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THE PLACE OF STATISTICS AND EXPERIMENTAL DESIGN IN ANIMAL LABORATORY RESEARCH

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ABSTRACT

Animal studies continue to have a vital role in science development because of large variations among individual animals,experimental designs and statistical analyses are particularly important in animal experiments .It involves some important steps in designing experimental designs and should adhere to the the ethical procedure and follow strictly the scientific method.For ethical and economic reasons, it is important to design animal experiments well, to analyze the data correctly, and to use the minimum number of animals necessary to achieve the scientific objectives—but not so few as to miss biologically important effects or require unnecessary repetition of experiments.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. 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.The description of statistical experiments 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. So, in order to bring the experiments using laboratory animals should be well designed,clearly presented and correctly interpreted if they are to be ethically acceptable and to avoid the statistical pitfalls and follow the guidelines using labouratory animals in experimental designing to get the accurate results and maximize the knowledge gained from statistics and animal experiments.

KeyWords: Animal studies, experimental designs, statistical pitfalls, guidelines and accurate data.

INTRODUCTION

Statistics is the science of rigorously quantifying uncertainty and applying it to life sciences i,e. biostatistics and has become indispensable due to capriciousness use of biological readouts. In animal research ,flawless biostatistics is essential for interpreting results and thus avoiding the unnecessary and unethical use of animals(Gosselin 2018). Experiments using laboratory animals should be well designed ,efficiently executed,correctly analyzed,clearly presented and correctly interpreted if they are to be ethically acceptable(Festing and lovell 1995).For ethical and economic reasons,it is important to design animal experiments well, to analysze the data correctly and to use minimum number of animals necessary to achieve the scientific objectives(Festing and Altman,2002).The 3Rs-replacement,reduction,and refinement-can be applied to any animal experiment by researchers to conduct those studies in as humane manner as possible(Parker et al.2014).In 19th century animal research has made major contributions to the health and welfare of humans and domestic animals.Immunization first developed against rabies by Pasteur using dogs ,sheeps and rabbits(Michael et al.2014).The randomized ,blinded controlled experiment was largely developed in the 20th century for agricultural research by Fisher, whose writings provide masterful insights into the process of designing and interpreting experiments (Maxwell and Delaney, 1989).DNA sequencing and the development of techniques to get genetically modified laboratory animals offer a new range of

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animal models along with the improved understanding of animal models(Anon,2014).Animal research is crucial for biomedical advances because animal models often show higher discrimination than many other experimental alternatives and have the necessary fidelity which may be required(Russel and Burch,1959). Replacement of 3R's addresses the substitution of animals by other non-sentient experimental entities (cell cultures, invertebrates, or mathematical models). For instance, it has been shown that lethal doses are better extrapolated from human cell cultures to human subjects than from animals to human subjects (Ekwall et.al. 1998). An advantage of animal research as opposed to clinical trials is that the researcher can plan and control many more variables in the experiment than when humans are involved. This is also a responsibility because the success or failure of our experiment depend more on our ability to carefully design the experiment (Schulz et.al. 2012). Experimental protocols should be refined to minimize any adverse effects for each individual animal. For example, appropriate anesthesia and analgesia should be used for any surgical intervention. Death is not an acceptable endpoint if it is preceded by some hours of acute distress, and humane endpoints should be used whenever possible (Stokes 2000). A number of sequential experimental designs that use fewer animals have been developed for this purpose (Lipnick et.al. 1995;).If the animals are to receive chemical or biological treatments, an appropriate method for administration must be identified (e.g., per as 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). 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).

NEED OF STATISTICAL AND EXPERIMENTAL DESIGN IN ANIMAL LABORATORY RESEARCH

Animal research is crucial for biomedical advances because animal models often show higher discrimination than many other experimental alternatives and have the necessary fidelity which may be required (Birch,1959).The principles of humane experimental technique provide key insight and clues about the possible behavior of drugs and treatments in other species like ours(leist and hartung,2013).The European directive proposes the 3Rs (Replacement,reduction and refinement) as an ethical approach to animal research,being conscious of benefits of animal experiments and harm infringed to them.Replacement addresses the substitution of animals by other non-sentient experimental entities (cell cultures,invertebrates for instance,it has been shown that the lethal doses are better extrapolated from human cell cultures to human subjects than from animals to human subjects(Ekwall et. al.1998).

