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Animal Experiments in Medicine

Handbook for Medical Students

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Introduction

In 2004 the Federation of European Laboratory Animal Science Associations (FELASA) published the European standards for animal experiments and a quality assurance system has subsequently been implemented in the European Union (details are provided at the website http://www.felasa.eu/recommendations.htm). In the autumn of 2006, the guidelines of the Scientific Committee of Animal Experimentation of the Hungarian Academy of Sciences were made public, which set the minimum requirements for training and education in laboratory animal sciences in Hungary. These include a 15-h theoretical course, a 40-h course of practical training, and an examination based on the theoretical part of the teaching program. Certification for those working with animals is a prerequisite at the University of Szeged, too. Students completing the theoretical course are required to attend the 40-h practical training course at their home institutes, and the participants (students, residents, postdoctoral fellows, Ph.D. students, etc.) become finally entitled to perform animal experiments legally. The chapters of this book are based on the themes of this theoretical course and include the following main topics:

1. Significance of animal experiments in research and medicine
2. Ethical aspects and legal regulations
3. Breeding and animal houses
4. Anesthesia, and pre- and postoperative care
5. Planning of experiments, data evaluation, and statistical analyses
6. Basic experimental techniques. Monitoring of vital signs: circulation, respiration and microcirculation research
7. Outbred, inbred, and transgenic animals. Immunobiology and cloning
8. Alternative methods: *in vitro* techniques and tissue culturing

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I. General background (*Dr. Mihály Boros*)

Motto: *"The sciences do not try to explain, they hardly even try to interpret, they mainly make models."* (John von Neumann)

1. The scientific method

We need to discuss and understand the connection of science with experimentation, and the linkage with animal experiments, but only a brief description can be presented here. The scientific method is the standardized "tool" that scientists use to find the right and wrong answers to questions. In science, a "fact" is defined as an observation that has been repeatedly confirmed and for all practical purposes is accepted as true. To obtain such facts, we have to follow a logical pathway with well-defined steps.

The starting point is the identification of a problem. A problem is a scientific question to be solved: what do we know already and what do we want to know? Asking the right question is of the utmost importance, as it must summarize the facts from past experience or observations on which we base our current hypothesis. Repetitive experiments should be avoided.

A hypothesis is a single statement, and another key to successful research. We have to propose an answer to the problem (*i.e.* the hypothesis), and design a project which includes experimentation. The experiments are then performed to test the hypothesis.

Next, we have to examine the results and test new evidence. The conclusion is a summary of the results of the experiments and a statement as to how the results relate to the hypothesis. If the results do not support the hypothesis, it should be rejected. Reasons for experimental results that are contrary to the hypothesis are analyzed and, if applicable, the conclusion can be followed by the postulation of ideas for further hypotheses and testing. If the results support the hypothesis, it is accepted, and the new results, data and novel findings may then be transmitted to others[1].

The scientific method is carried out collectively: individual experiments or experimenters can be wrong, but science is self-correcting. The hypothesis-driven scientific method is best characterized by the route observations → hypothesis → prediction → testing → acceptance/rejection → new observations.

The principle of parsimony[2] underlies all scientific modeling. It warns us to choose the simplest one from a set of otherwise equivalent models. When this is done there is less chance of introducing inconsistencies and redundancies.

A further important feature is that a scientific hypothesis must be testable, as otherwise the problem can not be solved experimentally. In practice, it is often easier to refute rather than to substantiate a hypothesis.

2. The model

By definition, a model is a tool via which to understand or describe our world. It is a schematic description of a system, theory or phenomenon that accounts for its known or inferred properties, and may be used for further studies of its characteristics. The goal in science is to describe and understand reality through experiments, and thus the "goodness of fit" through usability testing is the most important characteristic of a model. The model is useful only if it can explain a large number of observations with a minimum number of assumptions and if it can predict the results of future measurements.

A further important characteristic is the possibility for measurements; questions should be developed in such a way that quantifiable metrics can be evaluated. Measurements involve the assignment of numerals to represent physical properties, and these are prerequisites for exactness and reproducibility. Because of the great significance of measurements and analysis,

it is important that measurements are repeated (by the same experimenter and by different experimenters) to test for reproducibility. Only if measurements are reproducible can they be considered reliable for model-building purposes. Hence, duplication (but not repetition!) is the cornerstone of scientific investigation.

Basic categories in model-making are separation (*i.e.* the system must be separated from the outside world), selection (we have to select a phenomenon, interaction, *etc*. from a pool of phenomena, interactions, according to the goal) and economy (the model must be the simplest, according to the goal: "*entia non sunt multiplicanda praeter necessitatem*" – Occam's razor advises that, if a choice is to be made between two explanations, always the simpler should be chosen.)[2]. Separation and selection introduce bias and errors into the model. Nevertheless, this is a prerequisite for model-making.

Information types can be *a priori* or *a posteriori*. *A priori* knowledge is obtained before the experiments; a *posteriori* knowledge includes "*a priori"* information and the knowledge obtained during the experiments. Deductive models utilize *a priori* information only; they are based on the general laws and principles of natural sciences and have the aim of the description of a single, well-defined phenomenon. Inductive models make use of experimental data only.

How can a hypothesis be proved through model-making? This is a question of validity, credibility and reliability - experimental biology and medical research are concerned with the choice of the right model. A relevant model is "good science".

Nevertheless, models are always simplified versions of reality, and model-making therefore has some inherent problems. The translation of model knowledge into predictions and decision criteria is sometimes difficult. Extrapolation from animal models to human conditions always requires an awareness of interspecies and other dependent variations.

3. Models in medicine and biology – a critical approach

The purpose of the scientific method is to construct an accurate, reliable, self-consistent, non-arbitrary representation of the world. Today, model-making is a general principle in medical and biological research. The basic categories are *in vitro* models (referring to the technique of performing a given experiment in a test tube in an artificial environment; literally "in glass" or "in a glass container")[3] and *in vivo* models (literally "in life", within a living organism) models. The *in vitro* models currently most often used in medicine are the following:

- Whole organ perfusion (*e.g.* isolated, perfused Langendorff heart)
- Superfusion of tissue slices
- Primary cell culturing
- Culturing of immortalized cell lines
- Studies on subcellular lines
- Molecular studies on gene and protein expression

This type of research usually aims at describing a single experimental variable. With fewer variables, results are generally more discernible. A majority of the *in vitro* models are *ex vivo* (literally "out of the living organism") systems (cell, tissue or organ), and a further sub-category is „*in silico*" (which is performed on computers or via computer simulation). Whilst these experiments have numerous merits and provide valuable data, they may not accurately reflect the integrative nature of the human body. *In vitro* research is important, productive and less expensive than *in vivo* research, but the results may not correspond to those inside the living organism. *In vivo* models include human (clinical trials) and non-human (animal) testing. Apart from legal, ethical, *etc.* issues, what are limits of clinical

testing? Human studies in general are noninvasive and there are numerous problems relating to the prior history. Non-human, *in vivo* animal studies can overcome the problems listed above for humans, but may not be good models because of size differences, interspecies variations and differing genetics (see later).

In vivo studies may employ acute (short-term) or chronic (long-term) models. The end-points are usually different, acute studies ending within 24 h, whereas chronic models involve long-lasting observations. In acute models, the effects of anesthesia and perturbations from the normal physiology should be noted, while in chronic models the problems include the use of indwelling catheters, transducers, *etc*.

The application of the scientific method in clinical practice (*i.e.* human models) was first addressed and regulated by the Nuremberg Codex (1947) and the Declaration of Helsinki of the World Medical Association (1964, 1975). These recommendations for the conductance of clinical research are still valid:

1. Clinical research must conform to the moral and scientific principles that justify medical research.
2. Clinical research should be based on laboratory and animal experiments or other scientifically established facts.
3. Every clinical research project should be preceded by careful assessment of inherent risks in comparison to foreseeable benefits to the subject or to others.

The reason for making models in medicine is to understand the structure and function of the human body. Animal models are applied because some experiments cannot be carried out *in vitro*, or on humans, or are better performed on animals. These models are useful and essential at present, and perhaps necessary for future medical advances too. Animal model-making today covers the whole area of medicine, and is utilized for:

- Treatment (past examples: rabies (first tested in dogs and rabbits), rickets (dogs), leprosy (monkeys and armadillos), *etc.*)
- Prevention: diphtheria (horses), polio (rabbits), rubella (monkeys) and measles (monkeys).
- Discovery: insulin (dogs), modern anesthesia (dogs) and DNA (mice and rats).
- Development: laparoscopic surgical techniques (pigs) and open-heart surgery (dogs).

Naturally, there are numerous limitations of animal experimentation. Of these, the most important perhaps is the fact that effects on animals do not always accurately predict the effects on humans. A further important question is the possibility of extrapolation (an example is the comparison of the lethal dose 50% (LD50) between common species: toxic levels may or may not be relevant for humans), and the genetic background of species differences (*e.g.* chocolate (theobromine) can be poisonous to dogs; cortisone is a teratogen in mice, and insulin is deadly to many animals). Some symptoms are difficult to discover in animals (*e.g*. minor aches and pains), and in some cases alternatives exist (see below).

It is very important to determine the possibility of extrapolation in every single case. Today, a medical scientist should always consider the three Rs[4] (see later) of **r**efinement (minimizing animal pain and suffering), **r**eduction (minimizing the number of animals used, *e.g*. using animals that have been genetically engineered to be susceptible to human conditions) and **r**eplacement (avoiding the use of animals in experiments). Current disputes mostly target the latter possibilities and degrees of *replacement* are often argued:

(i) *Relative replacement*: experiments that eliminate the suffering of animals but not their use (*i.e.* absolute refinement)

(ii) *Absolute replacement*: experiments that do not require biological material derived from animals.

Replacement possibilities include:

- Information (the reduction of the unnecessary duplication of animal work; this is scientific and economical reality)
- Computer-based (*in silico*) systems, and mathematical modeling
- Physical-chemical techniques (*e.g.* the commercial Eytex test system can predict whether a chemical will irritate the eyes, replacing the Draize test)
- Use of lower organisms such as bacteria and fungi or embryos (*e.g.* the Ames test)
- Human studies (*e.g.* volunteers and population/patient studies)
- Cell, tissue and organ cultures

The deciding factor is always that simple models with the possibility of furnishing clear answers should be selected.

3.1. The purpose of making animal models

- The species itself (veterinary research)
- To investigate animal behavior (*e.g.* stress-copying mechanisms)
- To test non-pharmaceutical products (*e.g.* toxicity tests for products ranging from pesticides to deodorants)
- To test pharmaceuticals (*e.g.* new drugs and vaccines)
- Educational purposes (*e.g.* university students)
- Basic medical purposes (*e.g.* research on cancer, AIDS, xenotransplantation, *etc.*). These are extrapolation models, or "mechanisms in humans" studies. Most of the animals are used for:
    - Behavior / psychology
    - Therapy ("preclinical studies")
    - Toxicology / safety assessment
    - Anatomy / surgery
    - Infection / immunity
    - Tumor development
    - Research and diagnosis
- The study of general biological mechanisms (biological research with nonmedical aims, and basic science). The main categories are as follows:
    - Descriptive studies (for physiology, pathology, ecology, *etc.*). Here the experimental design is secondary; the lack of hypotheses makes quality assessment difficult.
    - Studies of the responses to an intervention (in physiology, pathology, behavior, *etc.*) Here the experimental design is of paramount importance.

3.2. Categories of animal models

Table 1. Spontaneous models - similar mechanisms are present in test species and humans

Test species	Defect / susceptibility	Human equivalent	Why used
Min mouse	Mutant apc gene	Familial adenomatosus polyposis (colon cc)	Human endpoint
Cat	Asthma	Asthma	Equal pathology

Irish setter	Factor VIII deficiency	Hemophilia A	Therapy trials
Woodchuck	Hepatitis B susceptible	Hepatitis B	Infection studies
Armadillo	Leprosy	Leprosy	Therapy trials

Table 2. "Induced" models (manipulated animal as model)

Method	Question
Behavior (stress, learning, *etc*.)	Is animal stress (behavior, learning, *etc*.) analogous to human stress?
Surgical, biochemical, *etc.* interventions (nerve cuts, ligatures on vessels, *etc.*)	Is extrapolation possible?
Genetic manipulation, transgenics, knockouts, knock-ins (see later).	Is it treatment or pretreatment?

Table 3. "Negative" models ("why does not" models)

Species	Question
Dog	Why no atherosclerosis?
Monkey	Why do HIV-infected chimpanzees not get AIDS?

Table 4. "Orphan" models. Species with characteristics that may reveal important biological knowledge (here mutation induction is an important research field)

Species	Characteristics
Pig	Many $CD4^+CD8^+$ T cells
Ruminants	Numerous γδ-T cells
Birds, cattle	Leucosis is infectious

3.3. Animal biology

The prerequisite for choosing the best model is a knowledge of the animal biology. Characteristic differences between rodents (rats) and humans are listed below, but comparisons should be made in every case.

Table 5.

Characteristic	Human	Rat
Biliary excretion	-	++
Plasma protein binding	++	Low; this affects the fate of xenobiotics (streptomycin, chloroquine, serotonin, adrenaline, noradrenaline, dopamine, *etc.*).
Respiration	Nasal-oral	Nasal
Gut flora	No cecum	Huge cecum
Skin	Naked	Fur
Albinos	Rare	95% (tyrosinase defect, nerve system affected, sensitive to light, retinal damage, reduced hearing, histology is different, low P450 activity)
Gallbladder	+	-
Activity	During daylight	Nocturnal (reverse lighting cycle?)
Social ranking	-	+ (male fighting)
Natural behavior	+	Animal crowding (in breeding and before experiments)
Digestive system differences		Teeth Stomach (empty or not?) Fiber content in feed
Special differences		Thigmotropism - sticking to each other Harderian glands (porphyrin signs around nostrils)
Differences in pathophysiology		*E.g.* signs of disease (fever or hypothermia): +/-

The fate of drugs should also be mentioned. Allometric extrapolation is always possible (the dose scaling factor is usually related to body weight – see below)[5], but the metabolism is temperature-dependent, the body surface also relates to species, *etc.*, and thus the choices of species affects pharmacology, toxicology, anesthesia, *etc.* research significantly.

Table 6.

	Body weight (kg)	Scaling factor
Human	60	1
Dog	8	2
Monkey	6	2
Rabbit	2	3
Rat	0.2	5
Mouse	0.02	8

4. Models in practice

The importance of differences between models and clinical reality (*i.e.* problems of clinical practice) is presented through examples.

4.1. Sepsis

Human sepsis is a severe, and often lethal disease, and the pathophysiology is still unknown. Important rodent models used to replicate clinical situations are briefly described below:

Table 7.

Model	Advantages	Disadvantages
Endotoxin (lipopolysaccharide-LPS) injection	Simple (on intraperitoneal injection, shock ensues in 4-6 h). Low doses produce hyperdynamic, and high doses hypodynamic reactions	Simple (sepsis is more than LPS as anti-LPS is effective in animals, but not in humans)
Cecum ligation and puncture (CLP)	Gives trauma such as appendicitis or diverticulitis, or necrosis of cecum. The time scale is adjusted to the parallel clinical situation. The cytokine responses are similar to clinical patterns. Resuscitation strategies, fluids, *etc.* can be incorporated.	Involves invasive surgery, (closure and recovery); difficult to standardize
Fecal inoculum	A consistent batch of feces can be prepared. Easy to administer (i.p. injection).	No significant trauma involved
Agar with bacteria	Slow release of bacteria from ”*nidus*”	Usually done with one bacterial strain
Intravenous bacteria	Easy to dose. The time course can be controlled. Systemic infection results.	Usually one bacterial species used (E. coli), which may be serum-sensitive and avirulent. Rapidly killed, and large quantities overwhelm host defense.
Pneumonia models	Easy to administer to the trachea or nose, a common site of bacterial infection. The dose can be controlled, and the model is	Some animals get local infection (and not systemic)

	reproducible. Inflammatory infiltration of cells occurs; the injury can be quantified.	

Comparisons between model and clinical reality[6-8]

Table 8.

Experimental models for sepsis	Clinical sepsis
Homogenous genetic pool, usually one sex	Heterogenous genetic pool with two sexes
Inbred	Outbred
Mouse, rat, rabbit, pig, dog	*Homo sapiens*
Healthy animals	May have other diseases
Young adults most often used	Often neonates or elderly
Coprophagy / unclean environment (less sensitive to endotoxin)	Usually clean
Experimental models for sepsis	Clinical sepsis
Pretreatment or early treatment common	Can not treat what is not there
Adjuvant therapy not common	Active treatment (fluids, antibiotics, inotropes, organ support)
Mechanical ventilation seldom used	Mechanical ventilation is often used
The route of infection is usually the blood	"Nidus"
Heparin, anesthesia and analgesia are used	Optimum care
The insult is uniform	The degree and site vary
Rapid onset	The onset is usually slow
Standard times of study	Individualized time course
The strain is usually avirulent	Almost always virulent
Various endpoints – surrogate markers, not often go to lethality	Clinical endpoints – organ function and mortality
Death usually ensues in 1-8 h	Death occurs in patients with sepsis, due to multiple organ failure that develops in days to weeks

4.2. Hemorrhage

Hemorrhagic shock is a severe, often lethal clinical condition where the therapy is controversial (important possibilities and resuscitation strategies include permissive hypotension, crystalloid solutions, hypertonic solutions, hemoglobin substitutes, whole blood, fresh frozen plasma, packed red blood cells with or without white blood cells, *etc.*).

Hypertonic resuscitation was recommended until the 1980s as this modality gave the best results in fixed volume and fixed pressure models (*i.e.* in controlled hemorrhage models – see below). After the 1990s the professional opinion changed and it is currently proposed that aggressive resuscitation is detrimental and the permissive hypotension gives the best survival (*i.e.* in uncontrolled hemorrhage models. Remark: this possibility was first suggested in the early 1900s[9]**).**

Table 9.

Protocols / experimental models to test therapeutic strategies	Conclusions
Constant pressure (Wiggers)[10] model • Removal of blood from the femoral artery into an open reservoir • Set the hydrostatic column to the desired mean arterial pressure (*e.g.* 40 mmHg) • Resuscitate at a set time (*i.e.* 60 min) or when a given % of shed blood volume autotransfuses (flows back to the circulation due to vascular collapse).	Most animal studies utilize males. However, low testosterone and high estradiol levels are protective and prevent immune suppression after shock. Females tolerate trauma-hemorrhage and sepsis better.[11]
Constant volume model • Hemorrhage with or without systemic anticoagulation • Calculated blood volume = % of body wt (≈60 ml blood/kg body wt) removed from the femoral artery at 1 ml/min to 40% of blood volume • Resuscitate at a given time (*e.g.* 60 min).	The blood volume (a significant determinant of survival in hemorrhage) depends on the body wt. It is very easy to overlook 20-30 g differences in body wt in treatment groups – and it may be concluded that a treatment protects when it is simply that the animals are slightly lower in body wt. Constant blood volume protocols for study of circulation require tight control of body wt.[12]
Uncontrolled hemorrhage models • Standardized vascular trauma (*e.g.* cut through the iliac artery)	Most relevant to clinical scenario (!). The central nervous system drive of autonomic reflexes is affected by anesthetics. Breathing is depressed by some anesthetics (some analgesics can suppress inflammation); -anesthesia must be closely controlled.[13,14]

It should be noted that the same clinical reality leads to different results in very similar models, in which merely some of the important characteristics are significantly different. It is obvious that the explanation for the alterations in a significant variable is then the discrepancy between model and reality.

5. Summary

- Experimental biology involves the choice of the right model. Preclinical trials with irrelevant or clinically inadequate models lead to faulty clinical trials.
- The most important issue is the relevant control.
- The design of the possible animal models and their potential limits should be carefully considered before the study is begun.
- A wide reading relating to the field is essential before a hypothesis is set up.

II. Ethical aspects and legal regulation of animal experiments (*Dr. Sándor Nagy*)

1. The importance of animal experiments

The importance of animal experiments in science, and especially in biomedical science, is very great. However, much of the information available on this field is erroneous, misleading or false. We present here the viewpoint of science, which is based, not only on its inner logic, but also on moral philosophical arguments.

Man uses animals for various purposes. Of these, at least two are essential for our survival. More than 95 per cent of all animals used by man serve for our nutrition, while only about 0.3 per cent are utilized in scientific research. The activity of every species, including man, is directed towards its survival. Throughout evolution, only species that could adapt to the changing environment have survived. The unique evolutionary advantage of man is wide-ranging knowledge and its confirmed and systematized form: science. Similarly to other species, we wish to survive, this, being the main obligation to our species. Accordingly, we cannot give up the main instrument of our survival: increasing our knowledge through science.

Exploring the world is an activity inseparable from mankind. An especially important part of this pursuit is research into the living world. Knowledge gained by the life sciences can serve human health and the quality of life, and also furnish an understanding of the interactions between man, the biosphere and that of other species. As life phenomena can be studied only in living organisms, the life sciences cannot do without animal experiments.

This chapter will deal with the role of animal experiments in medical science. Medical science is a branch of the life sciences, the goal of which is to protect human health and quality of life. This goal is served by providing a scientific basis for medical curative and preventive activity. However, medical science is constrained by the moral principle that humans must not be subjected to *experiments*, but only to *investigations,* in a limited way, with reasonable justification, under very carefully considered and strictly controlled conditions. The Declaration of Helsinki issued by the World Medical Association permits clinical investigations on humans (*e.g.* for the testing of new drugs), only, if they are based on data obtained by means of animal experiments.[15]

The use of animals is also necessary in toxicological tests protecting human health. A large number of various substances, produced by industry to satisfy human needs are released into the environment.Toxicological testing is indispensable before such release, in order to protect society.

Experiments on animals not only serve immediately practical (therapeutic or preventive) purposes. The results of basic research, leading to knowledge on the functioning of living organisms and the characterization of their constituent compounds, can often be utilized only in the long run. This applies to all fields of basic research. The need for such researches is, evident, however, as these lay the foundation of the applications.

Mozart, probably the greatest genius in the known history of mankind, died in 1791, whereas the death of the prominent historical figure Alexander the Great occurred 2114 years earlier. Both men suffered untimely deaths in their mid-thirties. Few would readily discern parallels between these events. Yet we now think that the diseases to which they succumbed could most probably have been prevented, cured or at least treated if they had lived today. More relevant to the present subject is the fact that the art and science of medicine at the time of Mozart's death were scarcely more advanced than they were at the time of Alexander's demise. Remedies applied to treat the sick were almost equally limited in their availability and efficacy. In other words, medicine and its scientific basis: medical science had undergone virtually no progress during two millenia. In essence, this progress began only in the 19th

century. About 80% of our present biomedical knowledge has accrued since 1800 and more than 50% since 1900.[16]

A significant proportion of this knowledge is due to animal experimental research, which fundamentally began in the 19th century. A remarkable parrallelism, and even a causal relationship can be established here. Physicians recognized that there was little hope of coping with human illness until they first understood how living systems function when they are healthy and under pathological conditions. Research, with the use of animals, has produced impressive results. Few are aware of the fact that the Hungarian Ignác Semmelweis, who devised the first efficient method for preventing puerperal sepsis, himself conducted animal experiments. Use of animals was essential in the founding and development of microbiology after 1850.

The significance of animal experimentation is perhaps most vividly exemplified by the fact that about 75% of all Nobel Prizes in medicine and physiology (first in 1901) were awarded for researches involving the use of animals. An incomplete listing follows here (the species used are shown in parentheses). 1901, Behring: for the development of a diphtheria vaccine (guinea pig). 1902, Ross: an understanding of the life cycle of the malaria pathogen (pigeon). 1912, Carrel: the development of vascular and transplantation surgical techniques (dog). 1923, Banting and MacLeod: the discovery of insulin and the pathomechanism of diabetes (dog, rabbit and fish). 1939, Domagk: the development of the first effective antibacterial drug (mouse and rabbit). 1945, Fleming, Chain and Florey: the discovery of penicillin (mouse). 1952, Waksman: the discovery of streptomycin, the first antituberculotic drug (guinea pig). 1954, Enders, Weller and Robbins: culture of poliomyelitis virus, which made possible the later development of the vaccine (chimpanzee and mouse). 1987, Tonegawa: an understanding of the basic mechanism of antibody synthesis (mouse embryo). 1999, Furchgott, Ignarro and Murad: discovery of the basic role of nitrogen monoxide in the cardiovascular system (rabbit, guinea pig and cattle).

2. Animal rights activism – antiscience movements

The birth of these movements coincides with the spread of animal experimental researches in the 19th century. The first such movement was founded by the Englishwoman Frances Power Cobbe, (**Fig. 1**). When traveling in Italy in 1863, she heard about the physiological experiments performed on dogs by Professor Maurice Schiff in Florence. In what is now recognized as the first organized protest against animal research, Cobbe raised a petition among the English expatriate community in Florence, condemning the research as cruel and requesting Schiff to cease all his animal experiments.

Figure 1. Frances Power Cobbe

On returning to Britain, Cobbe began writing and recruiting support among influential people, including some in parliament. In 1875 she founded the first „antivivisection" society that still exists today. This led to Lord Caernarvon, a supporter of Cobbe, introducing the *Cruelty to Animals* Bill into the House of Lords and it was passed by parliament. A group of London physiologists and doctors organized a lobby against the bill and enlisted the support of the British Medical Association and the General Medical Council. A short, but furious, political struggle resulted in a number of alterations to the bill producing a compromise that placed nine substantial restrictions on animal experimentation, but permitted the granting of certificates that could give exemption from six of them. In this form the Bill was passed in 1876.

This pattern was repeated in a number of other countries with „antivivisection" societies being formed and lobbying for legislation to restrict animal experiments. The intensity of their activities varied over the following decades, but it increased significantly after 1960. Unfortunately, many of these activities have become interwoven, ever since their 19th century origins, with antiscience trends. A certain Sir George Duckett made the following statement in 1875: „Medicine has probably reached its limits and cannot know anything and cannot gain anything from repetition of experiments on animals". Since then, relying on animal experiments, medicine has given us antibiotics, vaccines, organ transplantation, the treatment of diabetes with insulin, heart surgery and a multitude of efficient drugs. Duckett's statement should be viewed in the light of this.

Opposition to science, one of the irrational trends, became a favorite play of some 20th- century philosophers who wish to blame science for the problems of mankind. This is a false view, of course, especially so in the case of medicine which fights against disease, suffering and death. It is not a necessity that animal protectionist movements, also stemming from humane intentions, should be combined with antiscience activism, all the less so as biomedical scientists pay respect to all forms of life, even if they need to sacrifice animals in their work for the interest of humans and in many cases for the interest of animals. There are more than 70 drugs today that can be used to treat both humans and animals. Poliomyelitis had been a dreaded disease. To develop the vaccine against it, many monkeys had to be sacrificed. When the vaccine was finally prepared, not only could this terrible disease be largely eliminated, but when a polio epidemic threatened the chimpanzees of the well-known animal activist Jane Goodall in Africa, the animal colony was immunized with the vaccine.

Opponents of animal experimental research often put forward two lines of arguments side by side: one utilitarian (here we discuss what, from a philosophical point of view, can be called vulgar utilitarism; for serious utilitarian philosophy, see section 4.) and morality-based reasoning. Interestingly, many fail to realize that these two cannot be applied simultaneously. Critics employing vulgar utilitarian argumentation hold that the differences between humans and animals are so great that the results obtained on animals cannot be applied to man. This statement has been refuted innumerable times by the history of science. In fact, human and animal organisms are essentially very similar. This makes it possible to apply results from animal experiments to humans. To suppose that the work of thousands of researchers worldwide is meaningless and that, for instance, almost three quarters of Nobel prizes in medicine have been awarded for fruitless effort is naïve, to put it mildly.

Critics arguing from a moral base maintain that the use of animals in scientific research is simply morally wrong, because we do not have the right to take animal lives. The moral basis of this argumentation is not stronger, however, but weaker than that of medicine striving to alleviate the huge suffering caused by diseases to mankind. Animal research is essential for the development of medical science. It may be noted, that only a fraction of 1% of all animals consumed is used for this purpose.

The vulgar utilitarian argumentation is often complemented by moral arguments, disregarding the fact that the two together are incompatible. In principle this type of utilitarian reasoning considers that animal experiments are useless and should therefore be stopped. If this proved untrue, they would, theoretically, not oppose them. If criticism is made on moral grounds, then vulgar-utilitarian arguments are unnecessary because moral principles are superior. A hypothetical example helps to illustrate this. If someone were to advocate experiments on people (as actually happened in Nazi Germany), this would undoubtedly be morally unacceptable, although perfectly logical from a purely vulgar-utilitarian standpoint. Obviously the two types of reasoning cannot coexist.

A characteristic of the animal rights movements, ever since their beginning, has been their disproportionately great attention to the fate of animals used for scientific purposes in contrast with that of animals used for all other purposes. This lack of balance is demonstrated by a study in which the authors utilized the official statistical data of the U.S. Department of Agriculture on the annual use of animals for various purposes. They compared the relative volume of the animal rights literature with the actual proportion of animals used in the particular fields. 25 books considered important by the animal rights activists were taken into account. The assumption underlying this analysis was that the number of pages devoted to a particular topic by the authors of these „important books" would reflect their collective perception of the relative importance of that topic in the overall scheme of concern for animals. Every page of each of the books was examined and the field of animal use against which a particular page expressed concern or protest was determined. A summary of the results may be given. In 1989 the ratios of animal use in the U.S. (expressed as percentages of the total use) were as follows: for food: 96.5%, hunting: 2.6%, killed in animal pounds: 0.4%, for research and teaching 0.3%, fur garments: 0.2%. In contrast, the percentages of pages expressing concern and protest in the animal rights publications in the different fields of animal use were as follows: for food: 30.6%, hunting: 2.3%, killed in pounds: 2.3%, and fur garments: 0.8%, and research and teaching: 63.3%. When these data are compared they are meaningful as concerns the question of what is motivating the animal liberators. In three categories, the proportion of concern expressed is reasonably well balanced with the percentage of animals used, *i.e.* those consumed for fur garments, killed in pounds or killed by hunters. By contrast, the ratio of concern/use is severely disproportionate in the other two categories. Although only 0.3% of all animals consumed in the US each year are used for research and teaching, these activities received almost two thirds of the criticism of the animal rights authors, whereas the use of animals for food, which accounts for 96.5% of the total consumed, received less than half as much criticism. Dividing the percentage of concern by the percentage of actual use, we get 0.32 for food, and 211.00 for research and teaching. The concern-to-use ratios show a discrepancy of 659-fold (*i.e.* 211/0.32).[17] The huge imbalance between the attention devoted to the activity and the actual extent of the activity is evident. In the background of this are emotional factors based on insufficient authentic information on animal experimentation.

Today, numerous animal rights movements exist in different countries. Their spectrum ranges from moderate to extremist (animal rights terrorism) **(Figures 2, 3, 4.)**. They have one common aspect: all wish to restrict or totally prohibit animal experimental research. The moderates hope to achieve restrictions by legislative action. The research community regards this group as one with which cooperation is possible. No cooperation appears possible with the extremist groups, as these do not exclude violence and illegal acts from their agenda.

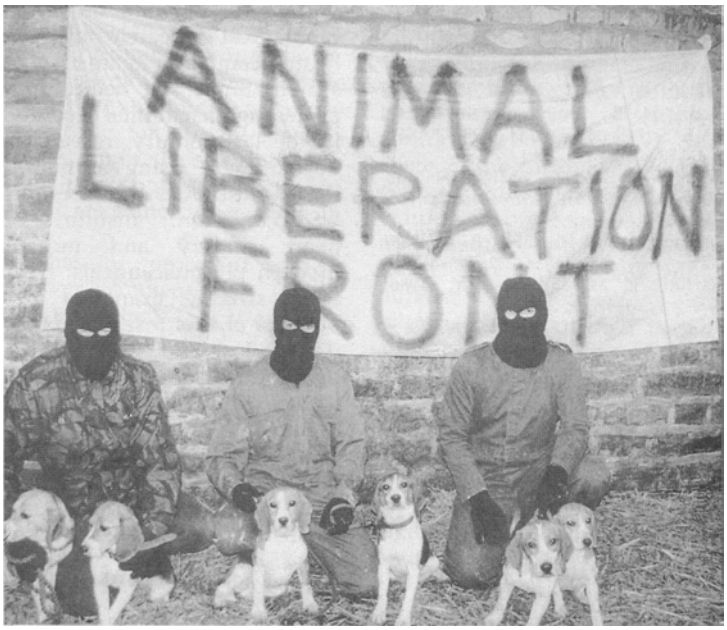


Figure 2. The Animal Liberation Front

Figure 3. Researcher's car bombed by animal rights extremists

Figure 4. Demonstrators against animal experiments

The increase in the number of animal rightists in recent decades is in part connected with the multiplication of television channels. Some of these channels exclusively present the lives of animals, demonstrating the really remarkable capacities of some species. Some city-dwellers rely solely on these channels for information on animals. Some may have personal experience only with their pet dog or cat. Such relationships can generate very strong emotional bonds. These bonds may be of enormous importance for lonely people or for those frustrated in interpersonal relationships. In many cases, these are misanthropic individuals. Many such types can be found among animal rightists. A quotation from Ingrid Newkirk (a prominent activist, *cf.* below): „I am not a morose person, but I would rather not be here. I don't have any reverence for life, only for the entities themselves. I would rather see a blank space where I am. This will sound like fruitcake stuff again but at least I wouldn't be harming anything."[18] Another significant factor is the „Walt Disney effect" attributable to the cute little anthropomorphic animal creatures that show human emotions in animated cartoons, however far this may be from reality.

Activity stemming from these emotional factors can take different forms in different people. It is regrettable if it gives rise to violent deeds. Fortunately, this has not yet occurred in Hungary (there have been threats), but in other countries organizations have suffered from animal rights terrorism. Some years ago the U.S. senate ordered an investigation of this problem. This revealed damages costing several hundred million dollars in facilities using animals, including break-ins, destruction and arson in research laboratories. Laboratory animals were set free by the thousand, and somewhat bizarrely, they included strains, that are incapable of living in the outside environment and soon perished there.

