Module 05 - Research Issues

Objectives of this Module

Upon completion of the module you should be able to:

- Identify the various aspects of an animal model that are part of its "definition"
- List the variables - the non-experimental factors - that can influence the animal models response
- Recognise the importance of accounting for all the variables in the experimental design, and controlling those variables (the non-experimental factors)
- For principal investigators and graduate students: develop a checklist of the variables that can affect their research program
- Describe and accept responsibility for their role in ensuring the successful conduct of the Experiment

Introduction

Imagine a group of experimental animals as the stars of a show being recorded for public presentation (the publication). All the work by the crew, behind the scenes, is aimed at making sure that the show goes on without a hitch the FIRST TIME it is staged. There are to be no re-takes. This is consistent with the ethical principles contained in the "Three Rs" of humane animal experimentation: Reduction; Refinement; Replacement. Following the identification of many of the variables that can affect how an animal responds in a research program, the roles various members of the "crew" have in making sure all the factors that can influence the model's performance are accounted for and controlled, will be presented. Then the "show" can go on without a hitch. The responsibilities of the various members of a research group in controlling both the non-experimental and protocol variables are listed below:

Responsibilities of the Principal Investigator

- Consider all pertinent non-experimental variables
- Outline need for controlling pertinent variables to all group members

- Ensure that all experimental variables are controlled or conducted using Standard Operating Procedures (SOP)
- Ensure monitoring and recording of controls on variables (through SOP)
- Ensure animal health quality is being checked before purchase
- Ensure health monitoring is being carried out according to facility rules
- Observe all facility SOP established to limit disease introduction

Responsibilities of the Graduate Students, Post-Doctoral Students and Research Staff

- Monitor and record controls on all variables
- Conduct all experimental procedures according to Standard Operating Procedures (SOPs)
- Employ skilled animal handling and manipulation techniques
- Observe all facility Standard Operating Procedures established to limit disease introduction

Responsibilities of the Facility Manager

- Understand demands for controls on non-experimental variables for all research underway
- Ensure consistent facility environmental operations
- Ensure high level of animal care training and expertise
- Ensure animal health quality before purchase
- Ensure health monitoring is done according to facility rules
- Implement Standard Operating Procedures for all animal facility operations

Responsibilities of the Animal Care Facility Staff

- Conduct all daily animal facility routines in a consistent manner, according to Standard Operating Procedures
- Conduct all animal care handling and manipulations in a consistent, gentle, humane way

Responsibilities of the Facilities Veterinarian

- To advise on and ensure health status of all animals
- To affect procedures that will maintain animal health quality

The following statements should provide an indication of the scope of this module.

1. Rats should be housed in small groups:

(a) to provide companionship for a highly social animal

(b) to minimise the stress each animal is experiencing

Rats, like most of the other rodents commonly used in research, are highly social animals and housing in small group's results in lower stress levels and a more uniform response to an experiment. Most rodents also groom themselves although mutual grooming does occur.

2. Relative humidity (RH) in an experimental animal room should be maintained in the range of 40-70% RH because:

(a) this is the most comfortable level for the animals

(b) micro-organisms have the lowest survivability in the air at that level of humidity

(c) air borne allergens are lower at this level than when it is drier in the room

Studies on the effect of relative humidity on micro-organisms (bacteria and viruses) have shown that when the relative humidity is around 50%, the survivability of these organisms in the air is much lower than at high or very low humidity. The level of allergens in the air decreases as the humidity increases. Comfort and well-being (including absence of respiratory irritation) are improved when the humidity is around 50%.)

Animal Models in Biomedical Research

Although the term "animal model" is commonly used, a definition may help clarify the context of the term. The American National Research Council Committee on Animal Models for Research and Aging drafted the following definition: "An animal model for biomedical research is one in which normative biology or behaviour can be studied, or in which a spontaneous or induced pathological process can be investigated, and in which the phenomenon in one or more respects resembles the same phenomenon in humans or other species of animals." Animal models used in biomedical research, particularly those used in the study of diseases and other conditions in humans, can be grouped as follows:

Spontaneous models - often called "natural" models. These include naturally occurring animal diseases or conditions that correspond to the same diseases or conditions in humans. Diabetes, hypertension, arthritis, immune deficiencies are just a few examples. Many hundreds of animal strains/stocks with inherited conditions have been characterized and conserved. The Jackson Laboratory, Bar Harbour, Maine (USA) holds one of the largest repository of these valuable animal models in mice (**http://www.jax.org/**).

