Chapter Q
Guidelines for the Care and Use of Laboratory Animals[1]

[1] By A.W. Herling, J. Maas, K. Seeger (1st edition), revised by U. Albus (2nd and 3rd edition).

Q.1 Regulations for the Care and Use of Laboratory Animals in Various Countries

See Table Q.1 on the following page.

Q.2 Techniques of Blood Collection in Laboratory Animals

Q.2.1 Introduction

Blood is collected from laboratory animals for various scientific purposes, for example, to study the effects of a test drug on various constituents, such as hormones, substrates, or blood cells. In the field of pharmacokinetics and drug metabolism, blood samples are necessary for analytical determination of the drug and its metabolites. Blood is also needed for some *in vitro* assays using blood cells or defined plasma protein fractions.

The techniques for blood collection depend on specific factors which differ from one experiment to the other. There is a difference between terminal and non-terminal blood collection techniques. The conditions of blood collection at the end of an experiment which includes death of the animal (terminal experiment) are completely different (anesthesia, volume of blood)

Table 1 Regulations for the care and use of laboratory animals

Country, Institutions	Legislation
Australia National Health and Medical Research Council	Australia is governed by a national Government, six State Governments, and two Territory Governments. Animal welfare within Australia is a State/Territory responsibility.
Commonwealth Scientific and Industrial Research Organisation	The Australian Animal Welfare Strategy (AAWS) from 2005
Australian Agricultural Council	http://www.daff.gov.au/ http://www.animallaw.info/
Austria Ministry of Agriculture	Bundesgesetz über den Schutz der Tiere (TSchG), BGBl. I Nr. 118/2004 http://www.vu-wien.ac.at/vetrecht/TSchG%20BGBl.pdf
Belgium	In 1991 the provisions of the ETS 123 convention were enacted in the Belgian legislation through the "Law of 18 October 1991 approving the European Convention on protection of vertebrate animals used for Experimental or other Scientific Purposes and its Annexes A and B done at Strasbourg on 18 March 1986". In 1994 the "Royal Decree of 14 November 1993 related to the protection of experimental animals" was enacted.
Canada	Canadian Council on Animal Care http://www.animallaw.info
China (Peoples Republic of China)	Regulations for the Administration of Affairs Concerning Experimental Animals, Nov. 1988
State Science and Technology Commission, Beijing	Implementing Regulations of the Administration on Medical Experiments on Animals, June 1989 http://www.nal.usda.gov/awic/legislat/internat.htm http://www.animallaw.info/
Denmark	LBK (Lovbekendtgørelse) nr. 726 af 9. sept. 1993: Bekendtgørelse om lov om dyreforsøg A minor change has been added to the above about animals that are released in connection to an experiment: • LOV nr. 386. 6 juni 1991 (animal protection law) • LOV nr. 1081 af 20. dec. 1995: Lov om ændring af lov om dyreforsøg • BEK (Bekendtgørelse) nr. 332 af 18. maj 1990: Bekendtgørelse om fremskaffelse af dyr til forsøg • BEK nr. 27 af 22. jan. 1996: Bekendtgørelse om ændring af bekendtgørelse om fremskaffelse af dyr til forsøg • BEK nr. 716 af 1. aug. 1994: Bekendtgørelse om forretningsorden for Rådet for Dyreforsøg • BEK nr. 333 af 18. maj 1990: Bekendtgørelse om forsøgdyrs pasning of opstaldning of om udryddelsestruede og vildtlevende dyr til forsøg m.v. • BEK nr. 739 af 6. dec. 1988: Bekendtgørelse over og indberetning om dyreforsøg Legislation revised in 1993 in conformity with EU directives http://www.retsinfo.dk/
European Union	Council of Minister's Directive 86/609/EEC (1986) on the Approximation of Laws, Regulations and Administrative Provisions of the Member States Regarding the Protection of Animals Used for Experimental and other Scientific Purposes http://europa.eu.int/ European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes. December 15, 1990 http://www.uku.fi/laitokset/vkek/Sopimus/convention.html
Federal Republic of Germany	Neufassung des Tierschutzgesetzes vom 18. Mai 2006
Ministry for Food, Agriculture and Forestry	Richtlinie vom 24. November 1986 zur Annäherung der Rechts- und Verwaltungsvorschriften der Mitgliedstaaten zum Schutz der für Versuche und andere wissenschaftliche Zwecke verwendeten Tiere (86/609/EWG) Gesetz zu dem Europäischen Übereinkommen vom 18. März 1986 zum Schutz der für Versuche und andere wissenschaftliche Zwecke verwendeten Wirbeltiere vom 11. Dezember 1990 http://www.bml.de/ and http://www.uni-giessen.de/tierschutz/

Table 1 (continued)