EXPERIMENTAL DESIGN

The experimental design depends on the objectives of the study. It should be planned in detail, including the development of written protocols and consideration of the statistical methods to be used, before starting work. In principle, a well-designed experiment avoids bias and is sufficiently powerful to be able to detect effects likely to be of biological importance. It should not be so complicated that mistakes are made in its execution. Virtually all animal experiments should be done using one of the formal designs described briefly below.

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1.BASIC DESIGNS

1.1:COMPLETELY RANDOMIZED DESIGN: In this design we have multiple groups,each one receiving a different treatment.Animals are randomly assigned to each one of the treatments.Animals are randomly the one of assigned to each one of the treatments. Example:We are testing a new drug against cholesterol levels in blood. Control animals have a concentration of 250 mg/dL with a standard deviation of 30 mg/dL.We will test two doses of our drug(D1 andD2). We will refer to the control animals as D0, and they only receive the vehicle of the drug (not the active compound). We will analyze 10 animals per group.(Doncaster and Davey ,2007).

1.2:USE OF COVARIATES: The use of covariates does not imply an experimental design in itself.It can be used with any design and it only requires the measurement of continuous nuisance factors that could affect our observations.A covariate are continuous variable that are known or expected to be related to the response variables of interest. Example: animals can be grouped or blocked as high medium and low groups according to their body weight,conversely the individual body weight can be used as a covariate to reduce the estimates of experimental error in the statistical model(Seo et. al.2018).

1.3:FACTORIAL DESIGNS: The factorial design involves the effect of many factors on the measurements.These factors are discrete(yes or no,several dose levels).If we study the same number of animals under every possible combination of the levels of all factors,the design is said to be balanced. Example: We are interested in the effect of a mammalian hormone for water balance in amphibians. We will study two amphibians species (toads and frogs),and we will examine the difference between performing the experiment when animals are dry before making the experiment and when they have been 30 minutes immersed in water before administering the hormone. The control group will not receive the hormone,but only the vehicle. After receiving the treatment, animals will be immersed in water for one hour. We will measure the change in weight of the animals after this time. We have three factors: species (S), moisture state (M) and hormone treatment (H), and we are interested only in the main effects. The linear model we will analyze will be $y = \mu+\alpha S +\alpha M +\alpha H +\varepsilon$ where y is the weight difference(Prkinson,2019).

1.4:NON-ORTHOGONAL DESIGNS: One way of estimating linear models is by progressively explaining variance of the observations by adding new terms that might be related to the variability observed in the data. Example: The three researchers participating in the previous study are now so kind to offer themselves to perform an extra operation so that we can better estimate the time reduction in the new surgical procedure, if it exists. Since we have 3 researchers and 2 operation procedures, each one of them will randomly perform one of them.

1.5:INCOMPLETE DESIGNS: Incomplete designs are useful when for experimental reasons we cannot test all treatments in all blocks. We may also use incomplete designs for complicated factorial designs in which not all combinations are to be tested. Example: We are studying the effect on asthma of two drugs (O and E) that are inhaled, at three different doses (D1,D2, and D3). We also want to study the effect of two different sprayers (SP1 and SP2). Additionally, Drug O must be given with a surfactant and we want to study two surfactants(S1 andS2). We are interested in the main effects of each one of the factors and not the interactions between the doses.

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Drug	Surfactant	Sprayer	Dose	Number of animals
O	S_1	SP_1	D_1	1
O	S_1	SP_1	D_2	1
O	S_1	SP_1	D_3	1
O	S_1	SP_2	D_1	1
O	S_1	SP_2	D_2	1
O	S_1	SP_2	D_3	1
O	S_2	SP_1	D_1	1
O	S_2	SP_1	D_2	1
O	S_2	SP_1	D_3	1
O	S_2	SP_2	D_1	1
O	S_2	SP_2	D_2	1
O	S_2	SP_2	D_3	1
E		SP_1	D_1	2
E		SP_1	D_2	2
E		SP_1	D_3	2
E		SP_2	D_1	2
E		SP_2	D_2	2
E		SP_2	D_3	2
Control		SP_1		5
Control		SP_2		5

FIG,1:We may design the above experiment with the following treatments (each row is a treatment),(Parkinson,2018).