Some examples of extreme statements from animal rights activists. Ingrid Newkirk (founder, People for the Ethical Treatment of Animals = PETA, an organization that is also active in Hungary): „ I find it small wonder that the laboratories aren't all burning to the ground. If I had more guts, I'd light a match."[19] Again: „Animal liberationists do not separate out the human animal, so there is no rational basis for saying that a human being has special rights. A rat is a pig is a dog is a boy. They're all mammals.[20] Michael W. Fox (vice president, The Humane Society of the United States): „The life of an ant and that of my child should be given equal consideration". [21] Alex Pacheco (director, PETA): „Arson, property destruction, burglary and theft are „acceptable crimes" when used for the animals cause". [22] Dan Matthews (spokesperson, PETA) (when asked about the consequences of stopping animal research): „Don't get diseases in the first place schmo".[23] Jerry Vlasak (Animal Liberation Press officer): „I don't think you'd have to kill too many {researchers}. I think five lives, 10 lives 15 human lives, we could save a million, two million, 10 million non-human lives".[24]

In general, animal rights activists exert their activity against animal experiments in three fields: a. moral philosophical persuasion, b. misrepresentation of facts about animal experiments, and c. vandalism, intimidation and terrorist acts. Moderates, employ only methods falling into category a. We will discuss only this category.

3. Do animals have rights?

Critical views condemning animal experiments, and alluding to moral philosophical grounds, display two trends. Firstly, animal experiments wrongly violate the *rights* of animals.[25] Secondly, proponents of this view contend that experiments impose on animals much avoidable *suffering.*[26] As will be shown here neither of these arguments is sound. The first relies on a mistaken understanding of rights; the second relies on a mistaken calculation of consequences and both deserve definitive dismissal

To understand whether animals have rights, we have to deal with the question of what a right is. A right, properly understood, is a claim, or potential claim that one party may

exercise against another. The target of a claim may be a single person, a group, a community, or (perhaps) all mankind. The content of a rights claim can vary greatly: repayment of a loan, nondiscrimination by employers, noninterference with free speech by the state, and so on. To comprehend any genuine right fully, therefore, we must know *who* holds the right, *against whom* it is held, and *to what* it is a right.

Alternative sources of rights add complexity. Some rights are grounded in constitution and law (*e.g,* the right of an accused to trial by jury); some rights are moral but give no legal claims (*e.g.* my right to your keeping the promise you gave me); some rights (*e.g,* against theft or assault) are rooted both in morals and in law.

The differing targets, contents and sources of rights, and their inevitable conflict, together constitute a tangled web. It is clear, however, that in the last analysis rights are based on morals and arise in all cases in a community of moral agents. Rights can be intelligibly defended only among beings who actually do or can make moral claims against one another. The possessors of rights are therefore persons, *i.e.* human beings.

Only humans confront choices that are purely moral; only they - and certainly not rats, cats or mice - lay down moral laws, for others and for themselves. Animals lack this capacity for free moral judgement. They are not beings of a kind capable of exercising or responding to moral claims. Animals therefore have no rights and they can have none. The holders of rights must recognize possible conflicts between what is in their own interest and what is just. In conducting research on animals, therefore, we do not violate their rights, because they have none to violate (although not pertaining directly to our subject, the same applies to our consuming animals as food).

It does not follow from this, however, that we are morally free to do anything we please to animals. Certainly not. In our dealings with animals, as in our dealings with human beings, we have obligations that do not arise from claims against us based on rights. Rights entail obligations, but many of the things we ought to do are in no way tied to another person's entitlement. Rights and obligations are not reciprocals of one another and it is a serious mistake to suppose that they are.

Examples are helpful. Obligations may arise from internal commitments made: physicians have obligations to their patients that are not grounded merely in their patients' rights. Teachers have such obligations to their students. Adults owe special care when playing (*e.g.* footballing) with young children, and children must pay special care when playing with young pets. Obligations may arise from special relationships: parents may feel obliged to pay the university tuition of their child or pay for his or her private language, music, *etc*. lessons if they reasonably can, even if the child formally may not have the right to all this support. We may be obliged to another for a special kindness done.

In our dealings with animals, few will deny that we are at least obliged to act humanely. To treat animals humanely, however, is not to treat them as humans or as the holders of rights. There are humans who are unable to make moral considerations (*e.g.* the brain-damaged, the comatose, or the senile). The capacity for moral judgement that distinguishes humans from animals is not a test, however, to be administered to human beings one by one. Persons who are incapable, because of some disability, to perform the full moral functions natural to human beings are certainly not ejected for that reason from the moral community. The issue is one of kind. It is about the essence of our being human. It follows from this that experiments must not be performed on humans and investigations demand their voluntary consent. Animals are such that it is impossible in principle for them, to give or withhold voluntary consent or to make a moral choice. What humans retain when disabled, animals have never had.

Animals display remarkable behavior at times. Conditioning, instincts and intelligence all contribute to species survival. Membership in a community of moral agents nevertheless

remains impossible for them. Genuinely moral acts have an internal as well as an external dimension. Thus, in law, an act can be criminal only when the guilty deed, the *actus reus* is carried out with a guilty mind, *mens rea.* No animal can ever commit a crime, and bringing animals to criminal trial (which did occur in medieval times) is a mark of primitive ignorance. Does a lion have a right to eat a baby zebra? Does a baby zebra have a right not to be eaten? Such questions do not make good sense; they mistakenly invoke the concept of right where it does not belong. Those who condemn biomedical research because it violates „animal rights" commit the same blunder.

4. Do we have moral grounds for using animals in science?

Abandoning reliance on animal rights, some critics resort instead to animal sentience – their ability to feel pain and distress. We should desist from the imposition of pain insofar as we can. Since all or nearly all experimentation on animals does impose pain and could readily be foregone (these critics say), it should be stopped. The ends sought may be worthy, but these ends do not justify imposing agonies on humans, and the agonies are felt no less by animals. This argument relies essentially on the views of Jeremy Bentham, an 18th century English philosopher **(Fig. 5).** [27] the founder of utilitarian philosophy. This philosophy is based on the calculation of the net product of pains and pleasures. The sum of all suffering („pains") in the world should be decreased and that of joys and well-being („pleasures") increased. Opponents of animal experimentation often quote from him the following sentence (made in reference to horses and dogs): „The question is not Can they reason? nor Can they talk?, but, Can they suffer?". Animals certainly can suffer and surely ought not to be made to suffer needlessly. But in inferring, from these uncontroversial premises, that biomedical research causing animal distress is largely (or wholly) wrong, these critcs commit two serious errors: first, they judge animal and human suffering equally, and second, they miscalculate the consequences of stopping animal experiments.

Figure 5. Jeremy Bentham

The first error committed by the proponents of this view is the assumption that all sentient beings have an equal moral standing. These critics contend that there is no moral difference between, say, a dog and a human; hence, the pains suffered by a dog must be weighed no differently from the pains suffered by humans. To deny such equality, according to this view, is to give unjust preference to one species over another; it is „speciesism". The prominent protagonist of this view is Peter Singer. [26] In his view the „speciesist" is similar to

the racist, or to the sexist. The racist violates the principles of equality by giving greater weight to the interest of members of his own race when there is a clash between their interests and the interests of those of another race. The sexist violates the principle of equality by favoring the interests of his own sex.

This argument is basically wrong and should receive outright refusal. Racism has no rational grounds whatever. Differing degrees of respect or concern for humans for no other reason than that they are members of different races is an injustice totally without foundation in the nature of the races themselves. Racists do grave moral wrong that has led to outright horror.

Between species, however, or more precisely, between humans on the one hand and other species (*e.g.* cats or rats) on the other, the morally relevant differences are enormous. Humans engage in moral reflection; humans are morally *autonomous* (Kant); and humans are members of moral communities, recognizing just claims against their own interest. Humans do have rights; theirs is is a moral status very different from that of cats or rats.

In itself, the epithet speciesist could even be acceptable (humans, like any other species favor their own interests), but the analogy between speciesism and racism is insidious and atrocious. Every sensitive moral judgement requires that the differing natures of the beings to whom obligations are owed be considered. If all forms of (vertebrate?) life must be treated equally, and if therefore in evaluating a (hypothetic) research program the pains of a rodent count equally with the pains of a human, we are forced to conclude (1) that neither humans nor rodents possess rights or (2) that rodents possess all the rights that humans possess. Both alternatives are absurd. Yet one or the other must be swallowed if the moral equality of all species is to be defended.

Humans owe to other humans a degree of moral regard that cannot be owed to animals. Some humans (physicians and biomedical scientists) take on the obligation to support and heal others and to fight against diseases, suffering and death with their science-based activity. The fulfillment of that duty may require the sacrifice of many animals.

If biomedical investigators abandon the effective pursuit of their professional objectives because they are convinced that they may not do to animals what the service of humans requires, they will fail, objectively, to do their duty. This would be an immense disaster. Then, the words of Ingrid Newkirk, cited on page 7 (…a rat is a pig is a boy…) would become reality. Even if it were true – as it is surely not – that the pains of all animate beings must be counted equally, a cogent utilitarian calculation requires that we weigh all the consequences of the use, and of the nonuse, of animals in laboratory research. Critics relying (however mistakenly) on animal rights may claim to ignore the beneficial results of such research, rights being trump cards to which interest and advantage must give way. But an argument that is explicitly framed in terms of interest and benefit for all in the long run must also attend to the disadvantageous consequences of not using animals in research, and to all achievements attained and attainable only through their use. The sum of the benefits of their use is utterly beyond quantification. The benefits of the elimination of horrible disease, the increase of longevity, the avoidance of great pain, the saving of lives (for humans and for animals) achieved through research using animals are so incalculably great that the argument of these critics, systematically pursued, establishes not their conclusion, but its reverse: *to refrain from using animals in biomedical research is, on utilitarian philosophical grounds, morally wrong.* [28]

When balancing the pleasures and pains (Bentham's terminology, see page 9.) resulting from the use of animals in research, we must not fail to place on the scales the terrible pains that would have resulted, would be suffered now, and would long continue had animals not been used. Nor may we ignore, in the balancing process, the predictable gains in

human (and animal) well-being that are probably achievable in the future, but will not be achieved if the decision is made now to desist from such research or to curtail it.

Medical investigators are seldom insensitive to the distress their work may cause to animals; in fact, they strive to minimize it, not least because this also serves the attainment of meaningful results. Opponents of research using animals are frequently insensitive to the cruelty of the results of the restrictions they would impose. Untold numbers of human beings would suffer grieviously as the consequence of this well-meaning, but shortsighted tenderness. If the morally relevant differences between humans and animals are borne in mind, and if all relevant consequences are weighed, the calculation of long-term consequences must give overwhelming support to biomedical research using animals.

5. Animal protection law

Measures and rules for animal protection and the prohibition of animal torture have been in effect in Hungary since the end of the 19th century. An all-inclusive law encompassing numerous fields of animal use, however, was passed by Parliament only in 1998. This is law XXVIII of 1998, *„On the protection and sparing of animals"*, which deals extensively with animal experimentation. Among its numerous attached rules, the following are related to this field: governmental regulation 243/1998. (XII. 31) „*On animal experimentation"*; ministerial regulation FVM-KÖM-GM 36/1999. (IV.2.) *„Rules for the breeding, keeping, transporting etc.of experimental animals"*; and ministerial regulation FVM 10/1999. (I.27.) *„On the Advisory Body for Animal Protection"*.

The law came into force before Hungary joined the European Union (EU), but the relevant EU recommendation was considered in its preparation. This recommendation is contained in the 123rd Convention of the Council of Europe, issued in 1986 and later modified.[29]

The main feature of the law as regards animal research is that licences are required to conduct experiments. The institution where the research is done must be licensed. Such institutions must have an Institutional Animal Experiments Committee (IAEC). The research program itself and its participants must have a licence. Licences have expiry dates. The chain of events involved in obtaining a licence is as follows. The head of a research project submits a request to the IAEC, which evaluates it, and – if it is found suitable – *approves* it. The approved request is then considered by the Scientific Ethical Council of Animal Experiments (SECAE). This latter body is elected from members of the Advisory Body for Animal Protection (ABAP). The research project is either *supported*, returned for modification, or rejected by SECAE. Supported projects are *licensed* by the particular County Animal Health and Food Controlling Authority of the location where the experiments are performed. This same authority *monitors* animal experimental institutions, facilities and programs, too.

According to the law, animal experiments are permitted only for the following purposes: a) the prevention, diagnosis and treatment of diseases of man, of invertebrate and vertebrate animals, and of plants, including assessement of the effects of drugs, chemicals and other products; b) study of the physiology of man, invertebrate and vertebrate animals**,** and plants; c) protection of the environment; d) scientific research; e) teaching and practical training; f) forensic investigations; and g) the breeding of special experimental animals.

Only persons who have appropriate qualifications (defined in a special regulation), suitable experience, and knowledge of the relevant ethical principles and legal constraints of animal experiments are permitted to act as project leaders in animal research activities.

Animal experiments can be performed only by persons who have received training that qualifies them for this activity. The same applies to caretakers of experimental animals.

6. Animal protection at the University of Szeged

The University is one of the institutions that are licensed for animal experiments. In accordance with its mission, it attaches great importance to scientific research, including animal research. Its standpoint on the latter can be found on the home page of the University at http://www.u-szeged.hu/object.178f81e5-d0b1-4228-8941-34ff8d20a67c.ivy. In the same place, other useful documents are also accessible, including the statement of the Interdepartmental Animal Experimental Scientific Committee of the Hungarian Academy of Sciences on the use of animals in scientific research, the bylaws of the IAEC of the University of Szeged and downloadable forms for the licensing process of animal experiments.

The University of Szeged, consistent with its humane calling, places great emphasis on enforcing the rules of animal protection in the animal research work conducted on its campus. At its own incentive, it established a Scientific Ethical Committe on Animal Protection in its medical shool as early as in 1992. This committee may be regarded as the predecessor of today's IAEC. An obligatory ethical codex of animal experimentation is in effect at the University.

7. Ethical codex of animal experimentation at the University of Szeged

It is the view of the University of Szeged that the usefulness for society, the indispensibility and the moral grounds of animal experiments are beyond question. Equally, it is important that the following principles should be observed in researches using animals.

I. The transportation, care and use of animals should be in accordance with the law on animal protection.
II. Procedures involving animals should be designed and performed with due consideration of their relevance to human or animal health, the advancement of knowledge, or the good of society.
III. It is a moral obligation of researchers to consider all projects from the aspect of the humane treatment of animals.
IV. Investigators and other personnel must be appropriately qualified and experienced for conducting procedures on living animals. Adequate arrangements must be made for their in-service training, including the proper and humane care of laboratory animals.
V. The living conditions of animals should be appropriate for their species and contribute to their health and comfort. The housing, feeding, and care of all animals used for biomedical purposes must be directed by a veterinarian or other scientist trained and experienced in the proper care, handling and use of the species being maintained or studied. In any case, veterinary care must be provided as indicated.
VI. The animals selected for a procedure should be of an appropriate species and quality and of the minimum number required to obtain statistically valid results. At the same time, use of a lesser number of animals should be avoided because this would be a wasting of animal lives. Where possible and reasonable, methods such as mathematical models, computer simulation and *in vitro* biological systems should be considered.
VII. When consistent with sound scientific practices, the following rules must be observed. Avoidance or minimization of discomfort, distress, and pain. Unless the contrary is established, investigators should consider that procedures that cause pain or distress in human beings may cause pain or distress in animals. Procedures with animals that may cause more than momentary or slight pain or distress should be performed with appropriate sedation, analgesia or anesthesia. Surgical or other painful procedures should not be performed on unanesthetized animals paralyzed by chemical agents (muscle relaxants).

VIII. Animals that are exposed to long and noxious effects during the experiment must not be reused for other investigations. Animals that would otherwise suffer severe or chronic pain or distress that cannot be relieved should be painlessly killed.

IX. Research using animals at the University of Szeged must only be performed with the approval of the IAEC under the licence of the animal health authority.

X. Investigators at the University of Szeged do not sign research contracts and do not support publications not conforming to these ethical principles.

III. Fundamentals in the keeping and care of experimental animals (*Dr. Balázs Gaál*)

An experimental animal can serve as a model for a human being in all those functions in which there is a considerable degree of genetic similarity. The experiments can be acceptable for everybody only if the results are unquestionable and reproducible, *i.e.* exact. This can be the case only if the experimental (treatment) and control groups differ in only one aspect, *i.e.* the aspect under investigation. There are significant differences in sensitivity between species because of their differing genetic backgrounds. This is the reason why different species are appropriate or not for a given experiment.[30]
An important requirement for laboratory animals (apart from price) is that they should be easy to keep. Today, rodents meet this demand best. Of all the mammals, mice and rats have been most frequently used in laboratories (approximately 90%), but in the last 20 years the proportion has decreased to 1/10. As compared with these species, the number of animals that can be kept as pets, such as guinea pigs, rabbits and dogs, is very low. The reasons for this are the increasing frequency of experiments without the use of animals, or the use of animals with particular characteristics (*e.g.* inbred, SPF, see below), and the rising prices.[30]

1. Biology of the most frequently used mammals

1.1. Mouse

Seventy per cent of all animals used in biomedical activities are mice. More than one thousand stocks and strains of mice have been developed, as have hundreds of mutant stocks that are used as models of human diseases. In terms of genetics, the mouse is the most thoroughly characterized mammal. The life span is 1.5-3 years. Mice are very active animals. They are sensitive to changes in temperature, draughts and high humidity. The incisors are open-rooted and grow continuously. Mice will bite or "pinch" with their sharp incisors if mishandled. Mice should be fed a commercial pelleted mouse or rodent diet and water *ad libitum*. Mice are polyestrous animals and breed the year round; ovulation is spontaneous. A fertile postpartum estrus occurs 14-24 h following parturition. The duration of the estrous cycle is 4-5 days. The average gestation period is 20 days. The average litter size is 10. The pups are weaned at 3 weeks of age.

1.2. Rat

The rat is the second most commonly used animal in biomedical activities, surpassed only by its relative, the mouse. Various stocks, strains and mutants are available, but much fewer than for the mouse. Most laboratory rats are outbred albinos. Rats are less active and aggressive than mice. They are typical rodents, with continuously growing incisors. Rats should be fed a commercial pelleted rat or rodent diet and water *ad libitum*. The life span is 2.5-3.5 years. They are sensitive to draughts, a high level of ammonia, low humidity and noise. They are susceptible to respiratory diseases. Rats too are polyestrous animals and breed the year round; ovulation is spontaneous. The duration of the estrous cycle is 4-5 days. The average gestation period is 22 days. A fertile postpartum estrus occurs within 48 h of parturition. The average litter size is 6-12. The pups are weaned at 3 weeks of age.

1.3. Hamster

Hamsters are the third most commonly used research animal. Ninety per cent of them are Syrian hamsters. Hamsters have a tendency to bite if roughly or improperly handled, startled, injured or awakened. Females tend to be larger and more aggressive than males. The life span is 1.5-2 years. The incisors are open-rooted and grow continuously. The cheek pouches of the hamster extend to the scapulae and can be everted. Hamsters should be fed commercial pelleted hamster or rodent diet and water *ad libitum*. The flank or scent glands

appear as dark patches on either flank. They are sebaceous glands that function in marking territory and mating behavior. The urethra of the female exists separately just ventral to the vulva. Breeding onset is at approximately 90 days. Hamsters are polyestrous animals and breed the year round; ovulation is spontaneous. The duration of the estrous cycle is 4 days. The average gestation period is 16 days. The average litter size is 5-9. Cannibalism is more common with hamsters than with other laboratory rodents. The young are weaned at 21 days.

1.4. Guinea pig

The most common stocks used in research are shorthairs. Guinea pigs panic easily in response to a new or frightening experience, scattering to the corner of the cage or freezing in place Teeth-chattering of nervous guinea pigs can also be seen. The life span is 4-5 years. All teeth are open-rooted and grow continuously. The guinea pig has a large cecum. Guinea pigs have a dietary requirement for vitamin C, and should therefore be fed a commercial pelleted diet formulated specifically for guinea pigs. Guinea pigs are polyestrous and breed the year round; ovulation is spontaneous. The duration of the estrous cycle is 15-17 days and estrus itself lasts 24-48 h. The average gestation period is 65 days. The average litter size is 3-4. The young are precocious at birth 3-4; weaning occurs at 14-28 days.

1.5. Rabbit

The breeds of rabbit most commonly used in research are the New Zealand White and the Californian, which weigh 2-5 kg, *i.e.* they are large breeds. The life span is 5-6 years. All the teeth are open-rooted and grow continuously. The rabbit cannot vomit, like the rat and horse. The cecum is large, and terminates in the vermiform process or cecal appendix. This appendix contains a large amount of lymphoid tissue. Rabbits are coprophagous. They consume soft, moist fecal pellets produced at night (night stool). Rabbits should be fed a commercial pelleted rabbit diet; water should be provided *ad libitum*. Diets such as these are nutritionally complete and do not require supplementation. The highly vascularized ears are important in thermoregulation. The uterus is duplex with separate cervical and uterine openings. Breeding onset in the medium-sized breeds is at the age of 5-6 months. Rabbits are induced ovulators with no regular estrus cycle. The vulva of the receptive doe is congested and purplish. The average gestation period is 32 days. Shortly prior to parturition (kindling), the doe builds a nest, pulling large amounts of hair from her mammary region and forelimbs. The average litter size is 7-8. Weaning occurs at 4-6 weeks.

1.6. Pig (potbellied pig, micropig, minipig)

Pigs are social animals and have a rigid dominance hierarchy. If pigs are group-housed, they will generally fight for the first 24-48 h to establish dominance, which is almost directly related to size. Pigs in general are friendly and docile, but will react severely to poor handling or a stressful environment. The life span of the potbellied pig (PBP) is probably 8-20 years with ~10-15 years typical. The PBP is sensitive to extremes of heat and cold and should be provided with a clean, dry, draught-free environment. Extended exposure to high temperatures combined with high humidity may be fatal to the PBP.

1.7. Cat

Cats are strict carnivores and predators. They have excellent vision and hearing, but their sense of smell is not as well developed as in the dog. Cats are generally solitary by nature, with strong territorial ties. At most they may form loose-knit social groups. Territories are marked by urine spraying and smearing surfaces with the scent glands located in the chin and in the head, in front of the ears. Cats are generally non social, but can adapt to group living. An ideal group is approximately 20 individuals, as this enables a hierarchy to form

which tends to be relatively stable. Cats have certain physiological features that are more in common with those in humans than the laboratory rabbit or rodent; hence, they have been extensively used in behavioral and biomedical research, particularly in the neurological sciences. Bred cats can generally live for 10 to 13 years. Cats cannot survive on diets adequate for other carnivores, unlike dogs (*e.g.* cats require taurine). The female cat (or queen) is seasonally polyestrous, but a small percentage can breed continuously. Queens can be run as a harem-mating system. The only way to detect estrus is via the behavior changes (and the vaginal smear). Estrus behavior lasts for 4-6 days. The queen is an induced ovulator. The gestation period is 59-65 days. The average litter size is 4-6 kittens. The kittens are generally weaned at 6-7 weeks.

1.8. Dog

The domestic dog is a species with a large number of breeds rather than genetically defined strains. There are marked differences between the various breeds in size, appearance and life expectancy. Dogs are highly social, smart, intelligent animals. Both nonspecific and human social contacts are extremely important for their well-being. Their social isolation and solitary housing are therefore considered to be important stressors. They do best when housed either in pairs or in small compatible groups in an environment affording some level of complexity and choice. Daily contact with caretakers is important. The dog has been used as a model of many human conditions in areas such as cardiovascular research, pharmacology and toxicology. The average life span is 8-12 years. Dogs used for research or teaching may be purpose-bred. The typical laboratory dog is the beagle, but other breeds are also used in large numbers. Dogs are carnivores with monogastric digestive systems. They should be fed only complete and balanced diets. Many commercially available dog foods contain all essential nutrients in their required proportions. These foods are manufactured in dry, semi-moist and canned forms. Reproductive data: the age of puberty is 7-10 months. The length of the estrus cycle (ovarian cycle) is 21-28 days. In most breeds the interval between estrus periods is 6–7 months. Females (bitches) ovulate spontaneously. The average length of gestation (pregnancy) is 63 days. The average litter size is 3–8. The puppies are generally weaned at 6–7 weeks. [30, 33-35]

2. Housing of experimental animals

2.1. General principles

Proper housing and good management of animal facilities are essential for the animal well-being, the quality of the research data, and the health and safety of the personnel. At a minimum, an animal must have sufficient space to turn around and to express normal postural adjustments, it must have ready access to food and water, and it must have enough clean-bedded or unobstructed area in which to move and rest. Animals should have opportunities to exhibit species-typical activity patterns. Whenever appropriate, social animals should be housed in pairs or groups, rather than individually, provided that such housing is not contraindicated by the protocol in question and does not pose an undue risk to the animals. Highly social and many other domesticated animals benefit from positive human interaction.

2.2. Physical facilities

The microenvironment of an animal is the physical environment immediately surrounding it (the primary enclosure), with its own specific factors (such as a cage or pen). The primary enclosure provides the limits of the animal's immediate environment. The macroenvironment is the physical environment of the secondary enclosure (such as an animal holding room, a barn, *etc.*). Basic types of establishments for animal keeping are breeding /

supply establishments and user establishments. Important elements of the primary and secondary enclosures are as follows:

- A cage is a permanently fixed or movable container that is enclosed by solid walls and, at least on one side, by bars or meshed wire, in which one or more animals are kept or transported; depending on the size of the container and the stocking density, the freedom of movement of the animals is relatively restricted.
- A pen is an area enclosed by walls, bars or meshed wire, in which one or more animals are kept; depending on the size of the enclosure and the stocking density, the freedom of movement of the animals is usually less restricted than in a cage; pens must be designed for the well-being of the species; they must permit the satisfaction of certain ethological needs (for example, the need to climb, hide or shelter temporarily).
- A run is an area enclosed by walls, fences and bars or meshed wire and frequently situated outside permanently fixed buildings, in which animals kept in cages or pens can move freely during certain periods of time.
- An animal holding room is a room situated inside a building where animals are housed in cages or pens, either for breeding and stocking, or during the conductance of an experiment.

The majority of holding rooms are usually designed to house rodents. Such rooms may also frequently be used to house larger species. Care should be taken not to house together species which are incompatible. Walls and floors should be smooth, impervious and have a non slippery, easily washable surface. Drains, if any, should be adequately covered. Such rooms should be provided with facilities for carrying out minor experiments and manipulations, where appropriate.

Service rooms are rooms for the storage of food, clean cages, bedding, cleaning and washing materials. The rooms for food and bedding must be cool and dry, and vermin- and insect-proof. The rooms for clean cages, instruments and other equipment must be adequately designed; the cleaning process must be arranged so as to separate the flow of clean and dry equipment in order to prevent the contamination of newly cleaned equipment. [30,31,34]

2.3. Housing of the most frequently used species

Rodents, guinea pig, ferret

These animals are usually kept in plastic cages with solid bottom and walls, of different sizes (shoe-boxes), with roofs made of wire, and with bedding. Guinea pigs and ferrets may be housed in small pens, made of plastic, metal or concrete, enclosed by low walls, with open roof and with bedding (wood shavings). Ferrets and guinea pigs are social animals, and it is therefore best to keep them in pairs or in groups. They need a complex, dynamic environment. For guinea pigs, it is necessary to provide a shelter in their cage or pen, where they can seek safety.

Rabbit

Rabbits are usually kept individually in small cages with a solid or meshed-wire bottom. They are gregarious animals, and should therefore be housed in compatible groups in pens with bedding (indoor or indoor-outdoor).

Cat

Cats are kept in individual caging, or in group pens or cages. The housing of cats in cages should be strictly limited. They should be let out for exercising at least once a day, if this does not interfere with the experiment. In the group situation, the most essential criterion for acceptable housing is the provision of an adequate number of hides and escapes for cats. Cat pens should be equipped with dirt trays, an amply raised shelf room for resting, and objects suitable for climbing and claw-trimming. In a closed pen, many non convertible windows are desirable.

Dog, swine (miniature pig)
There is a great range of possibilities as concerns housing: it may be situated in indoor or outdoor pen areas, with open access to the outside run, or in an indoor environment with some sort of complexity built into it. Single housing may be necessary because of the demands of an experiment. Dogs can be given opportunities for activity by having access to a run or being moved into another area (such as a large cage or outdoor pen), which provides more space for movement.
Monkeys (non human primates)
Non human primates are social animals which, when deprived of companionship for an extended time, develop unmistakable signs of depression and frustration (as do dogs and pigs). They are physiologically and anatomically adapted to live in a complex, dynamic environment. Housing such animals in pairs offers a practicable alternative to group-housing. Raised resting surfaces or perches are also desirable for monkeys and dogs.

3. Husbandry

The person in charge of the establishment must ensure regular inspection of the animals and supervision of the accommodation by a veterinarian (or other competent person).

3.1. Food, drinking water and bedding
Feed

Animals should be fed palatable, non contaminated and nutritionally adequate food, daily or according to their particular requirements, unless the protocol in which they are being used requires otherwise. Nowadays, the feed is 'standard' mixed feed, pressed into pellets for almost all species (dry laboratory-animal diets). Packing, transport and storage must be such as to avoid contamination, deterioration or destruction. The major problems with feed may be an increase in the number of bacteria, rancidity, and the appearance of toxins caused by fungi.

The feed distribution process may vary according to the species, but it must be such as to satisfy the physiological needs of the animal: for rodents *ad libitum* (as much they like), but for cats, dogs and pigs in portions. Feeders should be designed and placed so as to allow easy access to food and to minimize contamination.[30-35]

Drinking water

Animals should have access to potable, uncontaminated drinking water according to their particular requirements. (During transport, it is acceptable to provide water as part of a moist diet.) Watering devices should be checked daily to ensure their proper maintenance, cleanliness and operation. The methods commonly used in watering involve bottles, dishes and automatic systems. Bottles are often used for small animals such as rodents and rabbits, while dishes are customary for cats, dogs or pigs. When bottles are used, they must be made from translucent material in order to allow their contents to be monitored. All bottles and accessories must be taken to pieces, cleaned and sterilized at appropriate and regular periods. It is better to replace water bottles than to refill them, because of the potential for microbiologic cross-contamination.

Bedding

Bedding must be dry, absorbent, non dusty, non toxic and free from infectious agents or vermin or any other form of contamination. Ordinarily, softwood shavings and chips are appropriate and used, made from non resinous wood. Special care must be taken to avoid sawdust or bedding material derived from wood which has been treated chemically. Bedding should be used in amounts sufficient to keep animals dry between cage changes.

Sanitation (cleaning)

Sanitation (the maintenance of conditions conducive to health) involves bedding changing (as appropriate), cleaning and disinfection. Cleaning removes excessive amounts of dirt and debris, and disinfection reduces or eliminates unacceptable concentrations of microorganisms. The standard of a facility depends very much on good hygiene. Clear instructions must be given for the changing of bedding in cages and pens, and adequate routines must be established for the cleaning, washing and decontamination of cages and accessories, bottles and other equipment. All components of the animal facility, including animal rooms and support areas, should be cleaned regularly and disinfected as appropriate to the circumstances.

Contacts with the animals

The performance of an animal during an experiment depends very much on its confidence in man, something which has to be developed. It is therefore recommended that frequent contact should be maintained so that the animals become familiar with human presence and activity (adaptation). The staff must be sympathetic, gentle and firm when associating with the animals. [30-35]

4. Factors in the macroenvironment

Temperature

The temperature must be measured continuously. The recommended (optimal) temperature range for mice, rats, guinea pigs and miniature pigs is 20–24 °C, while for dogs, cats and rabbits it is 15–21 °C. Some conditions might require an increased environmental temperature, such as postoperative recovery, the housing of some hairless rodents and the housing of neonates.

Humidity

Extreme variations in relative humidity (RH) have an adverse effect on the health and well-being of animals. The RH must ordinarily be maintained at 55±10%. Levels below 40% or above 70% for a prolonged period must be avoided.

Air ventilation

The purposes of ventilation are to supply adequate oxygen (fresh air); to remove thermal loads caused by animal respiration, lights and equipment; to dilute gaseous and particulate contaminants (*e.g.* ammonia or dust); to adjust the moisture content of the room air; and, where appropriate, to create static-pressure differentials between adjoining spaces. The air in the room should be changed at frequent intervals. A ventilation rate of 15–20 fresh-air changes per hour is normally adequate. The use of recycled air to ventilate animal rooms must be avoided. The ventilation system must be designed so as to avoid harmful draughts. The air used for ventilation should be heated in winter and cooled in summer. Frequent bedding changes and cage-cleaning, coupled with husbandry practices, such as a low animal density within the room, and lower environmental temperature and humidity, can also reduce the concentration of toxic or odor-causing gases in the animal room air.[30-35]

5. Microbiological environment (microorganisms and infections)

Infectious agents may affect animal populations in various ways. Some are pathogenic and may induce clinical signs with variable morbidity and mortality. However, most microorganisms induce no or only mild disease. Silent, asymptomatic infections (adventitious pathogens and opportunistic organisms) are often activated by experimental procedures (stress, immunosuppressant, *etc.*) or environmental influences (transportation, toxic gases,

etc.). Frequently, certain strains of a given species are more sensitive to an infection, whereas the same agent may cause only milder or different symptoms in other strains, or the infection may be asymptomatic.

