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Biological Variation, Reproducibility and Predictability of *in Vivo* Drug Testing

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Introduction

Experimental research in animals is justified due to its expected predictability for conditions in other species, primarily human beings, but except for the limited number of veterinary drugs developed and tested, drugs are not tested on animals in order to benefit the animal species involved and thus not directly predictable.

Within the same species and even within the same strain or substrain of a species the variation can be rather large. This phenotypic variation is not only determined by the genotype but also by environmental factors as simple as housing conditions, feeding and microbiology (*Svendsen and Hansen*,1998). Within the last decades a major goal has been to set up rules to control these environmental factors, to minimise the biological variation and thereby improve the reproducibility of animal research. Good laboratory practice (GLP) regulations, which were introduced in preclinical safety testing of new pharmaceutical agents nearly twenty years ago, has aimed at standardisation by description and control of environmental factors (*Lissau,* 1994). This has lead to a higher level of international accept of test data due to the expected reproducibility.

Integration of study results obtained by animal experimentation is generally accepted as valid. However, in studies contradictory results may be obtained and sometimes great difficulties have been experienced in reproducing experiments of other investigators. Although the experimental animal cannot be calibrated to the same extent as laboratory apparatuses, laboratory animals have been widely characterised and broad insight into biological mechanisms has been achieved by animal experimentation.

Factors with an impact on biological variation, reproducibility and predictability have been identified in pharmacological and toxicological research and testing. This paper focuses on variable factors related to 1) the animal, 2) the environment and 3) the experimental procedure. These are variables which need to be controlled by standardisation in order to reduce or eliminate their impact, if animal studies should be reproducible and predictable.

Animal-related factors

Although the choice of animals, i.e. species, strain, sex etc., for an experiment is among the most important decisions to be made before initiation of an experiment with laboratory animals, one of the main contributing factors in cases of difficulties with reproduction of published results is the failure of authors of scientific papers to give essential informations on the animals used, the conditions they were housed under and the procedures they were subjected to (Table 1).

*Handling laboratory animals.*Animals should be handled in a standardized way to avoid variation. Detailed instructions on several techniques in various animals have been described by Iwarsson et al (1994). In European countries having signed the Council of Europe's Convention ETS 123 concerning training of personnel participating in animal experiments, no one is allowed to take part in the practical procedures of an animal experiment unless one has passed a basic course in laboratory animal science; a course, which should include practical training in animal handling. Harmful procedures should only be performed

Table 1. Essential information on animals, housing conditions and experimental procedures, which should be given in scientific papers (*Claassen*, 1994).

Animals
Species
Breed or designation of stock or strain (using international nomneclature)
Source
Genetic status (if not obvious from stock/strain designation):
- Strain
- or stock
- or hybrid
- or mutant

Age and/or weight at start of experiment
Sex
Reputed microbiological status:
- Conventional (microbiological status not specified)
- or specified pathogen free (pathogens or groups of patogens from which animals are free, must be specified)
- or Gnotobiotic (germ-free) or associated with pure cultures; all micro-organisms must be specified)

Method of delivery/transport
Quarantine or acclimatisation period

Husbandry during experiment
Measures to protect microbiological status:
- Open system (no special protective measures)
- Closed system (animals kept behind barriers or locks)
- Isolator system

Housing equipment:
- Type, material, dimensions, possibly cage type

Number of animals per cage or housing unit
Bedding:
- Type
- Quality
- Pretreatment

Environmental temperature (°C range):
- Regulated
- Not regulated

Relative humidity (% range):
- Regulated
- Not regulated

Lighting:
- Natural
- and/or Artificial (time of day and intensity)

Air changes per hour:
- Proportion of fresh and recirculated air
- Period of conditioning to husbandry conditions and procedures usind during experiment

Feeding
Feed:
- Type and composition, possibly brand name and identification number
- Pretreatment

Feeding schedule, (quantity, frequency)
Water:
- Type
- Quality
- Pretreatment
- Watering schedule (quantity, frequency)

Experimental procedure
The description of the experimental procedure depends on the purpose of the experiement, but the following information must always be provided:
- Number of animals and any pretreatment.
- Time schedule of the experiment (e.g. time of day of the investigation, time interval between sampling and processing)
- Statistical procedures

under anaesthesia. This goes for some sampling procedures, as well, but this is not always that simple, as certain types of anaesthesia may stress the animal more than the procedure, and therefore it may be more acceptable to use a non-stressful restraining device. For example using ether anaesthesia for periorbital punctures in mice stresses the animals more than the puncture itself (*van Herck et al*, 1991).