1.6:IMBALANCED DESIGNS: Imbalanced designs are useful when we cannot study all possible combinations of treatments and blocks for economical or ethical reasons or any other considerations. Imbalanced designs can also be analyzed by Least Squares. Example: We want to determine the effect on the growth of animals with three different hormone doses (D1, D2, and D3) and a control (C). We will measure five animals per group. We think that the litter animals come from may cause a difference. For this reason, we will take four animals from five litters. The most efficient design (the one that allows the comparison of any pair of treatments with equal variability) would be the balanced and complete one(COS,2018)

2.ADVANCED DESIGNS

2.1:LATIN SQUARES: Latin squares is a special kind of design in which there is a single treatment factor with L levels, and two blocking variables,each one with as many levels as the treatment factor(Giesbrecht and Gumpertz,2004). Example that calls for a Latin squares design is if we are using mice whose cages are placed on racks. It has been reported that the amount of water intake of the animals depended on the row position of the cage within the rack,and that the body temperature depended on the column of the rack. If we want to block these two effects, we may use a Latin squares design within each rack with 5 rows and 5 columns(Gore and Stanley, 2005).

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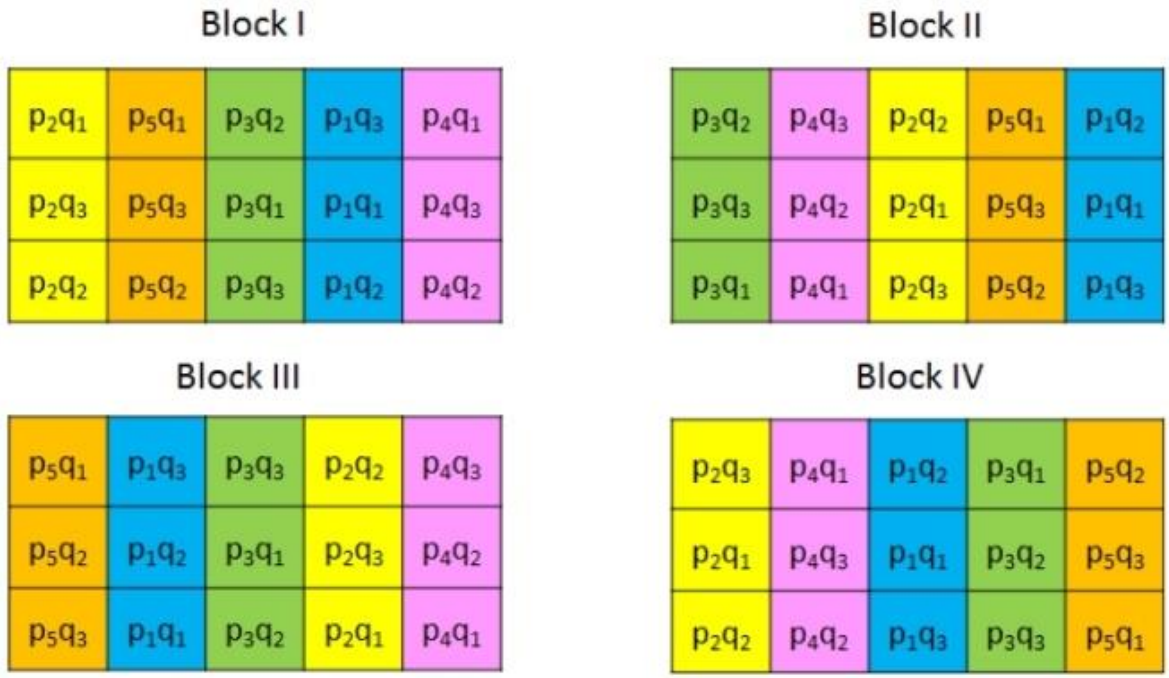
2.2:GRAECO-LATIN SQUARES: Graeco-latin squares and they results in superposition of two latin squares and they allow us to simultaneously perform two different experiments with just one treatment factor and two nuisance factor,or to consectively perform experiments(Example: We are studying the effect of four different cleaning products on the stress of the animals in an animal facility. Four centers participate in the study, and each one of them has four rooms with cages. Simultaneously, we are making a different study, also on the stress of animals, with four different types of cages(COS,2018).

