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General Principles, Designs, and Statistical Analyses in Experimental Animal Studies

Deney Hayvanı Çalışmalarında Genel Prensipler, Tasarımlar ve İstatistiksel Analizler

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ABSTRACT

Research using animals contributes significantly to many research and development studies, especially in the biomedical field. Within the scope of the study, conducting animal experiments in accordance with scientific principles and ensuring the ethical use and welfare of animals are issues that should be taken into consideration. In this context, the scientific contribution to be achieved by conducting these studies in line with scientific and ethical principles will be directly proportional. In many studies, while investigating the biological significance, it is seen that the effects of many factors are ignored, the answer to the biological question is investigated with simple experimental designs, or the accurate statistical analyses are not chosen. Therefore, in this study, the principles that a researcher planning an animal study should follow within the scope of the research (animal ethics, 3R, and other R rules, determination of sample size, randomization, and blinding) are briefly mentioned. Then, completely randomized design, regression design, split-unit design, hierarchical (nested) design, mixed effects design, and appropriate statistical analyses for these designs, which are thought to be useful in these studies, are discussed. It is thought that this review will be useful as it contains important summative information that will guide all researchers in planning animal studies accurately and quickly.
Keywords: Animal study; experimental designs; 3R rules, 12R's.

ÖZ

Hayvanların kullanıldığı araştırmalar, özellikle biyomedikal alanda birçok araştırma ve geliştirme çalışmalarına önemli düzeyde katkı sağlamaktadır. Çalışma kapsamında hayvan deneylerinin bilimsel prensiplere uygun yürütülmesi, hayvanların etik kullanımı ve refahının sağlanması dikkate alınması gereken hususlardır. Bu bağlamda bu çalışmaların bilimsel ve etik ilkelere uygun yürütülmesiyle elde edilecek bilimsel katkı doğru orantılı olacaktır. Birçok çalışmada biyolojik önemliliğinin veya sorunun araştırılmasında bazı faktörlerin etkisinin göz ardı edilerek basit deney tasarımlarıyla biyolojik cevabın araştırıldığı ya da doğru istatistiksel analizlerin seçilmediği görülmektedir. Bu nedenle bu çalışmada hayvan çalışması planlayan bir araştırmacının araştırma kapsamında uyması gereken prensiplerden (hayvan etiği, 3R ve diğer R kuralları, örneklem genişliğinin belirlenmesi, randomizasyon ve körleme yaklaşımları) kısaca bahsedilmiştir. Ardından da bu araştırmalarda faydalı olduğu düşünülen tamamen rasgele tasarım, regresyon tasarımı, split-unit tasarım, hiyerarşik (iç içe) tasarım, karma etkili tasarım ve bu tasarımlara uygulanacak istatistiksel analizler üzerinde durulmuştur. Bu derlemenin hayvan çalışmaları planlayan tüm araştırmacıları doğru ve hızlı yönlendirecek önemli özetleyici bilgiler içermesi nedeniyle faydalı olacağı düşünülmektedir.
Anahtar kelimeler: Hayvan çalışması; deney tasarımları; 3R kuralı; 12R kuralı.

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INTRODUCTION

Experiments using animals have contributed significantly to many research and development studies, especially in the biomedical field throughout the history of science. Although the results obtained from animal experiments cannot be directly translated to humans (1), they provide important information and clues about the possible behavioral attributes of pharmaceutical agents and therapeutic modalities tested in humans and other species (2).

Conducting animal experiments in accordance with scientific principles and ensuring the ethical use and welfare of animals are matters that must be taken into consideration. In many countries, the 3R rules (Replacement, Reduction, and Refinement) are recommended as an ethical approach in legislation regarding animal research (3,4). Researchers are further advised to adhere to guidelines such as Planning Research and Experimental Procedures on Animals: Recommendations for Excellence (PREPARE), Design and Execution of Protocols for Animal Research and Treatment (DEPART), and Animal Research: Reporting of In Vivo Experiments (ARRIVE) when conducting animal experiments (3-6).

In research planned within the framework of scientific principles, the researcher formulates one or more hypotheses to seek the appropriate answer to the biological question at hand. The selection of the appropriate experimental design must be made to test these hypotheses. There are many experimental designs used in animal research. However, the selection of the appropriate experimental design, considering the constraints and limitations of the study, will guide the researcher toward obtaining an accurate answer to the biological question. The choice of statistical analyses varies according to the selected experimental designs. Statistical analysis serves as a crucial tool for examining data and determining whether observed differences stem from sample variations or genuine disparities within the underlying population (2,7-10).