Country, Institutions	Legislation
Finland	Act on Animal Protection (247/1996, amend. 1194/1996 and 594/1998), Statute on Animal Protection (396/1996, amend. 402/1998), Statute on the Transport of Animals (491/1996, amend. 1398/1997 and 955/1998) and Statute on Animal Experimentation (1076/1985, amend. 395/1996)http://www.nca-nl.org/
France	No uniform national animal welfare law, but several decrees. "Loi à la protection de la nature", 10 juillet 1976 "Décret aux expériences pratiquées sur les animaux" 19 octobre 1987 Arrêtés interministériels (3 arrêtés), 19 avril 1988 http://www.nca-nl.org/
Italy	Legislative Decree no. 116 of January 27, 1992, enforcing European Recommendations contained in Directive 86/609/EEC Specific Law of October 12, 1993 http://www.nca-nl.org/
Ireland Department of Agriculture and Food	Cruelty to Animals Act, 1876 European Communities (Amendment of Cruelty to Animals Act, 1876) Regulations, 1994 http:/www.agriculture.gov.ie/
Japan Prime Minister authorized to set standards for minimum pain provision	Law Concerning the Protection and Control of Animals (Law No. 105, October 1, 1973, revised June 15, 2005) http://www.alive-net.net/english/en-law/L3-outline.html
New Zealand Animal Welfare Advisory Committee (AWAC), established in 1989 by the Minister of Agriculture	Animal Welfare act 1999, Commenced January 1, 2000 http://rangi.knowledge-basket.co.nz/gpacts/public/text/1999/an/142.html The codes of recommendations and minimum standards for the welfare of animals, endorsed as a national code on 25 May 1995 http://www.maf.govt.nz/mafnet/profile/legislation/
Netherlands Minister of Health, Welfare and Sport	Dutch experiments on animals act entered into force on 5 February 1997 http://www.nca-nl.org/
Norway Ministry of Agriculture	Norwegian Animal Welfare Act, 1974 New version of June 16, 1995 http://www.dyrebeskyttelsen.no/english/law.shtml
Republic of Czechia Czech National Assembly	"Law for the Protection of Animals" no 246 Sb, 15. April 1992, inclusive the amendments no. 162 Sb, 19. May 1993 http://www.nca-nl.org/
South Korea 1. Ministry of Agriculture, Fishery and Forestry 2. Ministry of Health and Welfare	1. "Animal Protection Act", May 1991 2. "Pharmaceutical Affairs Law" Korean Good Laboratory Practice Guidelines for Breeding and Caretaking of Test Animals
Spain	Real Decreto 223/1988 http://www.boe.es
Sweden The Swedish National Board for Laboratory Animals (CFN) Ministry of Agriculture, Stockholm	National Board for Laboratory Animals Ordinance Amending the Board's Ordinance (LSFS 1988:45, as last amended by SFS 1998:1344 of October 22, 1998) Containing Regulations and General Recommendations Concerning Ethical Examinations of the Use of Laboratory Animals for Scientific Purposes, etc. http://www.nca-nl.org/
Switzerland	"Tierschutzgesetz vom 9. März 1978 (TSchG), under revision in 2006 "Tierschutzverordnung", 27. Mai 1981 http://www.bvet.admin.ch/
Taiwan Council of Agriculture; In future: Provincial/ Municipal Governments	Taiwan Animal Protection Law, November 4, 1998. Very generally, so that additional regulations are going to be required. http://www.gio.gov.tw/info/98html/aplaw.htm

Table 1 (continued)

Country, Institutions	Legislation
United Kingdom	"Protection of Animals Act", 1991 "Animals (Scientific Procedures) Act", 1986 http://scienceandresearch.homeoffice.gov.uk/animal-research/legislation/
U.S.A. U.S. Department of Agriculture, Animal and Plant Health Inspection Service (APHIS), Animal Care (AC)	Guide for the Care and Use of Laboratory Animals, January 1996 http://www.nap.edu/readingroom/books/labrats/ The Animal Welfare Act, signed into law in 1966, amended four times (1970, 1976, 1985, 1990), can be found in United States Code, Title 7, Sections 2131 to 2156 http://www.nal.usda.gov/awic/legislat/usdaleg1.htm
Most countries	Local ethical commisions at universities and other institutions of biomedical research Useful Internet-links on international animal protection: http://www.nca-nl.org/ http://www.animallaw.info/ http://www.nal.usda.gov/awic/legislat/internat.htm http://www.uni-giessen.de/tierschutz/

from those of single or repeated blood collections from a conscious animal. Terminal blood collection under anesthesia allows the use of techniques which are not acceptable for non-terminal blood collections.

Q.2.2 Aspects of Animal Welfare

Minimizing any pain and distress in laboratory animals during the procedure have to be as important as achieving the desired experimental results. This is important not only for humanitarian reasons but also as part of good scientific practice. Blood collection may be stressful to the animal due to the handling and the discomfort associated with a particular technique. Many biochemical and physiological changes are associated with stress which affect the results, e. g. increases in the blood levels of catecholamines, prolactin and glucocorticosteroids can influence certain metabolic parameters, such as glucose, as well as the counts of erythrocytes, white cells, and packed cell volume. Therefore, stress should be reduced to an absolute minimum if it is not possible to avoid it at all, this is not only in the interest of animal welfare but also in the interest of good science to obtain representative data. To minimize stress during blood collection, e. g. from dogs or cats, it may be helpful for the animal as well as for the operator first to do some dummy runs and provide rewards to the animal.

During non-terminal blood collection it is important not to withdraw too much blood which could reduce total blood volume and lead to false results. A reduced total blood volume is accompanied by a reduced hemoglobin content and oxygen transport capacity (Gainer et al. 1955) as well as by a fall in blood pressure, and an increase in the concentrations of stress-related hormones. It may be further accompanied by other factors such as necrosis of the gastric mucosa.

The welfare of the individual animal should not be endangered by removal of too large a volume of blood or by too frequent collections. This may be the case more often when small laboratory animals, e. g. mice, gerbils, rats or hamsters are used. In these cases the study protocol should be adapted to use more animals to minimize distress for the individual animal.

Q.2.3 Total Blood Volume

The total blood volume is very difficult to determine (McGuill et al. 1989) and depends on species, sex, age and health as well as nutritional condition. Total blood volume is smaller in larger animals than in smaller animals of the same species in relation to body weight. It is also smaller in older and obese animals compared to normal weight and young animals. Total circulating blood volume is in the range of 55–70 ml/kg body weight.

Q.2.4 Terminal Blood Collection

Terminal blood collection represents (i) exsanguination as a single process of blood removal to collect as much blood as possible and (ii) multiple blood sampling during a terminal experiment under general anesthesia. Basically, exsanguination should only be performed after the animal has been rendered unconsciuos by another method, e. g. physical stunning or general anesthesia. This is due to the fact that stress occurs

with extreme hypovolemia and accessing deeper blood vessels causes pain. Due to the anesthetized condition of the animal and the terminal nature of the experiment, methods can be used for exsanguination which can never be recommended for non-terminal blood collections with recovery of the animal. These include

- blood withdrawal from the V. cava caudalis or the aorta after laparatomy when as much blood as possible should be removed in a sterile manner,
- exsanguination after decapitation, incision of the jugular vein or carotid artery or techniques in the slaughterhouse, when a non-sterile collection is possible,
- retro-orbital bleeding of smaller laboratory animals like mice, gerbils, hamsters and rats which can also be a method of exsanguination.

Q.2.5 Non-terminal Blood Collection

Non-terminal blood collections can be differentiated into single and multiple blood withdrawals. Possible peripheral veins for blood withdrawal are listed in Table Q.2.

