Practical Aspects of Experimental Design in Animal Research

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Abstract

A brief overview is presented of the key steps involved in designing a research animal experiment, with reference to resources that specifically address each topic of discussion in more detail. After an idea for a research project is conceived, a thorough review of the literature and consultation with experts in that field are pursued to refine the problem statement and to assimilate background information that is necessary for the experimental design phase. A null and an alternate hypothesis that address the problem statement are then formulated, and only then is the specific design of the experiment developed. Likely the most critical step in designing animal experiments is the identification of the most appropriate animal model to address the experimental question being asked. Other practical considerations include defining the necessary control groups, randomly assigning animals to control/treatment groups, determining the number of animals needed per group, evaluating the logistics of the actual performance of the animal experiments, and identifying the most appropriate statistical analyses and potential collaborators experienced in the area of study. All of these factors are critical to designing an experiment that will generate scientifically valid and reproducible data, which should be considered the ultimate goal of any scientific investigation.

Key Words: experimental design; laboratory animals; research methods; scientific methods

Introduction

Experimental design is obviously a critical component of the success of any research project. If all aspects of experimental design are not thoroughly addressed, scientists may reach false conclusions and pursue avenues of research that waste considerable time and resources. It is therefore critical to design scientifically sound experiments and to follow standard laboratory practices while performing these experiments to generate valid reproducible data (Bennett et al.1990; Diamond 2001; Holmberg 1996; Larsson 2001; Sproull 1995; Weber and Skillings 2000; Webster 1985; Whitcom 2000). Data generated by this approach should be of sufficient quality for publication in well-respected peer-reviewed journals, the major form of widespread communication and archiving experimental data in research. This article provides a brief overview of the steps involved in the design of animal experiments and some practical information that should also be considered during this process.

Experimental Design: Initial Steps

Literature Search

A thorough search of the scientific literature must be performed to determine what is known about the focus of the study. The search should include current and past journal articles and textbooks, as well as information available via the internet. Journal searches can be performed in any number of appropriate journal databases or indexes (e.g., MEDLINE, TOXLINE, PUBMED, NCBI, AGRICOLA). The goals of the literature search are to learn of pertinent studies and methods, identify appropriate animal models, and eliminate unnecessary duplication of research. The "3Rs" of animal research (Russell and Burch 1959) should also be considered at this stage: reduction of animal numbers, refinement of methods, and replacement of animals by viable nonanimal alternatives when these exist. The literature search is also an important component of an institutional animal care and use committee (IACUC[1]) protocol submission to provide evidence that the project is not duplicative, that alternatives to the use of animals are not available, and that potentially painful procedures are justified.

Scientific Method

The core aspect of experimental design is the scientific method (Barrow 1991; Kuhn 1962; Lawson 2002; Wilson 1952). The scientific method consists of four basic steps: (1)

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[1]Abbreviation used in this article: IACUC, institutional animal care and use committee.

observation and description of a scientific phenomena, (2) formulation of the problem statement and hypothesis, (3) use of the hypothesis to predict the results of new observations, and (4) the performance of methods or procedures to test the hypothesis.

Problem Statement, Objectives, and Hypotheses

It is critical to define the problem statement, objectives, and hypotheses clearly. The problem statement should include the issue that will be addressed experimentally and its significance (e.g., potential application to human or animal health, improved understanding of biological processes). Objectives should be stated in a general description of the overall goals for the proposed experiments and the specific questions being addressed. Hypotheses should include two distinct and clearly defined outcomes for each proposed experiment (e.g., a null and an alternate hypothesis). These outcomes may be thought of as the two experimental answers to the specific question being investigated: The null hypothesis is defined as no difference between experimental groups, and the alternate hypothesis is defined as a real difference between experimental groups. Development of a clearly stated problem statement and the hypotheses are necessary to proceed to the next stage of the experimental design process, although they obviously can (and likely will) be modified as the process continues. Examples of a problem statement and various types of hypotheses follow:

- Problem statement: Which diet causes more weight gain in rats: diet A or diet B?
- Null hypothesis: Groups are expected to show the same results (e.g., rats on diet A will gain the same amount of weight as rats on diet B).
- Alternate hypothesis: Experimental groups are expected to show different results (e.g., rats will gain more weight on diet A than diet B, or vice versa).
- Nontestable hypothesis: A result cannot be easily defined or interpreted (e.g., rats on diet A will look better than rats on diet B). What does "better" mean? Its definition must be clearly stated to create a testable hypothesis.

