAM test would allow the exclusion of unsuitable prototypes and facilitate the selection of a biomaterial type, thus reducing the side effects caused by improper materials and allowing a reduction in animal numbers.

Poster

Tissue response to natural scaffold materials and synthetic polymers: The HET-CAM test as analysis tool for early phase tissue reaction

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Biodegradable scaffolds are crucial for transplantation of tissue engineered cells. Animal models for scaffold evaluation usually cover the mid-term and late tissue reactions, but the early phase tissue response occurring during the first days after implantation is not documented. The HET-CAM test was used to compare the early phase tissue reaction in synthetic and natural scaffold materials.

All samples were tested on the CAM for three days followed by digital documentation and histological analyses.

All samples were completely integrated into the connective tissue of the CAM and showed different stages of degradation: The Hyalograft C® scaffold was completely disintegrated into single fibres while the Chondrocell® scaffold and the hyaluronic acid/polycaprolactone prototype had maintained their original

shape. Both synthetic materials led to an inflammatory tissue response and thrombus formation in single blood vessels was observed. Fibrous tissue present in the surrounding of the biomaterial showed a beginning encapsulation of the implant. The normal CAM structure was altered and atypical cells were observed. The collagen implant only provoked a mild tissue response indicated by the presence of single lymphocytes within the scaffold.

While only mild reactions were observed after implantation of the collagen scaffold, transplantation of synthetic biomaterials led to alterations of CAM structure and the presence of atypical cells. Although the long-term impact of these observations remains to be evaluated, CAM testing is a valuable tool for short term analysis and intermediate *in vitro/in vivo* biomaterial characterisation.



Poster

Spontaneous granulosa cell tumour of gerbil (*Meriones unguiculatus*) as a model for teaching and research in pathology

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Spontaneous granulosa cell tumour (GCT) of gerbil is a frequent pathology in this species and follows the natural tumour development along the animal life. We analysed a total of 241 GCT from 167 females. The largest incidence of GCT is in gerbils 2 to 3 years old, being most frequent in virgins. The ovarian macroscopy is normal – with incipient microscopic GCT – or shows developed tumour (cystic, solid or mixed). Based on the microscopy GCT are classified in incipient, cystic, solid and mixed. All tumours consist of nests and cords of cubical tumour cells; there are luteinic cells, Call-Exner bodies, pseudofollicles, incipient cysts, necrosis, and mitosis, except for the last one in the incipient tumours. Invasion compromises more frequently

the ovarian hilus, periovarian fat, and fimbriae. The largest number of metastasis is in the omentum. All the tumours with necrosis are malignant, as they also show metastasis. Malignant GCT represents 88% of the present sample. Natural death happened in just four virgin females with malign GCT, 2 to 3 years old. The information acquired and documented in this study is being used in lectures of Basic Pathology – Neoplasm – given to graduate students in our University. This tumour can be used as animal model of the disease giving opportunity of studies in the development of this ovarian tumour, including genetic, hormonal and immune modulation, as well as action of antineoplastic drugs.

Lecture

Alleviation of pain and discomfort in adjuvant arthritis rats

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Rheumatoid Arthritis (RA) is an inflammatory auto-immune disease that affects approximately 1% of the population and results in disability and joint deformity. As no cure exists, new therapeutic mechanisms are explored with animal models such as Adjuvant Arthritis. In this model rats are injected intradermally in the base of the tail with Freund's Complete Adjuvant, resulting in an inflammatory response with necrosis at the injection site and a systemic immune reaction resulting in swollen joints causing severe pain and discomfort comparable to RA.

Attempts to reduce pain and discomfort in the animals are limited so far to extra bedding material, long-spouted water bottles and maximally two animals per cage.

In order to reduce both animal discomfort and the number of animals we are investigating the possibilities to refine data acquisition and analysis by applying the Cat-Walk system and infrared thermography. Additionally, a new generation of analgesics is being screened for their effectivity to reduce pain without affecting the immunological parameters of the arthritis model. Preliminary data suggest induction of early arthritis coincides with irregularity of the paw pattern and prolonged crossing time on the Cat-Walk system, indicating possibilities for refinement in data acquisition. Further results will be discussed.

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Poster

The importance of control groups and normal in the assessment of animal wellbeing

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The original concept first put forward by Morton and Griffiths in 1985 was that the observation of clinical signs and their degree of deviation from normality could indicate the level of animal suffering. However, it is easy to be led astray if only affected animals are observed, and they are only compared with other controls in the experiment. What is vitally important is that both control and experimental animals are compared with nor-

mal naïve animals. Some examples of such misleading comparisons will be given, and the use of simple systems of comparison offered in their place.