Q.2.5.1 Single Blood Removal

A single withdrawal of up to 15% of total blood volume does not influence the well-being of the animal. However, the removal of 15 to 20% might be accompanied by side effects such as fall in cardiac output or blood pressure. Haemorrhagic shock can be induced by the withdrawal of 30–40% of total blood volume and the loss of 40% causes mortality in up to 50% of pigs and rats (McGuill et al. 1989).

A single removal of up to 15% of total blood volume may be repeated after 3–4 weeks from normal and healthy animals with no detectable adverse effects. This does not mean that the animal does not experience any adverse effects, but it does not show any.

Symptoms of hypovolaemic shock are fast pulse, pale mucous membranes, hyperventilation and a subnormal body temperature including cold skin and extremities. In these animals therapeutic intervention consists of volume substitution with warm isotonic intravenous infusion.

Q.2.5.2 Multiple Blood Removal

Multiple withdrawal of blood samples should not exceed 1% of total blood volume every 24 h (0.6 ml/kg/d). More frequent withdrawals and/or removal of larger volumes of blood causes anemia.

Symptoms of anemia are pale mucous membranes of the conjunctiva or inside the mouth, intolerance to exercise and an increased respiratory rate in cases of severe anemia. Anemia can be easily detected by de-

Table 2 Blood vessels for venous blood withdrawal

Species	V. coccygica + tt	V. auricularis	Orbital venous	V. jugularis	V. cephalica V. saphena	V. femoralis	V. mammarica
Mouse	cc + cc	–	a	–	–	–	–
Gerbil	cc + cc	–	aa	a	–	–	–
Hamster	–	—	aa	a	–	–	–
Rat	ccc + cc	–	a	aa	–	–	–
Guinea pig	–	c	–	a	–	–	–
Rabbit	–	ccc	–	c	–	–	–
Cat	–	–	–	aa/c	ccc	cc	–
Dog	–	–	–	ccc	ccc	a	–
Rhesus monkey	–	–	–	aa/c	ccc	cc	–
Pig	–	iii	–	iii/cc(cvc)	–	–	–
Sheep	–	–	–	ccc	c	– c	–
Goat	–	–	–	ccc	c	–	– c
Cattle	cc	c	–	ccc	c	–	– cc
Horse	–	–	–	ccc	–	–	–

c/cc/ccc conscious animal, c/i/a possible alternative, i/ii/iii immobilized animal, – not recommended or impossible, a/aa/aaa anaesthetized animal, cvc cranial vena cava, ccc/iii/aaa recommended route, tt amputation tail tip, cc/ii/aa acceptable route repetition of letters indicates the preferred condition

termination of erythrocyte cell count and packed cell volume (haematocrit), hemoglobin level as well as reticulocyte count in a blood sample. In case of anemia the animal should be treated with iron and vitamin B12 and should be monitored for the above mentioned blood parameters during therapy until normal values are reached again.

Q.2.6
Technical Aspects of Blood Removal

A common method in mice and rats for collecting up to 0.1 ml capillary blood is to remove the tip of the tail. For repeated blood sampling the blood clot on the tail has to be removed to get fresh capillary blood. This method is sufficient for multiple blood collections to determine, e. g. blood glucose or total radioactivity after the administration of radiolabeled drugs. In tailless animals such as guinea pigs and hamsters, cardiac puncture under general anesthesia may be the preferred technique.

Blood collections from larger animals will preferably be performed from a superficial vein. The person holding the animal and raising the vein plays a key role in collecting blood without undue stress to the animal by talking to and stroking the animal. Some animals, e. g. dogs and some primates, may be trained to present a limb for blood removal without the use of any physical restraint.

It is important to locate the vessel accurately before insertion of the needle or the catheter. In most cases obstruction of the venous return is necessary for distension of the vessel and to successfully insert the needle. The bore of the needle should be as large as possible to ensure rapid blood withdrawal with minimal risk of blood clotting within the needle. When the sample is taken too quickly by a syringe, the vein will collapse. After the needle has been withdrawn, continuous pressure should be applied immediately to the puncture site and maintained for at least 30 s. The animal should be monitored 15 min later to check for after-bleeding.

Q.2.6.1
Permanent Venous Cannulation

For multiple blood collections a permanent venous access by chronic cannulation is often recommended. In most cases, particularly in rats, it is necessary to restrain the animal in harnesses or jackets to prevent it from damaging or removing the cannula. In these cases the signs of stress are often apparent by an increase in serum levels of stress hormones. However, a few days after implantation of catheters, hormone levels are normal in restrained rats (Tsukamoto et al. 1984; Wiersma and Kastelijn 1985). Such animals are usually housed alone and the tethering restricts normal movements such as lying on the back and rolling over. Such restrictions may be considered as potential sources of stress. This can be prevented by having the catheter exit the back of the animal for only 2 cm and capping it with a steel needle. At the time of the experiment, a longer catheter is attached for blood collections.

A simple device for serial blood collection has been described by Sir-Petermann et al. (1995).

One has to balance very carefully the distress and discomfort of the individual animal with a permanent cannulation under restraint conditions for a longer period with multiple blood samplings without permanent cannulation. In the first case, multiple values from the same animal can be obtained showing perhaps individual differences among a group of animals. In the latter case it may be necessary to use a larger number of animals but there is less discomfort for the individual animal.

Short-term cannulation (less than a day) of a peripheral blood vessel in larger animals is easy to perform. A butterfly needle can be inserted under aseptic conditions and multiple blood samples can readily be collected. Long-term cannulation (longer than two days) in larger and smaller animals often presents complications such as blockage of the cannula by thrombi. The infusion and administration of substances via the permanent cannula are much easier than the removal of blood. Thrombi attached to the end of the cannula function as a one-way valve. Clotting can be prevented by repeatedly filling the catheter with saline containing heparin.

Q.2.6.2
Retro-Orbital Bleeding

Blood sampling by orbital puncture is a controversial technique. The puncture of the orbital venous plexus is often performed in tailless animals, e. g. hamsters. This technique is also used in rats and mice, when larger volumes are required which cannot be obtained from the tail vein. Basically, retro-orbital bleeding should always be performed under anesthesia. Pasteur pipettes, micropipettes or microcapillary tubes are used and pushed with a rotating movement through the conjunctiva laterally, dorsally or medially of the eye to the back wall of the orbit. In general, inflammatory reactions can be seen histologically in the puncture

track four days after punction. After 4 weeks the lesions have healed without detectable scars (van Herck et al. 1992). However severe side-effects such as retro-orbital haematoma with subsequent pressure on the eye cannot be completely excluded. This pressure can damage the optical nerve. The animal may be unable to close its eye. Bleeding from the orbital venous plexus should only be performed with recovery of the animal in exceptional circumstances when there is no other method available. The technique should be performed only by a well-trained staff and only one eye should be used.

