Review Article

THE FUNDAMENTALS OF EXPERIMENTS WITH ANIMALS – APPLICATIONS IN EXPERIMENTAL SURGERY

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ABSTRACT

With the objective of contributing to research in experimental surgery, this article presents an analysis of the principal parameters laid down by Brazilian and international ethics and animal welfare committees and which must be adhered to for publication in international peer-reviewed journals. Standardization of the genetics, sanitary status and environment of the species *Mus musculus* (mice), *Rattus norvegicus* (rats), *Oryctolagus cuniculus* (rabbits) and *Sus scropha domesticus* (pigs), appropriate transportation, acclimatization, enrichment of the environment, training of animal science technicians, information management, biosafety, diet, anesthesia, postoperative care, analgesia and euthanasia, in combination with well-planned research protocols are presented as the basic elements for achieving results with a high degree of acuity, reproducibility and precision.

Key words: Experimental surgery. Mice. Rats. Rabbits. Pigs

Introduction

The use of laboratory animals in scientific research is a dilemma that causes some of the greatest conflict in the entire bioethics debate. The principle of humane experimentation proposed by Russel & Burch[1] and known as the three Rs, proposes *reduction* of the number of animals used in each experiment, *refinement* of experimental techniques in order to avoid unnecessary pain and suffering and *replacement* with alternative methods, should be an imperative. Furthermore, technological refinements in the design of experimental models has also led to a reduction in the number of animals/ experimental groups by significantly reducing observed variation in each experiment. In this context, researchers should think about the real need for a biological model and the relevance of the proposed study before deciding to undertake a project involving animals.

Reducing the number of animals in biomedical research should not compromise the detection of biological effects and neither should it lead to repetition of experiments. The study design and sample size calculation, the control of variation, the statistical hypothesis being tested, the choice of statistical test used for data analysis and interpretation of the results all contribute towards refinement, making it possible to obtain more information without increasing the number of animals used.[2] Literature reviews suggest that the number of laboratory animals used in past experiments could have been reduced while still obtaining the same statistically valid information. It is also of fundamental importance that experimental results be published, irrespective of whether they have statistical significance, to avoid conducting redundant studies.[3]

Intra-sample variation can be reduced by using animals that are genetically and sanitarily homogenous, in addition to controlling environmental variables. Standardized procedures, accurate methods for measuring responses and well-defined objectives make it possible to accept or reject the initial hypothesis even with a reduced number of animals. The sample size is based on mathematical relationships between the size of the effect being studied (the biologically significant difference), the standard deviation (usually obtained from a pilot study), the level of significance required (usually between 0.05 and 0.01) and the test power (usually set between 80 and 90%).[3]

Sample size calculations have been discussed in detail by Scheibe.[4] Table1[5] shows the number of animals needed to achieve significance at $p<0.05$ for a range of differences between control and experimental groups and with coefficients of variation (CV) of 15% or 20%. As will be observed in Table 1, the primary objective should be to measure small variations as a function of the intervention studied, which is extremely important for reducing the intragroup standard deviation to a minimum.

In addition to the bioethical aspects of reducing the number of experimental animals, there are also legal issues involved. In

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Table 1. Number of animals needed to obtain statistically significant results (Extracted from Eckelman WC et al. 2007[5])

Percentage difference between experimental and control groups	Percentage CV due to biological variation	Number of animals	Significance (P<0.05)
20	20	2-7	Not significant
20	20	8	Significant
20	15	5	Significant
25	20	5	Borderline significance
30	20	5	Significant
25	15	5	Significant

CV = coefficient of variation

Brazil, the federal law regulating procedures and responsibilities related to the use of laboratory animals took 13 years to be passed, but on the 8th of October of 2008, Act number 11,794,[6] was indeed passed, and on the 15th of July of 2009 it was regulated by *Decreto* 6,899,[7] mandating the establishment of a national board to control animal experimentation, to be known as CONCEA – (*Conselho Nacional de Controle de Experimentação Animal*) and of animal usage ethics commissions, to be known as CEUAS (*Comissões de Ética no Uso de Animais*). Prior to this, the lack of federal legislation prompted some Brazilian states to enact their own legislation. For example, the state of São Paulo passed State Law number 11,977[8] in 2005, establishing a charter for the protection of animals, and the city of São Paulo passed Municipal Law number 13943[9] in 2004 prohibiting that the dogs and cats picked up by the city's zoonosis control authority be sent to research or teaching institutions. In the city of Rio de Janeiro, legislation has been passed and then repealed.[10] Florianópolis was the first Brazilian city to regulate the use of animals in research and teaching.[11]

Despite legislative obstacles, over recent years there has been a growing increase in the number of publications about surgical techniques employing experimental animals.[12] Therefore, with the objective of contributing to systematization of research into experimental surgery, this article provides a detailed analysis of the relevant literature found in a range of databases (Medline, Scielo, Lilacs, Embase), focusing on the principal parameters laid down by Brazilian and international ethics and animal welfare committees[13] and which must be met as a prerequisite for publication in high-impact journals.