Experimental models. Experimental models are models in which a condition or disease is experimentally reproduced by the scientist. Examples include producing diabetes using the chemical streptozotocin to damage the insulin producing cells in the pancreas; using a chemical carcinogen to produce a certain type of cancer; producing a stroke model through surgery.

Genetically modified models. A special group of induced animal models, involving manipulation of the animal's genetic code to produce the condition that the scientist wants to study. Genetically modified animals may carry inserted foreign DNA in their genome, or have genes replaced or removed ("knock-out" models). These models can help scientists study the genetic basis of disease, susceptibility and resistance, etc.

Negative models. Some animals are resistant to a particular condition or disease. Examining why
this is the case may provide answers to questions about disease resistance and its physiological basis.

Orphan models. Orphan models are conditions appearing naturally in an animal, for which there is no known human counterpart. Historically scrapie in sheep was such a model, but now is useful as a model for the human spongiform encephalopathies that are of so much concern (eg. BSE, "mad cow disease" and CWD, chronic Wasting Disease in deer).

Choosing the Appropriate Animal Model

Before an animal model is chosen the principal investigator must consider alternatives to the use of live animals in his/her experiment. In line with the *CCAC Guidelines on Animal Use Protocol Review*, protocol forms should include a declaration that the principal investigator has considered all non-animal alternatives before making the decision to use animals in his/her research. The most obvious choice of animal species and model for a specific research program may be the same model used by other researchers for the same research. However, with ever increasing numbers of animal models available including new spontaneous mutations, and genetically modified animals constantly being developed, the investigator must consider all factors when selecting the best model for his/her research. Some of the factors that will influence which animal model the investigator selects are:

- Appropriateness of the model or organ system for the proposed study
- Genetic aspects of the model
- Natural vs. experimentally produced models. Both natural models and induced models of disease are useful depending on the objectives of the study.
- Responses of the animal to procedures
- Environmental aspects important to that particular animal model
- Background information available on the animal and specific model
- Species availability
- Numbers needed, according to statistically appropriate design
- Age and sex needed
- Life span of the animal model
- Size of the animal model
- Cost of both the animal model and its ongoing care
- Facilities required to care for the chosen model appropriately
- Husbandry expertise. Some models require not only special housing, but also special care.

Among these factors are scientific considerations as well as purely practical ones. All are important to the success of the research program. Once the most appropriate animal model for the research program has been chosen (whether it is a specific mouse strain that will require specialised care and housing, or a conventional farm animal such as a pig), the research scientist should review the many influences (the non-experimental factors) that might potentially affect the outcome of the study. Those factors should be identified and accounted for at all stages to ensure they do not increase the variability or adversely affect the outcome of the experiment.

The many non-experimental factors that can influence the response of the animal model in an experiment can be grouped as follows:

- Animal factors
- Physical / environmental factors
- Husbandry, animal care, and handling factors
- Research manipulation factors

Animal Model in an Experiment

There are many animal-related factors that influence the response of the animal model in an experiment, and thus must be accounted for in the preparation and design of the study. Some of these are listed here:

- Genetic make-up
- Inbred vs. outbred vs. mutants vs. genetically modified
- Age, sex, reproductive status
- Microbial flora
- Biological rhythms
- Presence of stress/distress
- Diseases
- Overt disease
- Latent (subclinical or silent) infections
- Genotype-related conditions

A number of animal factors are pre-selected by the investigator: the age, sex and reproductive state of the animal, and its metabolic state (e.g., young and rapidly growing, adult, aged). The health status must also be considered when selecting the source of the animals. Of the many factors that can cause unknown or undesirable complications to the response of the selected animal model, disease states are very significant. Disease organisms, overt or latent (subclinical or silent), can significantly interfere with valid experimental results. Three examples are presented here:

Example 1: Infection causing disease

Example 2: "Ordinary" bacteria causing disease in immune deficient models

Example 3: Silent infection affecting research results

Example 1: Infection causing disease. SDAV (sialodacryoadenitis virus) infection in rats is a highly infectious disease with a short clinical course that is usually non-fatal. Most rats recover quickly with subsequent immunity to re-infection. SDAV infection alters several factors of the immune system and thereby may interfere with research. There is inhibition of phagocytosis, reduced interleukin-1 production by pulmonary macrophages, and depletion of

Rat with SDVA

epidermal growth factor. Because of the site of the infection, it could also complicate studies involving the eye, salivary glands, lacrimal glands and respiratory tract.