The types of organisms that can infect (experimental) animals include bacteria, protozoa, fungi, viruses, helminthes and arthropods. Many agents (even in the event of clinically silent infections) may exert impacts on the physiological parameters and hence on the results of animal experiments. It is obvious that experimental data obtained from diseased animals should, if at all, be used only with maximal caution. The use of laboratory animals that are free from unwanted microorganisms is an important prerequisite for the acquisition of reliable and reproducible results with a minimum of animals and is a significant contribution to animal welfare.[30]

5.1. Microbiological quality and classifications of experimental animals

The presence of unwanted microorganisms and the suitability of an animal population for a specific experiment can be demonstrated only by comprehensive health monitoring before and during experimentation. The quality of animals is most commonly characterized in terms of the microbiological (hygienic) status and of the system used in raising animals to ensure that a specific microbiological status is maintained. There are three major types of maintenance (hygienic levels):

- Isolator-maintained: The animals are kept in a sterilizable chamber with a sterilized air supply, a mechanism for introducing sterilized materials, and a series of built- in gloves.
- Barrier-maintained: The animals are bred and kept in a dedicated space, behind a barrier. For these facilities, personnel enter through a series of locks and are usually required to shower and use disinfected clothing. All equipment, supplies and conditioned air are sterilized or disinfected. Barrier facilities are designed to exclude organisms for which animals are the primary or preferred hosts, but will generally not exclude organisms for which humans are hosts. Barrier maintenance can also be achieved at the cage or rack level with equipment that can be sterilized or otherwise disinfected.
- Conventionally-maintained: The animals are raised in areas that have no special impediments to the introduction of microorganisms. This method of maintaining animals cannot ensure the stability of the microbiological status, because unwanted organisms can be introduced at any time. Micro-isolation cages are generally used to protect animals in otherwise conventional rooms.

Various classifications have been developed to define the microbiological quality of laboratory animals. The most important classifications (hygienic categories) are as follows:

- Germ-free (GF) animals that are derived by Cesarean section or embryo transfer and reared and maintained in an isolator with aseptic techniques.
- Specified-pathogen-free (SPF) animals that show no evidence (usually by serology, culturing or histopathology) of the presence of particular microorganisms. An animal can be classified as SPF if it is free of one or many pathogens. Commercial suppliers have coined various terms to indicate the SPF status. All terms are related to specified organisms from which the animals are stated to be free and for which they are regularly monitored.
- Virus-antibody-free (VAF) animals that are free of antibodies to specified rodent viruses. Animals might not be free of viruses other than those specified and might not be free of other microorganisms.
- Conventional animals in which the microbial burden is unknown, uncontrolled or both.

- Clean conventional or minimal disease (MD) animals that are maintained behind a low-security barrier and demonstrated to be free of selected pathogens. Use of this term should be avoided because of the lack of precision of its meaning.

5.2. Sources of infections / microbial contamination

The sources of microbial contamination include vermin, experimentally infected and spontaneously ill laboratory animals or their tissues or tumors, air, food, water, bedding, ancillary equipment and personnel. (The highest microbiological risk is man himself.)
Good facility management practices and constant surveillance are necessary to minimize the introduction of unwanted microbes.

5.3. Zoonosis

Infectious diseases that may be transmitted from other animals, either wild or domestic, to humans, or from humans to animals, are called zoonoses (the latter is sometimes called reverse zoonosis). The list of potential zoonoses related to working with animals in research, teaching or testing is quite long, though in reality the risks are very low when the common small laboratory animal species are dealt with in the laboratory. There are a number of reasons for this low risk. Firstly, commercial suppliers of laboratory animals have done an excellent job of producing disease-free animals. Further, institutions have generally developed good occupational health and safety programs that include active veterinary monitoring and care programs. The risk of exposure to zoonotic diseases is greater for those who work with experimental animals from random sources (including cats, dogs and most livestock), and for field researchers studying wild animals in their habitat. Working with non human primates in the laboratory is a special case because of the many zoonotic concerns.[30-32]

5.4. Animal quarantine and stabilization

Whenever possible, the health status of every animal should be ascertained before it is brought into the facility. Quarantine is the separation of a newly received animal from those already in the facility until the health of the new animal has been evaluated. Effective quarantine minimizes the introduction of disease agents into established colonies and prevents the possibility of zoonoses.

The quarantine period should be of sufficient duration to allow the expression of diseases present in the incubation stages. Some or all of the following should be achieved during the quarantine and stabilization period: diagnosis, control, prevention and treatment of diseases; physiological and nutritional stabilization; and grooming to include ectoparasite control (many zoonotic agents require an arthropod vector).

The period of quarantine is in some cases laid down in the national animal health regulations. In others, it varies according to the circumstances and should be determined by a competent person, normally the veterinarian appointed by the establishment.
Even when the animals are believed to be in sound health or, in the case of a higher microbiological status, after transportation (receiving), it is good husbandry for them to undergo a period of acclimatization (physiological, psychological, and nutritional stabilization) before being used in an experiment.

5.5. Symptoms, diagnosis, standard procedure for infected, sick animals

At the beginning of an illness, there are only general symptoms. It is usually too late to save the stock (group of animals) when specific signs are present. Clinical examination is difficult in some species (*e.g.* rodents). An animal that appears sick must be isolated immediately (in an SPF facility, it must be removed), and its surroundings must be

disinfected. Apparently sick animal must be examined. In a conventional environment, isolated cases or the whole animal population can be treated.

6. Social environment and other environmental factors

The social environment usually involves physical contact and communications among members of the same species (conspecifics), although it can include non contact communication among individuals through visual, auditory and olfactory signals.

6.1. Space recommendations and group size

An animal's space needs are complex, and considerations of only the animal's body weight or surface area are insufficient. Space needs are given high priority in animal welfare regulations, where the minimal space needs and cage sizes are specified in detail. Some species benefit more from wall space (*e.g.* rodents), shelters (*e.g.* primates) or cage complexities (*e.g.* cats and primates) than from simple increases in floor space. Whenever appropriate, social animals should be housed in pairs or groups, rather than individually, provided that such housing is not contraindicated by the protocol in question and does not pose an undue risk to the animals. Space for group-housed animals should be based on the individual space needs, the behavior, the compatibility of the animals, the number of animals, and the goals of the housing situation.

6.2. Other factors

Effects between and within species (sound, olfactory and microbiological effects) should be considered. Because of these effects, all animal species should be kept (housed) in separated areas (rooms and barns). Noisy animals, such as dogs, pigs and nonhuman primates, should be housed away from quieter animals, such as rodents, rabbits and cats. Other effects involve light, color and the biological rhythm.[30,31,32]

7. Transport conditions

All animals must be acquired lawfully. The use of purpose-bred research animals might be desirable and, for the most frequently used animals, is obligatory by the regulations. As a rule, vendors of purpose-bred animals regularly provide information that describes the genetic and pathogen status of their colonies or individual animals. All transportation is a stressful experience for animals and as a consequence, the physiological parameters may be seriously modified. For normalization of these modifications, rodents need at least 2 weeks, while animals with a highly developed nervous system (*e.g.* primates) need at least one month. All parts of the transportation process must be carried out according to the prevailing animal health, transport and welfare regulations. All transportation of animals should be planned to minimize the transit time and the risk of zoonoses, to protect against environmental extremes, to avoid overcrowding, to provide food and water when indicated, and to protect against physical trauma. Animals must be in good health for transportation and it is the duty of the sender to ensure that they are so. Female animals which are likely to give birth during the transport must be excluded. Consignments of animals must be received and unpacked without avoidable delay. After inspection, the animals must be transferred to clean cages or pens and be supplied with feed and water as appropriate.[30-32]

IV. Pain and distress (*Dr. Balázs Gaál*)

Pain and distress are elements of a particular kind of biological adaptation in nature. Because of the difficulties in determining and measuring them, frequently only abnormal behavior patterns point to pain and distress. Distress is an aversive state in which an animal is unable to adapt completely to stressors and the resulting stress. Pain is normally defined as an unpleasant sensory and emotional experience associated with potential or actual tissue damage (human definition). Long-lasting (chronic) pain (usually produced by disease, injury or surgery) and long-lasting discomfort are important causes of distress, which can lead to anxiety and depression (passivity and apathy). Why is it important to recognize and reduce pain? Pain has different negative consequences (”stress response”), which lead to various physiological malfunctions, such as delayed wound healing, the delayed recovery of organic functions, decreases in immune functions, *etc.* Experimental data may therefore be false, or modified (unless a stressor, such as pain, is the subject of the experiment).

This topic is given high priority in animal welfare regulations: the pain and distress of animals should be minimized whenever possible: *"all experiments shall be designed to avoid distress, unnecessary pain and suffering to the experimental animals."* Accordingly, pain and distress must be reduced to a minimum for both ethical and scientific reasons.[30,36,37]

1. Recognition of pain

It has been proved that animals can feel pain, even at a very young age. Pain is difficult to assess in animals because of the inability to communicate directly about what the animal is experiencing. In the absence of evidence to the contrary, it is assumed that something that is painful in a human will also be painful in an animal. Acute and chronic pains can give rise to different symptoms. The objective signs of pain, which can be measured (defined), are as follows (mainly in cases of acute pain):

- sudden vocalization,
- pupil dilatation,
- increases in respiratory and heart rates,
- sudden urination or defecation, *etc.*

Mostly in the event of chronic pain, the behavior of animals changes, but, it is difficult to recognize these alterations (and different species display different behavior patterns). The behavior patterns are as follows:

- an anxious appearance, and increased aggression and guarding,
- limited or/and slow movement, and a hunched position (back and neck),
- adoption of an unusual posture, *e.g.* a rigid posture,
- a weight loss,
- failure to groom,
- withdrawal, and extended sleep periods,
- a lack of appetite, refusal of food and water,
- apathy and depression,
- vocalization. [30-32,36,37]

2. Alleviation of pain

There are two possible ways to alleviate pain: 1. anesthesia, or the exclusion of the perception or consciousness of pain, *i.e.* the absence of sensation, and 2. analgesia, *i.e.* the suppression, restriction or reduction of pain. As laid down in animal welfare regulations: *”all experiments shall be carried out under general or local anesthesia”*. This does not apply in cases when:

- anesthesia is judged to be more traumatic to the animal than the experiment itself;
- anesthesia is incompatible with the object of the experiment.

In such cases, appropriate legislative and/or administrative measures shall be taken to ensure that no such experiment is carried out unnecessarily. If anesthesia is not possible, analgesics or other appropriate methods should be used in order to ensure as far as possible that pain, suffering, distress or harm are limited. Provided such action is compatible with the object of the experiment, an anesthetized animal which suffers considerable pain once the anesthesia has worn off shall be treated in good time with pain-relieving means.[40]

3. Anesthesia

a. Local (regional) anesthetic drugs act to disrupt nerve conduction temporarily. When applied around a nerve, they produce analgesia in the region served by a nerve. However, these drugs exert no depressant effect on the brain. The major forms are: superficial (surface), conductive and infiltrative local anesthesia.

b. General anesthesia is defined as: all of the methods when the exclusion of the sensation of pain is accompanied by unconsciousness. The basic ways to perform general anesthesia (introduction and maintenance) can be:

- Injectable anesthetics can be administered by various routes, depending upon the specific compound. The most frequently used routes of administration in laboratory animals are intraperitoneal, intramuscular and intravenous.
- Inhalant anesthesia with anesthetic gases. Frequently, an injectable anesthetic is used to induce anesthesia, and the inhalation agent is used for maintenance.

4. Preanesthetic medications (premedication)

It is often advisable and sometimes necessary to premedicate an animal before the induction of anesthesia. In general, anticholinergic drugs, tranquilizers, opioid narcotics (general anesthetics), neurolept-analgesics and dissociative agents are used for premedication. These drugs are used to relieve anxiety and produce calmness, to reduce the dose of anesthetic needed and to provide postoperative pain relief. All premedication drugs, except the anticholinergics, are considered CNS depressants.

5. Assessing the depth of general anesthesia

Involuntary reflexes (such as the palpebral reflex, the ear pinna reflex, and the pedal / withdrawing / reflex, patellar reflex) and the objective signs of pain (such as increases in heart rate and respiratory rate, a rise in blood pressure, pupil dilatation, salivation, movements of limbs, head and tongue, *etc.*) are used primarily as a means of determining the depth of general anesthesia.

6. Basic tasks during anesthesia, recovery

A variety of things must be done to prepare for anesthesia:

- Withhold food and water from large animals for 12 h prior to anesthesia induction, and from small animals for 2 h to prevent regurgitation and aspiration. It is not necessary to withhold food and water from rodents prior to anesthesia.
- Intubate the trachea whenever possible, even if injectable anesthetics are being used. Intubation can be achieved in animals as small as a rat. This will prevent aspiration pneumonia and allow the assistance of respiration if the animal stops breathing.
- To minimize the effects of surgery and anesthesia on hydration (decreasing blood pressure, increasing fluid requirements and hemorrhage), an intravenous catheter is placed whenever possible to provide access for fluids and medication. Fluids are

supplemented intravenously if possible, or otherwise intraperitoneally or subcutaneously during and after anesthesia.

- Animals frequently become hypothermic during anesthesia. Hypothermia depresses all physiological functions, and resulting in prolonged recoveries. Heat loss should be prevented by insulating cold surfaces with a blanket, and heat should be supplemented heat with a thermal blanket or with pre-warmed fluids.
- The depth of anesthesia must be monitored carefully and closely to ensure that the animals do not become too deeply anesthetized and die, and to ensure that they do not become too lightly anesthetized (see above) and experience pain from the surgical procedure. Evaluation of the normal physiological functions (vital signs) in combination with an evaluation of the reflexes is a means of assessing the animal's relative health status under anesthesia. Vital signs routinely monitored are: the heart rate and rhythm, the respiratory rate, depth and rhythm, the arterial pulse, the body temperature, the mucous membrane color, the capillary refill time, the pupil size and the response to light and the muscle tone.
- Vital signs should be evaluated at least every minute during the induction stage, and as often as possible during the maintenance and recovery stages, but not less frequently than once every 5 min. Electrical and mechanical monitoring devices (*e.g.* ECG and pulse-oxymetry) facilitate the monitoring of a greater number of vital signs, with constant checks on the anesthetized patient.
- Monitoring and support must continue until the animal has recovered completely from the anesthesia.

During the recovery period, it is important that attention be paid to the following:

- The prevention of heat loss (towels, heating pad, *etc.*).
- Checks on vital signs (at least every 5 min).
- Turning the animal from side to side once every 15 min unless contraindicated.

Complete recovery means that the animal is able to hold itself in a normal upright position, its body temperature has returned to normal and all physiological indices are within the normal limits. Anesthetic recovery can be rapid for gaseous agents and short anesthetic episodes. The recovery time can be prolonged if animals were anesthetized for a long time or if injectable agents were used.

7. Analgesia

The complete exclusion of the sensation (experience) of pain can be achieved only through general anesthesia, but with the use of analgetics it can be considerably decreased and the negative consequences of pain can be avoided. The basic principle of analgesia today is that the prevention of pain is more effective than its treatment ("preemptive analgesia"). This means that it is best if analgesia can be provided to animals prior to the painful procedure, rather than waiting until after the clinical signs of pain are observed. In this way, the hypersensitivity of the nervous system can be prevented. It is more efficient if two or more drugs are used in combination. The drugs tend to have synergistic effects.

Some anesthetics (narcotics) do have a pain-alleviating effect, but most of them do not. When the latter drugs are used, hypersensitivity of the nervous system (peripheral and central) likewise develops, in spite of the anesthesia (the absence of a pain sensation), and leads to clinical pain after recovery. Accordingly, prior to general anesthesia it should be considered what kinds of analgetic drugs are needed (see premedication above).

8. Drug groups which can be used as analgesics

These comprise opioids, local anesthetics, nonsteroidal anti-inflammatory drugs, alpha-2-agonists and dissociative agents. Some of these groups, when used in combination with each

other or with other anesthetics, are suitable for the achievement of general anesthesia (see premedication above).

V. Administration of different agents. Basic principles of invasive (surgical) procedures (*Dr. Balázs Gaál*)

1. Handling and restraint of experimental animals

The use of proper restraint and handling techniques reduces the level of stress to the animals and also to the researcher. Handling stress is an experimental variable and should be minimized whenever possible. As a result of improper handling, animals can inflict serious injuries on humans and to themselves. If a study involves significant handling of animals, it is recommended that the animals be accustomed to the handling. The basic principles of handling are gentleness and firmness. A chemical restraint should be considered for any prolonged or potentially painful procedure.

Mouse

Tail restraint is adequate for examining or lifting animals and transferring them to another cage. Grasping the loose skin of the neck and back may be used to perform minor, nonpainful procedures such as injections.

Rat

A rat may be picked up by grasping the base of the tail, similarly as for mice. A suitable method to lift and restrain a rat is to place the hand over its back, slipping the thumb ventrally between the forelimbs into the intermandibular space. The hind legs should be grasped to restrain the animal for injections or other minor procedures.

Hamster

As it does not have a tail, a hamster must be grasped firmly by the loose skin of the back, or handled in a manner similar to the rat.

Guinea pig

The guinea pig is lifted by placing one hand around the animal's trunk and supporting the hind limbs with the other hand. It is important to support the rear quarters.

Ferret

Friendly ferrets can be lifted and handled by grasping the trunk. Active or biting ferrets may be restrained by grasping the skin of the neck firmly.

Rabbit

To lift a rabbit, one should grasp a large fold of loose skin over the shoulders with one hand, and the rear feet with the other hand. Failure to grasp or support the rear feet may result in injury to the animal and/or to the handler (rabbits have powerful rear limbs). To carry a rabbit, it is useful to support the animal's body between the forearm and abdomen of the handler, with the rabbit's face hidden under the handler's elbow.

Dog

A slip lead is recommended for working with dogs. The size of a dog determines the method used to lift it. The initial step is the same for all dogs. The dog is grasped above the neck, with one hand around its body, with the hand supporting the dog's chest. The dog can be restrained in a lateral recumbent or sitting position for injections and minor procedures. An intractable dog may need to be muzzled with a commercial or a gauze muzzle.

Cat

Cats are often cooperative enough to be restrained easily on a table by the loose skin at the back of the neck and hips, or with one hand restraining the body and the other restraining the head. A fractious cat should be wrapped in a heavy towel for restraint, with any needed limbs carefully withdrawn for treatment. A face mask (muzzle) can be used to avoid biting, and a squeezecage to restrain the animal for injections.

Swine

Pigs in general are friendly and docile, but will react severely to poor handling. Handling and restraint in pigs relies greatly on treating the pigs in a humane manner. There are several levels of restraint and handling, from touching and coaxing the animal to restraining it for chronic procedures. When approaching a pig, be sure that it is made aware of your presence. If pigs are startled, they may cause injury to themselves or others in the pen. The giving of food is one of the most effective forms of basic restraint in the pig. Smaller pigs may be easily picked up with their body supported while their legs hang. To perform the procedure in larger pigs, place one arm under the chest cranial to the thoracic limbs and the other arm cranial to the pelvic limbs under the abdomen. When a pig is to be moved, a small board is used to apply pressure to its side. Several designs for slings to restrain pigs have been described and can be used.

Nonhuman primates (monkeys)

No matter how small, these animals can be dangerous. Chemical immobilization is normally used. Injections can be given to a confined animal with the help of a squeeze cage. Tether systems are recommended if drugs must be administered to animals, or if blood must be collected frequently.

Laboratory dogs and monkeys can readily be trained through positive reinforcement to accept routine procedures such as an intramuscular injection, an intravenous injection and oral dosing. Such training helps to reduce the stress involved in these procedures; forceful restraint is not needed. [30,32,38]

2. Administration of different agents (fluids and drugs)

When drugs, vaccines, injectable anesthetics or other agents are to be administered, one or other of several different routes may be selected. The route is governed by the nature of the agent being administered, the animal and the purpose of the administration, among other factors.

- Enteral (gastrointestinal) administration. The possible routes are oral (through the mouth), gavage (into the stomach via a gastric feeding needle), per rectum (through the anus into the rectum), or into the bowel via a tube.
- Parenteral administration. Parenteral routes of administration involve mainly injections into various compartments of the body: intradermal (into the skin), subcutaneous (under the skin), intramuscular (into a muscle), intravenous (into the vascular system through a vein), intraarterial (into the vascular system through an artery), intraperitoneal (into the abdominal cavity), into the spinal cavity, into the trachea, or into the nasal cavity. Sites used for the collection of blood from veins may also be used for intravenous administration. Intraperitoneal administration is one of the most frequently used parenteral routes in rodents.
- Special procedures: transplantation (*e.g.* embryo transfer, skin-grafting), implantation and blood transfusion.[30,32]

3. Basic requirements of invasive interventions (injections, blood collection and surgery)

- Painlessly, as far as possible. If pain can be avoided, this is a must! Animals cannot understand why they have to bear pain. Invasive experimental interventions are accompanied by anxiety, fear and suffering; rewarding and praising are therefore important after every regularly repeated intervention.
- Aseptic intervention. This is necessary in order to avoid contamination of the samples, and to avoid infection of an animal, even if it is euthanized after the experiment (intervention).
    - The animal should be surgically prepared by careful shaving to remove all hair from the surgical field.
    - The surgical field should be cleaned and disinfected with an appropriate preparation.
    - All surgical instruments and chronic instrumentation must be sterilized with steam or gas. Cold chemical sterilization is appropriate for minor surgical procedures.
    - Investigators should follow standard surgical practices (scrubbing, cap, mask, gloves, *etc.*).
    - Sterile drapes should be positioned on the animal to define the surgical field.
    - During the course of surgery, procedures for preserving sterility should be strictly followed.
- Postsurgical care
    - Surgical wounds should be protected by plastic collars and/or dressings (or in some other way).
    - Surgical wounds and sites of instrument entry into the body should be observed, cleaned and treated daily. Topical antibiotics can be applied. Surgical dressings should be changed every day.
    - Basic biological functions, including urination, defecation and appetite, are good indicators of an animal's overall physical well-being. These are easy to observe and should be monitored regularly and often.
    - Stitches should be taken out after 10–14 days.
    - Postsurgical (clinical) pain must be relieved.[30-32]

VI. Euthanasia (*Dr. Balázs Gaál*)

This is defined as a method of killing an animal rapidly and painlessly. Experimental animals must be euthanized painlessly when this is decreed by the rules. Another reason for euthanasia is to gain organs and tissues or to examine morphological changes caused by an experiment. The legal animal welfare rules lay down that: *"At the end of any experiment, it shall be decided whether the animal shall be kept alive or killed by a humane method, subject to the condition that it shall not be kept alive if, even though it has been restored to normal health in all other respects, it is likely to remain in lasting pain or distress"*.[40]

The method used must produce rapid unconsciousness and subsequent death without evidence of pain or distress, or the animal must be anesthetized before being killed. This should be carried out by properly trained personnel. The method used should be safe for the attending personnel, easy to perform, and cause death without producing changes in tissues. Careful, gentle handling is important in order to minimize distress to the animal. Whenever possible, animals should not be exposed to the euthanasia of others, especially of their own species. The resultant distress (because of the frightened behavior and the release of certain odors, or pheromones) may lead to physiological changes in other animals, which may affect research results. The basic methods and agents are as follows.

1. Pharmacological – chemical methods

- The injection of a lethal substance, usually a large dose of a general anesthetic agent (pentobarbital) i.v.
- An overdose of potent inhalant anesthetic gas (halothane or isoflurane).

These methods are suitable for most species, including the pig, dog, cat, rodents, rabbit, birds and primates.

- Carbon dioxide inhalation (compressed CO_2 from cylinders). This can be suitable for all species, but in practice, its use is limited to rodents and other mammals weighing less than about 500 g.

2. Physical methods

- Decapitation (small animals and rodents); this requires special equipment.Dislocation of the cervical vertebrae (mice and rats).
- Exsanguination (farm animals and swine).

The use of sedation or anesthesia prior to these methods is recommended.

3. Not recommended methods

Freezing, the use of ether, chloroform, strychnine, magnesium sulfate, muscle paralyzers, *etc.*[30-32]

VII. Experimental design, evaluation, data handling and analysis (*Dr. József Kaszaki*)

The aim of this chapter is to give a practical overview of experimental design, implementation, data handling and analysis, from conception to realization, and from experimental results to conclusions.

1. Background

The design of an experiment starts with a summary of the available knowledge, via a literature search. It is important to be fully acquainted with the details of the issue. The researcher should update his or her knowledge on the subject and read everything concerning the planned experiment. He/she should strive to understand the relationship between published theories and experimental results, and acquire the necessary methods and measurement techniques. Today, it is not necessary to visit a library, as various scientific databases and international journal homepages can be accessed by using the internet. The best is Pubmed, with an excellent searching coverage. The accessibility of this homepage is: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi. Searches can be made by giving keywords, combinations of words, authors and journal data. A list of hits with articles is obtained and the abstracts can be read. In some cases, the article itself can be downloaded as a PDF file by using the Adobe Acrobat Reader free software. However, in most cases registration and a password are needed for direct access to the journal homepage. A*dvice: read articles for a week and work for only a day, rather than vice versa.*

2. Scientific questions and hypotheses

Chapter I deals with this topic in detail, and thus only the essence of the process will be summarized briefly here with regard to the unknown parts of the topic and the literature search, an attempt should be made to postulate a question and create a hypothesis. What is a hypothesis? If a hypothetical process of the examined phenomenonis outlined, this is a hypothesis. The basis of the theory is known from the literature, but the relationships and details of the examined phenomenon should be demonstrated or proven through our experimental data. If the question, the hypothesis, the experimental design and the execution of experiment are all were correct, the results of the measurements will hopefully give an answer to the postulated question.

3. Design of the experiment

A detailed experimental design requires the selection of a model and object, the collection of methods, planning of the experimental setup, protocol and groups, and a special consideration of the control.

3.1. Main types of experimental models

Selection of the experimental model is a cardinal point in the process of the experimental design. The question arises of what type of model is suitable to test the conception. What level of organization is desired, a cell-tissue model, an organ or a whole living animal? The main types of experimental models are *in vitro (ex vivo)* and *in vivo,* which means a rank of order in examinations, from the simpler toward the complex.

In vitro: Examination of a cell or tissue under artificial circumstances. Primarily, this is the field of cell or tissue cultures (Chapter XI deals with this topic in detail).

Ex vivo: Examination of vital signs of an organ or organ system taken from a living organism. To maintain the vital functions, an artificial medium and circumstances are utilized, with the physical and chemical parameters equivalent to those of the blood (*e.g.* isolated vessel rings or a heart perfused according to Langendorff).

In vivo: **E**xamination of an entire organism, awake or anesthetized animal, under normal or pathological circumstances.

3.2. The object of an experiment

- A single cell
- A cell culture, a tissue or a tissue culture (see later)
- An organ
- A living (awake or anesthetized) animal.

An *in vivo* model can involve a small animal (*e.g.* a rodent) or a large animal (*e.g.* dog, pig or sheep). The advantages of small animals (rodents) are the higher number of experiments performed at the same time, the short breeding time and genetic specialties (transgenic or knockout animals). The disadvantages are the limited or very expensive instrumentation (*e.g.* for hemodynamic measurements), and to a certain extent human compatibility of the results on rodents is doubtful.

In large animals, all these points are advantages, *i.e.* a wide range of instrumentation for hemodynamic measurements, *etc.* is available and the human compatibility is more evident. The disadvantages of large animal models are the restricted number of experiments (usually only one animal can be examined at a given time) and the longer breeding time, which can exclude genetic examinations.

3.3. Methods for experiments

In a wider sense, the experimental methods can be grouped according to the scientific fields as morphological, biochemical, physiological, pharmacological, genetic, *etc.* methods. Since this chapter focuses mainly on *ex vivo* and *in vivo* experimental models, the methods are summarized in a narrower sense for these experimental models.

3.3.1. Anesthesia (see in details in Chapter IV)

3.3.2. Airway maintenance

Securing free airways is a basic condition in every anesthetized animal experiment. Anesthesia alone or an unnatural position of an animal (*e.g.* a supine position for pigs) can result in inadequate respiration, which can influence other physiological functions. Airway maintenance is best achieved by endotracheal intubation, *i.e.* an appropriately sized tube is inserted into the trachea through the mouth. In rodents, the airway maintenance is performed surgically. Methods of respiration support are discussed in detail in Chapter X.

3.3.3. Surgical techniques and instrumentation

The aims of a surgical intervention can to be explore and visualize the examined area (*e.g.* the abdominal cavity or thoracic cavity) or to implant sensors, probes or transducers such as arterial or venous catheters or blood flow probes.

3.3.4. Methods for recording physiological parameters

To monitor physiological parameters, sensors, probes or transducers are used, which are connected to instruments and (computerized) data-acquisition systems. The basis of each developed monitoring technique is the conversion of changes into electric signals. The method is usually based on the following steps: detection of the change → conversion → enhancement and filtration → data output (analog or digital) → data storage.

The most important physiological parameter is blood pressure. This measurement is one of the prerequisites to register the condition of the circulation. It can be monitored by direct (invasive) or indirect (noninvasive) means (methods of circulation and microcirculation monitoring are discussed in detail below and in Chapters VIII and IX).

3.3.5. Analysis of biological samples

The technology of obtaining biological samples is a cardinal point of the analysis, which can be performed immediately (online) or following storage (offline). Cooling (refrigeration) is essential for biochemical determinations. The medium for refrigeration can be an ice-cold solution (0-4 °C), dry ice (carbon dioxide snow) (-70 °C) or liquid nitrogen (-180 °C). The temperature of the sample or of sample storage is important for the biochemical determination, *e.g*. sample storage below -70 °C is adequate for enzyme activity measurement, but not for RNA content determination. Biological sampling can involve blood, liquor, urine, *etc.* or whole tissue (*i.e*. biopsy). Usual parameters of *blood samples* are cell components (red blood cells, leukocytes, *etc*.), components of the plasma (metabolites, anions, cations, glucose, lactate, *etc*.), rheological parameters (sedimentation, hematocrit and plasma viscosity) and blood gas analysis (pH; pCO_2, pO_2, oxygen saturation and base excess). Parameters from *tissue biopsies* are biochemical parameters (*e.g*. enzyme activities) and histology (light microscopy, electron microscopy, *etc*.)

3.4. The experimental setup

The experimental setup is an organized collection of devices, instruments and methods for physiological or biochemical monitoring. It includes the scheme of the surgical instrumentation, the equipment to maintain the experimental state (respirator and infusion pump), instruments for monitoring and a collection of applied biochemical methods. **Figure 6** demonstrates a rat setup for monitoring of the mesenteric circulation.

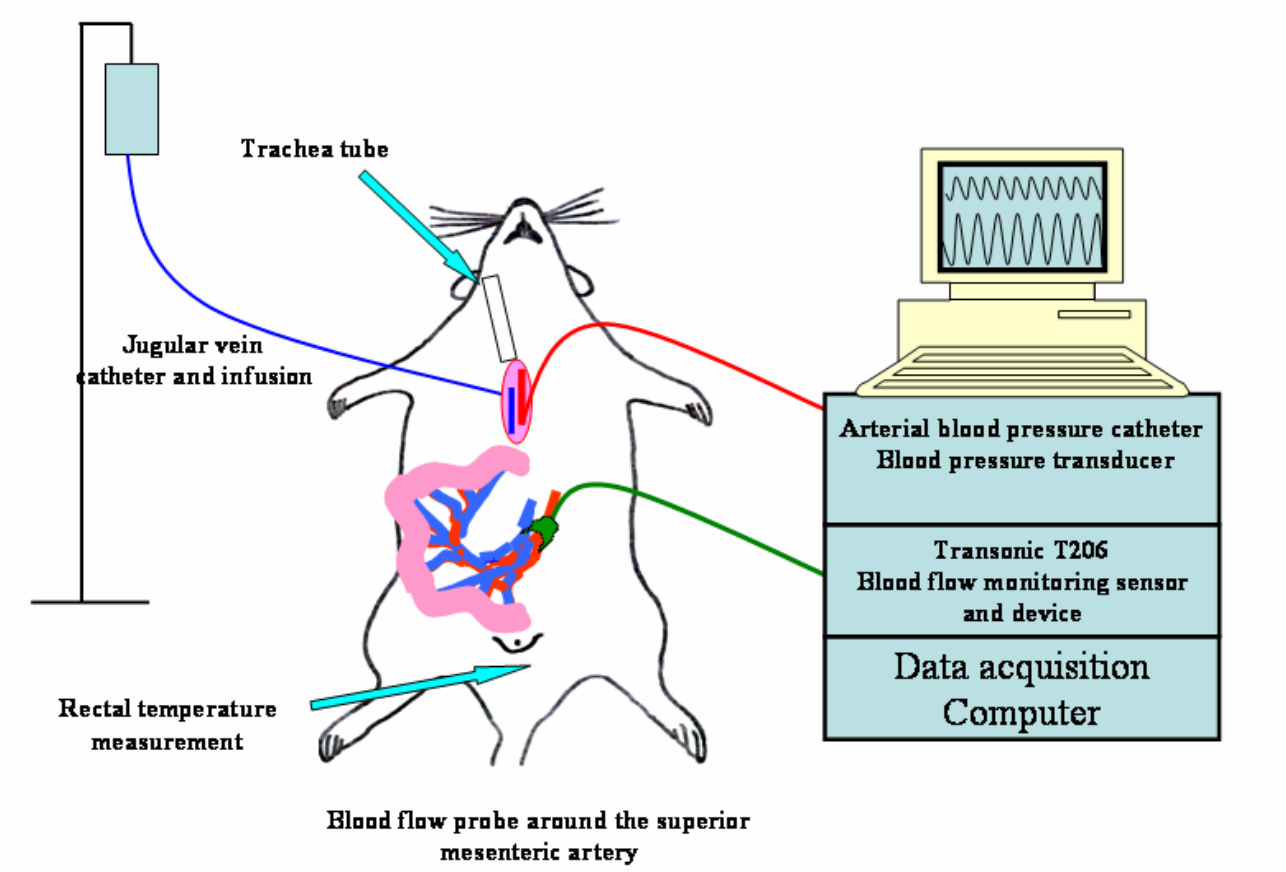


Figure 6. Experimental setup in a small animal (rodent) model.

3.5. The experimental groups

The most important point of the experimental planning is the application of an adequate control. What can we regard as a control? A control has to show a difference between an

intervention-caused change and a situation without any intervention. A control can be an initial period of a process, *i.e*. a value measured before an intervention under normal circumstances, which is termed the baseline. This latter case is a self-controlled experiment. A self-control, however, is often inadequate, mainly in longer experiments, when other variables (*e.g.* long-term anesthesia) can influence the outcome of the process. Hence, in this case an extra group should be observed to check the time-dependent changes, and the same process must be monitored, but without any interventions (control group).