Choice of experimental model and genetic standardization

For any specific objective there is a range of biological models appropriate to simulate the target species of the research. Animal species used as models for human beings should have biological, anatomical and physiological similarities. Genetic definition should be the first element to be taken into account, once the experimental model has been chosen. In the case of rodents, there are countless strains of mice (*Mus musculus*) and rats (*Rattus norvegicus*). The genetic background and monitoring of experimental animals should be described, for example, the name of the lineage, whether it is heterogenic (outbred), isogenic (inbred), congenic, recombinant, mutant or transgenic.[14]

In the case of rabbits (*Oryctolagus cuniculus*) and of pigs (*Sus scropha domesticus*), breeds should be defined. The white New Zealand rabbit is the breed most often used in experiments and the pig breeds most used are Large White, Landrace, Yorkshire, and Hampshire. In comparative studies using experimental animals, differences in physiology, genotype, phenotype and maturity at a given weight and age should all be taken into account. Animals must be sourced from the same supplier throughout an experiment, since even isogenic animals can vary from one animal house to another.

Traditional pig breeds have the disadvantage of increasing in weight from 1 kg at birth to 100 kg by 4 months, reaching around 150 kg at 9 months. As a result, they are most appropriate for acute experiments or experiment lasting a maximum of 3 weeks.[15] Miniature pigs (minipigs) have been recommended for longer experiments due to their small size and weight. Depending on feeding regime, they weigh 0.5 kg at birth, 12-45 kg at 4 months of age and reach 45-100 kg when adults. The breeds most often used in research are Yucatan, Hanford, Sinclair, Pitman-Moore and Goettingen. The "Minipig br" has been bred in Brazil.[16]

Whatever the experimental model chosen, a critical analysis must be made of the limitations imposed by the physical and anatomical differences from humans and of the diseases that can potentially be contextualized.

Sanitary standardization of the biological model chosen

Conventional animals may be asymptomatic carriers of pathogenic organisms which could interfere with experimental result, particularly long-term observations of post-surgical results, making it difficult to interpret and reproduce data. The sanitary category of laboratory animals should be appropriate to the experimental objective.

Environmental standardization

The experimental animal housing facilities should have a standardized environment in terms of temperature, humidity, illumination, light/dark cycles, air quality (achieved by effective filtration and 10 to 15 changes per hour in order to prevent recirculation). These climatic conditions should be maintained within the experimental environment by automatic adjustments.[17,18]

Variations in ambient temperature influence metabolic processes provoking, for example, changes in the blood supply to the organs and enzyme activity in the liver. There are also interactions between ambient temperature and drug toxicity. The LD_{50} of certain pharmaceuticals varies with ambient temperature.[16] A reduction in ambient temperature below an animal's capacity to regulate body temperature increases its susceptibility to infections. It has been concluded that it is of fundamental importance to record ambient temperature daily in order to allow for correct interpretation of results. Ambient temperature should be kept within the limits for rodents (22 °C ± 2 °C), rabbits (18 °C ± 2 °C) and adult pigs (16 °C ± 2 °C).[14]

Water is a better conductor of heat than air and as the water vapor content increases, the conductivity of air also increases.

Variations in temperature and humidity can lead to pathological changes in the airways and skin and also to infections. In rats, relative humidity below 40% triggers ring disease: constrictions in the tail restrict the blood supply and the distal tail becomes edematous and may develop necrosis. The recommended animal room humidity is around 45% ± 15%.[17]

Lack of adequate ventilation together with high demographic density and poor cage hygiene leads to an increase in the concentration of ammonia in breathing air, irritating the epithelium of the upper airways and increasing susceptibility to infectious diseases. If the concentration of carbon dioxide rises above 8%, the result is unconsciousness followed by death.[17]

When an investigator does not have access to animal housing facilities with the correct climatic conditions, one option is a ventilated cage system into which the filtered air is blown at constant temperature and humidity directly into each cage, without causing turbulence, providing a microenvironment free from external contamination and with lower concentrations of gasses, thanks to an appropriate exhaust system. Individual ventilation directly into the cage had a positive effect on reproductive performance and reduced the incidence of pneumonia in Wistar rats and reduced the concentration of ammonia inside the cage, when compared with rats kept in an environment with diluted ventilation, as in conventional animal room.[19]

The wood shavings used as bedding for animals must be free from chemical substances, such as insecticides or fungicides, and the wood should be soft and absorbent, so that it does not release volatile substances that could affect the microsomal enzymes of the liver. Both the quality of the wood shavings and the cage cleaning routine can affect experimental results, by reducing ammonia concentration and the stress suffered by animals.[17]