Genetics. If the environmental conditions are kept constant, the physiology, biochemistry and behaviour of the animals are determined by the genotype. Responsiveness of laboratory animals may, however, differ in various ways due to genetic factors.

The variation of parameters in an inbred strain is generally smaller than that of an outbred stock. Between inbred strains there are large strain differences some of which are well defined, e.g. their histocompatibility types, while other differences related to the physiology, behaviour, pharmacokinetics, pharmacodynamics and toxicology must be considered on the basis of experience. Within some inbred strain, e.g. Lewis rats, different substrains exist, which are very different from one another (Table 2). Outbred stocks are in general poorly defined and it is more the rule than the exception that there is large variation inside a colony, between different stocks and even between stocks carrying the same name. In pharmacological and toxicological studies outbred animals are more popular than inbred animals. In pharmacology outbred animals are preferred basically because they are cheeper than inbred strains. In toxicology it is sometimes argued that outbred animals are considered a more appropriate model for the genetically heterogeneous human. However, as the variation within an outbred stock cannot be precisely defined, and therefore cannot be differentiated in the experimental design, it should be considered that specific toxicity endpoints might be identified more precisely and with a lower number of animals in susceptible inbred strains (*Festing*, 1987, 1990), especially, if a factorial experimental design is used *(Beynen et al*, 1993b).

Table 2. Differences in genetic markers between three different substrains of Lewis rats. The LEW/Mol strain is bred by M&B Ltd., Ll. Skensved, Denmark (*Hansen, 1989*), the LEW/Han strain was bred at the Central Institute for Laboratory Animal Breeding, Hannover, Germany until 1992 (Central Institute for Laboratory Animal Breeding, 1990), while the LEW/Kyo strain is bred at the Institute of Laboratory Animals, Faculty of Medicine, Kyoto University, Kyoto, Japan *(Bender et al*, 1984).

MARKER	LEW/Mol	LEW/Han	LEW/Kyo
Coat colours			
a	a	a	.
b	B	B	.
c	c	c	c
h	h	h	.
p	P	P	.
r	R	R	.
Proteins			
Acon-1	*b*	*b*	*b*
Acp-2	.	*a*	.
Adh-2	.	*a*	.
Ahd-2	.	*c*	*c*
Akp-1	.	*b*	*b*
Alp-1	.	*b*	*b*
Amy-1	.	a	a
Es-1	.	a	b
Es-2	a	d	a
Es-3	a	d	a
Es-4	.	b	b
Es-5	b	.	.
Es-6	b	b	b

MARKER	LEW/Mol	LEW/Han	LEW/Kyo
Es-7	.	b	b
Es-8	b	a	b
Es-9	.	c	a
Es-10	a	b	a
Es-12	.	a	a
Es-13	.	a	a
Es-14	.	b	.
Es-15	.	b	.
Es-16	.	b	.
Es-18	.	a	.
Fh	a	a	a
Gc	.	a	a
Gdc-1	.	a	a
Glo-1	a	a	a
Gox-1	a	a	.
Hbb	a	b	b
Lap	b	b	b
Mgd	.	b	.
Mup-1	b	b	b
Pep-3	a	a	a
Pgd	a	b	b
Pg-1	.	a	.
Pk	.	b	.
Svp-1	b	b	b
Histocompability and blood types			
RT1	1	1	1
RT2	*a*	*a*	.
RT3	a	a	.
RT6	.	a	.
RT7	.	a	.
RT8	b	b	.