Morton, D. B. and Griffiths, P. H. M. (1985) Guidelines on the recognition of pain, distress and discomfort in experimental animals and a hypothesis for assessment. *Vet. Rec.* 116, 431-436.

Lecture

Working with genetically engineered rodents: A comprehensive program of veterinary care

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Technological advancements enabling investigators to genetically engineer mice and rats quickly and efficiently have created a deluge of models available for research today. The rise in magnitude and complexity of genetically modified animals has resulted in an increased number of models with reproductive and adverse phenotypic challenges requiring special care to appropriately house and maintain these valuable models. This presen-

tation will describe a systematic approach to providing clinical care to a large and diverse population of transgenic mice and rats. Topics to be covered include clinical observation training, managing adverse phenotypes including clinical assessment and disposition, record keeping and data reporting, and determination and refinement of endpoints.



Lecture

Promoting laboratory animal welfare and refinement in neuroscience and behavioural research through an Institute for Laboratory Animal Research (ILAR) report

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Over the last twenty years in the United States, the main vehicle for promotion of the 3Rs has been inclusion of language reflective of the 3Rs in federal regulation, including the Animal Welfare Act, the US Government Principles for the Utilisation and Care of Vertebrate Animals used in Testing, Research, and Training, and Public Health Service Policy on Humane Care and Use of Laboratory Animals. While an important step in the general acceptance of the 3Rs within the research community, these regulations do not provide practical guidance to scientists on implementing the 3Rs. Such guidance is particularly needed for the neuroscience research discipline, which has seen a doubling in membership to its professional society over the last decade. In

order to address this, ILAR published a report “Guidelines for the care and use of mammals in neuroscience and behavioural research.” This report provides practical information on applying the 3Rs to common research methodologies, such as food and fluid regulation. Neuroscience specific methodologies, such as implanting neural probes, are also discussed. In addition, this report introduces the scientist to advances in refinement strategies, such as using behaviour to monitor animal pain. Since its publication in 2003, more than 24,000 copies of the report have been distributed and it is publicly available on the National Institutes of Health (NIH) website.

Poster

Lack of references to refinement measures in published animal-based research of genetic diseases

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Animal models are valuable research tools in studying debilitating human diseases; but for ethical reasons it is particularly important to focus on refinement in this kind of research. Since the possibility to publish is an important driving force in research, scientific journals strongly influence which approaches are chosen by researchers. To study to which extent refinement is reported, we analysed publications from 2003-04 using a recognised severe model, R6/2 Huntington’s disease mice. These mice show a rapid disease progress, with motor deficits progressively worsening until premature death at about 14-18 weeks. Of a total of 27 papers, 24 reported experiments in which mice reached the age where clinical symptoms appear. We distinguished between A) acute or *ex-vivo*, B) live-animal research with a fixed cut-off point and C) survival experiments. The

papers were reviewed for references to: Welfare assessment, humane endpoints, housing adaptations and compliance with official recommendations. Despite that the majority of the papers reported compliance with official recommendations (Tot: 19/24, A: 7/9, B: 4/6, C: 8/9), less than a third reported that humane endpoints (Tot 6/24, A: 0/9, B: 1/6, C: 5/9) and/or housing adaptations (Tot: 7/24, A: 0/9, B: 3/6, C: 4/9) were applied. Even for survival studies, where animals develop severe symptoms and ultimately become unable to eat and drink, half of the papers failed to report such refinements. Journal publishing policy could play an important role in self-regulating research; however on the basis of the reported observations we conclude that this potential is presently poorly utilised.



Poster

Replacing the use of live stimulus animals by scent cards in the social recognition test for mice

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In the conventional test of social recognition used in behavioural phenotyping of mice, mice to be tested are confronted with juvenile stimulus mice; however van den Bos et al. (*ATLA* 30, 299-304, 2002) have demonstrated that for rats, live stimulus animals can be replaced by scent stimuli. We present a pilot study of a similar approach in mice. Adult male mice (N=12; equal numbers of two different genotypes) were housed in same-genotype pairs, and cards were scent-marked by leaving them in the mouse cages for 2 weeks. The behaviour of individually tested mice was studied in test cages with clean or scent-marked cards. In the first test, mice were exposed to one clean card (C) and one card with an unfamiliar scent mark (S1). After

a 5 minute interval, the test was repeated with the same scent card as in the first test, together with a different, unfamiliar scent card (S2). The time spent in contact with the cards was measured, with the hypothesis that mice showing social recognition in the second test would spend more time in contact with the new than with the recently encountered scent card. Indeed, mice spent more time (Wilcoxon-signed-ranks test; $Z=-2.43$; $p=0.015$) with S2 (70.22 ± 5.90 s) than with S1 (43.01 ± 5.92 s), confirming the hypothesis. The results indicate that social recognition in male mice can be demonstrated using scent cards rather than live animals, thereby reducing the number of animals needed for testing.