A comparison of the routines at four different animal houses that bred Sprague-Dawley and Wistar rats, investigating the parameters of frequency of cage cleaning, ammonia concentration and type of bedding, indicated that weekly cleaning reduced ammonia concentrations and aggressive conflicts. The type of bedding did not affect ammonia concentration, but rats given aspen wood shaving bedding exhibited more pathological changes in the lungs than those given paper bedding.[20]

The hypothalamus-pituitary system is stimulated by light, via the optic nerve, to produce and release the hormones necessary to regulate the body. Light also contributes to regulating the body, with influence from the Earth's rotation. Countless bodily functions and activities are time-dependent, i.e. have a circadian rhythm. Many experimental results are affected by circadian rhythms.[17]

The potential influence of circadian changes to laboratory routines on certain parameters of interest in immunology have been described. These include the weight of the thymus and spleen, the number of leukocytes in bone marrow, peripheral blood and the peritoneum in male mice of C57BL/6J, BALB/c and CB6 F1 strains with identical laboratory conditions with the exception of the light/dark cycle. Many parameters were affected by inversion and it was concluded that great care should be taken when extrapolating results to other strains or species. All comparisons between different strains, especially those from several different laboratories, should be related to specific and standardized parameters.[21]

The light intensity in experimental animal houses should be 300 to 450 lux at 1 meter above the floor and 600 lux at 1 m from the ceiling, directly below the light source. The cages on the top shelves should be protected from direct light. Light/dark cycles should be 12/12. Albino animals should not be exposed to light intensities greater than 60 lux inside the cage to avoid provoking pathological changes in the retina and increases in endocrine activity. The light source should be fluorescent rather than incandescent.[17,18]

Many species of animal can hear ultrasound. The sound conducted by impulse through the CNS causes measurable reactions in several organs and systems (for example: increases in heartbeat and respiratory movements, increased blood pressure, hormonal hyperactivity etc.). Noise in the animal house can lead to abnormal behavior and body reactions that may mask or simulate results. Sudden and irregular noises are equally stressful. Noise levels should be below 60 dB.[17]

The noise inside two different types of cage, stainless steel and polycarbonate, were monitored using microphones inside the cages connected to recorders, while either a calm or a nervous technician cleaned the cages out. The results showed that both the material and the technician's style had an impact on the sound levels inside the rats' cages.[22]

Water can be treated using an autoclave, by filtration, hyperchlorination or acidification. Hyperchlorination may irritate mucosas, but interference in results is an unknown. The degree of acidification recommended, pH between 2.5 and 3, may affect the teeth and possibly interferes with results.[17] For pigs, the recommendation is to install automatic water systems.[14]

In general, animals are social beings and should not be prevented from interacting with other members of their species. If the experimental protocol demands individual cages, each animal should still be allowed to see others. Pigs, in particular, which are docile when trained via positive interactions with humans, may develop gastric ulcers in stressful situations such as food or water privation or abrupt environmental variations.[15] The type of housing affected biochemical parameters measured in the serum of rabbits. Animals housed in pairs exhibited less variation in weight gain and in serum alkaline phosphatase concentration.[23]

In experimental surgery, individual housing is unavoidable. When mice were housed individually or in pairs in the same cage separated by a screen and compared with mice housed in groups, by means of monitoring via telemetry from 1 week before abdominal surgery until 3 weeks after surgery, the results indicated that mice housed in groups suffered the least adverse effects while individual housing appears to be a better option than housing in pairs separated by a screen.[24]

Transportation and acclimatization

Transportation from the supplier to the research center must be carried out in a manner that preserves both the health and welfare of the animals being transported. Each transportation must be carefully planned in order to for each extreme temperatures and traffic congestion, even if this means traveling longer distances. Within the research center, transportation should be accomplished using cages protected by filters, inside appropriate containers, in order to avoid people becoming exposed to

allergens present in urine, saliva, hair and bedding, or to microorganisms or chemical or radioactive substances that may have been inoculated into the animals, and also to prevent escape. Acclimatization of animals to the experimentation area should be observed. With the objective of determining the time necessary for rats to acclimatize to a new environment, radio telemetry was used to monitor heartbeat, body temperature and activity, using previously implanted transmitters. Animals' weights were also recorded both before and after transportation. With the exception of temperature, all parameters were altered significantly. The results indicated that 3 days are necessary for rats to become acclimatized to a new environment.[25]

The influence of environmental enrichment

With the objective of improving animal welfare, several researchers have demonstrated positive results by enriching environments. This is modifying the environment of an animal in captivity to increase its physical and psychological welfare, providing the stimulus for animals to meet their species-specific requirements. Examples of enrichment items are accessories that appear like nests, tubes to hide in, refuge areas etc., both for rodents and for rabbits. Pigs kept in environments enriched with hoses, toys and strips of rubber exhibited less aggression.[18]

The effect of environmental enrichment on the acute physiological stress caused by keeping mice in individual cages for short periods has been investigated. Telemetry was used to monitor heartbeat, body temperature and plasma corticosterone concentration. The results suggest that the response to stress was reduced in the group kept in an enriched environment. There was no effect on thymus weight or the concentration of tyrosine hydroxylase,[26] which is an enzyme involved in the biosynthesis of neurotransmitters.