Sex related differences. Often only one sex of a species is included in a study protocol. This implies the risk that the results obtained are interpreted for both sexes. Males are often preferred on the basis of the assumption that females may give results with greater variability due to the endocrine changes during the estrus cycle. In fact biochemical, physiological and behavioural fluctuations across the estrous cycle often have the consequence that the response to an external stimulus may vary across the cycle, fluctuations which may involve the neuronal systems, drug-induced behavioural changes and pharmacokinetics. Furthermore, males and females may react differently in relation to behaviour, pharmacokinetics, pharmacodynamic effects and toxicological effects. Sex differences occur over a wide range of behaviours, e.g. activity, aggression, pain and taste sensitivity, food intake and body weight regulation, the learning and retention of certain kinds of mazes, avoidance responses, taste aversion, and performance on certain schedules of reinforcement (*Beatty*, 1979). Differences in drug metabolism and disposition may contribute to the sex differences in pharmacological and toxicological responses. Differences in receptor affinity, receptor density and neurotransmitter systems may add to the dimorphism in the response. Sex related differences have been described in relation to more than two hundred substances. Other extensive sex differences have been reviewed in relation to pharmacokinetics by Kato (1974) and in relation to toxicology by Calabrese (1984).

Age related changes. During development numerous changes occur in the morphology, the physiology and the biochemistry of the animal. The most extensive changes occur in the first 4-6 weeks after birth after which maturation takes place. This is followed by a stable phase of maturity which subsequently merges into senescent over a relatively long period. These changes may result in variation in pharmacokinetics (*Birnbaum*, 1991), pharmacodynamics and toxicology (*Calabrese*, 1986). Species, strain and sex differences are important variables in age related pharmacodynamic responses. The changes in pharmacokinetics and pharmacodynamic response often leads to age-dependency of toxicological effects.
Animals are often purchased from vendors according to weight, but different stocks, or the same stock from two different vendors may have different growth curves. Therefore, animals of the same weight may have different age, and standardisation according to age will often reduce the reproducibility of the study more than standardisation according to weight.

Stress. The stress response, including behavioural reaction and activation of autonomic and neuroendocrine functions, is the consequence of disturbance of the animal by physical or psychological stimuli. Changes in the autonomic and neuroendocrine systems may result in functional changes of different biological systems. The neuroendocrine response include a broad range of pituitary hormones with an increased or decreased release. The literature on the impact of different kinds of stress on experimental results is immense. There is a stress response to simple experimental procedures, such as cage handling, transportation, animal handling, exposure to novelty and emotional communication. Therefore, animals should be given time to adapt after transportation, surgery etc. Various indices of the stress response suggest that the majority of stress effects are short lived. However, some stress experiences may be maintained for a longer period or in rare cases become chronic. In general, adaptation to the stressor takes place after repeated exposure and repeated handling.
It is very important to focus on stress since differences in stress between experiments or between laboratory animal strains otherwise may become an uncontrolled factor. The neuroendocrine and autonomic reactivity of an animal is highly dependent on species and strain. Significant strain differences have been described with many examples. Animals stressed by a certain factor, e.g. transportation, may impose stress upon nearby animals not subjected to that factor (*De Laat et al*, 1989).

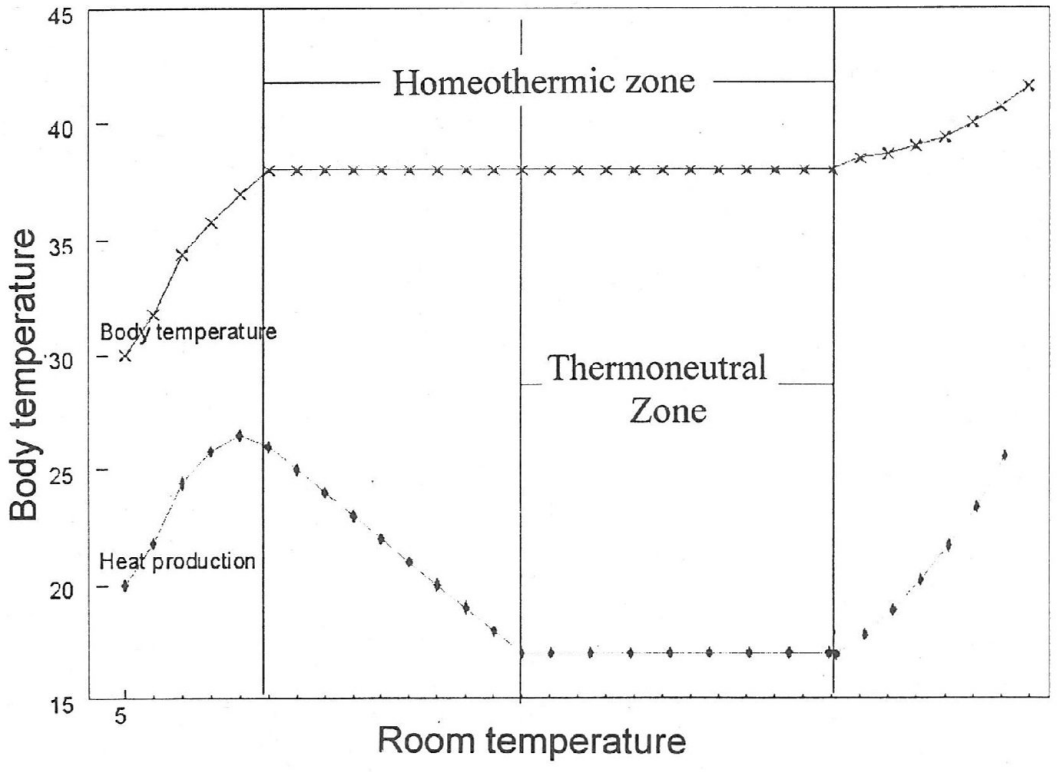
Environmental factors
The environment along with the genotype makes up the phenotype of the animals (*Poole*, 1987; *Reese*, 1991). Examples of environmental factors are the microbiology of the animal, housing and feed related factors.