We can examine not only the changes in a pathological stage, but the effect of some treatment in that condition. In this case, a negative and a positive control group are used. The concept of the negative control is the same as that of the control group outlined above. This is a condition in which only minimal variations in the examined parameter can be observed during an experiment. In contrast with the negative control, the positive control produces the maximal variation in an examined parameter during the experiment. In addition, a positive control group can be an intervention group, and compared with a third, treated group. **Figure 7** demonstrates this situation. Experimental pleuritis was induced in mice and the inflammatory process was characterized by an elevation in the white blood cell count in the pleural cavity. This is an intervention group, which is now considered a positive control. The mice in the negative control group were injected with the vehicle (saline) of the pleuritis-inducing substance. The negative and positive control data were then compared with those on the mice in which the intervention (pleuritis) was treated with a drug (acetylsalicylic acid).

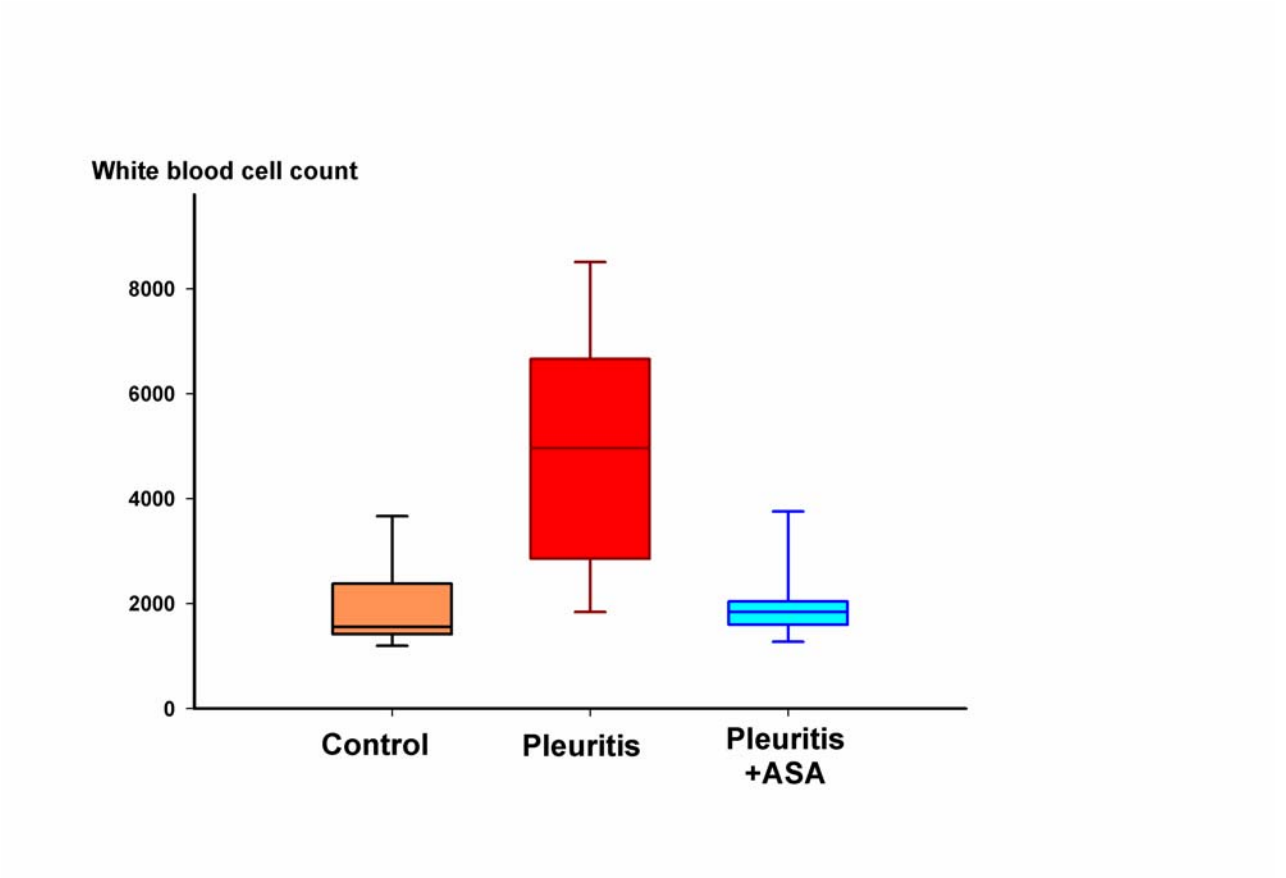


Figure 7. Demonstration of data on negative and positive controls and a treated group in a box-whisker plot (acetylsalicylic acid = ASA).

Experimental groups should be well planned for each experiment. An experimental intervention can be pretreatment (the influencing effect is started before an intervention) or a

treatment (it is started during/after the intervention). On the basis of the above scheme, experimental groups are either pretreated or (post)treated types.

3.6. The experimental protocol

The experimental protocol consists of a scheduled sequence of experimental periods, usually a control period, an intervention (surgical, pathological or pharmacological, *etc*.) and an observation of the consequences of intervention. These periods can extend to the pretreatment or posttreatment periods. It is important that the time-scheduled sequence of measurements and sampling should be performed in every group because of the exact between-group comparisons.

When planning the experimental protocol regarding the number of measurements or sampling, it is important to take into account two (partially contradictory) viewpoints. A sufficient (*i.e*. more than necessary) number of samples should be taken to map the examined process, mainly in the event of a long experimental protocol. The other viewpoint is that the statistical analysis which will be used for data evaluation should already be taken into account in the phase of experimental design: if the experimental protocol is crowded with unimportant (more than necessary) samples and measurements, the data evaluation will be very difficult, whilst the observed change will be lost in the sea of irrelevant data. To avoid this problem, it is worth while to review the types of the different experimental protocols on which the subsequent statistical analysis is based. It is important to emphasize that in the next section "sample" or "measurement" denotes the same event as concerns data evaluation.

3.6.1. Types of experimental protocols

Comparison of samples ("before and after")

This is the simplest type of comparison. Altogether two samples are taken or two measurements are performed on the same animal, before and after an intervention (**Figure 8**). These samples are interdependent as concerns the statistical analysis, because the sampling was performed on the same animal.

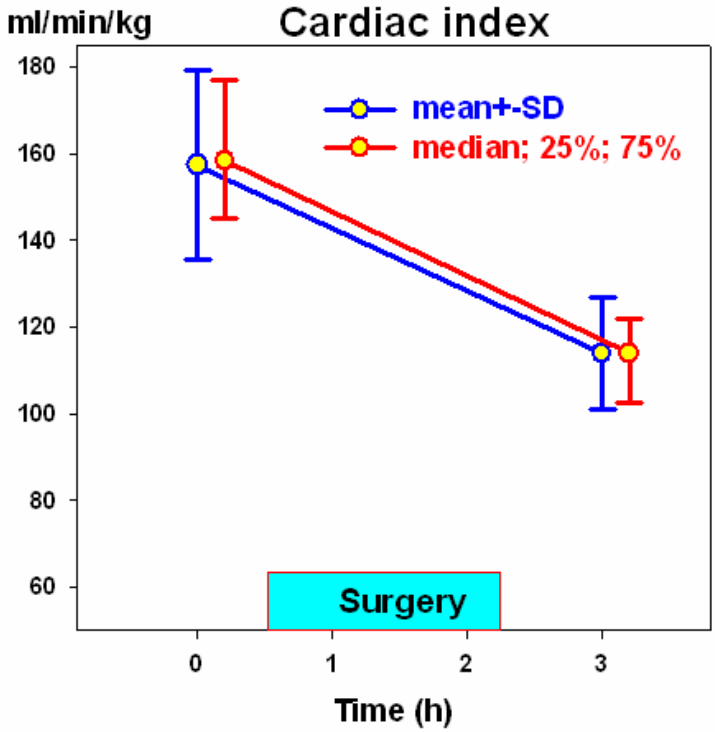


Figure 8. Two dependent measurements are performed on the same animal, before and after an intervention.

3.6.2. Comparison of two samples

Sampling is performed in two individual animals at the same time (**Figure 9**) and the difference is compared (*e.g.* a control and a treated group). These two samples are independent as concerns the statistical analysis, because two different populations of data are compared.

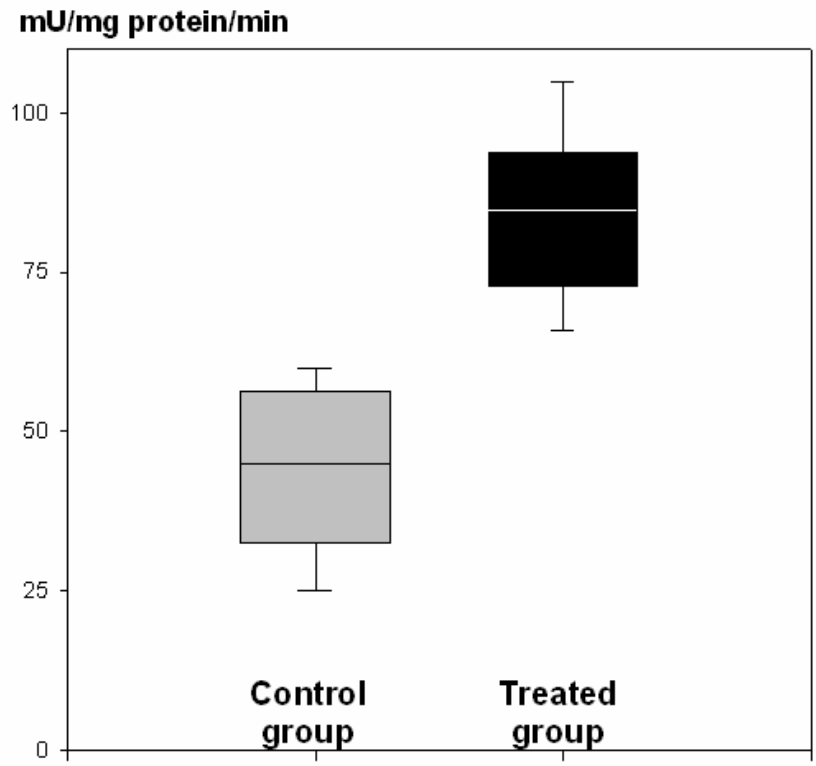


Figure 9. Comparison of two independent samples

3.6.3. More than two groups are compared - one sample was taken

The pleuritis model described in section 3.5 is a typical example of the comparison of more than two groups a negative control, a positive control and a treated group being compared (**Figure 7**). These samples are independent from each other as concerns the statistical analysis, because they derive from different populations.

3.6.4. Repeated measures in one group

This type of experimental protocol is used if a time-dependent process is examined. It is valid for a minimum of three measurements, where the variation in an examined parameter is compared with the baseline value or to a control period (**Figure 10**). These measurements are dependent on each other as concerns the statistical analysis, because the measurements were made on the same animal.

Figure 10. More than two, repeated, dependent measurements in an experimental group.

3.6.5. Repeated measurements in more than two experimental groups

This situation is similar to the previous one, but a time-dependent process is examined in more than two different experimental groups (**Figure 11**). Data evaluation is performed according to two viewpoints.

1. Within-group analysis, where the variation in an examined parameter is compared with the baseline value. These measurements are dependent on each other as concerns the statistical analysis.

2. Between-group analysis, where the variation in an examined parameter are compared with each other, at the same time, in all groups. In this latter case, the data are independent of each other as concerns the statistical analysis.

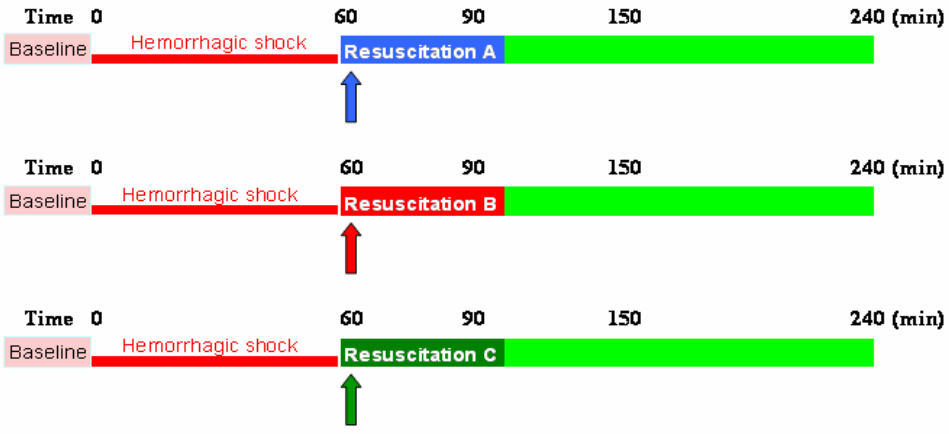


Figure 11. Repeated measurements in the case of more than two experimental groups.

4. Implementation of the experiment

The conductance of an *in vivo* experiment needs patience and persistence, because attention must be paid to several important things simultaneously, and these measurements should be accurate and standardized. Two questions should be asked: what kinds of measurements are needed and what do we have to look at? In a preliminary study, it is practical to measure as many parameters and as many times as possible, because we do not know exactly the variations in the examined process and can not foresee the reactions of the experimental object. The direction or tendency of changes will be outlined with the increasing number of successful experiments (in the optimal case) and this permits optimization of the number of measurements and examined parameters. Extra preliminary study data could be selected later, but the missing data can be supplied only through new (and sometimes very expensive) experiments.

The report is an important *("sine qua non*") constituent of an experiment (logbook). It is vital to register and write down accurately every event, including the date and time of the measurements, the sampling parameters, the pharmacological treatments (doses, volumes, duration of infusions, *etc*.) and all other observations. The significance of the report can not be overemphasized (words slip away and only the writing remains): it is necessary to write a report which will be understandable for everybody even years after the experiments. Some examples of what must be borne in mind during an *in vivo* experiment:

- The maintenance of anesthesia: Attention must be paid to the signs of awakening (blinking, stretching muscles, or an increase in heart rate) and a supplementary dose of anesthetics must be given at scheduled time or whenn needed.
- Body temperature: In anesthetized animals, rectal or blood temperature monitoring is very important. Certain anesthetics, surgical intervention and pathological events can influence the regulation of temperature, which can be compensated only by using a heating pad.
- Infusion: Continuous and controlled fluid replacement is mandatory in anesthetized animals and after surgical interventions. The negative fluid balance caused by surgery or anesthesia should be compensated; the normal dose of crystalloid fluids in larger animals is 7-10 ml saline/kg bodyweight/hr.
- Blood gases: If possible, the monitoring of blood gases should not be neglected (partial pressure of O_2, pCO_2, and oxygen saturation). These parameters can indicate a respiratory insufficiency or disturbed homeostasis.

5. Data evaluation and statistical analysis

5.1. Making a database

Any database operator software (*e.g*. MS Excel) can be used for a data summary and evaluation. Data from an individual experiment are arranged as a data record, the first column of which contains the code, the datum, group of the experiment and the name of data source file (the constant cell content in these columns inside the record). The time of the measurements and the values of the measured parameters are given in additional columns (with variable cell content inside the record). **Table 10** demonstrates an example of a database.

Table 10. Set up of a database. (Sham-op = sham-operated; MBP = mean arterial pressure; HR = heart rate; BTemp = body temperature; CO = cardiac output).

Date	Code	Group	Data Source	Time	MBP	HR	BTemp	CO
2006.10.13	Col1	Sham-op	CO ID1	0	76,42	389,61	37,55	73,6
2006.10.13	Col1	Sham-op	CO ID2	30	96,56	489,8	38,28	60,1
2006.10.13	Col1	Sham-op	CO ID3	60	92,63	454,55	37,85	61,3
2006.10.13	Col1	Sham-op	CO ID4	90	98,86	478,09	37,75	71,2
2006.10.13	Col1	Sham-op	CO ID5	120	85,95	585,37	37,43	87,7
2006.10.13	Col1	Sham-op	CO ID6	150	79,02	444,44	38,03	68,6
2006.10.13	Col1	Sham-op	CO ID7	180	83,46	463,32	37,5	74,8
2007.01.31	Col32	Colitis	CO_ID1	0	154,1	421,1	37,9	75,0
2007.01.31	Col32	Colitis	CO_ID2	30	152	412	37,2	83,1
2007.01.31	Col32	Colitis	CO_ID3	60	151,5	408,6	36,7	68,9
2007.01.31	Col32	Colitis	CO_ID4	90	155,3	375,1	35,4	74,5
2007.01.31	Col32	Colitis	CO_ID5	120	147	409	37,5	87,2
2007.01.31	Col32	Colitis	CO_ID6	150	138,5	454,1	38,6	94,3
2007.01.31	Col32	Colitis	CO_ID7	180	136,2	447	37,0	103,1

5.2. Graphical presentation of data

Discret (categorical) data (*e.g*. man/woman; alive/dead), which have only a single value are usually presented in a column or pie chart. However, the majority of the data will be continuous, *i.e*. they may have any value, and measurement points or calculated statistical data should be presented. In this case, histograms, box-whisker plots or mean-deviation plots (in the case of one variable) can be used. An example of the application of a point chart is a correlation test, when a point set represents the relationship of two parameters. A *box-whisker* chart presents the 5-95% range of the data, the lower and upper deviation (25-75 percentiles), and the median value (**Figure 7**). A mean-deviation chart can be a point line (**Figure 8**) or a column plot.

5.3. Statistical analysis

First, it should be mentioned that the size of this chapter does not permit an in-depth description of the statistical analysis. The aim is to present a short, understandable basic concept and a brief review of the statistical tests applied in practice.[41]

5.3.1. Basic concepts and definitions

Population: The sum of the examined things, a complete, holistic examination of which can not be performed or is not economical.

Statistical sample: The observed data relating to a set of individuals selected from a population. The element number of the sample is equal to the number of individuals within the sample. The greater the number of measurements we have, the better the evaluation of the characteristic of the population based on the sample. There are spatial, time-dependent and economic limits of a determination. Determination of the optimal element number of a sample is important; it depends on the problem, our previous knowledge and the methods applied.

Sampling must be *representative* (*i.e*. the selected samples should well reflect the examined population) and *independent*. The latter term means that the data are not independent of each other if the same thing is measured several times: in this manner, the number of elements of a sample can not be increased.

Distribution of the sample (*histogram*) is the difference between the largest and smallest sample elements. It is divided into several (> 5) intervals. Columns are inserted in every interval, and there are as high as the number of observations in that interval. The more sample elements there are, the greater the number of intervals, and the histogram therefore approximates to the theoretically optimal distribution. The sample may originate from a population with a normal (parametric) distribution if the theoretical distribution has a bell-shaped (Gaussian) curve (**Figure 12**). If the sample is from a population whose distribution deviates from Gaussian (characterized by skewness), these samples have non-normal (or nonparametric) distribution (**Figure 13**).
Number of elements: This is the number of samples.
Mean: If $x_1,x_2,...,x_n$ indicate the elements of a sample, the mean is an average of the sample elements, *i.e.*:

$$\bar{x} = \frac{\sum_{i=1}^{n} x_i}{n}$$

Standard deviation (SD): This is the deviation of the sample, *i.e.* the average deviation of the sample elements from the mean. In the event of normal distribution, 95% of the sample elements can be found in the interval mean±2SD. Formula:

$$SD = \sqrt{\frac{\sum (x_i - \bar{x})^2}{n-1}}$$

Standard error (SE v. SEM): This is the deviation of the mean: SE=SD/√*n*. If new samples are taken from the same population, SE indicates how much the mean deviates from the calculated value. In the event of normal distribution, mean±2*SE indicates the interval in which the mean moves in 95% of the cases.
Percentile: The 10% percentile means the value for which 10% of the sample elements are lower.
Quartiles: There are the 25% and 75% percentiles.
Median: The middle element of the sample, *i.e.* the value from which 50% of the sample elements are lower or equal. Its calculation: the median is the middle element if the elements of the sample are ordered by size, or the mean of the two middle values.
Range: The difference between the biggest and smallest values (sample elements).
Skewness coefficient: This shows wheather a distribution is parametric or not (skew). For a negative skewness coefficient, there is left-side skewness, *i.e.* the values larger than the mean are more frequent.
Statistical estimation: This is a parameter of the distribution of a population estimated via the samples. The theoretical mean of the sample is approximated from the mean calculated from the sample elements. The c*onfidence interval* is the interval which usually includes the real value of the estimated parameter with high probability.
Hypothesis test: This is a method to decide wheather there is sufficient evidence to confirm the examined (alternative) hypothesis. There are two opposite statements, the zero hypothesis and the alternative hypothesis. An experiment is made to prove that an alternative hypothesis is true beyond any doubt and to show that the zero hypothesis is with high probability false.
p value: In the event of the existence of a zero hypothesis, this is the probability of the tentative (alternative) statistical result. Computer systems are able to calculate this p value approximately, which is more informative than $p > 0.05$ or $p < 0.05$.
Significance, significant change: This involves a divergence from the zero hypothesis exceeding a given level. There is a statistically significant difference if the differences in the mean values between the treatment groups are greater than would be expected by chance.
Significance does not mean that the compared, expected values will certainly differ, but only that the probability of coincidence is low (5%). If $p > 0.05$, this means (that it is not significant) that we did not detect a difference at the 95% level.[41]

5.3.2. Statistical analysis in practice

Statistical analysis of data begins with a distribution test. If there are sufficient sample elements (several hundred), the distribution type can be assessed with high accuracy by the skewness coefficient or the theorem of deviation sameness (these tests are made automatically by most statistical software). However, it is very rare to have hundreds of elements from one sample. In practice, we usually have about 5-20 sample elements and with such a small element number the distribution type can not be established accurately. In this case, we commit a smaller fault if we assume a nonparametric distribution of the data.

Statistical methods (tests and assays) can be applied by the type of distribution. In the event of parametric distribution (Gaussian curve; **Figure 12**), we have to use parametric statistical assays and the data are characterized by the mean value and standard deviation. In the case of nonparametric distribution (skew Gaussian curve; **Figure 13**), nonparametric statistical tests have to be used and the data are characterized by the median and 25%-75% percentiles. Following this, the type of the experimental protocol determines the applicable statistical methods. With two samples, we can use paired or unpaired statistical tests, depending on the sample dependence or independence (summarized in **Figure 14**). In cases of more than two and/or a repeated measures protocol, the applicable statistical methods are summarized in **Figure 15**.[41]

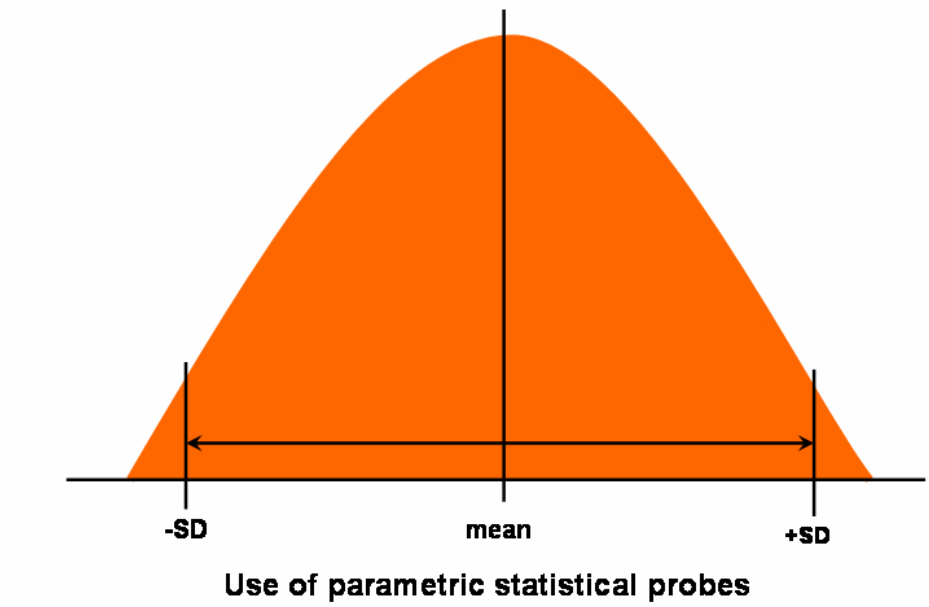


Figure 12. Parametric distribution is characterized by the mean and the identity of the standard deviation

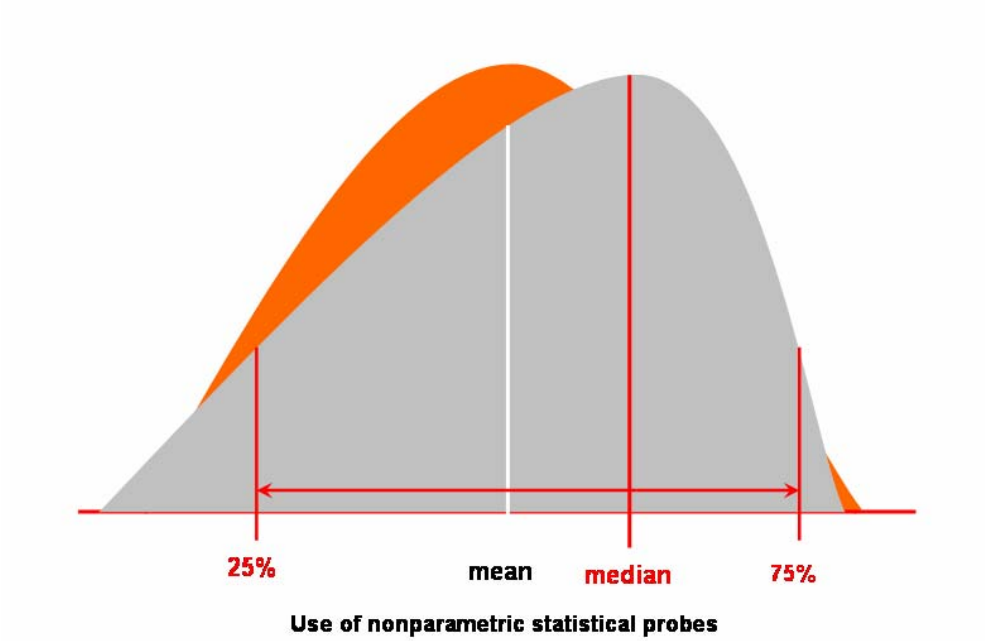


Figure 13. Distorted Gaussian curve; nonparametric distribution is characterized by the median and quartiles (25%-75% percentiles).

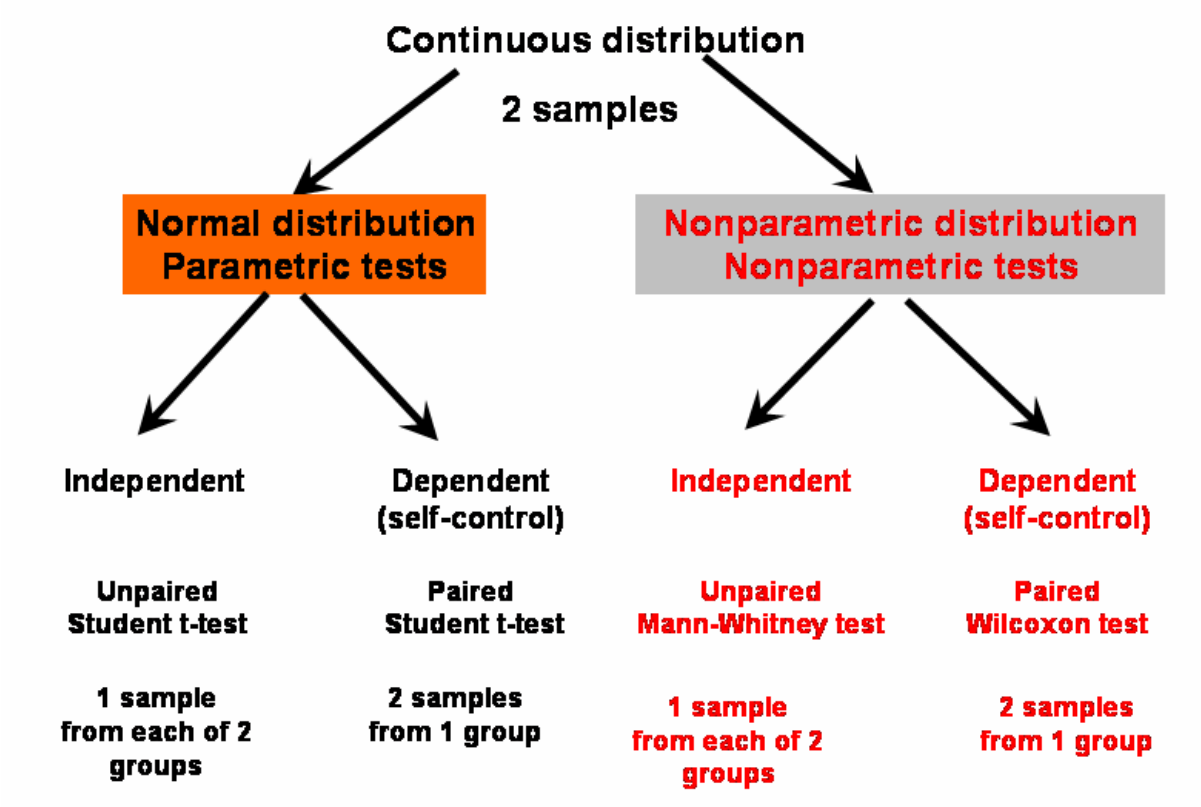


Figure 14. Summary of applicable statistical methods in the case of two samples.

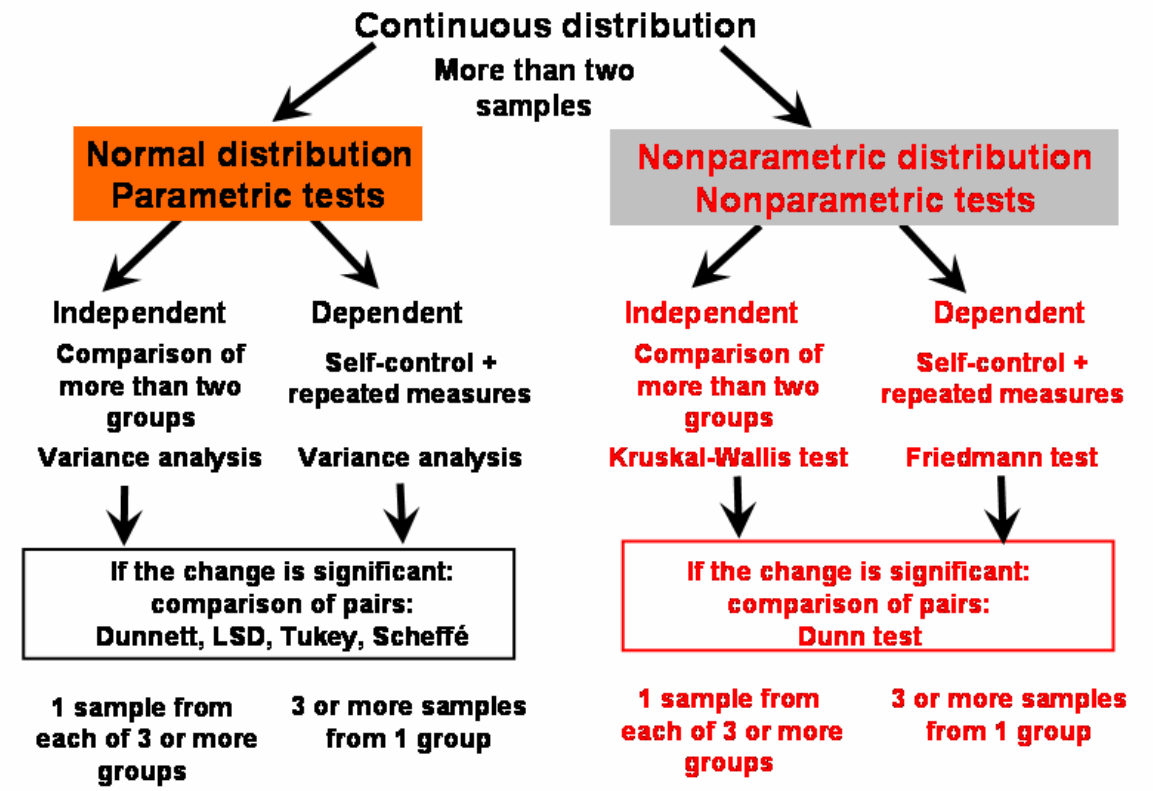


Figure 15. Summary of applicable statistical methods in cases of more than two and/or a repeated measures protocol.

VIII. Experimental techniques in practice. Monitoring of vital signs: circulation (*Dr. József Kaszaki*)

The basis of each developed monitoring technique is the conversion of changes into electrical signals. The method is usually based on the following steps: detection of the change → its conversion into an electrical signal → enhancement and filtration → data output (analog or digital) → data storage. A sensor/transducer is a device that converts energy from some other form (*e.g.* heat, light, sound, pressure, motion or flow) into electrical energy. The basis of the conversion is that some semiconductors change their resistance to movement, which is then quantified by a Wheatstone bridge. The characteristics of a transducer are:

- The *sensitivity* is the minimum input parameter that creates a detectable output change.
- The *range* is the difference between the maximum and minimum values of the applied parameter that can be measured.
- The *precision* is the degree of reproducibility of the measurements.
- The *resolution* is the smallest detectable incremental input parameter that can be detected in the output signal.[42]

1. Noninvasive cardiovascular monitoring

1.1. Electrocardiography

In animal experiments, electrocardiography (ECG) is a key method. It provides information on the normal state and pathological changes in the myocardium, the heart rate, the atrial or ventricular rhythm and the status of myocardial oxygenation. The ECG waves in experimental animals may be significantly different from the human forms (*e.g.* the converse T wave in pigs).

1.2. Noninvasive measurement of blood pressure

Measurement of blood pressure is one of the prerequisites for an understanding of the condition of the circulation. Primarily, it can be made in indirect (noninvasive) ways in rodents (mice and rats). A cuff sensor is placed around the bald tail of the animals.