Training technicians in animal experimentation

Due to bioethical considerations, all procedures with animals should be carried out by trained professionals. The Federation of European Laboratory Animal Science Associations (FELASA)[27] recommends four categories of professionals. Category A contains four levels, with professionals starting with theoretical and practical training and, as they accrue experience, progressing up through the difficulty scale to performing routine tasks, taking more than 5 years to be fully trained. For categories B and C, FELASA has proposed courses to qualify technicians and researchers to work with animals. Since these categories have constant turnover, there is robust demand for training.FELASA defines category D as a person who has the qualifications and experience to carry out the following tasks: manage all animal, human and physical resources; make provisions for health and welfare of animals; provide advice, instruction and assistance to investigators on laboratory animal; ensure compliance with all the laws; be responsible for the development and education programs; contribute to the in-depth development of innovative concepts in the humane care and use of laboratory animals.[28]

Information management

In response to inadequately planned research, incompetent execution of projects, insufficient documentation of methods and results in pre-clinical trials, the Food and Drug Administration (FDA-USA) proposed a regulatory basis for good laboratory practices in 1979 and the Organization for Economic Cooperation and Development (OECD)[29] proposed principles to be adopted internationally in 1981. In Brazil it is the national institute for standards, measurement and industrial quality (INMETRO - Instituto Nacional de Metrologia, Normalização e Qualidade Industrial)[30] that is responsible for accrediting laboratories in accordance with the guidelines laid out by the OECD.

The principles of Good Laboratory Practices (GLP) are not directly related to the progress of scientific projects, but strict adherence to GLP will eliminate many sources of errors and uncertainties. Systematic errors and artifacts can be avoided through the application of technical validity and approved standardized operating procedures,.

Biosafety

When genetically-modified organisms are being used, the directives set out by the Brazilian technical committee on biosafety (CTNBio - *Comissão Técnica Nacional de Biossegurança*).[31] If the risks are known, a safety program can be designed, covering everything from correct airflow in installations to emergency procedures and elimination of waste.

CTNBio guideline number 1 classifies genetically modified animals into four levels of biosafety and animal usage installations are also classified on the same scale. The biosafety level of animal houses and laboratories must always be greater than or equal to the highest biosafety level of the genetically modified animals being used. Accreditation of animal houses and laboratories is the responsibility of institutions' internal biosafety commissions (CIBio) which must provide this information to the CTNBio in their annual reports.

Diet

Diet has a profound impact on experimental results, not only in terms of providing sufficient nutrients, but also as a result of non-nutrients. Both animal and vegetable ingredients can contain a range of physiologically active substances, naturally or as a result of contamination. Some of those with relevance for animal experimentation are: mycotoxins, nitrosamines, pesticides, organic forms of heavy metals, saponins and phytoestrogens, many of which have carcinogenic properties.[14]

The content/composition of the diet should always be investigated for its effective potential for absorption. For example, proteins derived from feathers are less absorbable than those from other sources. Essential amino acids must also be present in sufficient quantities and in the correct proportions.

Anesthetic considerations

Mice and rats

Precautions that should be taken with rodents prior to administering anesthetic include a review of the sanitary history of the colony and an assessment of animals' physical appearance. The most common administrative route for anesthetic agents is parenteral. Drug volume, administration location and irritant properties of the agent should be taken into consideration. In order to minimize errors with intraperitoneal injections, 4 hours' fasting and a 20-22 needle recommended. Water should not be restricted. Table 2 presents the dosages, administration routes and main precautions for anesthetics, according to the Johns Hopkins University "blue book".[32]

Rabbits

In this species, the cecum can act as an anesthetic reservoir, affecting the effect of drugs. It is recommended that dosages are calculated on the basis of metabolic weight (body weight x 0.75). Age, sex, race and strain, body weight and time of day all affect the response to anesthetic drugs. There is no need for fasting because rabbits have a high metabolic rate and low risk of vomiting. Cannulae with a diameter of 2 to 4 mm recommended for endotracheal intubation, which should only be performed by trained personnel since the degree of difficulty is high and local anesthetic must be applied to the larynx to prevent laryngospasm. Animals anaesthetized with barbiturates or other agents that reduce respiratory function will require oxygen supplementation. The depth of anesthesia is assessed by the response to ear-pinch and by palpebral and corneal reflexes. Rabbits have high concentrations of catecholamines in their circulation and sudden sentience can increase the concentrations of catecholamines in circulation and cause fatal cardiac arrhythmia.[32]

Pigs

Pigs are very sensitive to restraint and to anesthesia. In some cases these events cause malignant hyperthermia which provokes muscle rigidity, tachypnea, tachycardia and rectal hyperthermia followed by dyspnea, cardiac arrhythmia, apnea and death. Pre-anesthesia preparation must include 12 hours' fasting and 4-12 hours with no water. Deep anesthesia provokes loss of tonus in the mandible and an absence of response to pinching the interdigital membrane. Ocular reflexes are not a good indicator of depth of anesthesia. Endotracheal intubation and oxygen supplementation in these animals. For prolonged surgical procedures it is recommended that the anesthetic be combined with analgesics and neuromuscular blocking agents.