Microbiological factors. Infections in the animals may cause pathological changes and clinical disease, but they may also have an impact on the animal which is not clinically visible, e.g. on immunology, physiology, oncology and reproduction. Infections may inhibit the induction of a certain animal model, may make it difficult to interpret the final results, may show a dose-related response or raise the variation in the experiment (*Hansen*, 1994). For example infection with Clostridium piliforme in mice irreversibly changes the pharmacokinetics of warfarin (*Friis & Ladefoged*, 1979) and mice infected with this agent will, when treated with carbon tetrachloride, get a dose-related number of focal necroses in the liver, while uninfected mice do not show such a response (*Takenaka & Fujiwara*, 1975). To avoid interference from infectious agents only microbiologically defined animals should be used, i.e. animals from colonies started on the basis of animals derived from caesarean section or embryo transfer, and housed in barrier-facilities, where they can be efficiently protected against infections. The commercial vendors should run a current screening for infectious agents according to recognized guidelines such as the FELASA guidelines (*Kraft et al*, 1994). Also during the experiments it is recommended to protect the animals against infections by certain hygienic precautions (*Hansen & Skovgaard-Jensen*, 1995). The impact of microorganisms on research has been reviewed by Hansen (1994) and Baker (1998).

Housing conditions. The room temperature, relative humidity and air change should be adapted to

the species housed in the room and the number of individuals, and should be adequately controlled. Normally animals are not housed at temperatures within their termoneutral zone, i.e. small changes in room temperature will be compensated for by the animals by a change in their metabolism (Figure 1) (*Svendsen*, 1994). The design of the animal cage, the bedding material, the number of animals in the cage and the subsequent microclimate in the cage may influence the animal characteristics, e.g. rats housed in dirty cages show an impaired activity of the drug metabolising enzymes aniline hydroxylase, cytochrome P-450 and morphine demethylase (*Vesell et al*, 1973). It is well known that individual contra group housing may affect pharmacological responsiveness related to behaviour (especially aggression), central depressants and nociception, e.g. hexobarbital is metabolised faster by isolated rats than by group housed rats (*Dairman & Balazt*. 1970). Animals kept on pine or spruce bedding have significantly higher activity of cytochrome P-450 than animals kept on beech or aspen bedding (*Cunliffe-Beamer et al*, 1981). The significance of housing factors has been reviewed by Clough (1982).

Figure 1. The relation between body and room temperature and it's regulation by heat production (*Svendsen*, 1994).



Dietary factors. Diet may change physiological parameters (*Nelson & Felicio*, 1984), induce pathological changes (*Meyer et al*, 1989; *Ritskes-Hoitinga et al*, 1989) and enhance tumour development (*Tucker*, 1985). A purified diet may be required if certain ingredients have to be reduced below the levels found in natural compounds such as barley, but most diets for experimental animals are natural ingredient diets. Animals in experiments are fed a certain commercial diet, which are considered adequate for the needs of the animal, but many different commercial diets are available, and they differ quantitatively as well as qualitatively to such an extent that it may influence biological responce. Mostly, the nutritional levels are calculated rather than documented by analysis, and although nutrients have been documented they may not be completely available to the animal. Within the same brands of diets often more than one type exist, e.g. one for growing animals and one just for maintenance. Documentation of absence of contaminants such as growth promotors pesticides, heavy metals and biological contaminants is essential for certain types of studies where such contaminants may affect the outcome of the study.