Identification of Animal Model

In choosing the most appropriate animal models for proposed experiments, we offer the following recommendations: (1) Use the lowest animal on the phylogenic scale (in accordance with *replacement,* one of the 3Rs). (2) Use animals that have the species- and/or strain-specific characteristics desirable or required for the specific study proposed. (3) Consider the costs associated with acquiring and maintaining the animal model during the period of experimentation. (4) Perform a thorough literature search, network with colleagues within the selected field of study, and/or contact commercial vendors or government-supported repositories of animal models to identify a potential source of the animal model. (5) Consult with laboratory animal veterinarians *before* final determination of the animal model.

Identification of Potential Collaborators

The procedures required to carry out the experiments will determine what, if any, additional expertise is needed. It is important to identify and consult with potential collaborators at the beginning of project development to determine who will be working on the project and in what capacity (e.g., as coinvestigators, consultants, or technical support staff). Collaborator input into the logistics and design of the experiments and proper sample acquisition are critical to ensure the validity of the data generated. Core facilities at larger research institutions provide many services that involve highly technical procedures or require expensive equipment. Identification of existing core facilities can often lead to the development of a list of potential intramural collaborators.

Design of the Animal Experiment

Research Plan

A description of the experimental manipulations required to address the problem statement, objectives, and hypotheses should be carefully devised and documented (Keppel 1991). This description should specify the experimental variables that are to be manipulated, suitable test parameters that accurately assess the effects of experimental variable manipulation, and the most appropriate methods for sample acquisition and generation of the test data. The overall practicality of the project as well as the time frame for data collection and evaluation are determined at this stage in the development process.

Practical issues that may need to be addressed include the lifespan of the animal model (for chronic studies), the anticipated progression of disease in that model (to determine appropriate time points for evaluation), the amount of personnel time available for the project, and the costs associated with performing the experiments (De Boer et al. 1975). If the animals are to receive chemical or biological treatments, an appropriate method for administration must be identified (e.g., per os via the diet or in drinking water [soluble substances only], by osmotic pump, or by injection). Known or potential hazards must also be identified, and appropriate precautions to minimize risk from these hazards must be incorporated into the plan. All experimental procedures should be detailed through standard operating procedures, a requirement of good laboratory practice standards (EPA 1989; FDA 1987).

Finally, the methods to be used for data analysis should

be determined. If statistical analysis is required to document a difference between experimental groups, the appropriate statistical tests should be identified during the design stage. A conclusion will be drawn subsequently from the analysis of the data with the initial question answered and/or the hypotheses accepted or rejected. This process will ultimately lead to new questions and hypotheses being formulated, or ideas as to how to improve the experimental design.

Experimental Unit

The entity under study is the experimental unit, which could be an individual animal or a group. For example, an individual rat is considered the experimental unit when a drug therapy or surgical procedure is being tested, but an entire litter of rats is the experimental unit when an environmental teratogen is being tested. For purposes of estimating error of variance, or standard error for statistical analysis, it is necessary to consider the experimental unit (Weber and Skillings 2000). Many excellent sources provide discussions of the types of experimental units and their appropriateness (Dean and Voss 1999; Festing and Altman 2002; Keppel 1991; Wu and Hamada 2000).