Lecture

A behavioural study on beagle dogs rehabilitated from a laboratory in India

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Introduction: 283 beagle dogs in the age group of 1-10 years, that were bred and used in a laboratory, in India, were rehabilitated and homed. Families who adopted the dogs observed severe behavioural/psychological trauma and/or physiological ailments. Through this study it is proposed to record observable signs of physical/physiological/psychological trauma in rehabilitated beagle dogs.

Methods: A random sample of 36 dogs was studied by way of 1) direct observation, 2) a comprehensive questionnaire and 3) interaction with the owner of the dog.

Over 40 physiological/psychological/physical parameters were recorded by way of a score sheet or descriptively and were ascertained for 0, 90, 180 and 360 days post rehabilitation. Some of the parameters that were studied included apathy, aggression, fear, compulsive disorders, response to human presence and

touch, phobias, stereotypes, ability to bond with human beings, physical deformities, physiological complaints etc.

Results: Apathy, extreme fear, tendency to hide, anxiety with an obvious differential response to men and women were observed in almost all the dogs. Stereotypies, pica behaviour, coprophagia and compulsive disorders were common. Almost all showed stress related skin disorders. A few beagles were aggressive.

Discussion: The paper discusses and qualifies the physiological and psychological status of these rehabilitated beagles, their biological implications, interplay of hormones in canine stress disorders, interferences in experimental responses, inferences of canine studies and implications in extrapolating the same to human health and well being.



Poster

The genetic absence epilepsy in Imp:DAK rats – a new model

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Spontaneous spike and wave discharges (SWD) on the cortical EEG occur in 40-60% of our Imp:DAK outbred stock of Wistar rats. The pattern of these high-voltage spindles in EEG records is similar to that occurring in petit-mal epilepsy seizures in humans.

Following the Hanover principles of mating and selection based on the EEG, in F4 generation we obtained two lines: 1) SWD[+] in which seizures occur in 100% of rats; and 2) SWD[-] without seizures in three-hour EEG recording period. We used 60 rats (30 male and 30 female) divided according to occurrence of seizures (30 of SWD[+] line and 30 of SWD[-]). To find differences between these two lines, we applied the following behavioural tests: open field test, radial maze test, conditional

passive and active avoidance tests and hot plate test. We performed also morphometry of some regions of the brain, and assessed the activity of acetylcholinesterase (AChE) in the brain and blood.

The SWD[+] rats showed impaired short-term spatial memory and long-term memory, and significantly lower sensitivity to pain. In the SWD[+] rats, morphometry revealed lower density of neurocytes and glia cells in II-IV layer of the brain cortex. The activity of AChE in midbrain, cerebellum, and blood of the SWD[+] rats was lower.

We conclude that these two lines may be used in pharmacological or neurotoxicological studies according to the aim and endpoints of the study.

Lecture

Advancing the 3Rs in pet nutrition research

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Pet food companies that manufacture premium products often conduct extensive research in the area of pet nutrition so as to 1) evaluate the nutritional adequacy of new products, 2) assess nutrient bioavailability of raw materials, and 3) evaluate the efficacy of new nutritional technologies. Since aspects of this research involve the use of animals, it is important that strict animal welfare guidelines are established, along with a research program aimed at developing alternative methodologies. At the Iams Company, several alternative methods have been developed to advance the Three Rs in all areas of pet nutrition research. Protocols have been developed which allow feeding studies, which have historically been done under laboratory-controlled conditions, to now be conducted in the homes of pet

owners. Reapplication of *in vitro* methods, which were developed for the health care sector, has reduced the number of evaluations that must be conducted in animals. Advances in analytical methodology have reduced the need to use animals for evaluating protein quality. Development of new state-of-the-art automated systems for the collection of faeces and urine can significantly reduce the need to cage animals. Over the past two decades, use of animal alternatives by many industries has not only advanced animal welfare, but has led to methods that provide better data, which are faster and cheaper to generate. Not only is investment in alternatives by the pet nutrition industry a positive step in improving animal welfare, it will also lead to faster, more cost-efficient and better results.