1.3. Pulse oxymetry

This is a continuous, noninvasive method that records the arterial oxygen saturation and heart frequency, and analyzes the absorption of infrared light of the examined circulatory area. The degree of saturation of the blood with oxygen can be calculated from the ratio of oxygenated hemoglobin and the total amount of hemoglobin (SpO_2 = percentage of hemoglobin saturated with oxygen). The spectrum of light absorbance of hemoglobin depends on the oxygenation (the arterial and venous blood have different colors). If blood is illuminated with light of a given wavelength, conclusion on the oxygen concentration can be drawn from the intensity of the reflected (transmitted) light. After reflection, only a part of the light reaches the detector, and only a small fraction of it (the pulsating part) carries the information. Since pulsation is characteristic only of arterial blood, the plus (variable) absorption due to the pulse added volume of arterial blood is used to calculate the arterial oxygen saturation (other factors can be filtered out). Under normal circumstances, the arterial oxygen saturation is constant (97-99%), while the saturation of the venous blood is on average 75%. The sensor is placed on the bald surface of the tail or the ear in rodents, whereas in larger animals (pig and dog) the tongue is the best place for the sensor.[43]

2. Invasive hemodynamic monitoring

2.1. Principles of invasive pressure measurements

The basic tool is a hollow tube (cannula or catheter) introduced into the lumen of the vessel, which mediates the venous or arterial pressure as a mechanical energy signal through a flexible, but relatively rigid-walled tube to a mechanical–electronic signal converter (transducer). The fluid

column (*e.g*. saline) in the cannula is in direct contact with the blood flowing in the vessel (according to the Pascal law, the pressure propagates constantly, without weakening in the nonflowing fluid column). In this way, the blood pressure signal is transmitted to the sensor membrane mediated by the fluid column inside the cannula.

Cannulas introduced into certain parts of the circulation need to be rinsed permanently with heparinized saline. If blood or air bubbles enter the catheter, they mast be removed, since they can influence the pressure measurement significantly. In contrast with a fluid, air can be compressed, and the pressure curve will therefore be blunted. The technique is accurate, but the zero level of the system must be defined at the beginning of every setting, and subsequently repeated.

2.2. Venous pressure monitoring

The usual possibilities for venous catheter insertion are the femoral vein or the external jugular vein by venous cutdown (surgical intervention). Venous catheters are used to give a supplementary dose of anesthetics, drugs or infusion and for blood sampling. A venous catheter positioned appropriately into the superior vena cava permits measurement of the central venous pressure (CVP). The CVP indicates the right ventricular preload (the rate of venous inflow to the heart), from which the circulatory blood volume can be estimated. In extreme situations, it demonstrates the severity of hypo- or hypervolemia. The cellular components of the blood (the red blood cell count, the white blood cell count and the thrombocyte count) and rheological parameters (sedimentation, hematocrit and plasma viscosity) can be determined from a venous blood sample.[42, 43]

2.3. Measurement of arterial pressure

An arterial line involves catheterization of the carotid or femoral arteries in animal experimental practice. It provides a direct measurement of blood pressure, blood gas measurement and continuous hemodynamic information. Maintenance of a sufficient average arterial blood pressure level is of significance as concerns maintaining adequate organ perfusion. From the pulsating pressure signal, the systolic, diastolic and mean pressure values and the number of pulse waves per minute (*i.e*. the heart rate) can be determined. The pulse pressure (the difference between the systolic and diastolic pressures) indicates the volume state; a high pulse pressure points to vasodilation-hypervolemia, while a low pulse pressure points to vasoconstriction-hypovolemia. The steps of the arterial pressure measurement:

1. catheterization of an artery;
2. connection to the pressure measurement system (it must be bubble-free);
3. secure fixation of the cannula;
4. zeroing of the transducer;
5. fixation of the transducer at the heart level;
6. start of the measurement.

2.4. Monitoring of the pulmonary circulation

In small animals, introduction of a catheter into the pulmonary circulation via peripheral vessels is impossible. This method can be performed in large animal models, however, by using a Swan-Ganz catheter. This flow-directed thermodilution balloon catheter is introduced through the femoral or jugular vein and the tip of the catheter is positioned into the pulmonary artery. It contains four lumens to monitor the right atrial pressure, the right ventricular pressure, the pulmonary artery pressure and the pulmonary capillary wedge pressure (distal, yellow branch). The CVP measurement is made through the proximal blue branch, which is also used to inject the thermal bolus into the right atrium for thermodilution cardiac output measurements (see later). The end of the blue lumen is located 29 cm from the tip of catheter. The red channel provides a means of inflating and deflating the balloon located near the tip of the catheter. The inflated balloon permits the catheter tip to be driven into the pulmonary artery. The thermistor (white) branch provides an

electrical connection to the temperature-sensitive thermistor head. It is located 3 cm from the tip of the catheter and is used to measure the blood temperature

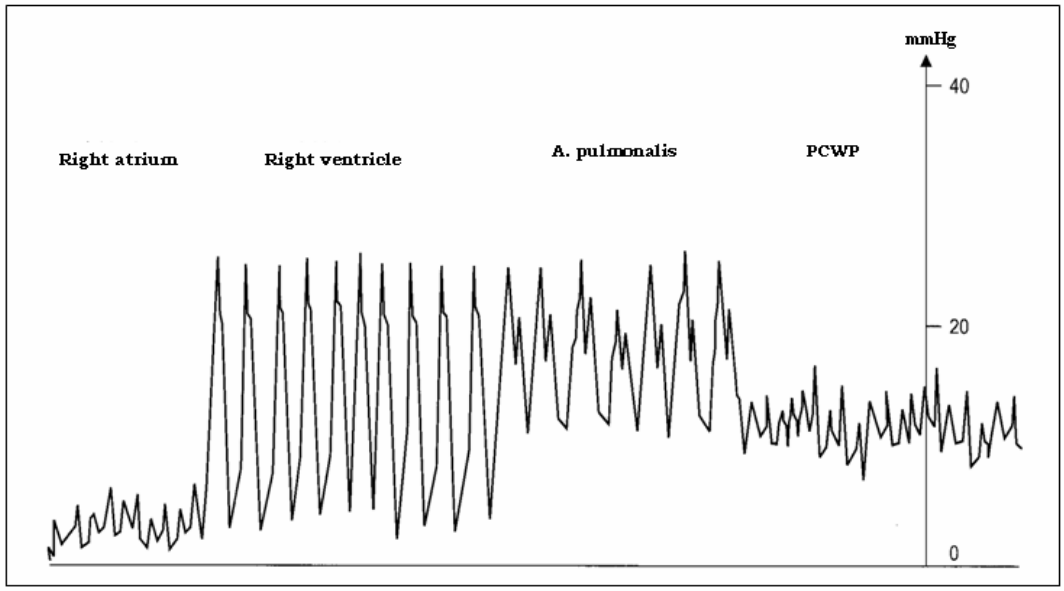


Figure 16. Forms of pulmonary artery and wedge pressure curves

The catheter is usually introduced through the femoral vein or jugular vein, under continuous pressure control (**Figure 16**). When the pressure signal of the right ventricle has been obtained, following balloon inflation (red lumen) the thermistor catheter tip can be led into the pulmonary artery. In the event of successful introduction, a 10-25 mm Hg pressure signal can be seen on the monitor. Following deflation of the balloon, the catheter is pushed ahead until collision. With repeated balloon inflation, the pulmonary capillary wedge pressure distally from the catheter tip can be measured; this is equal to the pressure in the returning branch of the pulmonary circulation (left atrium). Swan-Ganz catheterization of the pulmonary artery permits simultaneous measurement of the central venous, the pulmonary artery and the pulmonary capillary wedge pressures, and the cardiac output.[42, 43]

2.5. Monitoring of the cardiac output

2.5.1. Pulmonary thermodilution method for cardiac output measurement

This method utilizes the Swan-Ganz catheter and can be performed on large animals. If the cardiac output is determined by pulmonary thermodilution, a standard volume (2.5–5–10–20 ml) of cold (at least 10 °C lower than the blood temperature) solution is injected through the blue branch into the right atrium, as quickly as possible. The exact temperature of the injected saline is measured with a reference thermistor of the catheter, while the moving cold blood bolus is registered by another thermistor positioned at the tip of the pulmonary catheter, at about 30 cm from the injection point. The computer software produces a thermodilution curve from the transient change in temperature of the blood. The cardiac output is calculated by integration of the curve area.[42-43]

2.5.2. Cardiac output measurement with a transpulmonary thermodilution method

In small animals, the cardiac output can be measured only in this way. The effect of the thermal bolus injected into the central vein is registered by a thermistor catheter positioned in the femoral artery (**Figure 17**). For pulmonary thermodilution, the arterial side of the pulmonary circulation is considered representative of the total circulation, while transpulmonary thermodilution extrapolates data from a longer compartment, from the total pulmonary circulation and a part of the arterial side of the circulation. This extended (both in time and in space) sampling decreases the effect of breathing, and another algorithm (based on the Stewart-Hamilton principle) is used to determine the cardiac output from the integration of the bolus-effect curve. Hemodynamic parameters calculated from the arterial pressure and the cardiac output are the cardiac index (CO/body area in ml/min/m^2), stroke volume (CO/heart rate in ml) and peripheral vascular resistance (mean arterial pressure-CVP)/CO).

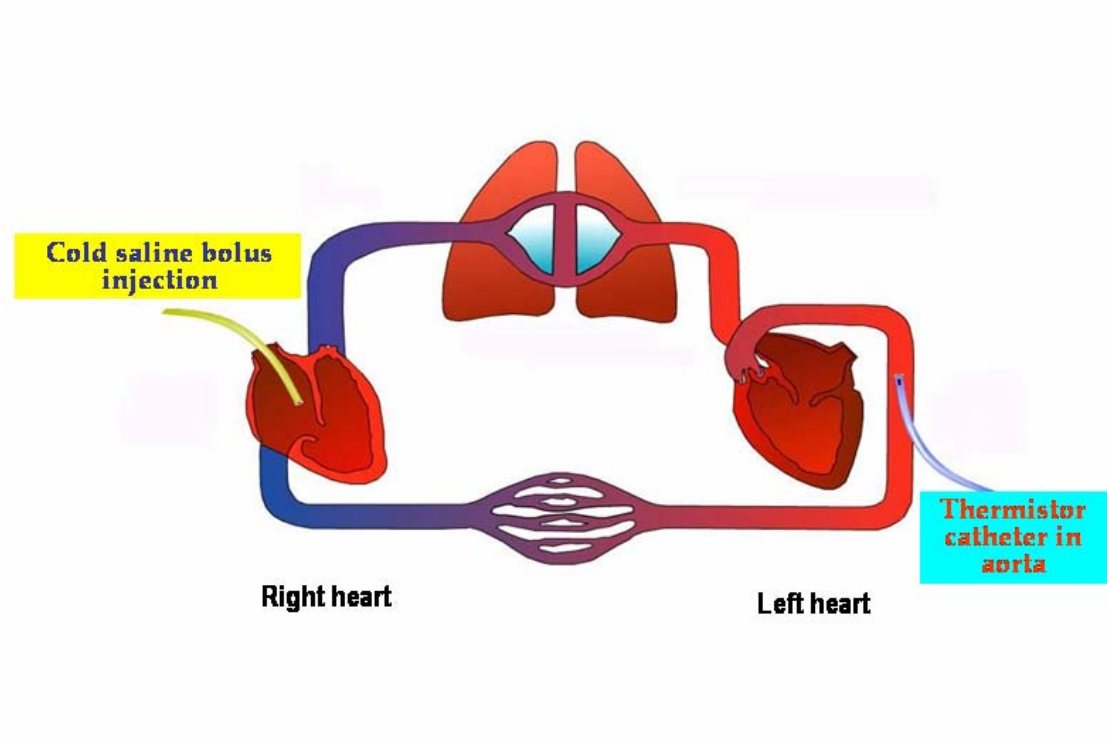


Figure 17. Scheme for the transpulmonary thermodilution method

2.6. Blood flow measurement

The aim of the measurement is to determine the amount of blood flowing through a cross-section of a vessel during unit time. The most frequent method is ultrasound flowmetry, when an ultrasound sensor (probe) is positioned around the examined artery. The measurement depends on the size of the probes; this measurement has probe-dependent lower and upper values. The technique is based either on the measurement of the transit time or on the Doppler principle.

Direct ultrasound flow measurements apply ultrasound waves and the transit time is determined by probes placed around the vessel. The ultrasound head and receiver are localized opposite each other (usually at 30–45°), or side by side (the double-beam method, when there is a reflecting surface on the other side of the vessel), and both heads transmit ultrasound waves to the other. One of the signals runs in the direction of the blood flow, while the other runs in the direction opposite to the flow. When the first beam arrives, the other beam starts moving oppositely to the flow. The transit time of the ultrasound waves differs for the flow-direction and the against-the-flow direction movement

2.7. Heart contractility

The role of the left ventricle is to move the diastolic volume (*i.e*. the blood) toward the systemic circulation. Factors influencing the ventricular work are the preload, afterload, heart rate, oxygen and energy supply, neurohumoral state and contractility. The heart contractility is determined with monitoring of the systolic phases of the normal heart cycles. The method is based on the *dP*/*dt max* values of the isometric contraction or on the end-systolic pressure-volume relation (ESPVR).

1. The *dP*/*dt max* value of the arterial pressure curve represents the rate of pressure increase in the left ventricle, and is thus a parameter of the myocardial contractility.

2. ESPVR evaluation requires the simultaneous measurement of one parameter related to the pump function (*dP*/*dt*, ventricular pressure) and another one related to the preload (end-diastolic volume), these two parameters producing pressure-volume loops. The slope of a straight line fitted to the end-systolic points of the pressure-volume loops is the elastance, which is an accepted value for heart contractility. In practice, this evaluation is possible only by means of a special catheter introduced into the cavity of the left ventricle, which simultaneously measures the signals of pressure and volume. It is important that *dP*/*dt* max is a load-dependent parameter, in contrast with ESPVR.

IX. Examination of the microcirculation (*Dr. Andrea Szabó*)

1. Introduction

The morbidity and mortality statistics demonstrate that cardiovascular diseases and circulatory disorders pose the most severe problem in healthcare worldwide. The reason for this may be that the etiologies and causative factors of these diseases are not well defined and only limited information is available on the pathophysiology. Likewise, the characteristics and mechanisms of the microcirculation are particularly poorly understood. This is surprising if we consider the importance of the physiological and pathophysiological functions of the microvessels of the body. The diagnosis or at least an awareness of the microvascular changes in some diseases would critically affect the final therapeutic interventions. Not only circulatory disorders (*i.e.* atherosclerosis) have microcirculatory manifestations; diabetes, for instance has severe skin symptoms, and microcirculatory disorders are the main factors in the pathogenesis of different inflammatory diseases too. Indeed, there are microcirculatory alterations behind each of the cardinal signs and symptoms of acute inflammation (*rubor* = redness, *calor* = heat, *dolor* = pain, *functio laesa* = functional disorder).

Today, the methodological background for clinical microvascular and microcirculatory measurements is still very poor, and animal models are used almost exclusively to characterize these changes. Intravital microscopy, which was designed for animal experiments is improving, however, and may be applied in clinical practice in the near future.

Studies of the microcirculation started only several decades ago, but these approaches have revolutionized our understanding of the pathogenesis of various circulatory disorders and particularly that of acute inflammatory reactions. It is important to note that these data, including information on mediators of inflammation, were obtained from animal models exhibiting a high degree of homology with human situations. These methods can also provide a good basis for an assessment of the efficacy of human therapeutic interventions.

2. Functional anatomy and characteristics of the microcirculation

The major components are the metarterioles, precapillary sphincters, “preferential channels”, small “true capillaries”, and postcapillary venules (**Figures 18 and 19**).

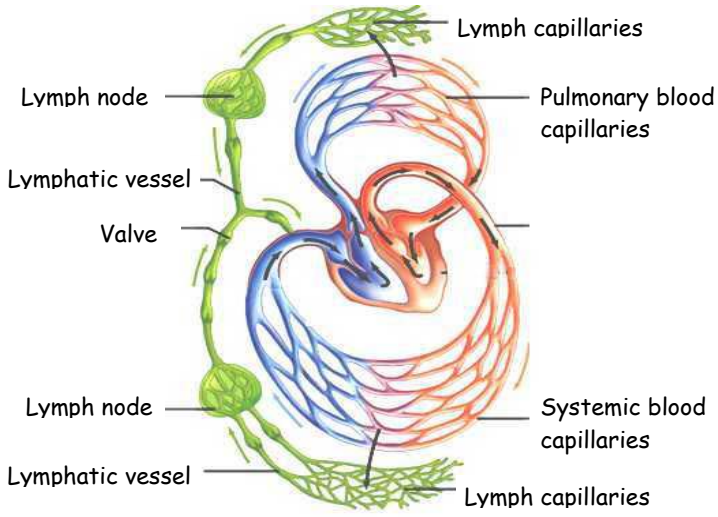


Figure 18. Organization of the systemic circulation and the microcirculation

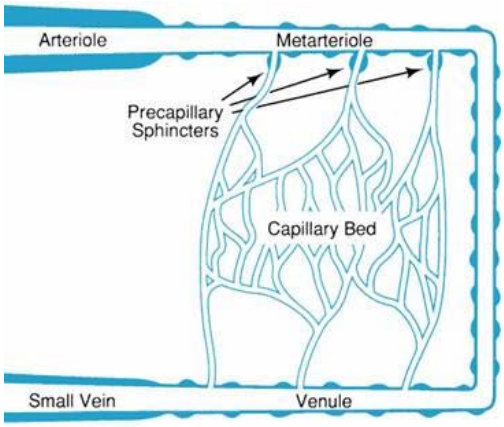


Figure 19. Schematic overview of the microvascular organization

Main characteristics

1 Progressive decrease in intravascular pressure from the arteriole to the venule.
2 The arterioles are surrounded by smooth muscle, which becomes discontinuous in the metarteries and ends in a smooth muscle ring called a precapillary sphincter. The perfused capillary area is controlled by precapillary sphincters. Opening of the precapillary sphincters increases the number of perfused capillaries.
3 Capillaries are composed of a single layer of endothelial cells surrounded by a basement membrane of collagen and mucopolysaccharides as well as pericytes (a few elongated cells with the ability to contract wrap around capillaries). Only ~ 30-50% of all capillaries are open under resting circumstances.

The main function of capillaries is transvascular exchange. The capillary network is excessively dense; the maximum distance between them does not exceed 100 μm and no cell is more than 3-4 cells away from a capillary. As they are thin-walled, they present a large surface / volume ratio and a thin diffusion barrier facilitating the delivery of nutrients (also oxygen) and the removal of waste products (also CO_2)(**Figures 20 and 21**). The capillary density varies from organ to organ (*e.g.* in the normal lung, the capillary density is 35 times greater than that in the skeletal muscle). Opening of the precapillary sphincters results in a decreased diffusion distance from the capillary to the cell. The diameter of capillaries is just large enough for RBCs (erythrocytes) to squeeze through, whereas polymorphonuclear neutrophil leukocytes (PMNs) sometimes get stuck. The transendothelial transport depends on the diffusion gradient and the Starling forces (**Figure 22**). These latter forces are dependent upon the capillary hydrostatic pressure *vs* the interstitial hydrostatic pressure and the osmotic pressure of the plasma. As a result, ~20 l of fluid is filtered and ~16 l is regained by the lymphatics. Edema occurs when the extent of filtration exceeds the capacity of the lymphatic circulation.

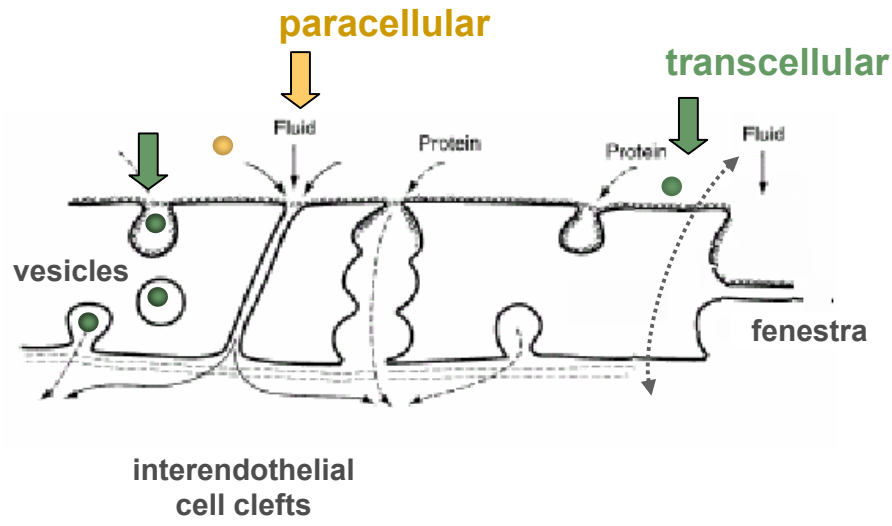


Figure 20. The transendothelial exchange

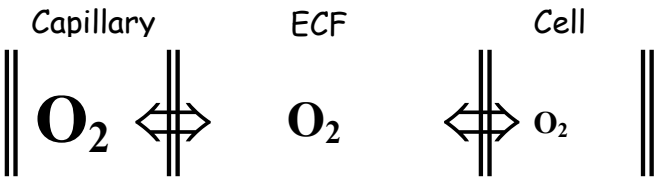


Figure 21. The oxygen concentration decreases from the capillaries toward the extracellular and intracellular fluids.

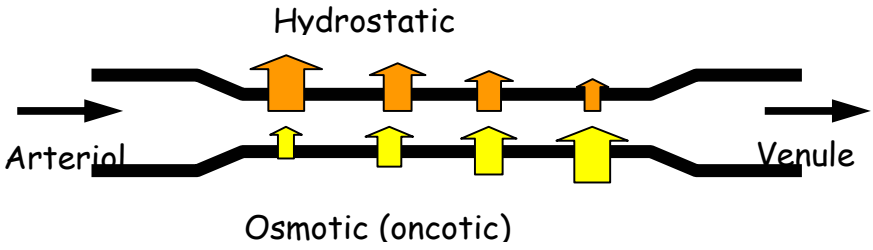


Figure 22. Scheme of the hydrostatic and osmotic forces along the capillary bed

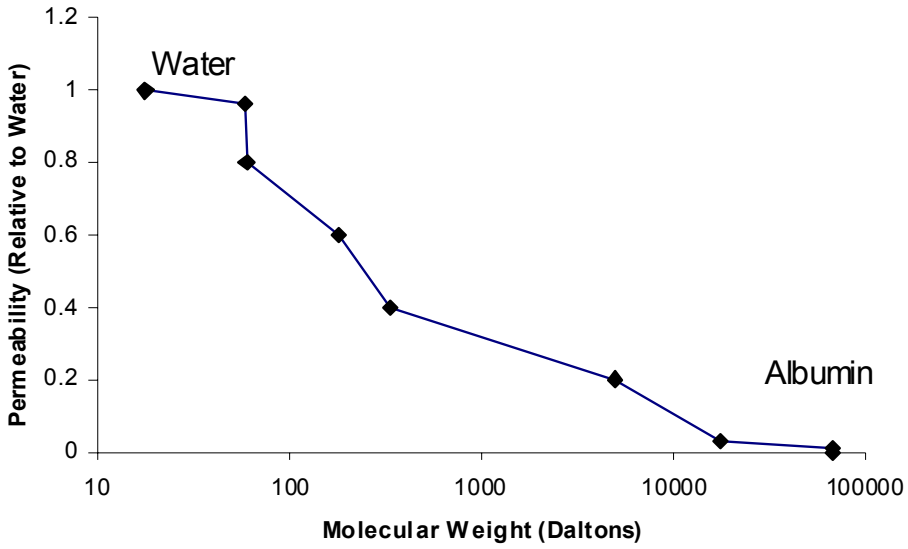


Figure 23. Diffusion through the capillary membrane
This Figure illustrates that the permeability of substances through the capillary membrane decreases roughly proportionately to the logarithm of the size. Accordingly, in microcirculatory permeability measurements, high molecular weight substances larger than the albumin are not permeable. Small molecules equilibrate rapidly between the intravascular and interstitial compartments, and therefore these cannot be used for microvascular permeability measurements.

3. Microcirculatory manifestations of acute inflammation

Inflammation is a provoked response to tissue injury (caused by chemical agents, cold, heat, trauma, or the invasion of microbes). It serves to destroy, dilute or sequester the injurious agent, and induces repair. It is a protective response, but can be potentially harmful. There is a close association between the microcirculation, inflammation and the immune response. The immune response is activated to recognize, mark and destroy an invader (it must distinguish self from non-self, failure of which would result in an autoimmune disease). The lymphocytes have a unique ability to recognize foreign substances (antigens), including those on the surface of invading pathogens, virus-infected cells and tumor cells (lymph nodes filter the lymph and help bring antigens into contact with lymphocytes, while other leukocytes, such as PMNs and macrophages, can engulf microorganisms and foreign particulate matter by phagocytosis – these cells are mobile.

The microcirculatory manifestations of acute inflammation include:

- Alterations in vascular caliber: vasodilatation leads to an increased blood flow, causing redness and swelling (*rubor* and *tumor*).
- Increased permeability: gaps occur due to (1) contraction of, for example, myosin and shortening of the individual endothelial cells, (2) direct endothelial injury, causing necrotic cell death, or (3) to the immature endothelial layer. Increased permeability for plasma proteins and cells leads to the exudation of protein-rich fluid into the extravascular space. Leakage, restricted to venules 20-60 mm in diameter, is caused by endothelial gaps. There is usually an immediate and transient response (30 min).
- Emigration of PMNs from the microcirculation: leukocyte rolling, adhesion and migration

lead to the accumulation of inflammatory cells, *e.g.* of leukocytes, leading to elimination of the offending agent (**Figure 24**). As a result, neutrophil-endothelial interactions are increased (**Figure 25**).

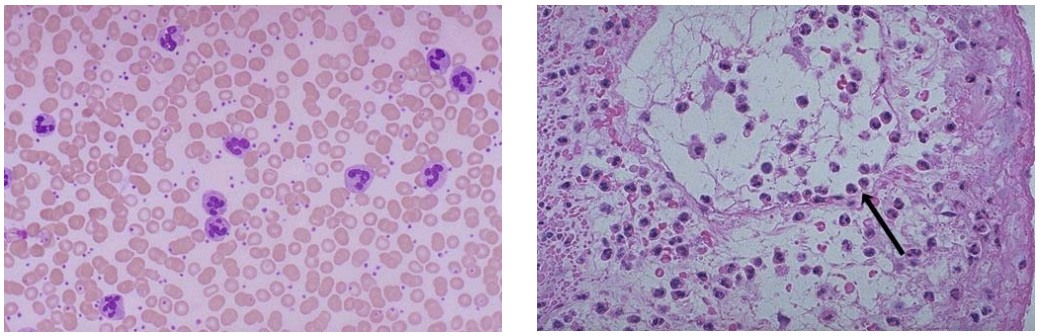


Figure 24. PMNs in the blood (on the left) and infiltrating tissue (on the right).

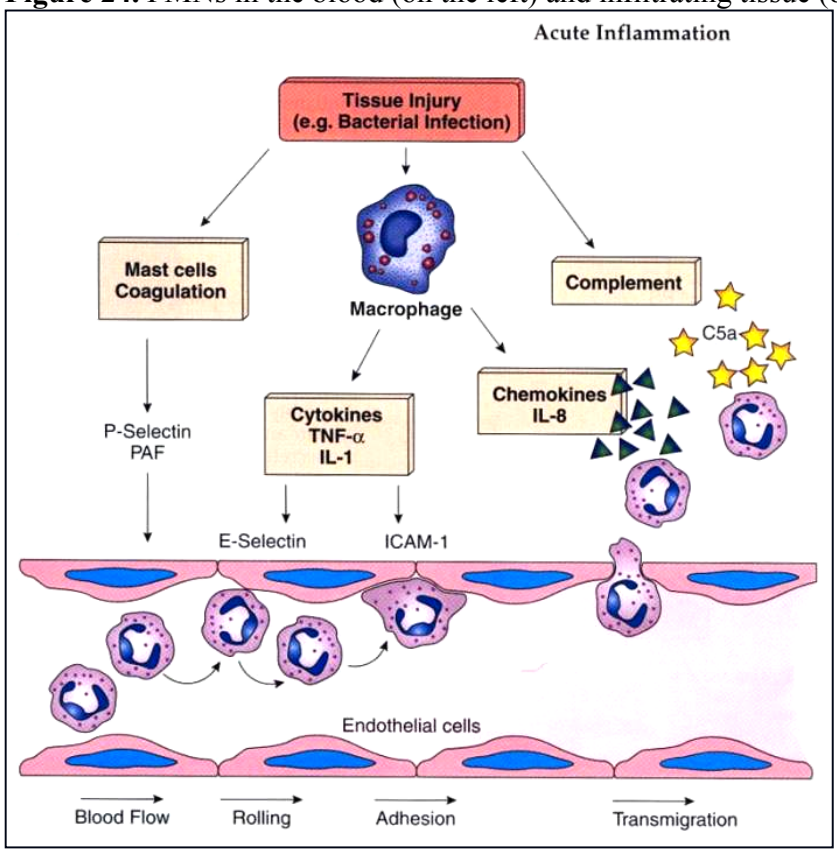


Figure 25. Mechanisms of neutrophil-endothelial cell interactions

Binding of chemical agents to specific receptors of the leukocyte cell membranes stimulates a variety of events, including chemotaxis. As a result, the PMN-endothelial cell interactions are increased.

3.1. Leukocyte activation and transmigration

In order for PMNs to leave the vessel lumen, the endothelial cells need to be activated and upregulate adhesion molecules that can interact with complementary adhesion molecules on PMNs. Primarily the selectins and integrins are thought to be responsible for PMN rolling and

sticking, respectively (**Figure 26**).

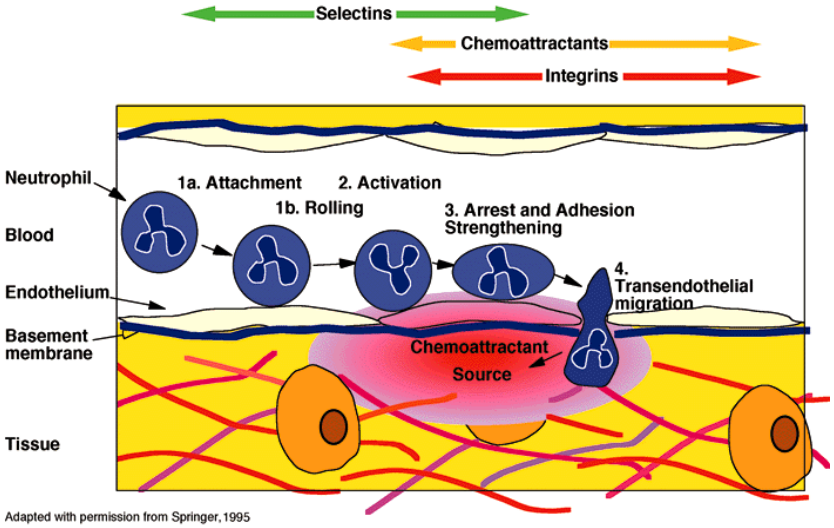


Figure 26. Mechanisms and adhesion molecules involved in leukocyte rolling, adhesion and transmigration

After adhesion, the PMNs move along the endothelial surface, and insert large cytoplasmic extension pseudopodia into endothelial gaps. These gaps are created by the actions of histamine and other chemical mediators and by the PMNs themselves. PECAM is an adhesion molecule that is partially involved in the process of transmigration. The entire cell passes through once the pseudopodia have penetrated through the basement membrane, the breakdown of which is aided by collagenase (**Figure 27**).

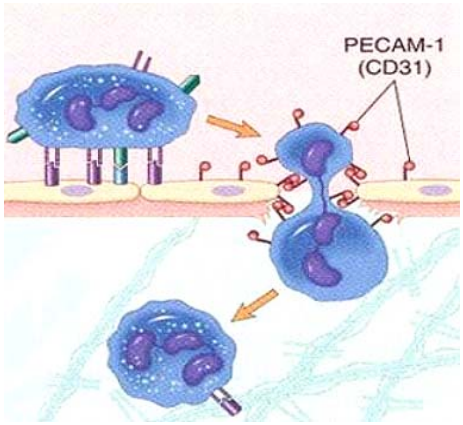


Figure 27. Migration of PMNs through the endothelium

Table 11. Some of the adhesion molecules that have been found to play roles in different steps of neutrophil-endothelial interactions

Step	Endothelial ligand	Leukocyte ligand
Rolling	Selectins/*ligands* E-selectin (endothelium) P-selectin (endothelium and platelets) *GlyCAM-1*	Selectins/*ligands* + *ESL-1* + *PSGL-1* + L-selectin
Adhesion=sticking	Immunoglobulin family VCAM-1 (vascular adhesion molecule 1) ICAM-1 (intercellular adhesion molecule 1) ICAM-1	Integrins + VLA-4 = β1 integrin) + LFA1 (CD11a/CD18) + MAC1 (CD11b/CD18) = β2 integrin
Transmigration	Endothelial PECAM-1 ("platelet-endothelial cell adhesion molecule" = CD31)	Leukocyte PECAM-1 = CD31)

3.2. Mechanisms of killing elicited by PMNs:

Phases:

- Recognition and attachment
- Engulfment and fusion of phagosomes and lysosomes
- Killing and degradation, mainly through the generation of oxygen radicals and their halogenation. Neutrophils have oxidative and nonoxidative mechanisms of killing:
  - The NADPH oxidase system, a membrane bound enzyme complex, reduces O_2 to superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH) = oxidative burst.
  - Bactericidal and cell-degrading enzyme contents of the lysosomal granules (*azurophil* and *specific* granules) fuse with phagosomes to form phagolysosomes.
  - The H_2O_2-MPO-halide system is the most efficient bactericidal system.

4. Experimental examination of the microcirculation

4.1 Structural (static) methods

4.1.1. Light microscopy and India ink perfusion

In animal models, this method involves the intravenous administration of a natural carbon solution (India ink, also called Chinese ink in American English), a substance once widely used for writing and printing. The tissue is then fixed, embedded in paraffin, sectioned and stained with hematoxylin and eosin. The purpose is to visualize the microcirculation for the examination of the efficacy of perfusion and for the discontinuity of the endothelial lining. Signs of microvascular damage are leakage from the capillaries, black spots on the histological slide, or nonperfused areas (**Figure 28**). A disadvantage is that the method is static: it relates to only one time point, and hence repeated sampling is required in order to evaluate time course changes.