2.3:CROSS OVER DESIGNS: In cross –over design we block time and individuals .In this way we eliminate the inter subject variability from the analysis because an individual is its own control and reduce the number of subjects if we keep fixed the statistical power,or increase the statistical power if we keep fixed the number of subjects.Example ,We are studying the pain reduction caused by an analgesic. There are two treatments: control(with only the vehicle)and treatment(with the drug). We plan to perform a cross-over design in which an animal receives first one of the treatments, and we perform the measure of pain reduction. Then, we wait for a wash-out period such that there is no interference between the first and second treatment or when a limited number of animals are available or when individual animal variation is to be removed,crossover designs are often used(Morris, 1999).

2.4:SPLIT UNIT DESIGNS: We have an experiment with two factors .one of them requires large experiental units,while the other one small ones.Additionally,the second factor can be applied to a small portion of the experimental units of the first factor. Example, We are investigating the effect of light and diet on the growth of mice. – The experimental unit for the light factor is the whole room, all cages receive the same treatment (number of light hours). – The experimental unit for the diet is the cage, all mice in the same cage receive the same treatment.All experiments with repeated measures belong to this class of designs(Sorzono).



FIG,2:Example of split-unit design with four blocks.In each of the blocks we apply five treatments of the hard

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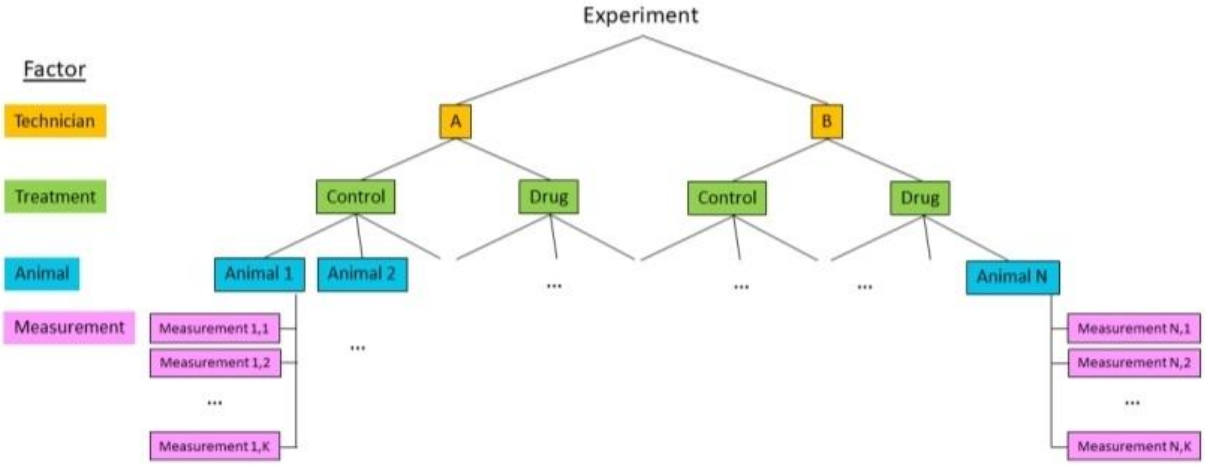
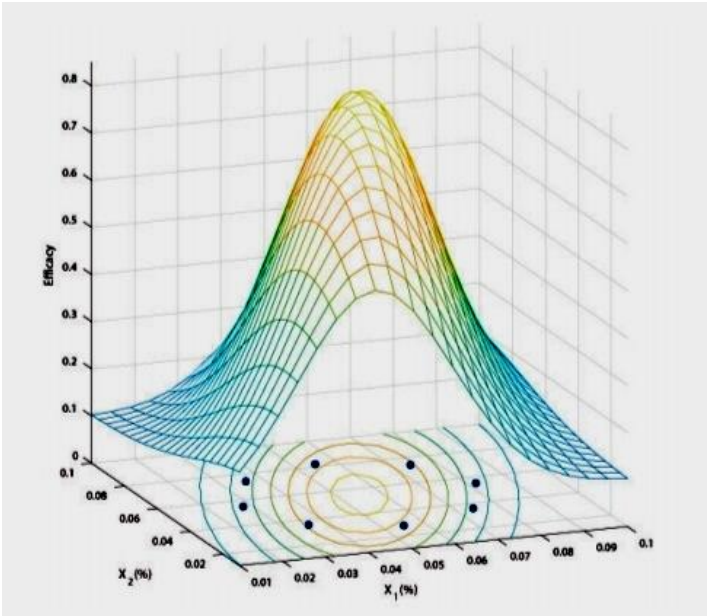
to change factor *P*(note that all treatments in the same column are the ame) and three treatments of the factor *Q*.The colour of each cell is given by the P treatment(Sorzono,2018).