Postoperative care and analgesia

The recovery room should be heated, noise-free and dimly lit. Suppression of suffering and pain must be one of the priorities in animal experimentation. Invasive surgical procedures are prone to causing pain and post-operative stress can seriously

Table 2 - Anesthetic agents, routes of administration and principal precautions for mice, rats, rabbits and pigs (Partially extracted from Use of Experimental Animals at Johns Hopkins University32)

Anesthetic agent	Dosage	Precautions
MICE		
Ketamine hydrochloride (C) [Ketalar®, Ketamin®] + xylazine hydrochloride (X) [Rompum®, Anasedan®]	100 mg/kg (C) IM + 5 -16 mg/kg (X) IP 80-100(C) mg/kg + 10 (X) mg/kg IP	Ketamine inhibits blinking, ocular lubrication is needed to project against corneal ulceration. Anesthesia time is 60-100 minutes and anesthetic depth varies.
Ketamine hydrochloride + Xylazine hydrochloride+ Acepromazine [Acepran®]	100 mg/kg (C) +20mg/kg (X)+3mg/kg (A) IP	Excellent survivability and reliable depth of anesthesia when compared to other combinations. The dose varies with the mouse strain being used.
Pentobarbital	60 mg/kg IP	Duration of effect is highly variable between different strains, sexes and ages.
Dexmedetomidine hydrochloride (M) [Precedex®] + fentanyl (F) [Fentanest®]	0.6mg/kg (M) + 0,06 mg/kg (F) IM	Duration of effect is between 20 and 30 minutes.
Ketamine hydrochloride + Dexmedetomidine hydrochloride (M)	Males: 50mg/kg (C) + 10 mg/kg (M) IP Females: 75 mg/kg (C) + 1-2.5 mg/kg (M) IP	This combination produces light anesthesia and good immobilization. For retroorbital bleeding, local ophthalmic anesthetic can be used.
RATS		
Acepromazine	0.5-1.0 mg/kg IM 2.0-5.0 mg/kg SC, IP	Provokes sedation in combination with ketamine.
Atropine sulphate	0.05-0.10 mg/kg SC 0.4 mg/kg SC, IM	Some strains have atropinesterase in serum
Ketamine hydrochloride + xylazine hydrochloride	75-95 mg/kg (C) + 5 mg/kg (X) IM or IP 40-75 mg/kg (C) + 5-10 mg/kg (X) IP	Anesthesia
Ketamine hydrochloride + Acepromazine (A)	75 mg/kg (C) + 2,5 mg/kg (A) IP	Anesthesia
Ketamine hydrochloride + Dexmedetomidine hydrochloride	75 mg/kg (C) + 0,5 mg/kg (M) IP	Anesthesia
Pentobarbital	30-45 mg/kg IP	Diluted in saline at less than 10 mg/kg

RABBITS		
Pre-anesthetic		
Glycopyrrolate [Robinul®]	0.01-mg/kg IV 0.1mg/kg IM, SC	Duration 60 min. Some rabbits produce atropinesterase. Effective anticholinergic agent in rabbits
Diazepam [Valium®, Dienpax®]	0.5-5 mg/kg	Cardiovascular side effects are minimal when used alone.
Midazolam [Dormonid®]	0.01-0.1 mg/kg IM, IV	Can mix with other drugs or solutions.
Anesthetic		
Ketamine hydrochloride + Xylazine hydrochloride	35-50 mg/kg IM (C) + 5-10 mg/kg IM (X) or 10 mg/kg IV © + 3 mg/kg IV (X) Continuous IV infusion: 25 mg/kg (C) + 5mg/kg (X). Give the first 1/3 over 1 min, and remainder slowly over the next 4 min..	Respiratory depression, hypotension and hypoxemia are common. Not suitable for intrathoracic and intraabdominal procedures. Local injection can cause irritation. Infusion provokes good muscle relaxation and analgesia, moderate depression of respiratory and heart rate and severe hypotension.
Ketamine hydrochloride + Xylazine hydrochloride + Butorphanol [Stadol®]	35 mg/kg + 5 mg/kg + 0,1 mg/kg IM	Longer loss of reflexes and less hypotension than with K/X combination.
Ketamine hydrochloride + Dexmedetomidine hydrochloride	25 mg/kg + 0.5 mg/kg (M)	Duration 90-180 min. Low mortality
Pentobarbital	20-60 mg/kg IV	Perivascular infiltration will result in local tissue necrosis. Opioids modify the duration of action of Pentobarbital.
Thiopental		Perivascular infiltration will result in local tissue necrosis.
Pigs		
Pre-anesthetic		
Atropine sulphate	0.07-0.09 mg/kg IM	Administer 15-30 min before induction of anesthesia
Acepromazine	0.11-0.22 mg/kg IM	
Glycopyrrolate	0.004-0.01mg/kg IM	Duration 30 minutes.
Ketamine hydrochloride + Acepromazine	33 mg/kg +1.1mg/kg IM	Duration 30 minutes, provokes moderate cardiodepression.
Ketamine hydrochloride + Xylazine hydrochloride	20 mg/kg IM + 2 mg/kg IM	An anticholinergic is recommended to overcome the cardiodepression provoked by the xylazine. Makes endotracheal intubation possible.
Anesthetic		
Thiopental	6.6-25mg/kg IV 3-6 mg/kg/h continuous IV infusion	Very short action, eliminated by the kidneys, severe cardiopulmonary depression
Pentobarbital	20-40 mg/kg IV 5-15 mg/kg/h continuous IV infusion	20-30 min duration of action, metabolized by the liver, provokes more cardiodepression than thiopental, slow recovery Using ketamine as pre-anesthetic increases the duration of the effect of pentobarbital.
Propofol	Induction with 0.83-1.66 mg/kg IV followed by boluses of 14-20mg/kg/h	Requires pre-induction. Used in cardiovascular protocols.