Q.2.6.3
Cardiac Puncture

The collection of blood by cardiac puncture has been performed in guinea pigs, gerbils and hamsters. In these species it is difficult to collect blood by alternative methods except retro-orbital bleeding. In general, cardiac puncture should be performed under general anesthesia with atropine as premedication to prevent cardiac arrhythmia. If cardiac puncture is used for a non-terminal blood withdrawal with recovery, the animal has to be separated from other animals until it is fully conscious. It should be carefully watched for adverse effects and sacrificed if found in distress due to complications like bleeding into the pericardium or into the thorax.

REFERENCES AND FURTHER READING

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Q.3
Anesthesia of Experimental Animals[2]

Q.3.1
Introduction

In biomedical research, experiments should only be done with a conscious animal if it is not possible to do the study in an anesthetized one. Anesthetic conditions should always be chosen to exclude stress, discomfort and pain for the animal which could have negative influences on the pharmacological results and reproducibility of the data. Therefore, an experimental design causing minimal discomfort to the animal is always preferable. This is important not only for humanitarian reasons but also for good scientific practice.

Many pharmacological experiments are performed under anesthesia:

- terminal experiments under anesthesia followed by euthanasia,
- experiments under anesthesia with recovery at the end of the study, and
- experiments in which an animal is surgically prepared under anesthesia and continuation of the experiment occurs with the conscious animal after recovery.

Generally, two possibilities exist for immobilization of aggressive animals and to prevent escape: (i) physical restraint (e. g. immobilization cages or immobilization tubes) and (ii) chemical restraint with anesthetic compounds. As a rule of thumb it is recommended to use physical restraint for animal studies in which no anesthesia would be used in comparable studies in man. Physical restraint can be used for short and painless interventions like administration of substances or blood sampling from a vein.

In general, physical restraint produces fear, distress and anxiety in experimental animals with the result of stress symptoms which could affect the results of the study. To minimize pain for the animals, to obtain correct and reproducible results and to protect the handlers from aggression by the animals, it is often necessary to use chemical restraint. However, the chemical used can affect the biochemistry or physiology of the animal.

It is possible to anesthetize special areas of the animal (local anesthesia) or the whole animal (general anesthesia). Local anesthesia plays only a minor role

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for experimental animals as compared to general anesthesia.

Q.3.2 Local Anesthesia

Local anesthesia is the regional and reversible elimination of pain with chemical compounds. Circulatory, pulmonary and renal functions are not disturbed and the animals are conscious. Surface anesthesia has to be distinguished from the anesthesia produced after a local injection. The most common compounds for surface anesthesia are tetracaine and proparacaine. Procaine, butanilicaine, lidocaine, mepivacaine and etidocaine are commonly used injectable local anesthetics. Local anesthesia is only recommended for gentle and calm animals (cattle, sheep). For most laboratory animals, general anesthesia is the method of choice.

Q.3.3 General Anesthesia

Q.3.3.1 Preparation

It is very important to check the general condition of the animal prior to anesthesia. This check should include a clinical examination (inspection, auscultation, palpation) of the animal concerned. Sometimes it could be useful to perform a few laboratory tests, e. g. hematocrit, hemoglobin, pH-value of blood and acid/base parameters.

Those animals with a vomiting reflex should be fasted prior to anesthesia. Most animal species should be fasted for a period of at least 12 h but pigs and cattle for at least 24 h. Water can be offered during the fasting period ad libitum.

Q.3.3.2 Premedication

Premedication is recommended prior to anesthesia for easier administration of the anesthetic and for elimination of side effects of the anesthetic used, such as disturbing autonomic reflexes.

Q.3.3.2.1 Hydration and Base Excess

Based on hematocrit, hemoglobin and erythrocyte values, the hydration of the animal should be normalized prior to anesthesia. Infusions of glucose or Ringer solution can be used for this purpose. To check the success of the treatment repeated determinations of the above mentioned values are necessary.

In cases of acidosis (pH of blood < 7.36), treatment of the animals with $NaHCO_3$ is recommended. If measurement of base excess is possible, the amount of $NaHCO_3$ can be determined from the following formula:

$$\text{Dose } NaHCO_3 \text{ (ml)} = \text{g body weight} \times \text{base excess}/0.6$$

Q.3.3.2.2 Atropine

To avoid cardiopulmonary problems and to decrease saliva production, atropine should be administered intramuscularly prior to general anesthesia. The recommended dose varies considerably and is usually between 0.05 and 0.1 mg/kg body weight. Cats and rodents have a higher activity of atropine-esterase in the liver, and these species need higher amounts of atropine (up to 0.25 mg/kg).

Q.3.3.2.3 Sedation and Pain Elimination

Indications for sedation and elimination of pain are to calm the animals and to stabilize the autonomic nervous system.

For sedation the following compounds are used:

- minor tranquilizers without autonomic effects:
- meprobamate, diazepam,
- major tranquilizers with autonomic side effects:
- propionyl-promazine, acetylpromazine, azaperone, dehydrobenzperidol, xylazine, detamidone.

Anaesthesia does not necessarily lead to analgesia (elimination of pain). Although general anaesthesia produces loss of consciousness and pain isnot perceived, the noxious stimuli will be transmitted to the CNS and will develop central hypersensitivity, leading to a postoperative heightened perception. To reduce the degree of central hypersensitivity analgesics have to be administered before noxious stimulation begins (preemptive analgesia). Analgesia should also reduce or eliminate peripheral inflammation, which aggravates central hypersensitivity.

For analgesia, the opioids used are mainly:

- methadone, meperidine, fentanyl.

In most species (dog, rabbit, guinea pig) a sedative effect is to be observed after administration of these compounds. In other species (pig, cat) an excitatory effect can occur.