2.5:NESTED DESIGNS: Nested designs are similar to split unit designs ,only that we do not find all possible combination between factors Example,We are investigating the effect of a drug on the concentration of a given protein in the liver.Which is a suitable model for this design(cos,2018).



FIG,3:Pictorial representation of a nested design(COS,2018).

2.6: RESPONSE SURFACE DESIGNS: These designs can be seen as the sampling plan for a surface regression.If we have multiple continous factors,x1,x2,…..xk,then these designs plan which samples to take from the different factors to optimally fit a response surface Y=f(x1,x2,…xk). Example, We are preparing a formulation for a drug that must be delivered as an emulsion. We may dissolve the drug in two compounds simultaneously. The goal is to determine the optimal concentration of each of the two compounds such that the efficiency of the amount released is maximized.sorzono.



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FIG,4:Example of response surface design(Sorzono,2018).

2.7:MIXTURE DESIGNS: Mixture designs also address regressions of the type y =f (x1,x2,….xk),where y is a variable of interest and x1,x2,…are the fractions of the mixture made of compound 1,compound 2,these designs are similar to surface response designs ,only that there is an extra constraint that all control variables must add up to 1.Example, We are interested in preparing a feed for laboratory animals that maximizes the density of the bones. We have three ingredients for the feed, and we want to determine the optimal fraction of the three ingredients we must use. Our variable of interest, Y, is the density of the bones, that is supposed to be a function of the fraction of the three ingredients:Y = f(X1,X2,X3)(Parkinson,2018).

STEPS IN EXPERIMENTAL RESEARCH DESIGN

A careful statistical experiment design involves three steps:

1.**OBJECTIVE DESIGN**: We should clearly set from the very beginning the objective of our experiment(e,g.,measure the effect on sugar concentration in blood of a new drug treatment for type ii diabetes animals.With this objective in mind,we should choose:

The species,stocks,and strains of animals that will better allow extrapolation to other species, like humans. Example ,the concentration of glucose in blood plasma measured 4h after food intake when the treatment, at different doses, has been given for 2 weeks every 8 hours. The test we will use to verify whether the treatment has an effect egg. a t-test for the difference in the mean assuming unequal variance in both groups and a target difference so that we can determine when the treatment is successful or not.

2.SAMPLE SIZE DESIGN: To be able to detect a difference of 100mg/do when the standard deviation is 40,with a statistical power of 90% and a confidence level of 95% ,we need 5 mice per group. The confidence level; of 95% implies that if we repeat this experiment many times with 5 mice in each group ,just by chance ,we will erroneously find in 5% of them that our treatment is useful to cause such a reduction in the blood glucose level, when actually it does not have any effect. The statistical power of 90$ means that in the many repetitions of our experiment we will erroneously find useless 10% of the treatments that actually have such a large effect. we will analyze our data once the experiment is performed(t-test).Too many animals in an experiment is a waste of economical ,laboratory and human resources, too few will spoil the experiment .Both cases call for our ethical responsibility because the treatments and conditions applied to the research animals are harsh.(Mathews ,2010).

3.EXPERMENTAL LAYOUT OUT DESIGN: There are multiple software that allow us to calculate the sample size and the experimental design. However they should be used with care. The difference between the careful experimental design before carrying out the experiment, and the experiments performed to see what happens or without taking the necessary precautions blocking and randomization. In the long term careful statistical designs save animal lives ,reduce the harm infringed on animals, reduce research time and costs, increase research quality ,allow publications and promote ethics in science (Schulz et. al.2012).

STATISTICAL PITFALLS IN ANIMAL RESEARCH DESIGN

1 PROBABILITY PITFALLS

3.1:We are not good at recognizing ambiguously denned probabilities: When we say that a test forgiven disease is 98% accurate, we normally failed to recognize that this statement alone is ambiguous. In a

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frequents approach, the probability is denned as the ratio between positive cases and all possible cases. For instance, the probability being born a male is the ratio between the number of all male new burns and the number of all newborns. When we say that the disease test is 98% accurate, we do not know which the numerator and denominator are(Kahneman,2002).