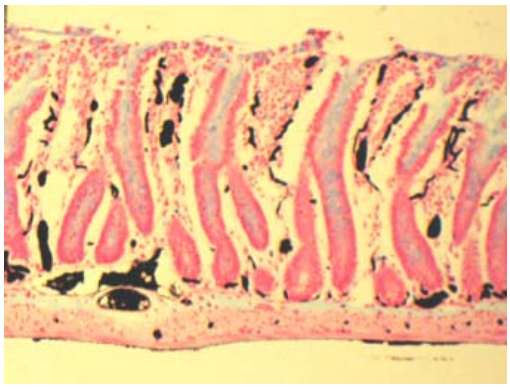


Figure 28. Discontinuity of the microvascular supply after ischemia of the small intestine (India ink spots as signs of capillary injury)

4.1.2. Electron microscopy

This reveals changes in different structures of the microcirculation at a cellular level (**Figure 29**). It likewise has a disadvantage that it is static, and does not reflect changes in higher units and intercellular communication.

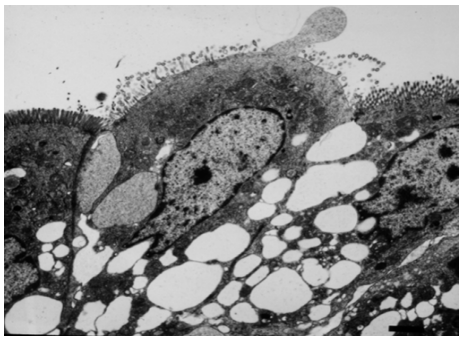
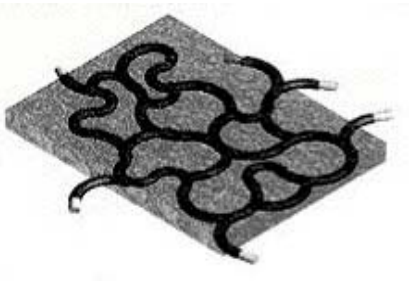


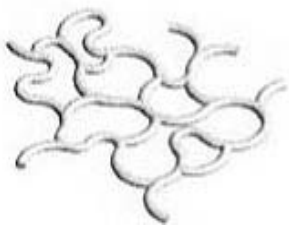
Figure 29. Electron microscopic changes in the intestinal epithelium after ischemia-reperfusion

4.1.3. Corrosion casting (combined with scanning electron microscopy)[44]

In order to prepare a vascular corrosion cast, the vascular supply to a specific organ or tissue is exsanguinated by perfusion, perfusion-fixed and injected with a rapidly-hardening methacrylate resin, which penetrates even the smallest capillaries. Once hardening of the resin is complete, the tissue is excised and placed in a concentrated alkali solution (KOH or NaOH), which corrodes away the tissue leaving the hardened resin (corrosion cast) intact (**Figure 30a**). Corrosion casts can further be analyzed with scanning electron microscopy. The purpose is to estimate the perfusion of a defined tissue mass. Again, the disadvantage is that the procedure is static, relating to only one time point (**Figure 30b**).



Vascular system in tissue. Injected plastic resin.



Plastic cast with tissue corroded away.

Figure 30a. Vessel network in the tissue at the time of resin injection and after corrosion

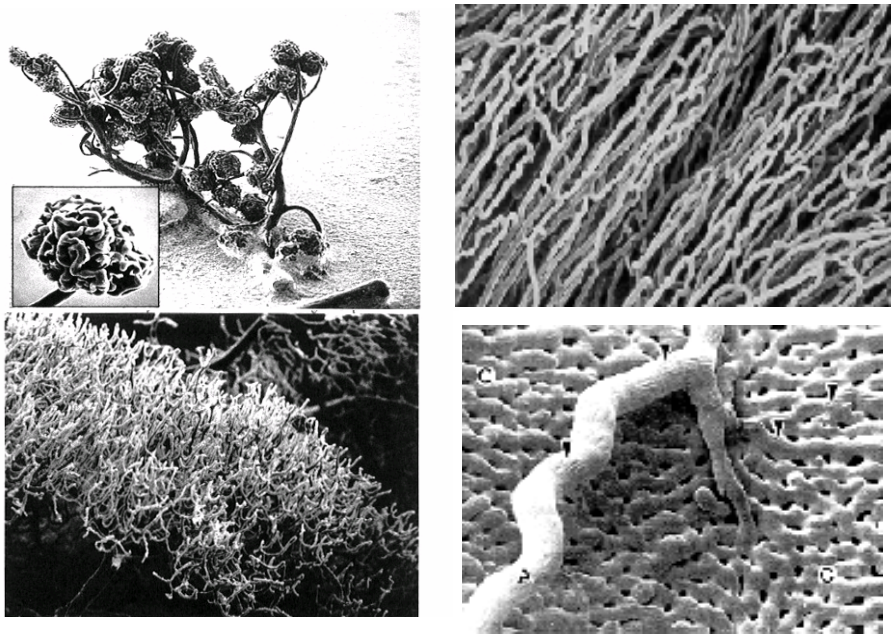


Figure 30b. Corrosion casts of the whole kidneys (upper left), kidney glomerules (left small insert), the stomach (lower left), the heart microvessels (upper right) and the eye (lower right)

4.2. Functional *in vivo* methods

4.2.1. Microsphere technique

Particles in the micro/nanometer range are injected intravenously and, after their spreading, their distribution is monitored, usually in one chosen organ. Depending on the labeling, the distribution between various histological structures, organ layers and organs can also be given. Separate perfusion of the body parts is also applicable.

The use of isotope-labeled microspheres is possible[45]:

- Determination from tissues *in vivo*: gamma cameras can detect changes in radioactivity above the chosen site/organ. If different isotopes are applied at separate time points, the tissue perfusion can be determined at a number of time points.
- Determination from harvested tissue: tissue from different organs can be harvested (usually

upon termination of the measurements, or rarely from biopsies), their radiation will be proportional to the organ perfusion, and the ratio of the perfusion of different organs can be calculated.

- Measurement from blood samples is also possible (together or separately from the tissue measurement) for organ-specific perfusion measurements, using pre- and post-organ comparison (also with a gamma scintillation counter).

Colored microspheres (particles with different colors) can also be injected:

- Determination from harvested tissue (from biopsies): using colorimetric/photometric measurements.
- Measurement from blood samples: the bead concentration of pre-organ and post-organ samples can be compared. As particles with different colors can be injected, separate time points of the experiments can be analyzed.

This has the advantage of functional and dynamic measurements, and the limitations of providing information on one or only a few time points, and the fact that the use of isotopes is hazardous.

4.2.2. Laser Doppler flowmetry[46]

The advantages of this technique are the dynamic use in any time frame, and the applicability in humans. Among its limitations are the facts that it can be used only in the upper layers of an organ, and the microcirculatory characteristics of single arterioles/venules/capillaries can not be determined (see principles in radiology manuals)(**Figure 31**).

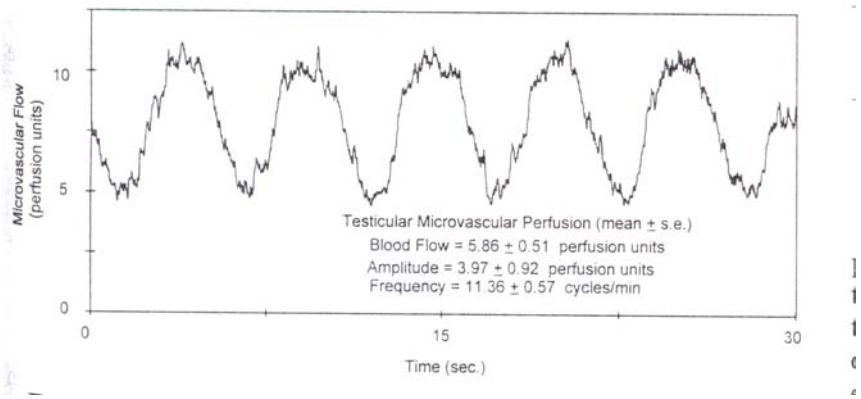


Figure 31. Laser Doppler flowmetric image of testicular flow

5. Direct observation of the microcirculation: intravital microscopy[47]

This involves a dynamic intravital, *i.e. in vivo* examination of the microcirculation in superficial layers of a tissue. As opposed to conventional histological examinations, the tissue is visualized not through a transillumination, but through an epi-illumination technique. This means that it operates not with light passing through tissue, but with illumination that is reflected from the tissue. Adjustment of the focus of the microscope allows the visualization of structures at different depths of the tissue (the maximum depth is 100-200 μm). The microscope is connected to a video camera and changes can be recorded virtually continuously with a video recorder; the evaluation is performed offline by the computer analysis of video frames.

5.1. Conventional fluorescent intravital video microscopy (IVM)

The tissue is illuminated with a mercury (HBO) lamp and the filter system of the microscope detects different-wavelength fluorescent signals. These signals are generated in the tissue via different forms of labeling of different blood components, *i.e.* RBCs, leukocytes, or the plasma itself. The various fluorescent components are detected with separate filters.

To label the *RBCs,* blood is taken from an animal, incubated with fluorescent molecules (Na-fluorescein), washed and administered intravenously to another animal (as there is no blood incompatibility in inbred rodents). The signals are bright spots flowing within vessels together with their own RBCs (**Figure 32**).

The *plasma* can be labeled with large molecular weight fluorescent markers that do not leave the circulation (larger than albumin). In this case, the plasma is bright in all vessel structures and the RBCs appear as dark spots.

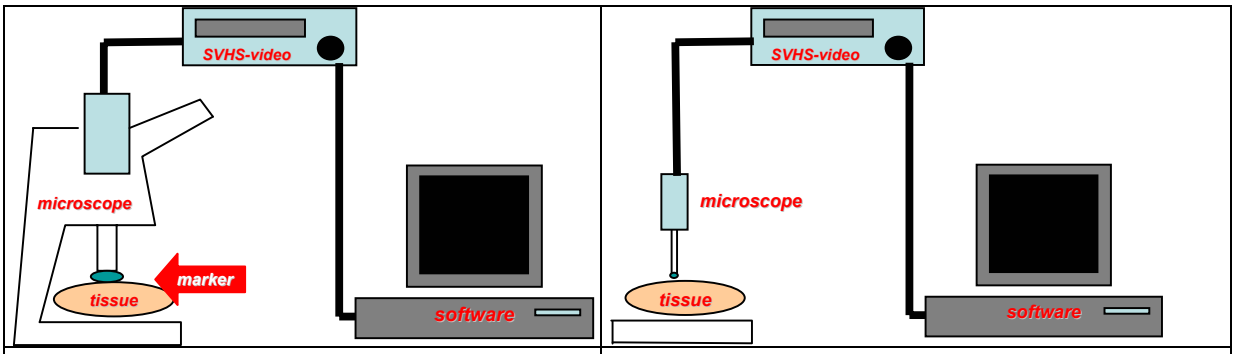
The labeling of *leukocytes* is very simple with a single intravenous injection of rhodamine 6G, a fluorescent dye. This compound is rapidly taken up by the leukocytes because it binds to their mitochondria.

5.2. Orthogonal polarization spectral (OPS) imaging[48,49]

The difficulties in the direct observation of the microcirculation of deeper layers are mainly due to the need for toxic fluorescent dyes applied for contrast amplification and the size of the instruments. An intravital system without a toxic fluorescent marker is the OPS technique, involving the use of polarized light. Its other major advantage stems from its size, since it can be held in the hand and can easily be brought even to the operating site. For an OPS image, 548 nm light is applied (in the range of hemoglobin and oxyhemoglobin), and thus every object which contains hemoglobin (*i.e.* the vessels) can be visualized. The camera is connected to a video recorder and images can be analyzed later (see at IVM)(**Table 12**).

Table 12. Comparison of the different intravital microscopic methods

Fluorescent (IVM)	**OPS imaging**
In vivo, dynamic	*In vivo*, dynamic
Operates with fluorescent markers	Operates with polarized light
Examination of superficial layers	Examination of superficial layers
Fluorescence staining: Leukocytes: rhodamine 6G RBCs: FITC (fluorescein isothiocyanate) Plasma: FITC-albumin, FITC-dextran	No staining, visualization of all hemoglobin-containing structures
Also for leukocyte-endothelial cell interactions	Not for leukocyte-endothelial-cell interactions
Video recordings, offline analysis	Video recordings, offline analysis



Not applicable in humans | Applicable in humans

5.3. Microcirculatory parameters with intravital microscopy

1. Perfusion

- Red blood cell velocity (RBCV; µm/s)
- Flow pattern changes (spatial and time-wise heterogeneity)
- Capillary perfusion changes (functional capillary density; length of functioning capillaries/area)

2. Leukocyte – endothelial cell interactions

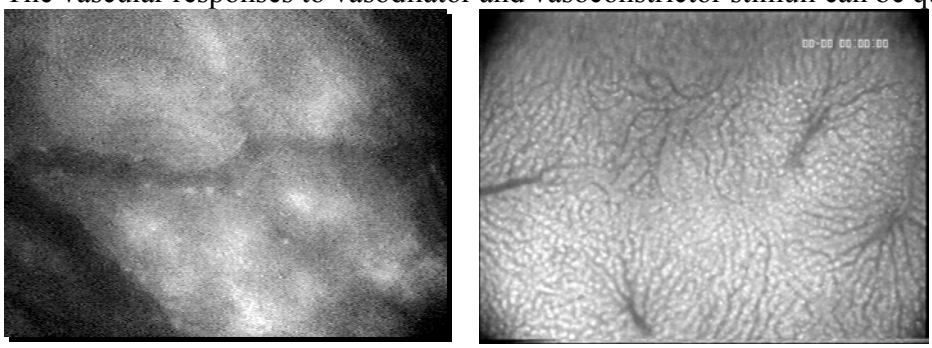
- "Free-flowing" (cells passing through the vessel segment that do not display any interaction with the endothelial cells; quantification: % of all)
- "Rolling" (cells flowing with 30-40% of the central line velocity, exhibiting rolling on the endothelial cell surface; quantification: % of all or cell number/vessel circumference/30 s)
- "Sticking" (cells not moving on the endothelial cell surface within 30 s; quantification: % of all or cell number/vessel surface)

3. Microvascular permeability

After the injection of FITC-albumin intravenously, the ratio of the intra- and extravascular light density (brightness) is measured, using computer software.

4. Vessel diameters

The vascular responses to vasodilator and vasoconstrictor stimuli can be quantified.



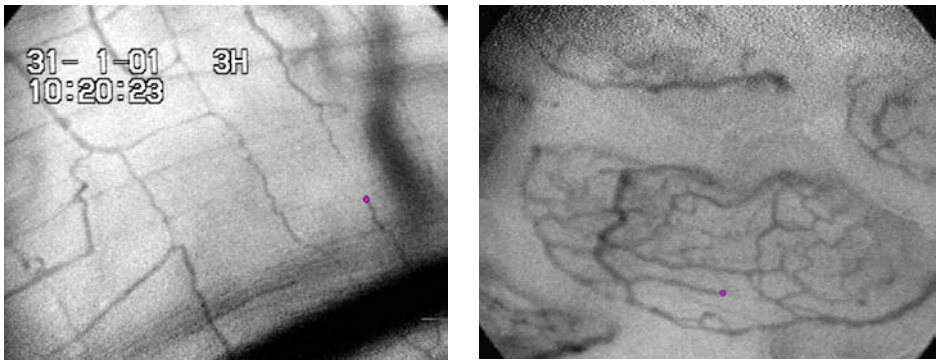


Figure 32. Fluorescent (upper left: leukocytes in a venule; upper right: liver) and OPS intravital microscopic images (lower left: muscle layer of the intestine, lower right: intestinal villi)

5.4. Tissue types examined with IVM

The limitation here is the tissue thickness. Since IVM provides information about only a superficial layer of the microcirculation, the tissue thickness greatly affects its applicability. All parenchymal organs can be examined, since the changes detected on the surface provide information about changes in deeper layers. Since small animals, such as rodents (mice, hamsters and rats), have very thin tissue, these are the most commonly used species for IVM.

If the intestine is taken as an example, from the serosal orientation the focus of the microscope can be adjusted only to detect the subserosal lymphatics and venules in human or dogs, but would reach deeper layers (the serosa, circular and longitudinal muscle layers, and even the submucosal vessels) in mice or rats. If it is desired to visualize the circulation of the intestinal mucosal villi, the lumen of the bowel can be open antimesenterically and the villi can be made equally accessible in dogs and mice. As concerns the skin, not even rats can be used. Only mice have a thin enough epidermis for the microcirculation under the skin to be observed.

Ideally, organs not exhibiting great movements are more accessible for IVM than moving, pulsating and contracting organs. For this reason, the heart and the lungs are rare subjects of these measurements. Because of the breathing movements transferred by the diaphragm to the liver, this organ requires special preparation for IVM. Specifically, the organ should be exteriorized and, after some rotation, the dorsal surface of the left liver lobe can be examined. Organs most often used for IVM include the muscle, intestine, periosteum, liver, pancreas, testis, urinary bladder, brain (through a cranial window) and lung (through a chest window, in breathing intervals).

5.5. Pathophysiology of the microcirculation

Perfusion: a decrease in *RBC velocity* (sometimes an increase), a decrease in *functional capillary density*, an increased microcirculatory heterogeneity, *leukocyte–endothelial cell interactions* (the proportion of free-flowing cells decreases, while proportions of rolling and sticking are increased), and the *microvascular permeability* is increased.

X. Animal models and experimental methods in respiratory research *(Dr. Ágnes Adamicza)*

1. Functional investigations

The functional investigation and monitoring of the respiration can be performed as follows:

- Basic monitoring (observation)
- Hemodynamics (pulmonary circulation, pressure and blood flow)
- Pulmonary mechanics (plethysmography, bronchoprovocation test and forced oscillation technique)
- Monitoring respiratory gases: noninvasive (capnometry and pulse oxymetry) or invasive (blood gas analysis)
- Biochemical analysis (blood, pulmonary tissue biopsy and bronchoalveolar fluid)
- Bronchoalveolar lavage (BAL)
- Measurements of pulmonary edema (gravimetric analysis, permeability measurements and transthoracic electrical impedance)

1.1. Basic monitoring

Basic monitoring includes observation of the respiratory movements (type, depth and frequency of breathing) or, in large animal models, auscultation (listening to the sounds arising within the lungs).

1.2. Hemodynamics

The pulmonary circulation can be investigated by measurement of the pressure and blood flow in the pulmonary artery (*e.g.* using a Swan-Ganz catheter in large animals). The usually measured parameters are the pulmonary artery pressure, the pulmonary capillary wedge pressure and the pulmonary vascular resistance.

1.3. Pulmonary mechanics

During breathing, air flow, pressure and volume changes occur in the lungs. Lung mechanics deals with the measurements and relationships of these parameters. Conventional lung mechanical parameters are the airway resistance, lung compliance, lung elasticity and pulmonary inflation pressure.

Airway resistance ($cmH_2O/l/s$) is the opposition to air flow caused by the forces of friction. It is defined as the ratio of the pressure to the rate of air flow. Accordingly, the gas flow is proportional to the pressure, and inversely proportional to the resistance. Resistance means the friction between the molecules of the gas and between the gas molecules and the walls of the airways. The rate of air flow is measured with a pneumotachograph, and the pressure is measured with a transducer.

Lung compliance (l/cmH_2O) means the distensibility of the lung tissue, a measure of the change in lung volume that occurs with a change in intra-alveolar pressure. The compliance decreases in response to lung fibrosis (the lung tissue is damaged by metal dusts or asbestos, etc.; the air sacs become inflamed and scarring begins in the interstitium (*i.e.* the tissue between the air sacs), and the lung tissue thickens and become stiff), during an airway blockage which impedes the air flow, in the event of an increased surface tension in the alveoli, in edema or in atelectasis. The compliance increases in emphysema.

The lung elasticity is the ability of the lung to return to its original shape and size, and is influenced by the fibro-elastic network surrounding the alveoli.[50]

The peak inflation pressure is the maximal value of the inflation pressure and can be measured at the end of the endotracheal tube or tracheal tube.

1.3.1. Plethysmography

The plethysmograph is a closed, airtight chamber or box, used to determine noninvasively the thoracic lung volume and airway resistance in research laboratories and in clinical medicine too. Several ports are used to attach the transducers, the pneumotachograph, the tracheal tube and the vascular cannulas to the plethysmograph. It is applied to measure the functional residual capacity (FRC, the volume in the lungs at the end of expiration) and the total lung capacity (TLC). During breathing, the volume of the thorax changes, so the pressure inside the chamber also changes.

The Boyle-Mariotte Law is used to calculate the volume of the lungs. This is an empirical law which states that at constant temperature for a given quantity of gas, the product of its volume and its pressure is constant:

$$PV = \text{constant}$$

or

$$PV = P'V'$$

where V' and V are the final and initial volumes, and P' and P are the final and initial pressures.

1.3.2. Bronchial provocation test

Bronchial provocation tests are widely used in the diagnosis of asthma. The pulmonary changes can be determined by measuring the enhanced airway reactivity to bronchoconstrictor agonists (acetylcholine, methacholine, histamine or environmental allergens). The airways are challenged with one or more concentrations of aerosolized agents, which evoke a hyperresponsive reaction. Mechanical tests are done before and after the challenge, or under experimental conditions the forced oscillation technique may be used.

1.3.3. Forced oscillation technique

The forced oscillation technique is used to study noninvasively the airway and lung parechymal mechanical properties separately.[51] Small-amplitude pseudorandom oscillations containing multiple frequency components (including the physiological frequency) are introduced into the trachea at end-expiration, and the respiratory system input impedance is measured. A model containing parameters that characterize the airway (resistance) and lung tissue (resistance and elastance) mechanics is fitted to the impedance spectra. The separation of the airway and tissue responses is based on the fact that the frequency-dependent characteristics of the airway and tissue components are different.

1.4. Monitoring respiratory gases

Noninvasive: Capnometry/capnography measures the exhaled (end-tidal) CO_2/pressure waveform of the exhaled CO_2, and the respiratory rate. Pulse oxymetry measures the O_2 saturation and the heart rate (used in clinical medicine).

Invasive: Blood gas analysis is used to determine the blood gas state (O_2 uptake and CO_2 elimination in the lung, and the blood pH), and the function of the lungs and kidneys in the acid-base balance, or pulmonary diseases. A blood sample is taken from an artery, and the analyzer measures the partial pressures of O_2 (pO_2) and CO_2 (pCO_2), and the acidity of the arterial blood (pH; a measure of the hydrogen ion concentration), and also calculates the actual and standard bicarbonate (calculated at pCO_2 = 40 mmHg), the base excess (the quantity of acid or alkali required to return the blood to a normal pH; positive = alkalosis, negative = acidosis) and the O_2 saturation (the percentage of hemoglobin-binding sites in the blood occupied by O_2).

1.4.1. The acid-base balance

Normal blood gas values: pO_2 90-100 mmHg, pCO_2 36-44 mmHg, pH 7.35-7.45, bicarbonate 22-26 mmol/l, base excess -2+2 mmol/l, and oxygen saturation 96-100%. In respiratory acidosis, pCO_2 is increased and pH is decreased; this results from obstruction in the airways, ARDS (see section 4.1) or depressed respiration. In respiratory alkalosis, a decrease in pCO_2 is accompanied by a pH > 7.45; it is associated with hyperventilation. Metabolic acidosis involves a decreased bicarbonate level and the pH is < 7.35; it is usually caused by gastrointestinal diseases. Metabolic alkalosis is the accumulation of base or the loss of acid, *e.g.* by vomiting.

1.5. Biochemical analysis

Functional investigations of the lungs can be performed by measurements of vaso- and bronchoactive mediators, enzymes (proteases or myeloperoxidase produced in the neutrophils), markers (these indicate the pathological condition of the lungs), proteins, surfactant, or differential cell counts (*e.g.* different white blood cells and macrophages) are determined in blood, in lung tissue biopsies or in BAL fluid.

1.6. Brochoalveolar lavage (BAL)

The examination of the BAL fluid is a simple means of monitoring and evaluating the severity of respiratory diseases, mainly inflammatory airway diseases. BAL can sample the proximal airways, the distal small airways and alveolar spaces. Under experimental conditions, BAL is performed with a cannula introduced through the tracheal tube, and aliquots of a small volume of sterile saline at body temperature are then instilled. After a few seconds, fluid is aspirated (suctioned) into a syringe. Aspiration can be repeated two or three times. The first aliquot (bronchial wash) can be analyzed separately, if the airways should be investigated; otherwise the samples are pooled. The recovery of BAL is quite variable; usually, it is 30-50% of the volume instilled, but it is decreased in chronic obstructive pulmonary diseases (COPD). Cytology or microbiology tests (cell counting, analysis of mediators, markers and cell culturing) should be performed on the BAL samples. BAL will result in a slightly decreased pO_2.

1.7. Measurements of pulmonary edema

Gravimetric analysis. The animals are overanesthetized, and the lung tissue samples are removed. After weighing, the samples are dried in an oven to constant weight. Lung edema is estimated via the wet-to-dry weight ratio.

Evaluation of lung capillary permeability. Evans blue dye (EB) is injected intravenously, where it rapidly binds to plasma albumin. During increased vascular permeability (edema), when extravasation occurs, the EB-albumin complex leaks into the surrounding tissues. At the end of the experiments, animals are overanesthetized and the complex is cleared from the pulmonary vascular system with saline perfusion. The extravasated EB can be extracted from the lung tissue by a solvent (formamide). The extracted EB concentration and the EB concentration in the blood can be measured by spectrophotometry. The permeability index is calculated as EB_{tissue}/EB_{plasma}.[52]

Transthoracic electrical impedance. If a small-amplitude, high-frequency current is introduced across the thorax, the changes in the impedance (voltage/current) to the signal will be altered, depending on the stroke volume during systole (this is the basis of cardiac output measurements by impedance cardiography). In the thorax, both the tissues with high conductivity (blood in the large vessels and the heart) and those with high resistance (the lungs, muscle, bones and fat) contribute to the value of the basal thoracic impedance (Z_0). Though the value of Z_0 displays great variability, the changes in it depend on the changes in

the fluid and air volume in the thorax. During thoracic bleeding or in pulmonary edema, it is decreased, whereas in pneumothorax it is increased. Thus, Z_0 is a very sensitive indicator of the changes in the air or fluid volume in the thorax, and its continuous postoperative monitoring in chest surgery patients can be a useful guide to the early detection of changes in the thorax when other parameters (hemodynamics or blood gases) do not change.[53,54]

2. Ensurance of open airways, mechanical ventilation

2.1. Endotracheal intubation

Intubation is the most general and secure way to provide open airways and it allows continuous monitoring of the respiratory function (*e.g.* the composition of inhaled or exhaled gases), the measurement of airway pressure, mechanical ventilation for a short time, suctioning of the mucus from the airways, BAL or giving medicines. A plastic or rubber tube of appropriate size (in large animals) or a short plastic catheter (in small animals) is inserted throiugh the mouth into the trachea or one of the main bronchi of an anesthetized animal. A laryngoscope (in large animals) or a forceps (in small animals) is used to expose the larynx. In mice, lighting the trachea through the thin skin makes intubation easy. At the distal end of a tracheal tube, an inflatable cuff inhibits the trickling of saliva or blood into the lung and the escape of the inspired air. The tension of the cuff can be controlled by a balloon on the other end of the tube.

2.2. Tracheostomy

Tracheostomy is a surgical procedure carried out under general anesthesia. A plastic tube (with an obturator, a cuff and a balloon in large animals) or a short plastic tracheal cannula (in small animals) is introduced into the trachea through an opening (*stoma*). It is performed when endotracheal intubation is contraindicated, or the airways must be maintained open for a longer time (for mechanical ventilation or for lung mechanical measurements). The technique of tracheostomy is as follows:

1. the animal is laid in a supine position,
2. the skin is scrubbed,
3. a transverse skin incision is made between the first and second tracheal cartilages,
4. the muscles are separated in the midline with blunt dissection,
5. a T-shape incision (stoma) is made: the trachea is cut with a scalpel between the first and second cartilages, and then the second (and third) cartilages are cut downward in the midline with scissors,
6. in large animals, an atraumatic suture is placed into the corners of the cut cartilages to retract the trachea during introduction of the tube,
7. after removal of the obturator, the cuff is inflated,
8. the threads are tightened together,
9. the skin is closed on both sides.

2.3. Thoracotomy

Thoracotomy is surgical opening of the chest under mechanical ventilation to perform measurements and interventions directly on the surface of the lung. Under experimental circumstances, it is carried out through the thorax with rib resection on one side (in large animals), or with cutting of the sternum in the midline (sternotomy) in small animals (rats or mice) (quick and little bleeding).

2.4. Mechanical ventilation

During mechanical ventilation, a machine called a respirator is used to inflate or deflate the lungs (positive pressure ventilation). The aim of mechanical ventilation is to

improve the oxygenation, to ensure the optimal ventilation or to eliminate atelectasis (the collapse of the alveoli due to the decreased alveolar ventilation or the occlusion of the alveoli). It is necessary to use mechanical ventilation when the spontaneous breathing of an animal is inadequate or blocked (in response to muscle relaxants) or in the case of thoracotomy. In volume-controlled respirators, during inflation the preset volume (tidal volume) is delivered into the lungs, in pressure-controlled respirators, the air flow proceeds until a preset peak pressure is attained. In the former types, the respiratory frequency or the minute ventilation can also be adjusted, and a valve protects the lungs from the overpressure. If an animal expirates against some cmH_2O pressure (positive end-expiratory pressure; PEEP), the alveoli are opened and filled with air, the oxygenation is improved, and the end-expiratory lung volume is increased. It is necessary to use PEEP during thoracotomy or in experiments on open-chest animals, where the alveolar spaces that are closed due to the supine position in the lower regions will be opened to ensure the homogeneous distribution of air.

3. Structural investigation of the lungs

The alveolar structure consists of the capillary endothelium, the alveocapillary membrane (0.5 μ in diameter; gas exchange), and a single layer of epithelium: type I epithelial cells (thin cells), and type II epithelial cells (pneumocytes, a cuboid cell shape, with microvilli on the surface, secretory granules = lamellar bodies in the cytoplasm). Capillaries cover 80-90% of the alveolar surface. The alveoli are covered by a thin film layer, called the surfactant. The surfactant is part of the humoral and cellular defense mechanism in the lung, and is secreted in the granules of the type II epithelial cells. This surface-active lipoprotein complex reduces the surface tension, increases the compliance (the lung is able to inflate much more easily), stabilizes the alveolar structure, prevents the alveoli from collapsing at the end of expiration, and ensures the optimal gas exchange. The extracellular matrix of the lung consists of insoluble protein fibrils and soluble polymer macromolecules (carbohydrate chains with proteins which can bind water). The insoluble fibrils are made up of structural proteins (collagen and elastin) that are synthesized by fibroblasts. Collagen and elastin are responsible for the stiffness and the elasticity of the lung tissue, respectively. The matrix provides a mechanical network to maintain the structural integrity of the lung tissue, and water and ions for the cell function through the interstitial fluid.

3.1. *In vivo* videomicroscopy, orthogonal polarization spectral imaging

In vivo videomicroscopy is used to study the alveolar stability (the change in size of the subpleural alveoli from peak inspiration to end expiration), the width of the alveolar septa, and pulmonary edema.[55]

The orthogonal polarization spectral imaging technique is suitable for visualizing the subpleural alveoli in the lung.

3.2. Histology

Light microscopy. Lung tissue is cut into very thin segments with a microtome, and the slices are then stained using hematoxylin (which stains cell nuclei blue) and eosin (which stains RBCs bright red, and the cytoplasm and connective tissue pink or red).

Electron microscopy. In electron microscopes, a beam of highly energetic electrons is used to examine the specimen and gain information on the structure and composition.

4. Animal models of human pulmonary diseases

To understand the mechanisms underlying a disease, we have to mimick the main functional characteristics of the disease in a representative animal model. In the animal

models of pulmonary diseases, the induced pathophysiological changes or the parameters of the lung function evoked by the changes can be measured. In these *in vivo* models, primarily small animals (mice, rats, guinea pigs and rabbits) are used to characterize the lung function of the different pulmonary diseases.

4.1. ARDS - acute (or adult) respiratory distress syndrome (shock lung or wet lung)

The main clinical symptoms of ARDS are tachypnea, hypoxemia, diffuse bilateral pulmonary infiltrates, pulmonary edema, decreased lung compliance, dyspnea (difficult breathing), hypotension and metabolic acidosis.

4.1.1. Clinical disorders associated with ARDS

ARDS can occur as a result of direct or indirect injury to the lungs. Direct injuries to the lung tissue include respiratory tract infection, pneumonia, aspiration of the gastric content, lung contusion, fat emboli or the inhalation of toxic substances. In these diseases, severe inflammation develops in the alveolar epithelium, and extends to the interstitial and endothelial layers. In several cases, systemic causes (indirect lung injuries), such as sepsis (40-45%), multiple trauma, various types of shock, burn injury, acute pancreatitis or a drug overdose can also evoke ARDS. Activated neutrophil and endothelial cells, and inflammatory mediators have important roles in the inflammatory processes.[56]

The pathophysiological mechanisms in ARDS are: damage to the capillary endothelium, damage to the alveolar epithelial cells, an increase in permeability, interstitial/alveolar edema (inflammatory cells), a surfactant dysfunction, atelectasis, a shunt (in the normal lung, the volume of blood passing an alveolus is matched with an equal amount of air; in a diseased state, there is a ventilation/perfusion mismatch, when the blood perfusion to an alveolus is normal, but the ventilation is reduced or ceases due to obstruction of the airways, atelectasis or mucus; there is no gas exchange), with decreases in functional residual capacity and compliance.

4.1.2. Lung injury markers

Capillary permeability: There is an increase, which leads to interstitial/alveolar edema. This can be concluded from measurement of the protein content in the edema fluid (taken from the endotracheal tube), or measurements of inflammatory mediators or cells in the BAL.