Minor tranquilizers, major tranquilizers and analgesic compounds are often used in common with anes-

Table 3 Anesthesia of experimental animals (values are in mg/kg)

Species	Premedication	Sedation	Short anaesthesia	Medium anaesthesia	Long anaesthesia
Rat	Atropine (0.2 s.c.)	Diazepam (2.5 i.m.)	Alfentanyle + Etomidate (0.03 + 2 i.m.) or Inhalation (Isoflurane)	Xylazine + Ketamine (5 + 100 i.m.) or Pentobarbitone (50 i.p.)	Xylazine + Ketamine (16 + 100 i.m.) or Urethane (1500 i.m.)
Mouse	Atropine (0.1–0.25 s.c.)	Diazepam (5 i.p.)	Alfentanyle + Etomidate (0.03 + 2 i.m.) or Inhalation (Isoflurane)	Xylazine + Ketamine (5 + 100 i.m.) or Pentobarbitone (50 i.p.)	Xylazine + Ketamine (16 + 100 i.m.)
Hamster	Atropine (0.1–0.2 s.c.)	Diazepam (5 i.p.)	Inhalation (Isoflurane)	Xylazine + Ketamine (5 + 50 i.m.) or Pentobarbitone (35 i.p.)	Xylazine + Ketamine (10 + 200 i.m.)
Guinea pig	Atropine (0.1–0.2 s.c.)	Diazepam (2.5–5 i.m.)	Inhalation (Isoflurane)	Xylazine + Ketamine (2 + 80 i.m.)	Xylazine + Ketamine (4 + 100 i.m.) or Pentobarbitone + Chloralhydrate (30 i.p. + 300 i.v.)
Rabbit	Atropine (0.1–0.2 s.c.)	Diazepam (1–5 i.m.)	Inhalation (Isoflurane)	Xylazine + Ketamine (5 + 25–80 i.m.)	Xylazine + Ketamine (5 + 100 i.m.) or Pentobarbitone + Chloralhydrate (30 i.p. + 300 i.v.)
Cat	Atropine (0.05–0.2 s.c.)	Diazepam (0.2–1 i.m.)	Acetylpromazine (0.5–1 i.v./i.m.) or Propionylpromazine (0.5–1 i.v.) or Xylazine (2 i.m.)	Xylazine + Ketamine (2 + 10 i.m.) or Ketamine (5 i.v.) or Inhalation (Isoflurane)	Pentobarbitone (35 i.v./i.p.)
Dog	Atropine (0.05 s.c.)	Xylazine (3 i.m.) or Acetylpromazine (0.5 i.m.) or Propionylpromazine (0.5 i.m.) or Droperidol (1 i.m.) or Diazepam (1 i.m.)	Thiopental (17 i.v.) or Metomidate + Fentanyl (4 + 0.005 i.m.) or Alfentanil + Etomidat (0.03 + 1 i.m.) or Inhalation/Intubation (Isoflurane)	Xylazine + Methadone (2 + 1 i.m.) or Xylazine + Ketamine (2 + 10 i.m.) or comb. with Diazepam (0.6 i.m.) or Propionylpromazine + Methadone (0.5 + 1 i.v.) or Acetylpromazine + Methadone (0.5 + 0.5–1 i.v.)	Pentobarbitone (30 i.v.) or Xylazine + Ketamine (2 + 15 i.m.) or Intubation (Isoflurane)
Pig		Azaperone (1–2 i.m.) or Chlorpromazine (1–2 i.m.)	Thiopental (10 i.v; 5% solution)	Azaperone + Metomidate (0.05–5 + 2.5–5 i.m. + i.v./i.p.) or Tiletamine + Zolazepam + Xylazine (2 + 2 + 0.5–1 i.m.)	Pentobarbitone (10–25 i.v.)
Sheep Goat		Xylazine (0.05–0.1 i.m.) or Diazepam (2 i.m.)	Xylazine + Ketamine (1–2 + 5–10 i.m.) or Thiopental (7.5–10 i.v.)	Pentobarbitone (20–30 i.m.)	Ketamine after pretreatment with Xylazine/ketamine 3–4 h or Intubation (Isoflurane)
Monkey[a]	Atropine (0.05–0.1 s.c.)	Diazepam (1.0 i.m.) or Xylazine (1–2 i.m.) or Ketamine (10–30 i.m.)	Inhalation/Intubation (Isoflurane)	Xylazine + Ketamine (2 + 10 i.m.)	Pentobarbitone (20–30 i.v./i.p.) or Intubation (Isoflurane)

[a] Anaesthesia of monkeys depends very much on the monkey species.

thetics. A compilation of such combinations is summarized in Table Q.3.

Q.3.3.3
Course of Anesthesia

The animal has always to be observed very carefully during anesthesia. Various systems can be checked with technical equipment, e. g. circulatory system (heart rate, pulse, blood pressure, ECG, peripheral perfusion, temperature) or pulmonary system (respiratory rate).

A very important procedure during anesthesia is the determination of the depth of anesthesia. There are four stages of anesthesia:

I. Stage of analgesia (from the first effect to unconsciousness):
   - heart and respiratory rate increase, normal dilation of pupils.

II. Stage of excitation (from the beginning of unconsciousness to the start of regular respiration): respiration irregular, dilated pupils, increased motor reflexes, nystagmus, opisthotonus.

III. Stage of tolerance (from the beginning of regular respiration to the termination of spontaneous respiration):
   This stage is divided into four steps:
   A) regular respiration, narrow pupils, most reflexes present
   B) skeletal muscles relaxed, narrow pupils, no eyelid reflex, corneal reflex present, flat respiration, good analgesia.
      This is the optimal stage of anesthesia for surgery
   C) only corneal reflex present, respiration very flat, pupils dilated
   D) no reflexes, respiration very flat, pupils very dilated

IV. Stage of asphyxia (after termination of the spontaneous diaphragmatic respiration):

V. no reflexes, no respiration: danger of death, immediate use of antidotes is necessary to prevent death.

By using combinations of different anesthetics – mainly by using combinations with muscle relaxing agents – the reactions of animals will differ from this scheme.

Q.3.3.4
Routes of General Anesthesia

In general, there are two different routes to induce general anesthesia: (i) injection and (ii) inhalation anesthesia. Sometimes combinations of both routes are used. The decision for one or the other route depends on the animal species, the purpose of the study and the necessity of control during anesthesia.