Rodents consume their food in separate meals and both food and water intake is almost exclusively-restricted to the dark phase of the light-and-dark cycle. The intake pattern is affected by strain, sex and age of the animal, by the housing conditions and by the drugs being tested.

Biological rhythms. Animals show biological rhythms, i.e. parameters may differ from time to time. Bio-rhythms such as pulse and respiration which are shorter than 24 hours are called *ultradian rhythms*. Body temperature, defecation, metabolism etc. follow a 24 hour cycle, the *circadian rhythm*, while the estrous cycle is an example of a rhythm longer than 24 hours, the *infradian rhythm*. Biochemical variables in blood and tissues, neuronal characteristics, pharmacodynamics and pharmacokinetics and subsequent toxicological effects vary according to circadian and infradian rhythms, e.g. the normal values of the serum parameters such as glucose differ from season to season (*Pessacq et al*, 1976). These variations have to be taken into consideration in the design of a study in order to obtain comparable data with least variation. It should also be noticed that the environment of the animals, e.g. the length of the lighting period in the animal rooms, will have an impact on the biological rhythms, although the infradian rhythms cannot be eliminated by the use of a standardised light-dark cycle.

Experimental factors

The responsiveness of the animal to the experimental stimuli is strongly related to the testing conditions. Important experimental factors may relate to the test compound and its administration, as well as to feeding and anaesthesia.

Factors related to administration of the test compound. Variations in bio-availability and biological activity are mainly the consequence of differences in the drug formulation administered, and should always be considered in relation to the type of formulation and the route of administration.

Intravenous administration. For a simple procedure such as intravenous drug administration variations can arise from rate of drug administration by injection or infusion, volume administered, and tonicity of injected fluid. The rate of intravenous injection may vary from a few seconds to some minutes. With the antidepressant fluvoxamine, Claassen (1994) observed clear reduction in arterial blood pressure in a cat after a dosage of 3 mg/kg given over less than twenty seconds whereas no reduction was observed when the same dose was given over a two minute period. Slowing down the intravenous infusion rate may prolong the time before onset of the effect and reduce the total dosage required for onset of effect (*Danhof & Levy*, 1984). The volume administered is important in particular for intravenous injections. Haemodilution and increase in cardiac output may take place as a consequence of the acute expansion of the blood volume, e.g. a volume of 2 ml/kg to a rat corresponds to about 3% of the blood volume. By interruption of the vessel wall by intravenous injection or infusion there is a risk of inflammation

and thrombus formation, especially when using chronic catheters or frequently repeated injections. Concentration and pH of the drug formulation are important determinants for inflammation and thrombosis. Often small thrombi are released from chronic catheters into the circulation causing subsequent pulmonary microembolism. Repeated intravenous injection into the caudal veins of the rat may result in a high number of animals with pulmonary emboli from fragments of hair and skin and formation of granulomatous inflammation. Blood concentrations may be lower than expected because the drugs may have been absorbed by the polymer material of the applied catheters. If later sampling is done via such catheters loaded with the compound, it may be released again resulting in erroneously high concentrations (*Garrettt & Chandran*, 1989).

Intraarterial administration. The possibility of pulmonary first-pass elimination is seldom recognised. However, higher plasma concentrations after intraarterial administration versus intravenous administration may be due to pulmonary first-pass elimination. e.g. as reported for propranolol in rats (*Iwamoto et al*, 1987). The first-pass elimination of serotonin and noradrenalin in the canine lungs is very high (92%), while the amines will gradually be released into the circulation if the metabolism is inhibited by a monoamine oxidase inhibitor (*Bakhle & Vane*, 1974).