3.2:We normally fail to consider the assumptions of probabilities: For instance, for the probability of being born male, we may make the following assumptions: 1) Each ovum has an X chromosome and none has a Y chromosome; 2) Half the sperm have an X chromosome and the other half have a Y chromosome; 3) Only one sperm will fertilize the ovum; 4) Each sperm has an equal chance of fertilizing the ovum; 5) If the winning sperm has a Y chromosome, then the embryo will be XY (male); 6) If the winning sperm has a X chromosome, then the embryo will be XX (female); 7) Any miscarriage or abortion is equally likely to happen to male or female fetuses. Our prediction with this model is that there is 50% chances of being a male or a female. We have come to this probability reasoning on a model of the world. However, reality is that in 2012 worldwide, 51.7% of the newborns were male, and 48.3% female(Thaler,2017).

3.3:We do not naturally calculate with conditional probabilities: We regularly monitor for the presence of a rare disease in our animal house. We have a test that correctly identifies 99% of the infected animals, and incorrectly gives a true positive in 0.2% of the non-diseased animals. There must be something wrong with these numbers, 99% and 0.2%, because they do not add up to 100%. This intuition is incorrect because they are not complementary probabilities. 99% is the probability of identifying the disease with the test (positive result of the test) knowing that the animal has the disease, while 0.2% is the probability of incorrectly identifying the disease knowing that the animal does not have the disease(Kahneman,2002)

2.DATA ANALYSIS PITFALLS

4.1: We get confused by variance and subpopulation:In very few cases,we need to analyze data with no variance. This could be the case for instance if we measure the time that an animal takes to perform a given task. We have an upper limit beyond which we stop the experiment, and in this particular case, all the animals reached that limit. The appropriate tool to analyze this data is through a survival analysis with censored data. The censoring will handle correctly the lack of variability in the dataset. In any case, the example just described should be analyzed with survival analysis.

4.2:We misunderstand the meaning of a p-value: If we compare two groups(treatment and control) and we get a p-value of 0.03. This means that ... • If the two population means were identical (null hypothesis), there is a 3% chance of observing a difference as large as you observed (or larger). • Random sampling from identical populations would lead to a difference smaller than what you observed in 97% of the experiments, and larger than you observed in 3% of the experiments(Simmons et. al.2011).

4.3:We fail to realize that non-parametricests are not assumption free: Nonparametric methods have several advantages or benefits over parametric methods: they may be used on all types of data including nominal,ordinal,interval and ratio scaled;they make fewer and less stringent assumptions than their parametric counterparts; they may be almost as powerful as the corresponding parametric procedure when the assumptions of the latter are met and when this is not the case, they are generally more powerful. This has led to their being used as a first resort when there are any problems with data distribution, such as non-normality. Note, however, that there is a restricted range of non-parametric equivalents of parametric tests, and while there are very efficient and effective equivalents for simple comparisons, there are no such simple equivalents for more complicated designs commonly encountered in ANOVA(Parkinson,2019).

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GUIDELINES FOR STATISTICAL ANALYSIS AND EXPERIMENTAL RESEARCH DESIGN USING LABORATORY ANIMALS:

The aim of these guidelines is to help investigators who use animals ensure that their research is performed efficiently and humanely,with the minimum number of animals. These guidelines and suggestions for further reading are based partly on previously published guidelines for contributors to medical journals (Altman et.al. 2000) and for in vitro experiments (Festing 2001). Although a useful set of guidelines for "appropriate statistical practice" in toxicology experiments has previously been published (Muller et al., 1984), with a more extensive set of suggestions for the design and analysis of carcinogenicity studies (Fairweather et al. 1998), general guidelines aimed specifically at experiments using laboratory animals in both academic and applied research do not appear to have been published recently. However, a recent book covers in more detail much of the ground discussed here (Festing et. al. 2002). Although responsibility for the quality of research rests clearly with those who perform it, we believe journal editors should ensure adequate peer review by individuals knowledgeable in experimental design and statistics. They should also ensure that there is a sufficiently full description of animals, experimental designs, and statistical methods used and should encourage the discussion of published papers through letters to the editor and, when possible, by suggesting that authors publish their raw data electronically (Altman 2002).