Endothelial markers: The levels of plasma adhesion molecules or vasoactive mediators increase.

Epithelial markers: The levels of surfactant or specific proteins of the surfactant in the BAL decrease.

Macrophages: The levels of cytokines in the BAL or the plasma are increased: NF-kappa-B, an inflammatory mediator, which is in an inactive form (IκB) in the cytoplasm under normal conditions. During activation (endotoxin and cytokines), DNA transcription and mRNA translation in the nucleus are inititaed, which is responsible for the continuous synthesis/activation of inflammatory mediators (cytokines, inductive nitric oxide synthase and adhesion molecules).

Neutrophils: These are proteases **(**elastase and collagenase) in the BAL, and myeloperoxidase in the BAL or lung tissue. There is a close correlation between the tissue myeloperoxidase activity and the histology scores.

4.1.3. Animal models of ARDS

- Intravenous administration (injection or infusion) of endotoxin or live bacteria: neutrophil sequestration in the pulmonary microcirculation, the release of inflammatory mediators and pulmonary edema.
- Intravenous injection or infusion of oleic acid (unsaturated fatty acid, a constituent of phospholipids of cell membranes): increased capillary permeability, protein-rich edema fluid, and ventilation/perfusion inhomogeneities. This is used to mimick the symptoms of ARDS complicated by pancreatitis or bone fractures.
- Intestinal ischemia-reperfusion induces local inflammatory processes (neutrophil activation and accumulation, and fluid filtration) in the intestinal microcirculation; hence, the barrier function of the mucosa is decreased and, due to the mucosal injury, bacteria or endotoxin are translocated from the lumen to the bloodstream. The activated neutrophils and endotoxin evoke inflammatory processes in remote organs, mainly in the lungs, where inflammatory cell sequestration, an increase in pulmonary capillary permeability, pulmonary edema or lung mechanical consequences occur.
- Acute pancreatitis induced by treatment with high a dose of cholecystokinin or its analog, cerulein, or with a combination of cholecystokinin and ethanol in rats or in mice.
- Sepsis induced by cecal ligation/puncture: after opening of the abdominal wall, the base of the cecum is ligated, and then punctured several times with a needle (the cecum is pressed to check the perforation). The symptoms appear 24 h after closure of the abdominal wall.

4.2. Emphysema

Emphysema is a type of COPD that involves irreversible destruction and abnormal, permanent enlargement of the alveolar spaces distal to the terminal bronchiolus. A chest X-ray and computerized axial tomographic scanning of emphysema in clinical medicine reveals hyperinflation of the lung, the loss of blood vessel markings, the reduced size of the heart, the presence of bullae (air pockets), and increased lung markings.

In emphysema, lung compliance is increased, and the lung is less able to contract. The lung cannot exhale air, and thus the next breath is started with more air in the lungs ("trapped air"). The alveoli are unable to fill with fresh air. This is an irreversible disease, which develops gradually, it can only be slowed, but not stopped. The main (exogenous) cause of emphysema is cigarette smoking. Other causes are air pollution or the (endogenous) imbalance of the protease-antiprotease activity.

4.2.1. Emphysema models

4.2.1.1. Intrapulmonary challenge with exogenous agents:

- Cigarette smoking was the first animal model (1990, 10 cigarettes/day for 1-12 months) in guinea pigs. Changes are observed in the trachea and large airways: an increased epithelial thickness, increased mucus secretion, an increased number of inflammatory cells (macrophages and neutrophils) in the alveoli, and an increased percentage of neutrophils in the BAL. However, there are difficulties in inducing emphysema in small animal models. Rats are resistant, and the susceptibility of mice is strain-dependent. There are anatomical differences: small animals are obligate nose breathers, and can tolerate cigarette smoking.
- In irritant gas models, inhaled nitrogen dioxide, ozone or diesel exhaust is used. Pulmonary changes: Increases in airway resistance and airway responsiveness, neutrophil infiltration to the alveoli, increased mucus production, and smooth muscle hypertrophy (increase in size of the smooth muscle cells).

- Proteases (elastase and papain: derived from plants; slow effect): Loss of elastin. Elastase-induced changes: Hemorrhage, neutrophil and macrophage infiltration, edema, air space enlargement, increases in compliance, FRC and TLC, decreased expiratory flow, and diminution of lung elastin content.[57]
- A low dose of endotoxin induces neutrophil sequestration, migration, elastase release and elastin destruction.

4.2.1.2. Genetic models: Mutant mouse strains develop an air space enlargement

- "Blotchy" mice have a deficiency of lysyl oxidase (a copper-containing enzyme, required for the production of collagen and elastin); abnormal collagen/elastin cross-linking.
- "Tight skin" mice have a mutation in fibrillin, which is involved in elastic fiber assembly and neutrophil/macrophage migration.

4.2.1.3. Gene-targeted mice

Gene targeting is the main tool of gene therapy to develop new treatments for a disease caused by a specific gene defect. It is used to transfer a missing gene into a mammalian cell or to modify a gene. Mice are the favorite subjects for experiments because gene manipulation can be easily performed, and the genome of mice is known.

1. Loss-of-function model ("knockout"; one or more genes are inoperable). Knockout of a protein leads to emphysema:
elastin-/-: fewer, dilated distal air sacs,
surfactant protein D-/-: increased macrophage elastase.

2. Gain-of-function model ("knockin"; overexpression of a gene): overexpression of specific proteins (IL-13: inflammation and increased macrophage elastase).

4.3. Asthma

Asthma is a complex, chronic airway disease that involves inflammatory mechanisms, increases in eosinophils, lymphocytes, neutrophils and mast cells in the airways, activation of inflammatory and structural cells in the lung, the release of inflammatory mediators, bronchoconstriction, increased mucus secretion, and bronchial hyperresponsiveness. The main structural change is the increased inner airway wall (the area between the epithelium and the smooth muscle) diameter. In asthma, eosinophils are recruited in the lung and the mediators released from the activated cells contribute to the airway inflammation and the bronchial hyperreactivity. Eosinophil granule proteins are cytotoxic and damage the epithelium, increase the vascular permeability, and activate the mast cell release of different mediators (histamine and cytokines). The inflamed airways become narrower through an allergy-induced spasm of the surrounding smooth muscles or the blocking of the airways with mucus.

4.3.1. Markers in asthma

BAL (this samples the proximal airways, the distal small airways and the alveolar spaces): lymphocytes and macrophages. Neutrophils and eosinophils are present mainly in the bronchial wash (this samples the more proximal airways). Examinations of sputum and blood, and measurement of exhaled nitric oxide. An increase in the concentration of exhaled nitric oxide has been found in asthma. The levels of exhaled nitric oxide parallel the inflammatory process in the asthmatic airway.

4.3.2. Asthma models

The most popular animal model of asthma is the ovalbumin (OA, a synthetic protein of egg white)-sensitization of small animals (rabbits, guinea pigs or mice). Rats differ from other animals in their pulmonary responses to the antigen, because the responses are mediated primarily by serotonin, and histamine cannot induce airway smooth muscle contraction in them. Large animal models (monkeys, sheep, and dogs) are used less frequently.[58]

The animals are sensitized by the intraperitoneal or subcutaneous injection of OA together with a second adjuvant, $Al(OH)_3$ (which contributes to the induction of an antibody reaction). The injection may be repeated 7 days later. After another 7 days, the animals are challenged with aerosolized OA, using a nebulizer, and the challenge is repeated for at least 7 days. Biochemical and physiological responses to sensitization, and lung mechanics (bronchoprovocation test) can be studied, and differential cell counts (eosinophils, leukocytes, granulocytes and erythrocytes) in the BAL can also be determined.

4.4. Pulmonary edema

In pulmonary edema, excessive fluid accumulates in the lungs, and the impaired gas exchange may cause respiratory failure. Edema can develop because of a heart and circulatory system failure or direct injury to the lung. Edema may be caused by an increased pulmonary capillary pressure, a decreased plasma oncotic pressure, an increased negative interstitial pressure, damage to the alveocapillary membrane, an increased capillary permeability or lymphatic obstruction. At first, the fluid extravasated from the vasculature passes only into the interstitium (interstitial edema), and further into the alveolar space (alveolar edema). Inflammatory cells (neutrophils and macrophages), RBCs, proteins and proteases appear in the edema fluid. Excessive fluid in the extracellular space can markedly impair the lung function due to the increased diffusion distance

4.4.1. Pulmonary edema models

Experimentally, pulmonary edema can be induced by intravenous administration (infusion or injection) of endotoxin (lipopolysaccharide, the outer membrane of Gram-negative bacteria). In response to endotoxin, endothelium/neutrophil interactions, neutrophil adhesion/activation or inflammatory mediators can be studied. In other models, the intravenous infusion of oleic acid (an unsaturated fatty acid, the phospholipid part of the cell membrane) causes an increase in capillary permeability, a protein influx into the alveoli and ventilation/perfusion mismatch. In this experimental model, the pathomechanisms of certain types of ARDS (pancreatitis and heavy bone fractures) can also be investigated.

XI. Cell and tissue cultures (*Dr. Csilla Torday*)

1. General background

The general public by and large accepts the experimental use of animals as necessary, but would like to eliminate any pain or distress in experiments involving living animals. Besides, there is a steady demand to replace animals in research by *in vitro* tests. Scientists too emphasize the need to reduce the pain and discomfort associated with experimental procedures, but understand that the replacement of *in vivo* experiments by *in vitro* methods is not an adequate solution. Some animal rights activists view the term "alternative" in the context of replacing all animal use with non-animal alternatives and fight to stop the use of animals in scientific experiments. Many of them are not experts in this field and do not know whether the alternatives are adequate or not to give answers to the questions arising in scientific research. There are questions that can be answered by *in vitro* experiments (for example, by using cell cultures), but these can not cover the whole area of scientific research. Working with living animals means the examination of questions in their complexity; *in vitro* experiments are able to answer only one special aspect of a question.

Clearly, the method of cell cultivation is very useful in scientific research as certain questions can not be examined via *in vivo* models. Additionally, cell cultivation is emerging technology in many fields (*e.g.* gene technology) and thus it is worthwhile to become acquainted with it, at least at a basic level.

2. *In vitro* procedures

2.1. Procedures that may be used for the replacement of live animals in research

- Biochemical tests and immunochemical techniques (*e.g.* for the identification of bacterial toxins)
- Organ, tissue or cell cultures (*e.g.* for biochemical research)
- Microorganisms (to screen compounds for carcinogenicity and /or mutagenicity)
- Computer simulation and computer-based relationship models

2.2. Procedures that have been used to replace animals

- Monoclonal antibody production (see below)
- Pregnancy testing
- Vaccine potency test
- Virus vaccine production

  A hybridoma is a cell hybrid resulting from the fusion of a cancer cell (usually a myeloma or lymphoma) and a normal cell (lymphocyte) in order to combine desired features of each, such as the ability of the cancer cell to multiply rapidly with the ability of a normal cell to dictate the production of a specific antibody. The hybridoma is immortal in the laboratory and makes the same products as its parent cells forever. The demand for hybridoma cell lines expressing highly specific monoclonal antibodies (MAbs) has increased dramatically in recent years due to the increased needs for MAbs used in diagnostic assays and. as novel therapeutic agents. These hybridoma cell lines are replacement alternatives for the production of most MAbs.

3. A brief history of tissue culture

1907 Ross Harrison described the technique of tissue culture, Alexis Carrel and Montrose Burrows later modified Harrison's technique.

1947 The American Tissue Culture Association was founded at a conference in Hershey, Pennsylvania

1949 G.W. Hyatt created the US Navy Tissue Bank to store bone tissue collected during orthopedic surgery.
1950 The first human tumor cell line, “HeLa”, was established from the cancerous cervical cells of Henrietta Lacks.
1962 Leonard Hayflick created the first normal human diploid cell line.
1980 Ananda Chakrabarty genetically engineered a strain of bacteria that could digest crude oil.

4. Tissue and cell cultures

Cell and tissue culture is a technique by which cells are removed from a plant or animal organism and grown under controlled conditions in a sterile medium containing all the necessary nutrients. Cell culture is also an established global manufacturing technique in the biotechnology industry.[59-61]

Cell cultures are usually used to screen compounds for carcinogenicity and/or mutagenicity; for the analysis of the cells themselves; to examine cell to cell communication; for an assessment of the cell's response to chemicals; and as a tool to produce cellular-derived protein products (biotechnology industry)

4.1. Cell/tissue culture terms

Cell culture: The maintenance of dispersed cells, derived from primary tissue explants or cell suspensions.
Tissue culture: The maintenance of a tissue in a way that may allow differentiation and preservation of the architecture and/or function.
Monolayer: A single layer of cells growing on a surface.
Subculture: The passaging of cells from one culture to another.
Primary culture: A culture started from cells, tissues or organs taken directly from an animal.
Organ culture: The maintenance of tissues, whole organs or parts of organs in a manner that may allow differentiation and preservation of the architecture and/or function.
Explant culture: An excised fragment of an organ which usually retains some degree of tissue architecture.
Cell line: This arises from the primary culture at the time of the first subculture –it has a "finite" lifespan.
Continuous cell line: A cell line which has been "transformed" – It has an "infinite" lifespan.

4.2. Types of cell cultures

- Primary cell cultures can be generated from embryonic or adult tissue, typically have a finite lifespan in culture. The advantage of primary cultures is that the cells have not been "modified" in any way (other than enzymatic or physical dissociation). The disadvantages of primary cultures are the mixed nature of each preparation, and the limited lifespan of the culture.
- Continuous cell lines are abnormal and are often transformed cell lines

5. Methods and conditions of cell cultivation

Laminar flow hoods

All media preparation and other cell culture work must be performed in a laminar flow hood. A vertical hood (biology safety cabinet) is best for working with hazardous organisms. The

filtered air blows vertically down from the top of the cabinet. In horizontal hoods, the filtered air blows out at the operator in a horizontal fashion. These are not useful for working with hazardous organisms, but offer the best protection for cultures. Both types of hoods involve the continuous displacement of air that passes through a HEPA (high-efficiency particle) filter that removes particulate matter from the air. The hoods are equipped with a short-wave UV light.

CO_2 incubators

Cells are grown in an atmosphere of 5-10% CO_2 because the medium used is buffered with sodium bicarbonate/carbonic acid and the pH must be strictly maintained in the physiological range, pH=7.2-7.4. The humidity must be maintained at about 100 % for cells growing in tissue culture dishes. Culture flasks should have loosened caps to allow for sufficient gas exchange.[59,60]

Microscopes

Inverted phase contrast microscopes are used to visualize cells.

5.2. Methods to prepare cell cultures

1. Preparation of solutions used for cell culture procedures.
2. Preparation of tissue for cell dissociation.
3. Dissociation of cells:
    - Mechanical dissociation: disaggregation of cells by aspirating tissue through a 10 ml syringe equipped with a needle of appropriate width. Gentle forcing of cell clumps through the needle into the syringe (trituration) without any enzymatic treatment.
    - Enzymatic dissociation: enzymatic digestion by collagenase, trypsin, trypsin-EDTA, dispase or protease treatment.
4. Filtration of cell suspension through a sterile nylon mesh to separate dispersed cells from the larger tissue pieces.
5. Washing of cells and centrifugation.
6. Resuspension and plating of cells.
7. Viable cell counts: a hemocytometer or a common microscope is used to determine total cell counts and viable cell numbers. Trypan blue is one of several stains recommended for use for viable cell counting in dye exclusion procedures. This method is based on the principle that live cells do not take up certain dyes, whereas dead cells do. Cells should be monitored daily for morphology and growth characteristics, fed every 2 to 3 days, and subcultured when necessary.

5.3. Cell attachment factors, cell adhesion molecules

These compounds are used to promote cell adhesion:

Collagen
Fibronectins (cell surface and plasma proteins)
Laminin (heteromeric glycoprotein)
Poly L-lysine (polycationic form of the polyamino acid in the range 70,000-150,000 kDa).
Poly-L-Ornithin (polycationic form of the polyamino acid with MW: 30,000-70,000).

5.4. Cell culture supplements

Certain compounds are used for media supplementation:

- Fetal calf serum (FCS) is frequently added to the defined basal medium as a source of certain nutritional and macromolecular growth factors essential for cell growth. FCS is the best supplementation for a basal medium, that is most frequently used for all types of cell cultures.
- Growth factors are naturally-occurring proteins, members of larger families of structurally and evolutionarily related proteins, that promote cell proliferation and cell differentiation The individual growth factor proteins are important for the regulation of a variety of cellular processes, acting as signaling molecules between cells (*e.g.* epidermal growth factor (EGF), basic fibroblast growth factor (bFGF or FGF2), nerve growth factor (NGF), neurotrophins, erythropoietin (EPO), cytokines and hormones).
- Insulin
- Transferrin
- Serum albumin

6. Cell culture media types and their uses

6.1. Basic constituents of media are inorganic salts, carbohydrates, amino acids, vitamins, fatty acids and lipids, proteins and peptides and serum. Each type of constituent performs a specific function.

6.2. Cell culture media types and their uses (Table 13.)

Media type	Examples	Uses
Balanced salt solutions	PBS DPBS Hanks BSS Earles BSS	They form the basis of many complex media
Basal media	MEM	Primary and diploid cultures
	DMEM	Modification of MEM containing increased levels of amino acids and vitamins. Supports a wide range of cell types, including hybridomas
	GMEM	Glasgow's modified MEM was defined for BHK-21 cells. The medium was developed by modifying Eagle's BME by adding 10% tryptase phosphate and twice the normal concentrations of amino acids and vitamins.
Complex media	RPMI 1640	Originally derived for human leukemic cells. It supports a wide range of mammalian cells, including hybridomas
	Iscoves DMEM	A further enriched modification of DMEM which supports high-density growth
	Leibovitz L-15	Designed for CO_2 -free environments
	TC 100 Grace's Insect Medium Schneider's	Designed for culturing insect cells

	Insect Medium	
Serum Free Media	CHO	For use in serum-free applications
	Ham F10 and derivatives Ham F12 DMEM/F12	NOTE: These media must be supplemented with other factors such as insulin, transferrin and EGF. These media are usually HEPES buffered
Insect cells	Sf-900 II SFM, SF Insect-Medium-2	Specifically designed for use with Sf9 insect cells

The Minimum Essential Medium (MEM), developed by Harry Eagle, is one of the most widely used of all synthetic cell culture media. MEM has been used for the cultivation of a wide variety of mammalian cells grown in monolayers. **Figure 33** shows the components of the most frequently used cell culture media Dulbecco's Modified Eagle Medium (DMEM).

Dulbecco's Modified Eagle Medium (DMEM	
component	**mg/L**
inorganic salts	
$CaCl_2$*2H_2O	**264,00**
$Fe(NO_3)3$*9H_2O	**0,10**
KCl	**400,00**
$MgSO_4$*7H_2O	**200,00**
NaCl	**6400,00**
$NaHCO_3$	**3700,00**
NaH_2PO_4*2H_2O	**141,00**
other components	
D-Glucose	**1000,00**
Sodium-Pyruvate	**110,00**
amino acids	
L-Arginine*HCl	**84,00**
L-Cystine	**48,00**
Glycine	**30,00**
L-Histidine-HCl*H_2O	**42,00**
L-Isoleucine	**105,00**
L-Leucine	**105,00**
L-Lysine-HCl	**146,00**
L-Methionine	**30,00**
L-Phenylalanine	**66,00**
L-Serine	**42,00**
L-Threonine	**95,00**
L-Tryptophane	**16,00**
L-Tyrosine	**72,00**

L-Valine	**94,00**
vitamins	
D-Ca-Pantothenate	**4,00**
Choline Chloride	**4,00**
Folic Acid	**4,00**
Inositol	**7,20**
Niacinamide	**4,00**
Pyridoxine-HCl	**4,00**
Riboflavin	**0,40**
Thiamine-HCl	**4,00**

Figure 33. The components of *Dulbecco's Modified Eagle Medium* (DMEM).

7. Culture dishes

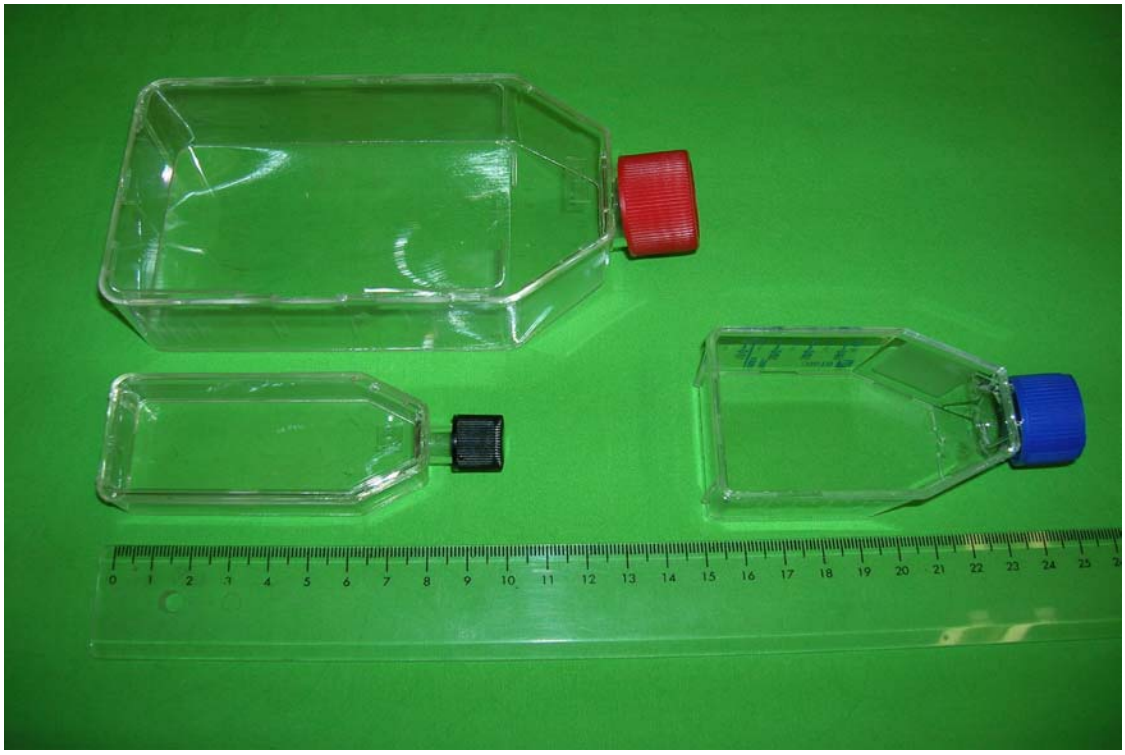


Figure 34. Cell culture flasks

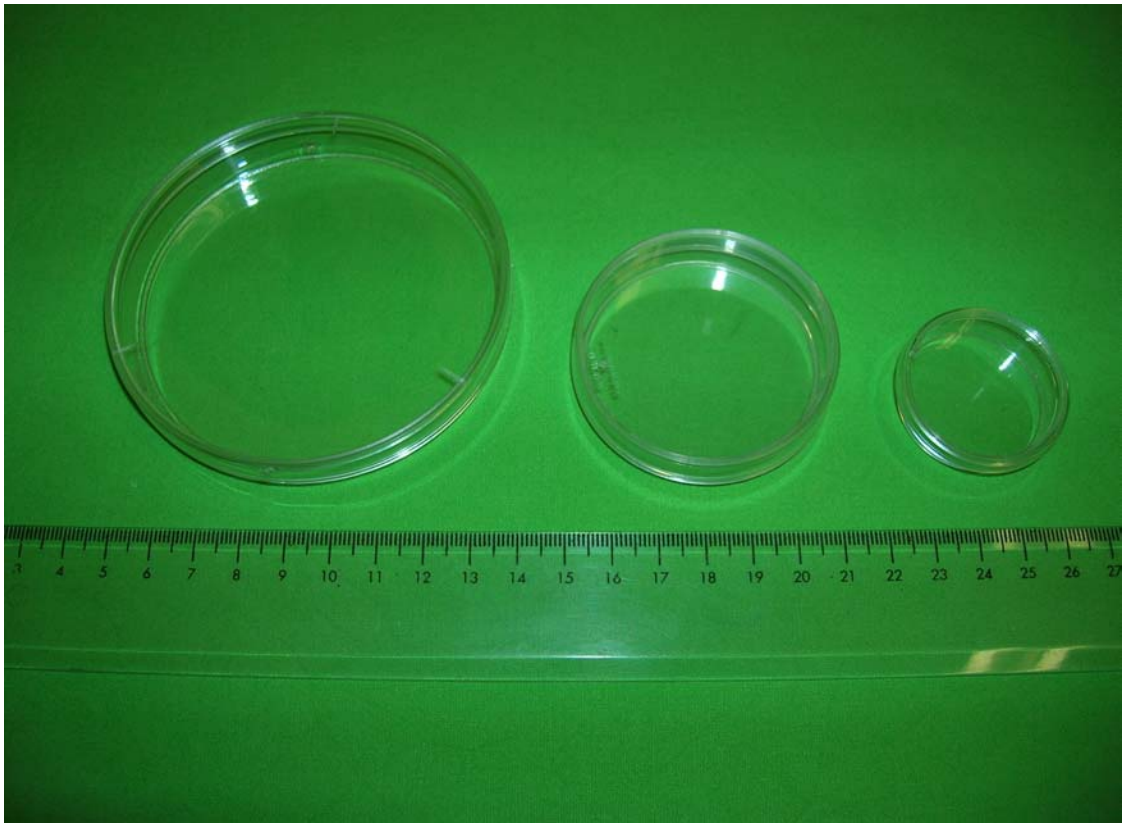


Figure 35. Petri dishes

Figure 36. Multiwell dishes

8. Subculture method

Proteolytic enzymes, trypsin, collagenase or pronase, usually in combination with EDTA, cause cells to detach from the growth surface. The enzymatic digestion is fast and reliable, but can damage the cell surface by digesting exposed cell surface proteins. The proteolysis reaction can be quickly terminated by the addition of complete medium containing serum.[59,61]

The steps of the subculture method are as follows:

1. Preparation of a trypsin - EDTA solution in a balanced salt solution (*e. g.* PBS without Ca^{++} or Mg^{++}).
2. Removal of the medium from the culture dish by aspiration, washing of the cells in a monolayer in a balanced salt solution (without Ca^{++} or Mg^{++}) to remove all traces of serum, and removal of the wash solution.
3. Addition of sufficient trypsin-EDTA solution in appropriate concentration to completely cover the cell monolayer.
4. Transfer of the culture to a 37 ^{o}C incubator for 2 min.
5. The coated cells are allowed to incubate until cells detach from the surface.
6. Monitoring of the cells under a microscope.
7. Progress can be checked by examination with an inverted microscope
8. The cells begin to detach when they appear rounded.
9. Dilution of the cells with serum, or with serum containing fresh medium and transfer to a sterile centrifuge tube.
10. Spinning of the cells, removal of the supernatant, and resuspension in culture medium (or freezing medium if the cells are to be frozen).
11. Addition of culture medium containing serum, and dilution into culture flasks or other culture vessels. Typically, 1:4 to 1:20 dilutions are appropriate for most cell lines.

9. Standard procedure for detaching adherent cells

1. Washing once with a buffer solution.
2. Release of cells from monolayer, surface treatment with dissociating agent, and observation of the cells under a microscope.
3. Incubation until the cells become rounded and loosen.
4. Transfer of the cells to a centrifuge tube and dilution with medium containing serum.
5. Spinning down of the cells, removal of the supernatant and replacement with fresh medium.
6. Counting of the cells in a hemocytometer, and dilution as appropriate into fresh medium.

10. Preservation and storage

10.1. Freezing cells [59-61]

1. Harvesting of the cells as usual and washing once with complete medium.
2. Resuspension of the cells in complete medium and determination of the cell count/viability.
3. Centrifugation and resuspension in ice-cold freezing medium: 90% calf serum/10% DMSO, at 10^6 - 10^7 cells/ml. maintenance of the cells on ice.

Note: A cryoprotective agent such as glycerol or DMSO lowers the freezing point. It is best to use healthy cells that are growing in the log phase.

4. Transfer of 1 ml aliquots to freezer vials on ice.
5. Transfer to the -80 ^{o}C freezer overnight.

Note: the cells are slowly cooled from room temperature to -80 ^{o}C to allow the water to move out of the cells before it freezes. The optimal rate of cooling is 1-3 ^{o}C per min.

6. Next day, they are transferred to liquid nitrogen, either in the liquid phase (-196 ^{o}C) or in the vapor phase (-156 ^{o}C).

10.2. Thawing of frozen cells

1. Removal of the cells from frozen storage and quick thawing in a 37 ^{o}C water bath by gentle agitation of the vial.
2. As soon as the ice crystals have melted, gently pipetting into a centrifuge tube containing prewarmed growth medium (10-20 ml complete growth medium per 1 ml frozen cells).
3. Pelleting of the cells by gentle centrifugation and discarding of the supernatant to remove cryopreservative (cryopreserved cells are fragile).
4. Careful resuspension of the cells in complete growth medium, followed by a viable cell count.
5. Plating of the cells. The cell inoculum should contain at least 3 x 10^5 viable cells/ml.

11. Production of artificial tissue ("tissue engineering")

There has been an enormous revolution in the biological sciences in the past twenty years, in the course of which a new area has emerged in biotechnology: namely human tissue engineering, a multidisciplinary field. Tissue engineering combines various aspects of medicine, cell and molecular biology, material sciences and engineering, for the purpose of developing tissue substitutes to regenerate, maintain or improve the function of damaged human tissues. Biotechnology engineering involves a uniquely interdisciplinary melding of engineering and medicine.

11.1. The history of tissue engineering

The first definition of tissue engineering was given by Langer and Vacanti[62], who stated it to be "*an interdisciplinary field that applies the principles of engineering and life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function or a whole organ*". MacArthur and Oreff6[65] defined it as "*understanding the principles of tissue growth, and applying this to produce functional replacement tissue for clinical use*."

1. Tissue engineering (regenerative medicine) involves the repair or replacement of structural tissues (*e.g.* bone, cartilage, blood vessels, bladder, *etc*.). Tissue engineering uses living cells as engineering materials; it could be artificial skin which includes living fibroblasts, cartilage repaired with living chondrocytes, or other types of cells used in other ways.
2. Tissue transplantation (stem cells) is the transplantation of cells that perform a specific biochemical function (*e.g.* an artificial pancreas, or an artificial liver).
3. Biological engineering is a broader field that generally encompasses tissue engineering and related fields (*e.g.* biomaterials).

Cells became available as engineering materials when it was discovered in 1998 how to extend telomeres to produce an immortalized cell line. Before this, laboratory cultures of healthy, noncancerous mammalian cells would only divide a fixed number of times, up to the Hayflick limit. Leonard Hayflick observed in 1965 that cultured cells divide about 50 times before dying. Near to this limit cells show signs of old age (exceptions: stem cells and cancerous cells). The limit of the cell division number varies from cell type to cell type and from organism to organism. The human limit is about 52 and has been linked to the shortening of telomeres, a region of DNA at the end of the chromosomes. The production of

engineered tissues is an emerging field which holds promise for the improvement of current medical therapies. Tissue engineering involves producing a 3D biocompatible scaffold with the proper amount of cells to implant, and then implanting the engineered tissue material *in vivo*.[64, 65]

11.2. Cell sources for tissue engineering

Autologous cells are obtained from the same individual into which they will be reimplanted. Autologous cells give the fewest problems with rejection and pathogen transmission.
Allogenic cells originate from a donor of the same species.
Syngeneic or isogenic cells are isolated from genetically identical organisms, such as twins, clones, or highly inbred research animal models.
Primary cells are from an organism.
Secondary cells are from a cell bank or from multipassaged primary cells.
Xenogenic cells are those isolated from individuals of another species. In experiments aimed at the construction of cardiovascular implants, animal (pig) cells have extensively been used.
Stem cells are undifferentiated cells with the ability to divide in culture and give rise to different forms of specialized cells. Depending on their source, stem cells are divided into "adult" and "embryonic" stem cells. The first class being multipotent and the latter mostly pluripotent; some cells are totipotent, in the earliest stages of the embryo. Stem cells may be a promising tool for the repair of diseased or damaged tissues, or may be used to grow new organs. [64, 65]

12. The scaffolding technique

Cells can generally be implanted or “seeded” into an artificial structure (usually referred to a scaffold) capable of supporting 3D tissue formation and serving at least one of the following purposes: cell attachment and migration, delivery and retention of cells and biochemical factors, and diffusion of vital cell nutrients and products expressed by the cells.

Biological scaffolds having the ability to support cell growth are constructed from natural materials (particularly components of the extracellular matrix; ECM). The ECM is a vital component of cellular microenvironments, furnishing cells and tissues the appropriate 3D architecture for normal growth and development. These scaffolds are often imperfect, both *ex vivo* and *in vivo*, to recapitulate the *in vivo* milieu and allow cells to influence their own microenvironments. The biodegradability of these materials is essential since scaffolds need to be absorbed by the surrounding tissues without the necessity of surgical removal. The scaffolding technique is of great promise for other areas of medicine, but it will be a long time before they are available for patient use.[66,67]
Types of biological scaffolds are protein scaffolds (collagen or fibrin-based), polysaccharide polymer scaffolds (glycosaminoglycans such as hyaluronic acid) and hydrogel scaffolds (polypeptide –based, *e.g*. PuraMatrix™).

12.1. Potential uses/advantages of collagen-based biological scaffolds

- A collagen gel matrix maintains its shape following cell seeding and culturing.
- This affords a highly permeable bio-scaffold design.
- Tissue implants may be produced for reconstructive/cosmetic surgery applications.

- Spinal cord repair implants can be generated.