Intramuscular administration, a common practice in the human and veterinary clinic, may be of limited use in experimental studies, particularly in rodents where strict intramuscular injection is difficult due to the size of the muscles. The intramuscular administration often leads to drug deposition at the injection site resulting in a relatively low bioavailability. Many intramuscular drug formulations have physical-chemical characteristics that are not physiological. The muscle tissue is rather fragile sensitive to unphysiological conditions such as high or low osmolarity and high or low pH values, in particular where the solutions have a substantial buffer capacity. Such formulations cause damage to the local muscle tissue at the injection site, and this may reduce the bioavailability. The characteristics of intramuscular injections have been dealt with in detail by Svendsen (1988).

Subcutaneous administration, is frequently used in experimental studies due to the simplicity of the injection procedure. However, the rate and extent of the bioavailability of the injected drug depend upon a great number of biological and biopharmaceutical factors. In contrast to the muscle tissue, the subcutaneous tissue is well supplied with lymph vessels, and lymphatic absorption may contribute significantly to the absorption process. Absorption from subcutaneous injections is also influenced by pH and osmolarity. High or low osmolarity may reduce the bioavailability (*Marvola & Lain*, 1980) as may high or low pH values. Poorly soluble substances may be solubilised in vegetable oil. In general, this will slow the absorption rate, and the absorption rate may vary with volume injected and concentration of substance in oil (*Hirano et al*, 1982). Absorption from aqueous suspensions vary to such an extent that any prediction is very difficult.

Intraperitoneal administration is attractive due to its simplicity. However, this route of administration in experimental work should only be used after careful consideration. In general, absorption occurs rather rapidly. It is often overlooked that parts of the substance is absorbed to the portal system and thus subject to hepatic first-pass metabolism. In addition, non-specific side effects may occur in response to both the vehicle and the compound.

Oral administration is one of the most frequently applied routes of dosing. Oral administration results in a relatively low peak drug level in plasma in comparison to parenteral dosing. Oral dosing in rodents is either by gavage or by admixtures in the diet. They are not equivalent and the latter method is primarily used in long-term chronic toxicity studies. For most compounds absorption is limited to the non-ionised form of the molecule. The rate of absorption after oral dosing may depend on the contents of the gastrointestinal lumen (*Nielsen*, 1997). With ad libitum feeding the stomach is never empty and the food contents parallels the eating behaviour which depends on the light and dark circle in the animal room. After fasting rats for sixteen hours the stomach will be empty provided that they are kept on a whire mesh with no access to bedding which they will otherwise eat. The gastric emptying rate is fairly con-

stant in the rat (*Newman & Booth*, 1981), although it may be under the influence of a circadian rhythm. Transit time in the first and second quarter of the small intestines is very short, while considerably longer in the third and fourth quarter and very long in the caecum and large intestines.

Factors related to the chemical or physical form of the test compound. Chemical (salt form, complexes, ester) as well as physical (crystal form, particle size) characteristics of the test compound itself is an essential factor. Absorption is influenced by the solvent in which the compound is dissolved/suspended, as well as by additional components such as co-solvents, surfactants or suspending agents.

The volume of drug solution or suspension influence absorption rate. In general, the absorption rate is enhanced by dilution of the dose. This is most probably related to a reduced transit time in the stomach (*Tsuzuki et al*, 1984).

Viscosity increasing agents may affect bioavailability aspects either from drug solutions or drug suspensions. In general the availability is reduced by viscosity increasing agents in drug solutions (*Sakiya et al*, 1981), whereas the availability is increased by viscosity increasing agents in drug suspensions (*Barzegar-Jalali & Richards*, 1979).

Co-solvents such as dimethylsulphoxide (DMSO), ethanol, glycerine, polyethylene glycol and propylene glycol may cause reversible structural and functional changes of the gastrointestinal mucosa. Drug absorption is highly dependent on the co-solvent and promotion as well as inhibition may occur. Co-solvents may have biological properties by themselves and they may affect drug metabolism. Poorly water-soluble drugs may be dissolved or suspended in oily vehicles. They delay gastric emptying and inhibit gastric secretion as other lipids do. Orally administered triglycerids are transported as chylomicrons via the lymph and may incorporate lipid soluble substances. This absorption route by-passes the liver and may affect the availability.