N Factor: Experimental Group Size

The assignment of an appropriate number of animals to each group is critical. Although formulas to determine the proper number of animals can be found in standard statistical texts, we recommend consulting a statistician to ensure appropriate experimental design for the generation of statistically significant results (Zolman 1993). Indeed, the number of animals assigned to each experimental group is often determined by the particular statistical test on the basis of the anticipated magnitude of difference between the expected outcomes for each group. The number of animals that can be grouped in standard cages is a practical consideration for determining experimental group size. For example, standard 71 sq in (460 sq cm) polycarbonate shoebox cages can house up to four adult mice, so group sizes that are divisible by four will maximize group size and minimize per diem costs.

Controls

A plethora of variables (e.g., genetic, environmental, infectious agents) can potentially affect the outcome of studies performed with animals. It is therefore critical to use control animals to minimize the impact of these extraneous variables or to recognize the possible presence of unwanted variables. In general, each individual experiment should use control groups of animals that are contrasted directly to the experimental groups of animals. Multiple types of controls include positive, negative, sham, vehicle, and comparative.

Positive Controls

In positive control groups, changes are expected. The positive control acts as a standard against which to measure difference in severity among experimental groups. An example of a positive control is a toxin administered to an animal, which results in reproducible physiological alterations or lesions. New treatments can then be used in experimental groups to determine whether these alterations may be prevented or cured. Positive controls are also used to demonstrate that a response can be detected, thereby providing some quality control on the experimental methods.

Negative Controls

Negative controls are expected to produce no change from the normal state. In the example above, the negative control would consist of animals not treated with the toxin. The purpose of the negative control is to ensure that an unknown variable is not adversely affecting the animals in the experiment, which might result in a false-positive conclusion.

Sham Controls

A sham control is used to mimic a procedure or treatment without the actual use of the procedure or test substance. A placebo is an example of a sham control used in pharmaceutical studies (Spector 2002). Another example is the surgical implantation of "X" into the abdominal cavity. The treated animals would have X implanted, whereas the sham control animals would have the same surgical procedure with the abdominal cavity opened, as with the treated animals, but without having the X implanted.

Vehicle Controls

A vehicle control is used in studies in which a substance (e.g., saline or mineral oil) is used as a vehicle for a solution of the experimental compound. In a vehicle control, the supposedly innocuous substance is used alone, administered in the same manner in which it will be used with the experimental compound. When compared with the untreated control, the vehicle control will determine whether the vehicle alone causes any effects.

Comparative Controls

A comparative control is often a positive control with a known treatment that is used for a direct comparison to a different treatment. For example, when evaluating a new chemopreventive drug regime in an animal model of cancer, one would want to compare this regime to the chemopreventive drug regime currently considered "accepted prac-

tice" to determine whether the new regime improves cancer prevention in that model.

Randomization

Randomization of the animals assigned to different experimental groups must be achieved to ensure that underlying variables do not result in skewed data for each experimental group. To achieve randomization, it is necessary to begin by defining the population. A homogeneous population consists of animals that are considered to share some characteristics (e.g., age, sex, weight, breed, strain). A heterogeneous population consists of animals that may not be the same but may have some common feature. Generally, the better the definition of the group, the less variable the experimental data, although the results may be less pertinent to large broad populations. Methods commonly used to achieve randomization include the following (Zolman 1993):

- Identifying each animal with a unique identification number, then drawing numbers "out of a hat" and randomly assigning them in a logical fashion to different groups. For example, the first drawn number is assigned to group 1, the second to group 2, the third to group 1, the fourth to group 2, and so forth. Dice or cards may also be used to randomly assign animals to experimental groups.
- Using random number tables or computer-generated numbers/sampling to achieve randomization.