Table 3 - Analgesics, doses and administration routes for mice, rats, rabbits and pigs (Partially extracted from Use of Experimental Animals at Johns Hopkins University[31])

Agent	Dose	Recommendation
MICE		
Buprenorphine [Subutex®, Temgesic®]	0.05-0.15 mg/kg, SC every 6-12h	For discrete to moderate pain. Duration of effect 3-5 h..
Butorphanol [Turbogesic®, Stadol®]	1.0-2.0 mg/kg SC every 4h	For moderate pain, less effective analgesia lasting 1-2 hours.
Morphine sulphate [Dimorf®]	0.98 mg/kg SC 2-5 mg/kg IM, SC every 4h	For intense pain.
RATS		
Acetominophen [Tylenol®]	1-2 mg/ml in drinking water	For discrete pain.
Acetylsalicylic acid [Aspirin®, Melhoral®]	100-150 mg/kg oral	For discrete pain, every 4 hours.
Buprenorphine	 0.01-0.05 mg/kg SC 0.02-0.5 mg/kg SC, IP, IM	For moderate pain. 8-10 hours' duration 6-12 hours' duration
Carprofen [Rimadyl®]	5-10 mg/kg SC, oral	For moderate pain.
Morphine sulphate	2.5-5.0 mg/kg SC	For intense pain, 2-4 hours' duration.
RABBITS		
Buprenorphine	0.01-0.05 mg/kg SC, IV	Duration 6-12 hours. Recommended for moderate pain.
Acetaminofenol	1 ml /100 ml in drinking water	Recommended for moderate pain.
Morphine sulphate	2-5 mg/kg SC or IM	Duration 2-4 hours. Provokes powerful analgesia with medium sedation and respiratory depression.
PIGS		
Acetylsalicylic acid	10 mg/kg oral route	For moderate pain, to be given with a stomach protector. Can be given in sweet syrup.
Buprenorphine	0.01mg/kg IV every 6 hours or 0.02mg/kg IV every 10h 0.005-0,1mg/kg every 12h IM	Less effective as analgesic in cases of inflammation, organ failure, or systemic disease.
Fentanyl	0.05mg/kg IM every 2 hours 50-100 μg/kg/h IV	For mini-pigs the transdermal route should be used.
Lidocaine and prilocaine cream	Topical, apply 2 mm of cream to skin 45 min prior to procedure	Prevents pain associated with taking blood and intravenous injections in the ear.
Ketaprophen[Profenid®]	1-3mg/kg orally every12h	Potent non-selective COX inhibitor, good analgesic and anti-inflammatory.

compromise animal welfare. Temperature should be around 25ºC to avoid hypothermia. International committees recommend round-the-clock nursing for the first 48 hours. Assessments for post-operative pain should be run systematically to determine whether animals require analgesics. These assessments involve a large number of indicators such as: evaluation of motor activity; changes in appearance, such as curling up, hairs standing on end, and secretions from the eyes or nose; changes in temperament, increases in aggression, reluctance to interact; changes to sounds emitted, gnashing or grinding of teeth, increase or reduction in sounds emitted; changes in the amount of food or water consumed, weight loss, reduced urinary and fecal output; and physiological changes to heartbeat, respiratory rate, blood pressure, oxygen saturation and skin color. The surgical site should be assessed for erythema, edema, discharges and etc.[33]

The use of opioids with pigs subjected to experimental surgery illustrates the impact of analgesia on experimental results. Two groups of pigs were both given the same pre-treatment and anesthetic, but one group also received epidural morphine prior to surgery and post-operative fentanyl transdermally. The second group recovered more quickly, gained more weight and had significantly different cortisol concentration immediately after surgery, although endorphin concentrations did not exhibit any difference between the groups.[34]