ETHICAL CONSIDERATIONS: The use of animals in scientific experiments likely to cause pain, distress, or lasting harm generates important ethical issues. Animals should be used only if the scientific objectives are valid, there is no other alternative, and the cost to the animals is not excessive. "Validity" in this case implies that the experiment has a high probability of meeting the stated objectives, and these objectives have a reasonable chance of contributing to human or animal welfare, possibly in the long term. The following "3Rs" of Russell and Burch (1959) provide a framework for considering the humane use of animals:

• Animals should be replaced by less sentient alternatives such as invertebrates or in vitro methods whenever possible.

• Experimental protocols should be refined to minimize any adverse effects for each individual animal. For example, appropriate anesthesia and analgesia should be used for any surgical intervention. Death is not an acceptable endpoint if it is preceded by some hours of acute distress, and humane endpoints should be used whenever possible (Stokes 2000). Staff should be well trained, and housing should be of a high standard with appropriate environmental enrichment. Animals should be protected from pathogens.

• The number of animals should be reduced to the minimum consistent with achieving the scientific objectives of the study, recognizing that important biological effects may be missed if too few animals are used. Some thought also should be given to the required precision of any outcomes to be measured. For example, chemicals are classified into a number of groups on the basis of their acute toxicity in animals. It may not be necessary to obtain a highly precise estimate of the median lethal dose (LD50 value) to classify them. A number of sequential experimental designs that use fewer animals have been developed for this purpose (Lipnick et al. 1995; Rispin et al. 2002; Schlede et al. 1992). Ethical review panels should also insist that any scientist who does not have a good background in experimental design and statistics should consult a statistician.

GENERAL PRINCIPLES: All research should be described in such a way that it could be repeated elsewhere. Authors should clearly state the following:

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• The objectives of the research and/or the hypotheses to be tested;

• The reason for choosing their particular animal model;

• The species, strain, source, and type of animal used;

• The details of each separate experiment being reported, including the study design and the number of animals used.

• The statistical methods used for analysis(MFW,2002).

STATISTICAL ANALYSIS: The results of most experiments should be assessed by an appropriate statistical analysis even though, in some cases, the results are so clear-cut that it is obvious that any statistical analysis would not alter the interpretation. The analysis should reflect the purpose of the study. Thus, the goal of an exploratory analysis is to identify patterns in the data without much emphasis on hypothesis testing, the goal of a confirmatory experiment is to test one or a few prestated hypotheses, and experiments aimed at estimating a parameter such as a genetic linkage require appropriate estimates and standard errors. The general aim, however, is to extract all of the useful information present in the data in a way that it can be interpreted, taking account of biological variability and measurement error. It is particularly useful in preventing unjustified claims about the effect of a treatment when the results could probably be explained by sampling variation. Note that it is possible for an effect to be statistically significant but of little or no biological importance. The materials and methods section should describe the statistical methods used in analysing the results. The aim should be to "describe statistical methods with enough detail to enable a knowledgeable reader with access to the original data to verify the reported results", (ICMJE 2001).

METHODS TO IMPROVE THE EXPERIMENTAL DESIGN AND STATISTICAL ANALYSIS IN ANIMAL LABOURATORY RESEARCH

The wrong use of statistical analysis experiments 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.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 and 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(Larsson 2001,Sproull 1995).

EXPERIMENTAL DESIGN: INITIAL CONSIDERATIONS

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

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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.(Festing,2003),(Johnson ,2002).

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),

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).

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.(Johnson and Besselsen ,2002),(Dell et.al 2002).

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).

CONCLUSION

The need for improved experimental design and statistical analysis of animal experiments, if they are to be considered ethically acceptable, has already been emphasized. The results of most experiments should be assessed by an appropriate statistical analysis even though, in some cases, the results are so clear-cut that it is obvious that any statistical analysis would not alter the interpretation.The method of statistical analysis depends on the purpose of study ,design of the experiments and nature of resulting data.The experimental design depends on the objectives of the study. It should be planned in detail, including the development of written protocols and consideration of the statistical methods to be used, before starting work.All animal experiments should be done according to the formal designs of experiments and inexpensive measures should be aimed to improve the quality of biostatistics in animal labouratory research,proper guidelines and formal experimental designed experiments should be followed in animal labouratory in order to avoid statistical pitfalls in animal labouratory research.

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