Q.3.3.4.1
Injection

By using this route of anesthesia the narcotic compound is dissolved in a liquid. The route of administration can be intravenous, intramuscular, subcutaneous or intraperitoneal. The mostly frequently used compounds are mentioned below:

Barbiturates

There are three groups of barbiturates: long acting, short acting and very short acting barbiturates. For laboratory animals short and very short acting barbiturates are used predominantly (sodium pentobarbitone, thiopental, hexobarbital).

Barbiturates are metabolized in the liver and mainly excreted via the bile. They are very fat soluble.Their short duration of action is caused by a distribution into adipose tissue. Fat represents a large compartment for these compounds with a relatively slow excretion. This can lead to prolongation of the narcotic effects after repeated dosing. The dosing of barbiturates should be adjusted according to the observed reactions of the individual animal as there are individual differences due to age, body weight, size, fat content and general condition of the animal. Barbiturates are not analgesic and should not be given without opioids.

Chloralhydrate

Chloralhydrate is a relatively old soporific compound. By using it for anesthesia cardiovascular side effects are often observed. The range of dosing is very narrow. Its use for laboratory animals is therefore limited. Intraperitoneal injections in rats can lead to paralysis of the ileus.

Combinations of Analgesic with Neuroleptic Compounds

This method is often used for dogs and rodents. Strong analgesics (morphine, methadone, meperidine, fentanyl) are combined with neuroleptics like phenothiazine, acetylpromazine or butyrophenone. The anaesthesia can be rapidly terminated by available antagonists.

Ketamine

Ketamine is a neuroleptic compound with a very fast onset of action following intramuscular administration. It can be used for nearly all species. A side effect of this compound is an increased tonus of skeletal

muscles but this can be prevented by the simultaneous administration of xylazine or diazepam.

Hypnotic Agents

Hypnotics are compounds which produce a very deep sleep without analgesia (metomidate). Therefore combination with neuroleptic compounds is recommended (e. g. combination azaperone with metomidate for pigs). As a single compound, metomidate can only cause anesthesia in birds.

Xylazine

Xylazine is frequently used for anesthesia in combination with other substances (Table Q.3). As a single compound it is only used to produce anesthesia in cattle.

Urethane

Urethane was formerly used as a hypnotic agent, It can, at the appropriate dose, produce a long acting (about 10 h) anesthesia in rats. Urethane is liver toxic and therefore its use is limited to some pharmacological models in which liver metabolism is of no importance. Due to its carcinogenic properties it should not be used anymore.

The important criteria of anesthesia are sedation, unconsciousness, analgesia and relaxation. These cannot be achieved with a single compound. Therefore, a combination of different compounds is necessary. The most common combinations for different species are listed in Table Q.3 with respect to the duration of anesthesia: short (up to 30 min), medium (up to 120 min) and long anesthesia (longer than 120 min).

Q.3.3.4.2 Inhalation

Inhalation anesthesia is more common for the bigger laboratory animals such as dogs, cats, sheep, goats and monkeys. However, it has gained an increased importance in small laboratory animals like rodents. The advantages of this form of anesthesia are the possibilities of controlling exactly the depth of anesthesia and of fast management of complications.

The parts of an inhalation system include:

- Bottle with oxygen (blue bottles)
- Valve to regulate pressure (reduces the pressure of the oxygen-bottle)
- Flowmeter (monitors the gas flow to the animal)
- Evaporator (evaporation of liquid anesthetic compounds)
- Oxygen-bypass (fast supply of oxygen to the animal in case of need)
- Tube to the system

Different techniques are used for laboratory animals:

Technique of Insufflation

Administration of anesthetic compounds is performed via a mask. Expiration occurs into the air of the room. Advantages are the simple procedure without valves and CO_2-absorber, and the very small dead volume of the system. Disadvantages are the waste of compounds, drying of the trachea of the animals, the impossibility of checking the respiration volume and the expiration of narcotic compounds into the room air (jeopardy to the staff).

Open System

Inspired and expired gases are separated by a valve. The inspired air consists of the fresh mixture of gases. The expiration reaches completely the atmosphere. The "Stephen slater" is the most used system of this group. It is recommended for smaller animals.

Half-Closed and Closed Systems

In closed systems all of the expired air passes to a CO_2 absorber. The CO_2 is removed chemically and the air is inspired again with newly evaporated anesthetic compounds mixed with oxygen. In a half-closed system, part of the expired air reaches the atmosphere. Advantages of closed systems include the economic benefit, the decrease of fluid and body heat loss from the animal and no risk to the laboratory staff. Disadvantages are the necessity to change the absorber every 8–10 h during anesthesia, the production of heat and the increase of resistance to breathing.

Summary

If it is possible inhalation should be done by intubating the animal. The risk of aspiration of stomach contents with the danger of an aspiration pneumonia can then be minimized. It is very important to use a tube with the correct diameter and length. An animal should be unconscious for intubation (see Table Q.3). In order to avoid gulp or cough reflexes it is recommended to administer succinylcholine, a muscle relaxant. Atropine can also be administered to decrease saliva production. Generally, all methods of injection anesthesia mentioned (Table Q.3) can be combined with an inhalation method. Such a "balanced anesthesia" is recommended for long and highly sophisticated operations.

Compound	Conc. with N_2O/O_2 (%)	Conc. without N_2O/O_2 (%)	MAC
Methoxyflurane	0.4–1	3.5	0.23
Isoflurane	1.0–2.5	1.5–3.5	1.4
Enflurane	0.5–1.5	2	2.2

MAC: minimal alveolar concentration for an anesthetic effect.

Table 4 Inhalation compounds and their characteristics

Q.3.3.4.3 Inhalation Compounds

The inhalation mixture has to include 21% oxygen. Sometimes it is better to administer 33% oxygen. Isoflurane, methoxyflurane and enflurane are widely used compounds for inhalation anesthesia. Ether is not longer recommended for anesthesia, due to the hazard of explosion and fire and because it is highly irritating to the respiratory tract. By using a mixture of N_2O and O_2 the amount of the evaporated compounds can be reduced drastically (Table Q.4).