The American College of Anesthesiologists[35] recommend calculating an acute pain score. Clinical judgment should be used to determine whether drug administration is needed. Recommended analgesics, their doses and routes of administration are given in Table 3, broken down by species.[32]

Euthanasia

Euthanasia is humane killing with a minimum of pain, fear and anguish and must be included in the experimental protocol and carried out at the end of the experiment. Notwithstanding, the legal and moral obligation to protect animal welfare and minimize discomfort must be safeguarded by means of a surveillance system, i.e., charts for recording clinical changes and pain scores and in order to identify problems and decided the point at which severely affected animals should be euthanized in order to avoid unnecessary suffering, even if this is prior to the date laid out in the protocol. Assessment should be based on weight loss, deterioration of clinical status and on specific symptoms related to the disease or condition induced. Animal welfare is a prerequisite for more realistic experimental results and so it is necessary to employ procedures that reduce animals' suffering and improve their welfare.[36,37]

Euthanasia agents, classifications, mechanisms of action, speed, ease of use, biosafety, indications per species, efficacy and comments have all been presented in detail in the AVMA Guidelines on Euthanasia.[38]

Conclusions

An animal's dramatype,[39] which is made up of its genotype, phenotype and environment, this last including all of the items discussed above, in combination with well-planned research projects, the use of sensitive techniques for detecting biological differences and appropriate data collection and statistical analysis, are all prerequisites for reducing the number of animals used in a study and for obtaining results with a high degree of acuity, reproducibility and precision.

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References

1. Russell WMS, Burch KL. The principles of humane experimental technique. UFAW, London: UFAW; 1992. [cited 2008 jan]. Available from: http://altweb.jhsph.edu/.
2. Festing MF, Altman DG. Guidelines for the design and statistical analysis of experiments using laboratory animals. ILAR J. 2002;43:244-58.
3. Puopolo M. Biostatistical approaches to reducing the number of animals used in biomedical research. Ann Ist Super Sanità. 2004;40:157-63.
4. Scheibe PO. Number of samples - hypothesis testing. Nucl Med Biol. 2008;35:3-9.
5. Eckelman WC, Kilboum MR, Joyal JL, Labiris R, Valliant JF. Justifying the number for each experiment. Nucl Med Biol. 2007;34:229-232.
6. Brasil. Presidência da República. Lei nº 11.794, de 8 de outubro de 2008. Regulamenta o inciso VII do parágrafo 1º do artigo 225 da Constituição Federal, estabelecendo procedimentos para o uso científico de animais; revoga a Lei nº 6.638, de 8 de maio de 1979; e dá outras providências.
7. Brasil. Presidência da República. Decreto nº 6.899, de 15 de Julho de 2009. Dispõe sobre a composição do Conselho Nacional de Controle de Experimentação Animal – CONCEA, estabelece normas para o seu funcionamento e de sua Secretaria Executiva, cria o Cadastro das Instituições de Uso Científico de Animais – CIUCA, mediante a regulamentação da Lei 11.794, de 8 de outubro de 2008, que dispõe sobre procedimentos para o uso científico de animais, e dá outras providências.
8. São Paulo. Lei Estadual nº 11.977, de 25 de agosto de 2005, institui o código de proteção aos animais do Estado de São Paulo e dá outras providências.
9. São Paulo. Lei Municipal nº 13.943, de 29 de dezembro de 2004, proíbe a entrega de animais capturados nas ruas para instituições e centros de pesquisa e ensino.
10. Marques RG, Miranda ML, Caetano CER, Biondo-Simões MLP. Rumo à regulamentação da utilização de animais no ensino e na pesquisa científica no Brasil. Acta Cir Bras. 2005;20:262-7.
11. Florianópolis. Decreto Municipal 5501, 2008, regulamentou a Lei 7486/2007.
12. Goldenberg S, Goldenberg A. Ferreira LM, Montero EDS. Clinical research. Acta Cir Bras. 2005;20:270-1.
13. NRC. National Research Council. Manual sobre cuidados e usos de animais de laboratório. Editores: AAALAC & COBEA. Goiânia: Gráfica UFC; 2003.
14. Poole TB. The UFAW Handbook on the care and management of laboratory animals. London: Longman Scientific & Technical; 1999.
15. Swindle MM, Smith AC, Laber-Laird K, Dungan L. Swine in biomedical research: management and models. ILAR J. 1994;36:39.
16. Mariano M. Minisuíno (minipig) na pesquisa biomédica experimental. O Minipig br. Acta Cir Bras. 2003;18:387-91.
17. Heine WO. Environmental management in laboratory animal units. Berlim: Pabst Science Publichers; 1998.
18. Ruys T. Handbook of facilities planning. New York: Van Nostrand Reinhold; 1991.
19. Teixeira MA, Chaguri LCAG, Carissimi AS, Souza NL, Mori CMC, Saldiva PHN, et al. Effects of an individually ventilated cage system on the airway integrity of rats (Rattus norvegicus) in a laboratory in Brazil. Lab Anim. 2006;40:419-31.
20. Burn CC, Peters A, Day MJ, Mason JG. Long-term effects of cage-cleaning frequency and bedding type on laboratory rat health, welfare, and handleability: a cross-laboratory study. Lab Anim. 2006;40:353-70.
21. Kolaczkowska E, Chadzinska M, Seljelid R, Plytycz B. Strain differences in some immune parameters can be obscured by circadian variations and laboratory routines: studies of male C57BL/6J, Balb/c and CB6 F1 mice. Lab Anim. 2001;35:91-100.
22. Voipio HM, Nevalainen T, Halonen P, Hakumaki M, Bjork E. RoLe of cage material, working style and hearing sensivitiy in perception of animal care noise. Lab Anim. 2006;40:400-9.
23. Nevalainen TO, Nevalainen JI, Guhad FA, Lang CM. Pair housing of rabbits reduces variances in growth rates and serum alkaline phosphatase levels. Lab Anim. 2007;41:432-40.
24. Van Loo PLP, Kuin N, Sommer R, Avsaroglu H, Pham T, Baumans V. Impact of living apart together on postoperative recovery of mice compared with social and individual housing. Lab Anim. 2007;41:441-55.
25. Capdevila S, Giral M, Ruiz de La Torre JL, Russell RJ, Kramer K. Acclimatization of rats after ground transportation to a new animal facility. Lab Anim. 2007;41:255-61.