Example 2: Ordinary bacteria causing disease in immune deficient models. Immune deficient or immune compromised models (e.g., nude or severe combined immunodeficiency (SCID) mice) are very susceptible to ordinarily non-pathogenic organisms such as *Pasteurella spp*. or *Staphylococcus spp*. Special caging and care procedures are vital to minimizing such infections.

Nude mice

Inadvertent infections in these special models can not only cause serious disease, but they will also interfere with the animal's immune response during research.

<u>Example 3: Silent infection affecting research results.</u>

Mouse parvovirus (MPV) is a clinically silent infection. In mice with normal immune systems the infection lasts up to 6 months, while in immunodeficient or immunosuppressed mice, infection and shedding of the virus may be lifelong. The virus requires rapidly dividing cells to replicate, and grows in gut mucosa with shedding of the virus in feces of infected mice. MPV persistently infects the lymphoid tissue, particularly the T cells and causes changes in immune system responses, either stimulating or suppressing depending on the studies being done. Infected T cells may not grow properly when cultured in vitro. Parvoviruses may also interfere with cancer research; T cells from infected mice may have diminished cytolytic capacity.

From these three examples, it should be obvious that infections, even subclinical infections causing no overt disease signs, can significantly affect the results obtained in a study. The efforts of many people, at many levels, to eliminate such infections from the laboratory animal models, have contributed significantly to minimising this unwanted variable in biomedical research that uses animal models.

Avoiding Infectious Disease in Laboratory Animals

VAF (Virus Antibody Free - free of specific viruses) status of rodents was developed to avoid research complications caused by infections. Actions that managers of laboratory animal facilities and researchers themselves can take to limit risk of infections ruining research include: ensuring the health quality of animals before purchase; conducting health monitoring according to facility rules; observing all facility SOPs established to limit disease introduction. Investigators must share the responsibility of ensuring the disease free status of their animal models. Knowing the health status of the animals and documenting it, are a very important part of the records that must be kept by the principal investigator, as part of defining the animal model in the publications arising from the research.

Physical and Environmental Factors

Room Temperature. Small laboratory animals respond to temperature variations by changes in behaviour (e.g., shivering, huddling) and metabolic rate (including increased food consumption if they require more body heat production). These changes could affect several metabolic processes including drug metabolism. Daily animal room temperature fluctuations should therefore be limited (by good heating/ventilation design) to +/- 2^{o}C. Daily temperature fluctuations should be monitored and recorded. For some studies it may be necessary to measure cage (microclimate) parameters and not just room environment.

Relative Humidity. Animal room relative humidity should be maintained at 55%+/-15%. This is important for several reasons: for the welfare/comfort of the animals (and the staff); for minimising disease spread by reducing the viability of airborne microbes; for allergen reduction. Prolonged relative humidity (RH) of less than 40% can cause ringtail in young, unweaned rats, and result in respiratory irritation.

Ringtail in a young rat.

Ventilation. Recommended air exchange rates in animal rooms are 15-20 air changes per hour. Such high rates, compared to human office or laboratory spaces, are necessary to remove animal generated heat, ammonia, carbon dioxide, and airborne particles (dust and allergens). Static microisolator cages may contain high levels of ammonia and carbon dioxide despite good ventilation rates in the room itself. Air pressure gradients from animal rooms to corridors or between zones in an animal facility are also important for containment of micro-organisms. Within a laboratory animal holding room there can be significant variations in ventilation, and levels of ammonia and CO2, as well as air flow. Randomising the location of animals in different treatment groups in a rack or in the room may help to avoid bias in the results due to these factors

Lighting. The lighting cycle for the animals has several aspects; the day/night cycle, the intensity of the lights, and the wavelength. Timer control of day/night cycle is necessary to maintain a consistent diurnal rhythm in the animal's metabolic state. The intensity and wavelength are also important to animals. Albino rodents in particular experience retinal damage when room light intensities are above 300 Lux. The sudden onset of lights in the morning affects some hormone levels - effects that may last for several hours. The use of dusk/dawn lighting systems, which gradually change light intensity between dark and light phases, is encouraged. For studies where the light levels might influence the research results, randomising the location of animals in different treatment groups on a holding rack may be useful in avoiding a light-induced bias in the results obtained.