In numerous studies, experimental designs are often formulated without adequately accounting for the effects of multiple factors when exploring biological significance or addressing problems. Instead of employing complex designs to investigate the biological problem comprehensively, researchers may opt for simpler experimental designs or fail to select an appropriate statistical analysis for the experimental design, potentially compromising the rigor and validity of the findings.

For these reasons, this study has mentioned the principles that a researcher should adhere to when planning an animal study within the scope of the research. Then, some special experimental designs including completely randomized design, regression design, split-unit design, hierarchical (nested) design, and mixed effects design are explained, and the statistical analyses that can be applied to these experimental designs are emphasized.

GENERAL PRINCIPLES FOR ANIMAL EXPERIMENTS

Animal Ethics

Animal ethics, a subset of bioethics, delineates the boundaries of permissible actions within the realms of human and animal sciences, particularly concerning the use of animals in research. It encompasses universal principles governing attitudes and behaviors towards animals, setting forth ethical guidelines and standards to ensure their welfare and humane treatment. Compliance with animal ethics enables the fulfillment of several objectives, including providing justifications for research and training studies involving experimental animals, organizing, and conducting experiments guided by scientific purposes and ethical principles, as well as safeguarding animal rights and preventing harm to animals (11,12).

Extending the Framework from 3Rs to 12Rs

All animal experiments should adhere to the principles of *Replacement*, *Reduction*, and *Refinement* (3Rs) within the framework of bioethics. *Replacement*, as one of these principles, involves prioritizing alternative methods such as in vitro experiments utilizing cell and/or tissue cultures whenever feasible, along with employing phylogenetically lower species (e.g., insects or other invertebrates), and utilizing methods such as computer simulation and inanimate systems whenever possible. *Reduction* entails minimizing the number of subjects by carefully selecting the appropriate number of groups, employing the most suitable experimental design, and conducting accurate statistical analyses. *Refinement* focuses on mitigating the adverse aspects of the method, such as unnecessary pain, suffering, and distress experienced by the animals, while simultaneously enhancing efficiency (9,12).

In the literature, it is evident that in addition to the traditional 3R rules, new guidelines have been introduced and expanded upon, leading to the development of the 12R rules. The initial 3Rs primarily concern animal welfare.

Apart from this, *Respect*, among the R's related to social value, refers to the respect shown to the animal's dignity (care of the animal), its welfare, as well as the rights and privacy of the animal owner or the community. *Responsibility* means the responsibility of the researcher and *Regulations* refers to compliance with applicable national legislation and regulations, as well as directives or standards, legal and/or professional registers. *Reproducibility*, among the R's associated with scientific integrity, pertains to utilizing a robust study design that effectively addresses research inquiries, employs appropriate animal numbers, and employs rigorous statistical analyses to ensure statistical validity. Transparency in reporting and sharing information is also crucial to facilitate reproducibility and enhance the generalizability of data. Moreover, *Relevance* underscores the justification or added value of the research, considering its potential benefits to animals, human health, society, and scientific advancement. *Transferability/Translatability* concerns the applicability and relevance of experimental models, simulations, or representations to real-world scenarios. *Righteousness* represents the intersection of animal welfare and social values in the effort to be fair, good, and worthy scientists and members of society and therefore respectable science. *Reliability* is about the robustness, quality, reliability, applicability, and generalizability of the data produced and the conclusions drawn, embodied in the culture of scientific quality and integrity. *Reckoning* refers to accountability for precautions to be taken during the planning and execution of an animal study and after its conclusion (13).

Determination of Sample Size

When determining the appropriate number of subjects for animal experiments, it is imperative to consider both scientific factors and ethical considerations such as financial constraints, study objectives, data structure, experimental design, and the characteristics of the experimental subjects. Hence, at this stage, it is advisable to adopt the 3Rs, insights gleaned from prior studies or pilot studies, power analysis, sequential sampling, resource equation method, or other strategies informed by practical experience (9,14-17).

Randomization and Blinding

Randomization is a vital procedure in animal studies that guarantees the complete random assignment of animals to experimental groups or conditions. This process can be facilitated using various tools such as computer software programs (e.g., Excel, SPSS, Minitab) or random number generators. Blinding is to ensure that information regarding specific treatments remains confidential to all relevant parties in the experiment who may be consciously or unconsciously influenced by this information. Thus, through the utilization of blinding and randomization, the potential for any conscious or unconscious interferences that could disrupt the experiment can be effectively minimized (2,15).