Q.3.3.5 Termination of Anesthesia

Inhalation anesthesia can be stopped by removing the supply of evaporated compounds. To hasten the elimination of anesthetic compounds, the concentration of oxygen in the system can be increased for a period of five min.

The elimination of injected compounds is difficult to influence. It may be possible to accelerate metabolism of the anesthetic by using agents which stimulate metabolism in the liver and excretion by the kidney.

It is very important to check the body temperature of the animal during and after anesthesia. In cases of low body temperature the use of heating lamps or pads is necessary. After termination of anesthesia the animals go through the same phases as mentioned above but in the reverse order (tolerance, excitation, analgesia).

During anesthesia it might be necessary to stimulate respiration or circulation. Stimulatory agents for respiration are doxapram, pentamethylentetrazole, nikethamide, methetarimide, lobeline or micoren. Stimulatory agents for circulation are adrenaline, effortil, dopamine and ephedrine. The application of pure oxygen via a mask is also recommended during an injection anesthesia. Antidotes to morphine and its derivatives are morphine-antagonists like naloxone. Yohimbine is an antagonist of xylazine. The antidote for diazepam is flumazenil. There are no direct antagonists for ketamine and barbiturates.

Q.3.4 Postoperative Analgesia

To effectively reduce pain in animals a pain assessment has to be made using behavior, stress response etc. Pain assessment will be facilitated by

- a good knowledge of the species specific behaviors of the animal being assessed
- a knowledge and comparison of the individual animal's behaviour before and after the onset of pain (eg pre- and post-operatively)
- the use of palpation or manipulation of the affected area and assessment of the responses obtained
- examination of the level of function of the affected area: e. g. leg use following injury or limb surgery, together with a knowledge of any mechanical interference with function
- the use of analgesic regimens or dose rates that have been shown to be effective in controlled clinical studies, and evaluation of the change in behaviour this brings about
- a knowledge of the non-specific effects of any analgesic, anaesthetic or other drugs that have been administered

Analgesics can be broadly divided into two groups, the opioids or narcotic analgesics and the non-steroidal anti-inflammatory drugs (NSAID). Clinical pain involves several pathways, mechanisms and transmitter systems. To provide the most effective pain relief, drugs of different classes should be applied, acting on different parts of the pain system, for example combining opioids and NSAIDs.

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Q.4 Euthanasia of Experimental Animals[3]

Q.4.1 Introduction

Biomedical research needs animals. This is most obvious in case of *in vivo* animal experiments. However, for other scientific purposes, e. g. *in vitro* studies, biological material is also necessary to study enzymes, membranes, receptors, cells, tissues, or organs which are obtained from dead animals. Therefore, animals have to be sacrificed in biomedical laboratories (i) at the end of an *in vivo* experiment, (ii) during experiments where sacrifice of the animals is not part of the study but must be done when pain, distress and suffering exceed acceptable levels or if it is likely for the animal to remain in pain or distress after cessation of the experiment, and (iii) to provide biological material for *in vitro* studies.

The following remarks are a summary of the*Recommendations for Euthanasia of Experimental Animals* of the *Commission of the European Communities* (1993) and the *Guidelines for Skillful and Human Euthanasia of Laboratory Animals* (1993) of Switzerland.

Q.4.2 Euthanasia

Euthanasia means a gentle death and should be regarded as an act of a human method of sacrificing an animal with a minimum of physical and mental suffering. The method of euthanasia should be appropriate for the species and the age of the animals. The method should be painless, avoid excitement and achieve rapid unconsciousness and death. Additionally, the method should be reliable, reproducible and irreversible.

Prior to euthanasia, it is important to recognize symptoms of fear, distress and anxiety; these symptoms are species specific. Depending on the species these symptoms may include distress vocalisation, attempts to escape, aggression, freezing, salivation, urination and defecation. Distress vocalization and release of certain odors or pheromones by a frightened animal may cause anxiety in other animals housed nearby. In this context it has to be stressed that many vocalisations of animals are in a range of frequencies which are out of the human hearing range. Therefore, animals should not be present during euthanasia of other animals, especially of their own species. If possible, an animal should not be killed in a room where other animals are housed, in particular in case of a bloody method of euthanasia, e. g. decapitation.

Euthanasia usually requires some physical control over the animals. Suitable control minimizes pain, distress, fear and anxiety in the animal and depends on animal species, size, state of domestication and method of euthanasia. Gentle handling, stroking and talking to the animal during euthanasia often have a calming effect on many animals. The use of sedating and immobilising drugs may be necessary in those cases, where capture or restraint may cause pain, injury or anxiety to the animal.

The person performing euthanasia is the most relevant factor during sacrificing an animal in order to cause a minimum of pain, fear and distress. A suitable method of euthanasia can be extremely harmful to the animal if it is badly performed. All persons performing euthanasia should be well trained, demonstrate professionalism and be sensitive to the value of animal life.

After euthanasia it is essential to confirm death. Signs of death are cessation of heartbeat and respiration, and absence of reflexes. Death must be guaranteed by exsanguination or removal of the heart, destruction of the brain, decapitation, evisceration or the presence of rigor mortis.

Methods for euthanasia of laboratory animals can be separated into physical and chemical methods.

Q.4.2.1 Physical Methods Recommended for Euthanasia of Laboratory Animals

Physical methods are stunning (concussion, electrical stunning, and stunning with a captive bolt), cervical dislocation, decapitation, and microwave irradiation. The different methods of stunning as well as cervical dislocation cause a rapid loss of consciousness which must be followed immediately by a method to force and guarantee death of the animal.

Concussion may be sufficient in smaller animals, e. g. rodents, to achieve unconsciousness and is performed by a blow to the head. Electrical stunning is a common method in the slaughterhouse predominantly for pigs. Only specific equipment must be used for this method either in the slaughterhouse or in laboratory. Stunning with a captive bolt is also a common and an effective method for larger animals in the slaughterhouse to achieve unconsciousness. Adapted equipment can also be used for larger rabbits in biomedical labo-

[3] By A.W. Herling (1st edition), revised by U. Albus (2nd and 3rd edition).

ratories. The correct localisation of the captive bolt is important in order to achieve immediate destruction of the brain. Cervical dislocation destroys the brainstem but the large vessels to the brain are often intact. All these methods have to be followed immediately by an act to force and guarantee death, e. g. exsanguination, removal of the heart or destruction of the brain.