Light "Pollution" Can Alter Tumour Growth Rates. In a study reported by Dauchy, et al., in the journal Laboratory Animal Science (1997), light contamination during the dark phase significantly altered the growth of a tumour (Morris hepatoma) in rats and changed some metabolic factors. In this experiment, the light "pollution" was a very low light level from a light in a hallway shining through a window into the animal room. This study reinforces the need to examine all factors that might cause variability, including the actual levels of light in the animal room, to ensure that the results obtained are meaningful.

Noise. The impact of noise on the behaviour or responses of laboratory animals in biomedical research has received too little study. The fact that loud "buzzer" noises may induce seizures in young rodents (this has been used to create a model of audiogenic seizures) is well known. Both intensity and sound frequency are important. Rodents and some other animals are particularly sensitive to ultrasonic frequencies, ones that we may not even be aware of since they are beyond the range of the human ear. Low frequency and other noises, for example from nearby construction, may also disturb the animals.

Feeds and Water. Unless otherwise specified by the investigator, SOPs in most animal facilities include provision of regular (perhaps certified) feeds. Water is usually municipal water with perhaps some treatment in the animal facility. If special dietary or water requirements are needed for the research project, the researcher must inform the animal facility management and laboratory animal technical staff.

Laboratory Animal Bedding. Unless otherwise specified by the investigator, SOPs in most animal facilities include provision of regular bedding materials for that facility. Any special bedding requirements have to be specified by the principal investigator. The phenomenon of resins in softwood bedding (e.g., cedar shavings) activating some of the hepatic enzyme systems (P450 enzymes) is well known. This may complicate results if the experimental outcome is related to hepatic enzyme activity.

Animal Care and Handling Factors

Animal Stress. Stress from many different sources can affect the animal's physiology, biochemistry and behaviour. Sources of stress in the handling and care of the animals include transportation, and the handling and manipulations done by animal care and research staff, and of course the procedures done as part of the research itself.

Transportation Stress. Laboratory animals are rarely used in the same location where they are raised, so usually they are transported to the facility where the research will be done. A number of studies have shown that animals experience varying degrees of stress as a result of the transport, and that it takes some time upon arrival at the research facility to return to a normal physiological state. Eating, drinking and growth tend to return to normal levels in about a week after delivery to the new location. Some subtle physiological and immunological changes may last longer. A common recommendation is to allow laboratory animals at least one week conditioning after transport to the research facility.

Housing Factors (Caging). The amount of space per animal, and the number of animals per cage may influence an animal's response in an experiment. Identical caging should be used for all animals in a study, to ensure that the space per animal and number of animals per cage remains consistent within a study. Studies have shown that the number of rodents per cage affects the stress level (either isolation or crowding), and their growth. As a general rule small rodents should be housed in small groups to minimise stress, and for social enrichment. If the research requires individual housing, this should be scientifically justified to the animal care committee.

Environmental Enrichment. The value of providing an enriched environment in improving the well-being of the animals must be emphasised. In the context of controlling research variables however, it must be noted that any changes (improvements) to the cages will have behavioural, physiological and anatomical effects, some of which result in permanent changes to the animal. (Module 10 provides a more in-depth discussion on environmental enrichment.) Therefore any improvements or enrichments should be uniformly and consistently provided to all animals for the duration of an experiment. Changes in the cage's physical environment should only be made following consultation with the investigator.

Routine (Daily/Weekly) Animal Care and Husbandry. The daily routines of the animal care staff may have a profound impact on the way an animal responds in an experiment. As a general principle all animals should be handled the same way, and the same time of day. Handling must be gentle, and consistent. Most animals quickly become familiar with their regular caretakers and their stress level rises when unfamiliar people handle them. The kind of handling each animal receives may in fact alter its behaviour or physiology, and thereby affect its response in a study. One example of this comes from a handling study conducted in pigs. Hemsworth and co-workers published a paper in 1986 describing a study in which the responses of pigs to three kinds of handling; pleasant handling, unpleasant handling and minimal handling, were compared.

The pigs exposed to pleasant handling approached people more quickly (not surprising). The females receiving pleasant handling were different with respect to age at first estrus and sexual receptivity when bred, and had a significantly higher pregnancy rate than the other two groups. Although not a statistically significant results, the males receiving pleasant handling reportedly had larger testicles.