EXPERIMENTAL DESIGNS AND STATISTICAL ANALYSES

Completely Randomized Design

In a completely randomized design, treatments are allocated entirely at random, ensuring that each experimental unit has an equal opportunity to receive any treatment. Within such designs, any variances observed between experimental units subjected to the same treatment are regarded as experimental errors. This uncomplicated design represents the fundamental framework upon which other experimental designs are built. Noise variables are assumed to affect all treatment groups equally (2,10). In studies involving more than two treatment groups, the model for the completely randomized design is shown in Equation [1]:

$$y_{ij} = \mu + \alpha_i + \varepsilon_{ij} \qquad [1]$$

y_{ij} refers to the outcome variable value of the *j*th animal in the *i*th group, μ denotes the grand mean, α_i refers to the main effect of the treatment, and ε_{ij} states the random error term. Classic analysis of variance (ANOVA) can be used to estimate this model (2).

For instance, consider a study aimed at investigating the effect of a drug on blood cortisol levels in rats with Cushing's syndrome. In this study, groups are established with three different doses of the drug, and animals are randomly allocated to each group. It can be asserted that a completely randomized design is well-suited for this investigation.

If a covariate exists within the design, impacting the outcome variable, it is incorporated into the experiment, and techniques for noise reduction are employed. The relevant model is given in Equation [2].

$$y_{ij} = \mu + \alpha_i + \beta\left(x_{ij} - \bar{x}\right) + \varepsilon_{ij} \qquad [2]$$

y_{ij} is the outcome variable value of the *j*th animal in the *i*th group, μ is the overall mean, α_i is the main effect of the treatment, β is the slope of the regression line between the covariate and the outcome variable, that is, the effect of the covariate, $\bar{x}$ is the covariate mean, x_{ij} is the covariate value of the *j*th animal in the *i*th group, and ε_{ij} denotes the random error term (2).

For example, in a Cushing's syndrome rat study, the plasma adrenocorticotropic hormone (ACTH) levels of the rats measured before the experiment or the body weights of the animals can be taken as a covariate in the experimental design. Thus, the completely randomized design is transformed into a completely randomized design with covariate.

Furthermore, a covariate can be added to the experiment to remove a confounder whose effect cannot be eliminated with some approaches (standardization or randomization). Thus, the power of the experiment can be increased. When a covariate is removed from the experiment, the amount of unexplained variation will increase, reducing power. In such instances, the covariate included in the experiment can be kept under control by using some statistical analysis such as analysis of covariance (10,18,19). Furthermore, Wang et al. (20) demonstrated the importance of including covariate in experiment to increase statistical power.

Regression Design

Regression design can be used when it comes to examining many different values of a variable, that is, different doses of a drug. By adding a generic f(x) function to the ANOVA linear model, the Equation [3] is obtained (2).

$$y_{ij} = f(D_i) = \beta_0 + \beta_1 D_i + \beta_2 D_i^2 + \varepsilon_{ij} \qquad [3]$$

In the regression model, D_i indicates the procedure applied to the *i*th animal, β_0, β_1, and β_2 show the regression coefficients, and ε_{ij} represents the random error term. Regression analysis is used in modeling the outcome variable y_{ij}.

For example, consider a study to investigate the impact of various doses of a drug on cholesterol levels. In this experiment conducted on rats, groups are established with 10 different doses of the drug, with 6 animals randomly allocated to each group. Regression design is suitable for this experiment.

Comparatively, regression design offers several advantages over a completely randomized design. Firstly, it entails lower costs due to the utilization of fewer animals in the experiment. Secondly, the estimated regression model enables the estimation of outcome variable values for intermediate doses, thereby providing valuable insights into dose-response relationships. Thirdly, it can be checked whether each of the estimated regression coefficients is significant (2).

Split-Unit Designs

Split-unit designs are typified by the random assignment of at least two treatment factors to distinct nested unit factors. Usually, one factor may be applied to larger experimental units, while others may be applied to a smaller subset of these units. Experiments incorporating repeated measurements are categorized within this design framework. These include experiments in which an animal is measured multiple times across multiple areas of its body or across multiple tasks (2,21). The model for a basic split-unit design with two factors is given in Equation [4].

$$y_{ijk} = \mu + \left(\gamma_i + \alpha_j^{(P)} + \eta_{ij}\right) + \alpha_k^{(Q)} + \alpha_{jk}^{(PQ)} + \varepsilon_{ijk} \quad [4]$$

Here, P indicates the factor applied to large units (j=1,…,j), and Q indicates the factor applied to small units (k=1,…,k). y_{ijk} is the outcome variable representing the jth P treatment and the kth Q treatment in the ith block. This model shows that the measurement is affected by block γ_i, the treatment applied to the large unit ($\alpha_j^{(P)}$), and some noise (η_{ij}) effective at the level of large units. All terms in parentheses indicate contribution to the larger experimental unit. $\alpha_k^{(Q)}$ shows the contribution of split-units given by the Q treatment, $\alpha_{jk}^{(PQ)}$ indicates the contribution of the interaction between P and Q factors, and ε_{ijk} shows the contribution of some noise at the split-unit level. A linear mixed modeling approach can be employed for the analysis of these models (2).