**Poster**

The prediction of respiratory sensitising potential of chemicals in a modified local lymph node assay

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The local lymph node assay is now widely used to identify the sensitising potential of chemical contact allergens: Mice are dermally exposed to test substances and lymphocyte proliferation induced in the draining lymph node assessed by incorporation of radiolabelled thymidine. Improvements of this assay, like flow cytometric determination of B lymphocytic phenotype or T-cell activation/memory phenotype have been described. In addition, differential cytokine profiling, based on expression analyses of cytokine mRNAs or ELISAs of cytokines produced by lymph node cells *ex vivo* have been applied. It is now generally agreed that respiratory sensitisers can be differentiated from contact allergens by the induction of distinct cytokine profiles. In the present investigation we applied a modified local lymph node

assay and measured cytokine production of *ex vivo* stimulated lymph node cells by a cytometric bead array and flow cytometry. The respiratory sensitisers trimellitic anhydride, phthalic acid anhydride and toluene 2,4-diisocyanate could be very well distinguished from the contact sensitisers Oxazolone, 2,4-dinitrochlorobenzene or α -hexyl cinnamaldehyde and the irritant sodium dodecyl sulphate. Interleukin-4 is a differential marker well suited to define respiratory sensitisation after topical application of chemicals in the modified local lymph node assay. Regarding the low numbers of animals used and the possibility to differentiate between contact and respiratory sensitisers, this test fulfils the criteria of reduction and refinement according to the principles of the 3Rs.

Poster

The rabbit as an animal model in vaccine safety research; effects of experimental procedures

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Vaccination with whole cell pertussis vaccine is and will continue to be part of the vaccination programmes in many parts of the world. In order to improve the whole cell pertussis vaccine, research is performed to gain insight in the side-effects and the components that causes these side-effects. In previous studies the results (particularly body temperature) were difficult to interpret due to the large variation in experimental outcome. A research-protocol has been set up in which stress-reducing measures, as housing condition and handling from birth on, have been taken up in order to guarantee the welfare of the animals as well as to reduce variation in experimental outcome. Social behaviour of the animals was continuously monitored with special interest in hierarchy. Behavioural tests were performed to determine tameness, anxiety and coping strategies. Testosterone

and corticosterone were measured to support the findings in the behavioural tests. Body temperature was continuously monitored by means of telemetry to measure the inoculation response. Leucocyte counts and immunoglobulins were measured to determine the effectiveness of the vaccine. Handling has a statistically significant effect on the overall behaviour of the rabbits. Experimental procedures had no effect on body temperature. Difference between vaccine concentrations could be distinguished, although the inoculation response between the handled and non-handled groups differed in quantity and quality. Based on the results in body temperature it can be concluded that the rabbit can be an appropriate animal model to investigate the safety of the whole cell pertussis vaccine.



Poster

Effects of housing condition on experimental outcome in a toxicology study

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Biotechnical and other experimental procedures are believed to have an effect on the outcome of an animal experiment. In this study we focussed on the effects of individual housing of Wistar outbred rats in a toxicology experiment. A parental generation was dosed, via the Benchmark dose approach, with a brominated flame retardant (TBBPA). The offspring was kept on the same doses as their mothers till the end of the study. Per gender the offspring were randomly single or social (N=5 per cage) housed. The experimental protocol followed the OECD415 guidelines, enhanced for endocrine and immunological endpoints. Part of the male animals was used in an immunisation study to test the immune response to Sheep Red Blood Cells (SRBC). The other animals were used in neurobehavioural studies. At the end of the study part of the animals went for necropsy. During necropsy a

large number of organs and tissues were dissected for further (bio)chemical or histological analysis. The following endpoints were addressed: sperm quantity and quality, cellular composition, immunological subpopulations and NK activity, thyroid hormones, clinical plasma components, TBBPA kinetics, *in vitro* biotransformation of TBBPA, activity of P450 factors, activity of steroidogenesis and histopathology of multiple organs. Although there were significant differences, a major problem in interpreting is the influence of age, bodyweight and gender, which markedly alter many of these variables. The consideration to house rats socially or individually should be based on the purpose of an animal experiment and the sensitivity of differences in parameters that serve this purpose.