26. Meijer MK, Sommer R, Spruijt BM, Van Zutphen LFM, Baumans V. Influence of environmental enrichment and handling on the acute stress response in individually housed mice. Lab Anim. 2007;41:161-73.
27. FELASA - Federación de Asociaciones Europeas de las Ciências del Animal de Laboratório. Estudios y la Formación de las Personas que trabajan com Animales de Laboratorio: Categorias A y C. [cited 2008 feb 13]. Available from: http://www.hulp.es/secal/secal.html.
28. FELASA – Federation of Laboratory Animal Science Associations. Guidelines for education of specialists in laboratory animal science (Category D). Lab Anim. 1999;33:1-15.
29. OEC/WHO. World Health Organization. Special Programme for Research and Training in Tropical Diseases. Good Laboratory Practice GLP. Quality Practices for regulated non-clinical research and development. [cited 2008 mar 15]. Available from: http://www.oecd.org/ehs.
30. INMETRO. Instituto Nacional de Metrologia, Normalização e Qualidade Industrial. [citado 16 mar 2008]. Disponível em: http://www.inmetro.gov.br/credenciamento/descricaoOrgs.asp#laboratorio.
31. Brasil. Presidência da República. Lei nº 11.105, de 24 de março de 2005. Regulamenta os incisos II, IV e V do parágrafo 1º do art. 225 da Constituição Federal, estabelece normas de segurança e mecanismos de fiscalização de atividades que envolvam organismos geneticamente modificados – OGM e seus derivados, cria o Conselho Nacionalde Biossegurança – CNBS, reestrutura a Comissão Técnica Nacional de Biossegurança – CTNBio, dispõe sobre a Política Nacional de Biossegurança – PNB.
32. John Hopkins University Animal care and use. [cited 2009 oct 2009]. Available from: http://web.jhu.edu/animalcare/rdf/index.html.
33. Stasiak KL, Maul D, French E, Hellyer PW, Vandewoude S. Species-specific assessment of pain in laboratory animals. Contemp Top. 2003,42:13-9.
34. Malavasi LM, Nyman G, Augustsson H, Jacobson M, Jensen-Waern M. Effects of epidural morphine and transdermal fentanyl analgesia on physiology and behaviour after abdominal surgery in pigs. Lab Anim. 2006;40:16-27.
35. Swindle MM. Pain assessment in swine. Technical Bulletin. [cited 2009 oct 7]. Available from: http://www.sinclairbioresources.com/Downloads/TechnicalBulletins/Pain%20Assessment%20in%20Swine.pdf.
36. FELASA - Federacion de Asociaciones Europeas de las Ciencias de Animal de Laboratorio. Recomendaciones para la eutanasia de los animales de experimentacion. [citado 13 fev 2008]. Disponível em: www.hulp.es/secal/secal.html.
37. Morton DB. Ethical aspects of the use of animal models of infection. In: Zak O, Sand MA, editors. Handbook of animals models of infection. San Diego: Academic Press; 1999. p.30-48.
38. AVMA Guidelines on Euthanasia. [cited 2008 may]. Available from: http://www.avma.org/issues/animal_welfare/euthanasia.pdf.
39. Obrink KJ, Rehbinder C. Animal definition: a necessity for the validity of animal experiments Lab Anim. 2000;34:121-30.

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