For example, assume an animal experiment to examine the effects of two different diets (high-fat, low-fat) and a drug administered at different doses (D1, D2, D3, D4) on the enzyme. In this experiment, there are 6 cages and 4 mice in each cage. Each mouse within each cage will receive a different dose, while all mice within the same cage will receive the same diet. Thus, each cage serves as a block for different doses and as an experimental unit for diets. Split-unit design can be used for this study.

Hierarchical or Nested Designs

These designs, which are similar to the split-unit design, may not include all possible combinations between factors. It encompasses repeated measurements, wherein the same animal undergoes measurement multiple times, often involving multiple assessments taken from the liver. Additionally, there may be specific time intervals between these measurements (0, 2, 6, 12, 24 hours). Thus, the animal becomes the factor hard to change in the experiment and the animal cannot be given more than one treatment. It is important to define the animal as the block variable in these designs because the animal can greatly influence the results and samples from the same animal are not independent. The model to be created differs depending on the number of factors in the experiment (2). The nested design model with three factors (A, B, C) is given in Equation [5].

$$y_{ijkl} = \mu + \alpha_i + \beta_{j(i)} + \gamma_{l(ij)} + \varepsilon_{ijkl} \quad [5]$$

y_{ijkl} is the lth observation value at the kth level of factor C nested at the jth level of factor B and the ith level of factor A. μ is the general average, α_i is the ith level of factor A, $\beta_{j(i)}$ represents the effect of the jth level of factor B nested at the ith level of factor A, $\gamma_{l(ij)}$ indicates the effects of the ith level of factor A, the kth level of factor C nested at the jth level of factor B. ε_{ijkl} represents the random error term. ANOVA can be used to analyze these models.

For example, consider a study examining the impact of a drug on the concentration of a specific protein in the liver. In this study, conducted by two different technicians, there are two distinct groups: experimental and control. Multiple measurements (3 times) will be taken from the liver of each animal, with only one technician examining each animal. Consequently, the technician and the treatment are nested within the animal. A hierarchical design is appropriate for conducting this study.

Mixed Effects Design

The model for this design includes at least one fixed and at least one random effect factors and the interactions between these factors. Fixed effect factors are the type of animal (wild type vs. transgenic), age group of the animal (2 months vs. 6 months vs. 1 year), time of the experiment, diet, supplier, and experimenter performing the observations or operations. Conversely, random effects refer to processes where the specific level of the process is expected to come from a larger population. In such cases, the focus is on understanding the variability of processes rather than their individual contributions. The mixed effects model can be given as in Equation [6] (2).

$$y_{ijk} = \mu + \alpha_i^{(A)} + \alpha_j^{(B)} + \alpha_{ij}^{(AB)} + \varepsilon_{ijk} \quad [6]$$

In this two-way mixed effects model, it is assumed that $\alpha_i^{(A)}$ is normally distributed with a random effect, $\alpha_j^{(B)}$ is a fixed effect, and the interaction $\alpha_{ij}^{(AB)}$ is assumed to be randomly distributed and normally distributed. Linear mixed effects modeling approach is used to analyze these designs.

For example, assume an animal study investigating the effects of different levels of neuro-exercise (no exercise, moderate and intense) and gender (female and male) on neurogenesis. The results of histopathological examination of tissues taken from four different regions of each mouse's brain will be used for evaluation. In this design, neuro-exercise and gender are fixed effects, while the mouse and the sections are random effects, so the results can be examined by creating a mixed-effects design model.

CONCLUSION

The aim is to attain more effective, cheaper, and more reliable results in animal studies. This necessitates planning, executing, and reporting studies within the framework of scientific and ethical principles. During the planning phase, the establishment of hypotheses can be achieved by selecting the appropriate experimental design and statistical analyses. Hence, this review offers valuable insights into the scientific and ethical principles fundamental to animal studies, along with details on various experimental designs and statistical analyses. As such, it is recommended for researchers, as it provides important summative information to guide the effective planning of animal studies.

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