While it is interesting to speculate whether these results can be correlated with human teenage behaviour, they do emphasise the point that how the animals are handled affects their behaviour, physiology, and anatomy. Consistent handling, by animal care staff, by research technicians, and by the investigators, will ensure that this source of variability is minimised. The weekly routine in any animal room must also be understood by the investigator. In many facilities, animal cages are cleaned once or twice weekly on specific days. Taking samples just after the cages have been cleaned may result in altered results because of the disturbance of the animals at that time. In addition, it must be noted that weekend and holiday animal care routines are generally different than regular week day routines. This may alter the animals' responses on weekend days if sampling is scheduled then.

Applying Basic Animal Behaviour in Your Research Project

An animal learns from experience what will be happening to it when it is handled. Animals very quickly learn handling routines and procedures. Assuming the handling is competently done, not only will an animal's stress level be reduced, it will be much more likely to accept the manipulation being done. During the conditioning period before an experiment begins, animals should be exposed to the routines that will be part of the study. This is particularly important if the study will involve special restraint, use of devices such as collars or jackets containing emerging catheters (e.g., rats with tethers for brain recordings). Familiarizing the animal to manipulations or restraint BEFORE a project starts is important for both welfare and scientific reasons. Rewards for good behaviour are an excellent way to enhance a cooperative attitude in an animal. Rewards such as "gummi-bears" or Fruit Loops are enjoyed by rats. Of course these would need to be acceptable to the research project.

Research Manipulation Factors. In addition to the many non-experimental variables that must be considered and controlled to ensure that the fewest animals need to be used and that the results are valid, there are the many research or protocol variables that the investigator and all research personnel must consider, and control. For some studies such as the surgical induction of cerebral ischemia (stroke) in a rodent model, there may be many variables to control; duration and depth of anaesthesia, body temperature during surgery and during recovery, duration of the application of the ischemia, timing of the analgesia, post-operative care and monitoring, etc. All of these may influence the outcome of the ischemia. It is recommended that for each specific research procedure a Standard Operating Procedure (SOP) be written and observed by everyone involved, to standardise as much as possible each and every animal manipulation.

Time as a Research Variable

The normal biological diurnal rhythms in an animal's biochemistry and physiology alter its responses depending on the time of day that treatments are applied or samples are taken. Thus efforts should be made to take repeated samples at the same time of day, every day. The duration of the manipulation(s) for each animal should also be maintained as consistent as possible. Biochemical and hematological changes start happening when an animal is taken from its cage. Studies have shown that some of these changes last for minutes to hours and will be reflected in the results obtained. For each animal in all treatment groups including control groups, time to sampling (from removing the animal from the cage) should be consistent.

Summary.

In this module we have tried to emphasise the importance of "routines" in all aspects of research with animals. By doing so, the research scientist accomplishes both scientific and ethical goals - using the fewest animals to generate valid, reproducible scientific data. The responsibilities of the various members of the research team in controlling both the non-experimental and protocol variables, as presented at the beginning of the module, are reviewed here in summary:

Responsibilities of the principal investigator

- Consider all pertinent non-experimental variables
- Outline need for controlling pertinent variables to all team members

- Ensure that all experimental variables are controlled or conducted using Standard Operating Procedures (SOPs)
- Ensure monitoring and recording of controls on variables (through Standard Operating Procedures)
- Ensure animal health quality before purchase
- Assure health monitoring is done according to facility rules
- Observe all facility SOPs established to limit disease introduction

Responsibilities of the graduate students, post-doctoral students and research technical staff

- Monitor and record controls on all variables
- Conduct all experimental procedures according to Standard Operating Procedures (SOPs)
- Employ skilled animal handling and manipulation techniques
- Observe all facility SOPs established to limit disease introduction

Responsibilities of the facility manager

- Understand demands for controls on non-experimental variables for all research underway
- Ensure consistent facility environmental operations
- Ensure high level of animal care training and expertise
- Ensure animal health quality before purchase
- Ensure health monitoring is done according to facility rules
- Implement Standard Operating Procedures for all animal facility operations

Responsibilities of the animal care facility staff

- Conduct all daily animal facility routines in a consistent manner, according to Standard Operating Procedures
- Conduct all animal care handling and manipulations in a consistent, gentle, humane way.

Responsibilities of the laboratory animal veterinary staff

- To advise on and ensure health status of all animals
- To affect procedures that will maintain animal health quality

Remember: "**Quality Animal Care = Quality Science**"