During the decapitation process the head is separated from the neck which causes an immediate interruption of the blood circulation to the brain and a fall in blood pressure in the brain with subsequent loss of consciousness. This is valid only for warm-blooded animals. In cold-blooded vertebrates it is recommended to stun the animals prior to decapitation due to their higher resistance against anoxia. For decapitation of smaller laboratory animals specific guillotines have been developed.

Euthanasia by microwave irradiation is used by neurobiologists for fixation of brain metabolites without destruction of brain anatomy. Only specific equipment developed for this purpose must be used (no domestic microwave ovens). It is essential to localize correctly the microwave beam onto the brain of the animal.

Q.4.2.2 Chemical Agents Recommended for Euthanasia of Laboratory Animals

Many chemicals can cause death due to their toxicity, but only a few are recommended for euthanasia. The most suitable chemicals for euthanasia are certain anesthetics in overdose. In this case, the anaesthetic agent causes unconsciousness, followed by death.

Volatile anesthetics such as halothane, enflurane, isoflurane and methoxyflurane should only be used in a gas scavenging apparatus. Carbon dioxide at high concentrations of 80 to 100% causes unconsciousness within a few seconds.

Injectable anesthetics, predominantly barbiturates such as sodium pentobarbitone, are the most widely used and the most appropriate agents for euthanasia for most animals. Three times the anesthetic dose causes generally rapid unconsciousness and death. Intravenous injection is the most reliable and rapid route. Intraperitoneal injection may also be used in smaller rodents but it needs more time for death to occur. The intracardial and intrapulmonal administration can only be recommended in unconscious animals, because it is painful and, in the case of intracardial injection, difficult to perform successfully on the first attempt.

The agent T61 is a mixture of a local anesthetic, a hypnotic and a curariform component. It is used only intravenously. Due to the curareform component it is not allowed in some countries but it has been demonstrated that unconsciousness and neuromuscular blockade occur simultaneously in dogs and rabbits. Nevertheless, prior sedation should be performed if possible.

Q.4.2.3 Methods and Agents Not to Be Used for Euthanasia of Laboratory Animals

Physical methods not to be used for euthanasia are exsanguination, rapid freezing, pithing, decompression, hyperthermia, hypothermia, asphyxia, drowning and strangulation. Chemicals not to be used are carbon monoxide, nitrogen, nitrous oxide, cyclopropane, chloroform, trichloethylene, hydrogen cyanide, magnesium sulfate, potassium chloride, nicotine, strychnine, chloral hydrate, and ethanol. Some of the above mentioned chemicals are not recommended for euthanasia because they are extremely noxious and dangerous to the experimenter.

Neuromuscular blocking agents such as curare, succinylcholine or suxamethonium which do not cause rapid unconsciousness prior to death should also not be used. Ketamine is a very good anesthetic with a wide therapeutic safety margin for most animal species. Therefore, it is unsuitable for euthanasia.

However, non-acceptable methods of euthanasia can be used if animals are anaesthetized or rendered insensible and unconscious by a recommended method. This is used for, e. g. exsanguination, rapid freezing, and pithing. Exsanguination must not be performed in sight or smell of other animals. Rapid freezing is important to minimize enzymatic processes prior to subsequent biochemical determinations in tissues and organs. Pithing is a quick method of brain destruction achieved by insertion of a needle through the foramen magnum.

Q.4.2.4 Recommended Methods for Euthanasia for Specific Animal Species

Mouse

- Decapitation
- Cervical dislocation with subsequent exsanguination
- Euthanasia within a 80% carbon dioxide atmosphere
- Euthanasia within an atmosphere of suitable volatile anesthetics
- Sodium pentobarbitone at a dose of 150 mg/kg i.p.

Rat

- Concussion, cervical dislocation (both with subsequent exsanguination), and decapitation, conducted only by well trained persons
- Euthanasia within a 80% carbon dioxide atmosphere
- Euthanasia within an atmosphere of suitable volatile anesthetics
- Sodium pentobarbitone at a dose of 100 mg/kg i.v. or 150 mg/kg i.p.
- Microwave irradiation

Hamster

- Decapitation; conducted only by well trained persons
- Euthanasia within a 80% carbon dioxide atmosphere
- Euthanasia within an atmosphere of suitable volatile anesthetics
- Sodium pentobarbitone at a dose of 300 mg/kg i.p.

Guinea Pig

- Concussion (with subsequent exsanguination), and decapitation; conducted only by well trained persons
- Euthanasia within a 80% carbon dioxide atmosphere
- Euthanasia within an atmosphere of suitable volatile anesthetics
- Sodium pentobarbitone at a dose of 150 mg/kg i.p.

Rabbit

- Stunning with captive bolt
- Concussion (with subsequent exsanguination); conducted only by well trained persons
- Sodium pentobarbitone at a dose of 120 mg/kg i.v.
- T61 at a dose of 0.3 ml/kg strictly i.v. via a catheter

Cat

- Sodium pentobarbitone at a dose of 100 mg/kg i.v. or 200 mg/kg i.p.
- T61 at a dose of 0.3 ml/kg strictly i.v. via a catheter; it is recommended to anesthetize the animal beforehand with 20–30 mg/kg ketamine i.m. or 1–2 mg/kg xylazine plus 10 mg/kg ketamine i.m.

Dog

- Sodium pentobarbitone at a dose of 100 mg/kg i.v.
- T61 at a dose of 0.3 ml/kg strictly i.v., it is recommended to anesthetize the animal beforehand with 1–2 mg/kg xylazine plus 10 mg/kg ketamine i.m.

Ferret

- Sodium pentobarbitone at a dose of 120 mg/kg i.p.

Cattle, Sheep, Goat, Horse, Pig

- Sodium pentobarbitone at a dose of 100 mg/kg i.v.; for larger animals prior sedation is recommended (Xylazin).
- All other acceptable methods which are used for slaughtering

Primate

- Sodium pentobarbitone at a dose of 100 mg/kg i.v.; it is recommended to anaesthetize the animal beforehand with 1–2 mg/kg xylazine plus 10 mg/kg ketamine i.m.
- T61 at a dose of 0.3 ml/kg strictly i.v., it is recommended to anesthetize the animal beforehand with xylazine/ketamine i.m.

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