Experimental Design: Final Considerations

Experimental Protocol Approval

Animal experimentation requires IACUC approval of an animal care and use protocol if the species used are covered under the Animal Welfare Act (regardless of funding source), the research is supported by the National Institutes of Health and involves the use of vertebrate species, or the animal care program is accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International (Silverman et al. 2000). In practice, virtually all animal experiments require IACUC approval, which entails full and accurate completion of appropriate protocol forms for submission to the IACUC, followed by clarification or necessary modification of any procedures the IACUC requires. Approval must be obtained before the animal purchase or experimentation and is required before submission of a grant proposal by some funding agencies. If the research involves hazardous materials, then protocol approval from other intramural oversight committees or departments may also be required (e.g., a Biosafety Committee if infectious agents or recombinant DNA are to be used, or a Radiation Safety Committee if radioisotopes or irradiation are to be used).

Personnel

Animal welfare regulations and Public Health Service policy mandate that individuals caring for or using research animals must be appropriately trained. Specifically, all personnel involved in a research project must be appropriately qualified and/or trained in the methods they will be performing for that project. The institution where the research is being performed is responsible for ensuring this training, although the actual training may occur elsewhere.

Pilot Studies

Pilot studies use a small number of animals to generate preliminary data and/or allow the procedures and techniques to be solidified and "perfected" before large-scale experimentation. These studies are commonly used with new procedures or when new compounds are tested. Preliminary data are essential to show evidence supporting the rationale of a proposal to a funding agency, thereby increasing the probability of funding for the proposal. All pilot projects must have IACUC approval, as for any animal experiment. As soon as the pilot study is completed, the IACUC representative will either give the indication to proceed to a full study or will indicate that the experimental manipulations and/or hypotheses need to be modified and evaluated by additional pilot studies.

Data Entry and Analysis

The researcher has the ultimate responsibility for collecting, entering, and analyzing the data correctly. When dealing with large volumes of data, it is especially easy for data entry errors to occur (e.g., group identifications switched, animal identifications transposed). Quality assurance procedures to identify data entry errors should be developed and incorporated into the experimental design before data analysis. This process can be accomplished by directly comparing raw (original) data for individual animals with the data entered into the computer or with compiled data for the group as a whole (to identify potential "outliers," or data that deviates significantly from the rest of the members of a group). The analysis of the data varies depending on the type of project and the statistics required to evaluate it. Because this topic is beyond the scope of this article, we refer the reader to the many outstanding books and articles on statistical analysis (Cobb 1998; Cox and Reid 2000; Dean and Voss 1999; Festing and Altman 2002; Lemons et al. 1997; Pickvance 2001; Wasserman and Kutner 1985; Wilson and Natale 2001; Wu and Hamada 2000).

Review

Detection of flaws in the developing or final experimental design is often achieved by several levels of review that are applicable to animal experimentation. For example, grant funding agencies and the IACUC provide input into the content and design of animal experiments during their review processes and may also serve as advisory consultants before submission of the grant proposal or animal care and use protocol. Scientific peers and the scientific literature also provide invaluable information applicable to experimental design, and these resources should be consulted throughout the experimental design process. Finally, scientific peer-reviewed journals provide a final critical evaluation of the soundness of the experimental design. The overall quality of the experimental data is evaluated and a determination is made as to whether it is worthy of publication. Obviously, discovering major experimental design deficiencies during manuscript peer review is not desirable. Therefore, pursuit of scientific peer review throughout the experimental design process should be exercised routinely to ensure the generation of valid, reproducible, and publishable data.

Summary

The steps listed below comprise a practical sequence for designing and conducting scientific studies. We recommend that investigators

1. Conduct a complete literature review and consult experts who have experience with the techniques proposed in an effort to become thoroughly familiar with the topic before beginning the experimental design process.
2. Ask a specific question and/or formulate an appropriate hypothesis. *Then* design the experiments to specifically address that problem/question.
3. Consult a biostatistician during the design phase of the project, not after performing the experiments.
4. Choose proper controls to ensure that only the variable of interest is evaluated. More than one control is frequently required.
5. Start with a small pilot project to generate preliminary data and work out procedures and techniques. Then proceed to larger scale experiments to generate statistical significance.
6. Modify original question and procedures, ask new questions, and begin again.

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