Feeding factors. Fasting causes severe changes in the physiological and biochemical processes which may be observed after fasting periods as short as 16-24 h. However, it is fairly common to withdraw food from animals for a period before the experiment. This will alter the metabolic state of the animal and induce adaptational changes in the endocrine and neuronal state. Withdrawal of food will result in decreased water intake probably altering the hydration state. Changes in pharmacokinetics and pharmacodynamics may be a consequence. Fasting of animals should therefore be limited to studies where fasting is an essential factor in the study. Animals with a vomiting reflex, e.g. dogs, cats, pigs, ruminants and primates, have to be fasted prior to anaesthesia, while rodents do not have this reflex and therefore do not have to be fasted before anaesthesia.

Food restriction may be necessary in studies on eating behaviour, operand behaviour and certain types of toxicity studies. Biochemical and physiological functions differ between restricted food and ad libitum food regimes. Aging, tumour development and other types of pathology are among those characteristics which differ remarkably (*Beynen et al*, 1993a).

Anaesthesia. Anaesthetics induce a state of unconsciousness, amnesia and pain insensitivity and at the same time interfere with numerous other life processes. These disturbances are important not only in in vivo experiments but also in ex vivo and post-mortem studies. Maintenance of a well-defined anaesthetic depth is important for the responsiveness of the animal and for the reproduction of the data obtained. This can best be achieved with inhalation anaesthesia. However, in most animal studies intravenous anaesthetics are used. An uncontrolled decrease of body temperature or blood pressure during anaesthesia may contribute to physiological and biochemical changes.

The cardiovascular and autonomic effects of anaesthetics are complex. In addition, anaesthetics influence metabolic processes, pharmacodynamics and pharmacokinetics. The characteristics of blood and tissue samples from anaesthetised animals may differ from those of conscious animals.

Experimental design

Experimental results are accepted or rejected after statistical evaluation. Concluding that there is a difference, when this in reality is not so, is called a *type I error*, while concluding that there is no difference, when there actually is one, is called a

type II error. The probability of avoiding a type II error is called *power*. Committing such errors will obviously not give a high level of neither predictability nor reproducibility of that study. Furthermore, resources may be wasted and unnecessary animal suffering caused if too many animals are used due to a poor experimental design. For example, Festing (1995) describes an experiment, in which a proper experimental design would have reduced the 216 rats used to 72 rats. On the other hand, using too few animals, may result in too little power, and therefore a careful determination of the correct size of the experiment is important. Such determinations may be based upon either mathematical equations, previous experience or the so-called *resource equation method* used for adding the numbers of animals needed due to the degrees of freedom, the environmental variation and the error (*Mead*, 1988). All the factors described in this chapter will add to the variation in the study or cause error and should either be eliminated, reduced or standardised. Error may be reduced by *stratification*, i.e. dividing animals into groups based on certain known characteristics, e.g. their weights. If there is no such information the study may be *randomised*, i.e. the animals are randomly assigned to test or control groups, thereby equally dividing the inter-individual variation between the groups. Bias in the treatment means may be prevented by standardising such factors as the housing conditions of the animals.

Different experimental designs may be used for eliminating variation in animal experiments (Table 3) (*Beynen et al*, 1993b, *Vølund* 1994).

Table 3. Examples of different experimental designs usable for controlling variation in animal experiments (*Beynen et al*, 1993).

Experimental design	**Explanation**
Randomised/parallel	Each group of animals is undergoing one and only one of the various treatments simultaneously.
Randomised block	The animals are grouped into blocks based upon certain characteristics.
Cross-over	Each treatment is applied to all animals for a predefined period followed by the next treatment.
Latin square	Each group of animals are subjected to each of the treatments, but in different order.
Factorial	Two or more factors varies independent of one another, e.g. different types of animals are subjected to different types of treatments.

General conclusion

The uncertainties in animal characteristics and test conditions so far have not hindered the insight into biological regulatory mechanisms. However, if animal studies should have the highest degree of predictability and reproducibility, only animals of well-defined health status, housed under well-defined conditions should be used. *In vivo* drug testing should be planned in order to eliminate the variation caused by improper standardisation from group to group or over time and the bias caused by erroneous techniques. This review has summarised the various factors which are critical for variation, and thereby for the reproducibility and predictability in experimental research in animals. The factors are many and some are more important than others. The importance may vary with the goal and the design of the study. It is the responsibility of the study director to take these factors into consideration in order to obtain data which present minimal biological variation and are reproducible. Taking this responsibility seriously will not only improve drug testing, but it will also be in accordance with the ethical obligation to gain as much information as possible whenever using animals for experiments.

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