12.2. Synthesis of tissue engineering scaffolds

A number of different methods have been described in the literature for the preparation of 3D porous structures to be employed as tissue engineering scaffolds. Molecular self-assembly is one of the few methods with which to create biomaterials with properties similar to those of the natural *in vivo* extracellular matrix (nanofiber cell-assembly). The encapsulation of stem cells in the self-assembled peptide scaffold allows these cells to differentiate into desired cell types expressing specific growth factors and cytokines. The application of these cell-scaffold systems into needed tissues affords a broad range of new applications including tissue repair and tissue engineering. Specific tissue types to be reviewed include cartilage, skin equivalents, neural tissue, blood vessels, myocardium and heart valves, and bioartificial livers.[66,67]

13. Fields of application of biological scaffolds

Worldwide research is currently being conducted with the aims of improving tissue engineering techniques involving bone marrow, liver, skeletal muscle, cartilages and the nervous system, and of producing artificial skin, artificial heart and circulatory assist devices and cardiac valve prostheses.

13.1. Nerve regeneration

These peptide scaffolds support nerve cell attachment and axon growth. Until recently, paralysis from spinal injuries and other nerve damage seemed to be irreversible, but biomedical engineers have now taken a very early step toward the reversal of paralysis by developing a biological scaffold [67] that supports nerve cell attachment, stimulates nerve cells to grow and produces a network for neurorepair and for neuroengineering. A new type of self-assembling biocompatible and biodegradable peptide scaffold allows nerve cells to grow, to form functional synaptic connections with other neurons, and to develop channels to communicate with one another.[68,69]

13.2. Cardiac muscle cell repair

Experiments are aimed at helping heart failure victims to rehabilitate by the supplementation of ischemized or dead heart tissue. An Israeli research team headed by Cohen recently created a new biological scaffold that allows healthy injectable heart muscle cells to replace died cardiac tissue.[68] Successful clinical trials of this technique on human beings in the future could revolutionize the field of cardiology, providing physicians with the ability to aid heart-attack victims to rehabilitate, helping them to increase their life expectancy and improve their quality of life. This scaffold was originally developed as a tool to create cardiac cell cultures, and to prepare human tissue outside of the body for experimental work. In these experiments with pigs, new muscle fibers grew into the scaffold, and a high proliferation rate of cells was found in the area where the scaffold was implanted. Signs of some kinds of healing processes and regeneration processes in the heart could be observed.[68]

13.3. Bone marrow stem cells

The loss of bone mass observed during aging enhances the risk of fractures. The process of bone repair in aging is slow and limited due to the reduced activity of the osteoblasts. The ability to enhance the healing of bone defects in aging can contribute to the prevention of the complications resulting from long-term immobilization that are especially fatal in old age. Osteoprogenitor cells were selected from rat bone marrow stem cells cultured

in DMEM on the hydrogel scaffold and transplanted into the area of a rat tibia segmental bone defect. It was revealed that, 6 weeks postimplantation, calcified material was present at the site of the defect, indicating new bone formation. It is concluded that committed osteogenic bone marrow stem cells contained in a biocompatible scaffold can provide a promising surgical tool for the enhancement of bone defect healing that will minimize the complications of bone repair in aging and disease.

13.4. Tissue-engineered cartilage

Certain peptides are able to self-assemble into stable hydrogels at low (0.1–1%) peptide concentrations. Such self-assembling peptides are characterized by amino acid sequences of alternating hydrophobic and hydrophilic side-groups. Sequences of charged amino acid residues include alternating positive and negative charges.[70] Articular cartilage defects resulting from traumatic injury or degenerative diseases may require novel regenerative medicine strategies for the restoration of biologically and mechanically functional tissue. Implanted chondrocyte cells within the wound bed may initiate a repair response through *de novo* cellular regulation. Delivery of chondrocytes to a cartilage defect may be facilitated by attachment to or encapsulation within a biocompatible scaffold. The tissue engineering scaffold defines a 3D template in which chondrocytes produce and deposit. The structural stability of the cell/scaffold system must be maintained by the scaffold until seeded chondrocytes have deposited a continuous network of ECM throughout the implant. Ideally, the scaffold would then degrade as the ECM network matures, guiding regeneration throughout the entire scaffold geometry. A successful cartilage replacement must integrate with surrounding normal cartilage, and the newly assembled ECM must provide tissue resilience to the tissue compression that occurs during normal joint loading.

XII. Inbred strains of animals, transgenic and gene knockout animals, cloning (*Dr. Tamás Jánossy*)

1. Introduction

In biomedical research, appropriate experimental models are essential for the study of biological properties and phenomena, and to answer correctly the scientific questions raised. Besides the selection of the optimal animal species, it is crucial that the genetic characteristics of the experimental animals should correspond to the aims of the experiment. Genetic factors determine the properties of the animals, including their sensitivities to different physical effects (*e.g.* light, heat, ionizing radiation, electric current, *etc.*), chemical substances (*e.g.* mediators, hormones playing a role in the functions, regulation and reactions of the organism, toxic substances, *etc.*), pathogenic microbes (bacteria, viruses, fungi, *etc.*) and foreign or self-antigens. Genetic factors may influence the susceptibility to pathological alterations and diseases, as well as the effectiveness and side-effects of treatments (*e.g.* drugs). Not only the induction, but also the intensity of the reaction is influenced by genes. Consequently, the responses of the individuals of a genetically diverse population can differ both qualitatively (a positive response or no response) and quantitatively (a low, moderate or high response). Therefore, if a disease caused by a pathogenic microbe is studied, a population (strain) of animals must be chosen in which all of the individuals carry the genes of susceptibility to the microbe. In conrast, a population resistant to the microbe should be selected to study resistance to the disease. If the incidence of a disease caused by a microbe is examined in a species, genetically diverse, nonselected experimental animals should be used.

The genetic characteristics of the experimental animals and the uniformity or variability of the population can be influenced by selecting an appropriate breeding method. The genetic constitution of the animals can be modified by genetic engineering (transgenic and gene knockout animals, *etc.*; see later).

2. Basic genetic terms and definitions

In the event of sexual reproduction, the progeny inherits a set of chromosomes from both parents, which are present in all body cells, but not in germ cells. As concerns the genes of the body chromosomes, every feature is encoded by a pair of paternal and maternal genes. The two genes with identical functions are located at the same site of each member of the chromosome pair [gene(tic) locus]. Polymorphic genes have two or more alleles. In the different alleles, the sequences of bases building up DNA differ from one another. If the progeny inherits a gene with the same DNA sequence, *i.e.* the same gene allele from both parents, which originates from a common ancestor of both parents, it can be regarded as a homozygote for the gene locus; conversely, it is a heterozygote. If the gene has both a dominant and a recessive allele, the property determined by the dominant allele is expressed in the heterozygotes, while the feature encoded by the recessive allele appears only if the individual is homozygotic for that gene, *i.e.* each chromosome of the pair carries the same recessive gene allele. In the event of codominant inheritance, the properties inherited from both parents are expressed in the heterozygotes.

2.1. Outbreeding

In this case, the breeding of related individuals must be avoided to the highest possible degree. Breeding of relatives is considered to occur if there is a common ancestor within four successive generations in the pedigree (family tree, or lineage). If the number of breeding pairs is less than 100, the possibility of breeding relatives must be totally excluded; if it is more than 100, rotation or random breeding can be used. In rotation breeding, to avoid the mating of littermates, males and females from the same litter are moved and mated among the cages consistently opposite each other, and this method is used from generation to generation. Outbreeding results in a genetically variable population, where the increase of inbreeding (the increase of homozygous gene loci) from generation to generation is less than 1%. This is named the inbreeding coefficient (F). In the overwhelming majority of genetic loci, the

individuals of an outbred population are heterozygous and the alleles of the paternal (A) and maternal (B) genes are different (AB). The population is *anisogeneic*, which means that the individuals have different genotypes.

2.2. Inbreeding

In this case, the strict breeding of relatives is applied. Most frequently, serial brother by sister matings are used (the members of the breeding pair are littermates, *i.e.* siblings), or the younger parent is mated with an offspring of the opposite sex. The chance of the development of a mutation in the younger parent and, due to this, the chance of the development of a genetic difference between the parent and the offspring is less than in the case of the older parent. Inbreeding results in increasing homozygosity (genetic drift): the genotype consists of identical gene alleles at more and more genetic loci (AA or BB, instead of AB). The homozigosity also increases in a closed breed. The breed is closed if no breeding animals are introduced from outside. In a balanced closed breed, half of the individuals are heterozygous (AB), while the other half are homozygous (AA and BB).

2.3. Inbred strain

If inbreeding (brother by sister or parent by offspring mating) is continued for more than 20 consecutive generations, an *inbred strain* develops, in which 98.6% of genetic loci are homozygous. In spite of the remaining genetic differences, this strain can be regarded as genetically identical, *i.e. isogeneic*. Animals from the same inbred strain are called *syngeneic*.

In isogeneic strains, any member of the strain accepts tissue grafts tranplanted from any other member of the same sex of the strain without any sign of rejection, because the histocompatibility antigens of all individuals of the strain are identical. Due to a weak histocompatibility antigen encoded by a gene locus on the Y chromosome, grafts from male donors can be rejected in female recipients. The skin grafting method of Silvers has proved to be excellent for the testing of isogeneity.

The development of complete homozygosity can be delayed by spontaneous mutations which occur rarely. Because of the possible spontaneous mutations, inbred strains must be maintained by brother by sister matings. Inbred strains can be most easily produced from small laboratory mammals (mouse, rat, guinea pig, hamster or rabbit), because these, especially mice and rats, are prolific and their gestation time is short.

3. History of inbred strains

The history of inbred strains is closely related to the history of tumor research (mice) and studies of multifactorial inheritance (guinea pigs). The rediscovery of Mendel's work led to the investigations of tumors as inherited diseases. In the initial experiments, spontaneous tumors developed in breeds of mice were studied. Jensen, Loeb, Ehrlich and Tyzzer attempted to transplant these tumors to other mice, but the grafts usually did not grow or regressed after transient growth. However, Jensen (1903) and Loeb (1908) successfully maintained two different mouse tumors by serial transplantations in relatively inbred mice. Little (1914) found that the rejection of tumors or the susceptibility to tumor growth is based on a number of dominantly inherited genes. This led to the discovery of histocompatibility genes and studies on cell-mediated immune responses.

In the meantime, Rommel (1906) started an inbreeding experiment involving guinea pigs. The descendants of these animals are still widely used as Strain 2 and 13 guinea pigs. His work was continued by Wright, who developed the mathematical theory of inbreeding. The introduction of the inbreeding coefficient is also linked to his name (1922).

Helen King started the inbreeding of rats in 1909 (PA and WKA strains). Little began the inbreeding of mice in the same year, which led to the development of the DBA strain. From his albino mice, Bagg produced the BALB strain in 1913, from which the BALB/c strain was developed in Snell's laboratory in the 1930s. In 1920, Strong developed the CBA and C3H strains, from a cross of a Bagg albino male with a DBA female, and, in 1921, the A strain from a cross between Bagg albino and Cold Spring Harbor albino stocks. Little founded the family of C57 mice (C57BL, C57BR, C57L and C58). The C57BL strain was split into the 6 and 10 sublines before 1937. Many of the most widely used mouse and rat strains were developed from 1920-1930. In 1929, Little founded the Jackson Laboratory in Bar Harbor (Maine), which continuously developed the inbred strains,

isolated new mutations and studied the characteristics of the strains. The Jackson Laboratory has the largest collection and is one of the main supplier of inbred mouse strains.

4. Nomenclature of inbred strains

The rules for the nomenclature of inbred strains of mice were created by the committee on nomenclature in 1952. The name of the strains should be denoted by 1-4 capital letters (*e.g.* mouse strains: A, AKR, CBA, DBA, *etc.*; rat strains: LEW, WAG, BN, PVG, *etc.*). Numbers are permitted only in the names of strains already known and named at that time (*e.g.* mouse: C3H, C57BL, *etc.*; rat : F344, AS2, M520, *etc.*). *Substrains* are formed when a strain is separated into two or more branches after 8-19 generations of brother by sister matings; if parallel substrains within the same colony are found to be genetically different. This is denoted by a symbol or number which is separated from the strain name with a slash (*e.g.* C57BL/6 and C57BL/10). A new *subline* is formed when a new colony is set up in a different laboratory, where it is bred for a long time. During this, genetic changes can occur. The name of the subline is indicated by the abbreviated name of the breeder or the laboratory (*e.g.* Sc = Scott, Sn = Snell, He = Heston, J = Jackson Laboratory, Ca = Cambridge). The symbols are added in historical sequence (*e.g.* C57BL/10ScSn). A new subline develops when the strain has been manipulated (*e.g.* the offspring has been fostered onto animals of another strain). The manipulations can cause changes in the strain (*e.g.* fostering can influence the carrying of viruses). Therefore, the different manipulations performed during breeding must be indicated in the name. The most frequent strain manipulations and their symbols are as follows:

- f (foster nursing): This indicates the fostering of the progeny by another strain, *e.g.* C57BL/10fC3H. This procedure can be applied when specified-pathogen-free (SPF) strains are developed. The fetuses are removed with the pregnant uterus under aseptic conditions before birth and transferred to a mother of an SPF strain.
- e: This indicates that the strain has been developed by the transfer of embryos to another strain.
- h: This indicates hand rearing.
- o: This indicates an ovary transplant.
- p: This indicates the preservation of ova by freezing in liquid nitrogen.

The subline symbols follow one another in chronological sequence (*e.g.* C57BL/10ScSnfC3H: the C57BL/10 strain was passed for breeding to J. P. Scott (Sc), who then passed it to G. D. Snell (Sn), and thereafter the offspring of the strain were fostered onto the mice of the C3H strain.

4.1. Abbreviated strain names

If the name of the strain is too long, it is reasonable to abbreviate it, but the full name must also be given in the text. The following abbreviated names of mouse strains are recommended: AKR = AK, BALB/c = C, C3H = C3, C57BL = B, C57BL/6 = B6, C57BL/10 = B10, *etc.*

4.2. Inbreeding depression

An inbreeding depression involves a decline in the reproductive performance, in the ability to survive, and in health in the first few generations of inbreeding. It is caused by the homozygosity of deleterious recessive genes which occurs at the beginning of inbreeding. Later, these are eliminated, and accordingly, no further inbreeding depression will occur in the established inbred strain.

4.3. F_1 hybrids

F_1 hybrids are the first generation of crosses between two inbred strains. All individuals are isogeneic and heterozygous for all genetic loci at which the two parental strains differ (*e.g.* histocompatibility genes). The *hybrid vigor* or *heterosis* which can be observed in the F_1 hybrids is the opposite of the inbreeding depression: it results from the covering up of deleterious recessive genes. The designation of F_1 hybrids is: (name of the maternal strain x name of the paternal strain) F_1, *e.g.* (BALB/c x C57BL/6)F_1; the abbreviated name is CB6F1.

5. Use of inbred strains in biological and medical research:

- Investigation of the properties of the species and genetic factors
- Examination of biological effects and responses
- Studies of physiological functions and mechanisms
- Investigation of immunological phenomena, immunogenetic factors, the function of the immune system, transplantation and tumor immunity
- Analysis of effects and mechanisms of action of drugs
- Creation of models of pathological conditions and diseases: studies on the pathomechanism and therapeutic possibilities

A wide range of species (mouse, rat, guinea pig, hamster, rabbit, chicken, *etc.*) and strains (mostly mice and rats) are available.

5.1. Coisogeneic strains

If a single-gene mutation occurs in an inbred strain, it can subsequently be maintained by establishing a new inbred strain. The new strain, which differs from the original strain at a single gene locus, is called a coisogeneic strain. The nomenclature of coisogeneic strains is indicated by the strain (substrain, subline) symbol, followed by a hyphen and the gene symbol in italics. For example, the CBA/Ca-*nu* mouse strain is a coisogeneic strain of the CBA/Ca strain, which carries the *nu (nude)* gene mutations determining hairless and athymic phenotype instead of the 'normal' alleles of the appropriate genes. The mutation may form the basis of a disease (see the following examples):

1. Mouse

Sex-linked anemia: *sla* gene
Diabetes: *db* gene
Obesity: *ob* gene
Amino acid metabolism disorders: *his* (histidinemia), *pro* (prolinemia) gene
Kidney disease: *kd* gene
Muscular dystrophy: *dy* gene
Embryonic disorder: *t* allele
Hairless and athymic: *nu* gene

2. Rat

Diabetes insipidus: *di* gene
Hairless and athymic: *rnu* gene
Bilirubinemia (jaundice): *j* gene

Coisogeneic strains make it possible to study the role of genes in determining the phenotype. Strains with disease-causing genes are suitable for the creation of experimental models of diseases.

5.2. Congenic strains, congenic resistant strains

In this case, the selected gene or genetic region of the donor strain (not necessarily an inbred strain) is introduced into an inbred recipient strain. The choice of the breeding method depends on whether the gene is dominant, codominant or recessive. Usually backcrossing is applied. In the cases of dominant or codominant genes, F_1 hybrids are first produced by crossing the two strains, and these are then backcrossed with the recipient strain. The offspring carrying the donor gene are selected, and backcrossed with the recipient strain. This must be repeated at least 12 times (the selection for the presence of the donor gene must be performed on each occasion). After the last backcross, a male and a female animal, which are heterozygotes for the donor gene, should be selected and mated, and from the offspring two homozygotic twins must be mated (intercross). The new congenic strain carries the donor gene (but not the appropriate gene allele of the recipient), and, due to the serial backcrosses, all the other genes (background genes) are identical with the background genes of the recipient.

In 1946, Snell applied this method to develop the congenic resistant mouse strains. Selection was based on the resistance to tumors (the tumor did not begin to grow or was rejected in the recipients) or the rejection of skin grafts. Using these strains, he proved that a polymorphic genetic region (major histocompatibility complex = MHC) is responsible for the rejection of allografts. In mice, this corresponded to the polymorphic gene described by Gorer, which encodes for blood group antigen II and was named H-2.

The usual nomenclature for congenic strains is as follows: the name of the recipient strain.the name of the donor strain. Most often, the abbreviated names are used, *e.g.* B10.D2. In this case, C57BL/10 (B10) is the recipient and DBA/2 (D2) is the donor strain. The background genes of the B10.D2 strain are identical with those of the B10 strain, while its H-2 genes are identical with those of the D2 strain ($H\text{-}2^d$), *i.e.* the B10.D2 strain differs from the B10, which carries the $H\text{-}2^b$ haplotype, only in the H-2 gene complex. H-2 congenic mice are very suitable for studies on transplantation immunology and immunogenetic research.

5.3. Recombinant inbred strains

Recombinant inbred strains are developed from the F_2 generation from the cross of two unrelated inbred strains with strict brother by sister matings through at least 20 generations. The first widely used recombinant inbred strains were developed from a cross between the BALB/cBy (C) and C57BL/6By (B6) strains and were designated CXBD, CXBE, CXBG, CXBH, CXBI, CXBJ and CXBK (D.W. Bailey, 1971). In the designation of these strains, the abbreviated names of the two parental strains are separated by a capital X, which is followed by a dash and a number (*e.g.* the names of strains developed from a cross between the AKR/J and C57L strains are AKXL-1, -2, -3, *etc.*). This rule is not used for the long-established, widely-known CXB strains. The recombinant inbred strains carry the mixed-up genes of the two parental strains. Because they are inbred, the genotype of all individuals of a strain is identical, *i.e.* they are isogeneic. They can be used for the identification and mapping of new polymorphic genetic loci and studies of the polygenic characteristics, which are determined by multiple genes (*e.g.* the life span, or the susceptibility to spontaneous and induced diseases), and also the biometric analysis of morphological, physiological, biochemical and behavior characteristics, and the investigation of drug effects.

5.4. Transgenic animals

Animals are considered to be transgenic animals if the genome contains foreign gene(s) introduced from outside. The term transgene originates from the expression *trans*ferred *gene*tic material. Gordon and his coworkers reported in 1981 that they could successfully transfer foreign DNA into mammals, thereby creating transgenic mice. Later,

transgenic rats, sheep, goats, cattle, rabbits and chickens were also produced. In biological and medical studies, transgenic animals make possible the *in vivo* investigation of functional effect of genes or their products, the development of experimental models of diseases, in agriculture, the development of animals with favorable characteristics (*e.g.* a higher yield of milk or meat, or higher resistance to infectious diseases), and in biotechnology and the pharmaceutical industry, the large-scale production and excretion of specific human proteins by milk (*e.g.* hormones, blood coagulation factors, enzymes, cytokines, *etc.*). The human proteins can be extracted from the milk with in high purity.

The gene construction is prepared *in vitro*, using recombinant DNA technology. Besides the structural gene (*e.g.* insulin gene), which is responsible for the function, it can contain a vector gene targeting the gene construction to the appropriate site of the genome, as well as promoter and enhancer DNA sequences regulating the function of the gene. They can be used to express genes only in particular tissues. For instance, lymphoid promoters and enhancers can be used to overexpress rearranged antigen receptor genes in lymphocytes, and insulin promoters to express insulin genes in the β cells of pancreatic islets. Promoter elements can also be included that respond to drugs (*e.g.* tetracyclin or dexamethasone), hormones (*e.g.* insulin) or cytokines, making it possible that the transcription of the transgene can be controlled at will. Genes rendering the cells resistant (*e.g.* the neomycin resistance gene) or susceptible to particular cell-killing drugs (*e.g.* the thymidine kinase gene, which makes the cells sensitive to gancyclovir) can also be inserted into the gene construction. Following transfection, they make possible the *in vitro* selection of the successfully transfected cells: neomycin kills the nontransfected normal mammalian cells, while gancyclovir kills those transfected cells which express thymidine kinase (see later). The gene construction can be introduced by three different methods: 1. microinjection; 2. transfection and transfer of embryonic stem cells; and 3. virus (adenovirus, retrovirus)-mediated gene transfer.

Creation of transgenic mice by microinjection

Following superovulation induced by gonadotropic hormone treatment, females are mated with a male. A few hundred copies of the transgene are injected into the male pronuclei of fertilized eggs obtained from the oviducts. The two-cell embryos are implanted into the oviducts of pseudopregnant females. Pseudopregnancy is induced by mating females with vasectomized males. One to 50 copies of the transgene insert in tandem into a random site of breakage in a chromosome and are subsequently inherited as a single mendelian trait. Since integration usually occurs before DNA replication, about 75% of the transgenic pups carry the transgene in all their cells, including germ cells. In most cases, integration of the foreign DNA does not disrupt the endogenous gene function. Each animal carrying the transgene is a heterozygote, from which homozygous lines can be bred. The microinjection method can be applied in every mammalian species. In mice, about 2.6% of the born offspring are transgenic; in other species, this proportion is 0.7-1.6%.

The nomenclature of the transgenic animals is as follows: The name of the recipient is followed by a dash and the symbol of the transgene, *e.g.* C57BL/10J-*TgN(XXXX)Y*, where *Tg* = transgenic, *N* = nonhomologous (or *H* = homologous) insertion, *XXXX* = the name of the gene, and *Yyy* = the code of the laboratory.

5.5. Gene knockout animals

The targeted insertion of a transgene in the middle of an endogenous gene usually disrupts the coding sequences and ablates the expression or function of that gene. This is commonly achieved by homologous recombination. In this case, a gene homologous to the targeted endogenous gene is constructed. The DNA sequence of the construct is almost identical with that of the endogenous gene. If this is inserted into a cell, it will preferentially

recombine and and replace endogenous sequences. The targeting vector can be designed such that homologous recombination will lead to the deletion of one or more exons of the endogenous gene. From the changes caused by the ablation of the gene function in the gene knockout animals, conclusions can be drawn concerning the physiological roles of the gene and gene products. This method can also be used to develop animal models of single-gene disorders.

Generation of gene knockout animals with the embryonic stem cell method. A targeting vector is used to first disrupt the gene in an embryonic stem cell line. This contains the neomycin resistance gene (*neo*) between the two homologous exons and, flanking one of them, also the thymidine kinase gene (*tk*). Embryonic stem cells are obtained from the inner cells of the blastocyst. These are pluripotent stem cells which can be propagated in tissue culture *in vitro*. They are also capable of differentiating, and thus they can produce all the cells of the mature organism, including the germ cells. The targeting vector gene added to the embryonic stem cell culture is inserted into some of the cells. In the event of homologous recombination, the exogenous gene inserted into the genome contains the *neo* gene, but not the *tk* gene. Accordingly, the transfected cells will be resistant to both neomycin and gancyclovir. During random, nonhomologous insertion, not only the *neo*, but also the *tk* gene is inserted. Such cells are resistant to neomycin, but sensitive to gancyclovir. If neomycin and ganciclovir is added to the cell culture following transfection, neomycin will kill the nontransfected cells, while gancyclovir will kill those transfected cells into which the vector has been integrated with nonhomologous recombination. Thus, only the gene knockout embryonic stem cells survive which have undergone homologous recombination. The presence of the desired recombination can be verified by DNA analysis [with Southern blot hybridization or the polymerase chain reaction (PCR)]. The selected cells are injected into blastocysts *in vitro* and implanted into pseudopregnant females. Animals that develop will be chimeric, *i.e.* some of the tissues will be derived from the gene knockout embryonic stem cells and others from the remainder of the normal blastocyst. The germ cells are also chimeric, but because these cells are haploid (they contain only one set of chromosomes), only some cells will contain the chromosome copy with the disrupted gene. If chimeric animals are mated with normal animals and either sperm or eggs containing the gene knockout chromosome fuse with germ cells of the normal partner, all cells in the offspring will be heterozygous for the mutation. If such heterozygous animals are mated, homozygous gene knockout mice, which do not express the gene, will be obtained with a frequency that is predictable by a simple mendelian segregation.

Homologous recombination can also be used to replace a normal gene sequence with another functioning exogenous gene. The *knockin animals* contain the transgene at a defined site in the genome, in contrast with random insertion observed in conventional transgenic animals. This method is used when the expression of the transgene should be regulated by endogenous promoter or enhancer regions.

The above conventional gene-manipulating techniques have proved to be of great usefulness, but they have some drawbacks. 1. The mutation of one gene during development may be compensated for by the altered expression of other gene products, and therefore the function of the modified gene may be obscured. 2. In a conventional gene knockout animal, the importance of a gene in only one tissue or at only one time during development cannot be easily assessed. 3. A functional selection marker gene, such as the *neo* gene, is permanently introduced into the genome, which can modify the phenotype of the animal. A refinement of the gene knockout method is made possible by application of the bacteriophage-derived Cre/*loxP* recombination system, which eliminates some of the above drawbacks. The Cre enzyme is a DNA recombinase that recognizes the *loxP* DNA sequences, and mediates the deletion of such gene segments flanked by two *loxP* sites. When a recombinant system based

on this principle is used, the *neo* gene can be deleted together with the linked homologous sequences from the gene knockout mice. The expression of the *cre* gene, *i.e.* the synthesis of the Cre enzyme and the gene deletion, can be restricted to certain tissues. A steroid-inducible promoter can also be used, so that Cre expression and subsequent gene deletion occur only after the animals are given an appropriate dose of dexamethasone. The Cre/*loxP* technology can also be used to create knockin animals. In this case, the *loxP* sites are placed to flank the *neo* gene and the targeting homologous sequences, but they do not flank the replacement (knockin) gene sequences. Therefore, after Cre-mediated deletion, the exogenous gene remains in the genome at the targeted site.

5.7. Cloned animals

The essential characteristic of the clone is that the genome of the offspring is fully identical with that of the starting organism. During cell divisions (*e.g.* the division of fertilized eggs and body cells), the clones of the same starting cell develop. The methods of cloning multicellular organisms are as follows:

1. The embryo splitting technique

This is a routine method for cloning domestic animals with good properties (*e.g.* cattle), whereby identical twins are produced artificially. An egg is fertilized artificially with a sperm, and the zygote is allowed to divide into an eight-cell embryo. The embryo is then divided into two four-cell, or most often into four two-cell pieces. No differentiation of the cells occurs for the eight–cell embryos, and thus the split embryos will be genetically identical as identical twins. Therefore, this method is also called embryo twinning. The offspring do not resemble their parents, but rather one another. The separated embryos are allowed to develop to a state when they can be implanted into the uterus of a pseudopregnant female animal. Thereafter, a normal pregnancy and delivery can occur.

2. Somatic cell nuclear transfer (SCNT)

This method was used by Ian Wilmut and Keith Campbell in the Roslin Institute in Scotland in 1997, when the sheep Dolly was created. SCNT requires two cells: a nucleus donor cell and an unfertilized oocyte or egg cell. The unfertilized egg cell is more likely than the fertilized egg cell to accept the implanted nucleus as its own. The egg cell is a more appropriate recipient cell than other cells, because it can be forced to be divided more easily. The steps of the nuclear transfer are as follows:

1. A body cell serves as a donor cell. In the case of Dolly, it was a mammary gland cell. The donor cell is placed in tissue culture and allowed to divide *in vitro*. The donor cell is then placed into tissue culture medium without fetal calf serum, in which cell divisions are stopped, and the cells enter the G_0 or dormant state. This is necessary for the recipient cell to accept the nucleus from the donor cell.
2. The nucleus must be removed from the egg cell. The nucleus contains most of the genetic information, but the mitochondrial DNA also stores genetic codes. The donor cell is placed next to the enucleated recipient cell, and 1-8 h after the removal of the nucleus from the egg cell, an electric stimulus is used to fuse the donor cell (or its isolated nucleus) with the enucleated egg cell and, at the same time, activate cell divisions and the development of an embryo. The genetic program of the differentiated donor cell is zeroed in the egg cell, and it returns to an undifferentiated state: it becomes pluripotent. If the embryo survives, it is allowed to grow in an oviduct of a sheep for 5-6 days (here the embryos survive better than in a tissue culture).
3. Once the cloned embryo has reached the blastomere stage of development, consisting of about 100 cells, the embryo is implanted into the uterus of a surrogate mother. The mother carries the pregnancy. The progeny is a genetically identical copy of the donor. This is

called reproductive cloning. Dolly was the only lamb born from 277 attempts. Blastomeres can also be used as a source of stem cells.

In 1998, Teruhiko Wakayama and Ryuzo Yanagimachi modified the method of Ian Wilmut (the Roslin technique) and elaborated the Honolulu technique at the University of Hawai, whereby they successfully cloned mice. The cloned offspring were able to reproduce normally and maintain the clone by sexual reproduction.

Incomplete reprogramming of the donor cell is thought to be a leading factor in the low rate of success of animal cloning. Chromatin transfer is a new cloning technique aimed at reducing these problems. It involves treatment of the cell of the animal to be cloned to remove molecules associated with cell differentiation before the nucleus is removed.[30, 72-74]

XIII. Models in Neurology. The experimental study of brain hypoperfusion (*Tamás Farkas Ph.D., Zsolt Kis Ph.D., József Toldi Ph.D., D.Sc.*)

Brain ischemia, or stroke, is the third most common cause of death in industrialized countries, with an estimated global mortality of 4.7 million per year. Furthermore, it is a major cause of serious, long-term disability, with more than 1 million adults annually reporting functional limitations resulting from stroke. The development of an ischemic brain injury depends on the interactions of many pathophysiological mechanisms, such as the altered release of neurotransmitters, the collapse of membrane ion gradients, the release of inflammatory cytokines, the triggering of neuronal death pathways, a failure of the blood-brain barrier, a compromised microcirculatory perfusion, the generation of reactive oxygen species, and a battery of other factors. This situation clearly underlines the importance of the research on this field. Several animal models of brain ischemia have been proposed, but, because of various limitations, only some of them will be discussed here.

1. Models of global ischemia

The term *global ischemia* refers to the fact that the total brain is hypoperfused, or is temporarily without any perfusion. The blood perfusion of the brain is suspended, for instance, when the heart stops to work. This situation lasts until the start of reanimation. The consequences of this situation in the central nervous system (CNS) well can be studied in the rat model of four-vessel occlusion (4VO).

The rat 4VO model

The blood supply of the brain is served by the 2 vertebral arteries and the 2 internal carotid arteries. To suspend the blood supply of the brain, all 4 arteries should be occluded, because of the Willis circle. If one of the 4 arteries is functioning, the brain is supplied by blood, though at a reduced level.

It may be noted that serious global ischemia can be produced in the gerbil by the occlusion of 2 common carotids, as they have no complete Willis circle: there is no connection between the basilar artery and the common carotids.

To prepare a rat 4VO model, both vertebral arteries should be exposed under anesthesia and then cauterized. After a recovery period of a few days, under brief anesthesia, both common carotids are exposed, and occluded for a short time (from a few seconds up to 4-5 min). During carotid occlusion, the brain is practically without blood supply.

This model is suitable for studying not only the consequences of a clinically dead state in the CNS, but also the mechanism of preconditioning. It is not a new observation that short ischemic periods act as ischemic preconditioning inducing ischemic tolerance. It is observed that, following ischemic preconditioning, the survival of the brain tissue is much better after a heavy ischemic attack. The mechanism, however, is not known in detail.

The rat 2VO model

This model is typically used to study long-term or permanent (chronic) brain hypoperfusion. It is rather simple to make the preparation: both common carotid arteries are ligated or occluded with a clip. This results in a severily hypoperfused brain. The model is applicable to study neurodegenerative diseases as consequences of chronic cerebral hypoperfusion[75].

The study of the consequences of global cerebral ischemia

a) ***Electrophysiological methods***

To study the effects of global ischemia, the animals are anesthetized. This can be done before or immediately after the induction of 2VO/4VO, or even days or weeks later[76]. The animals are secured in a stereotaxic apparatus, which allows the precise location of different brain areas with ease. Small holes are drilled with a dental borer over the skull in order to place the electrodes for stimulation and recording. In rats, the hippocampal formation is extremely responsive to ischemia, which is accompanied by a disturbed metabolism (e.g. decreased cytochrome enzyme activity during 2VO) and functioning. All these features can be well demonstrated through electrical stimulation of the hippocampal CA3 region and parallel registration of the evoked activity (population spikes) from the contralateral CA1 region. Under eligible circumstances, it is possible to follow up the population spikes over a long period (for 3-4 hours). In acute experiments, the population spike amplitudes can be compared with those measured before ischemia (self-controlled experiment), or within experimental groups; in chronic experiments, the same comparison is possible only by using a group of sham-operated animals. When a 2VO/4VO model is utilized, there is a strong positive correlation between the neuronal injury (histology), the hippocampal malfunction (behavioral tests) and the decrease in the population spike amplitudes (electrophysiology).

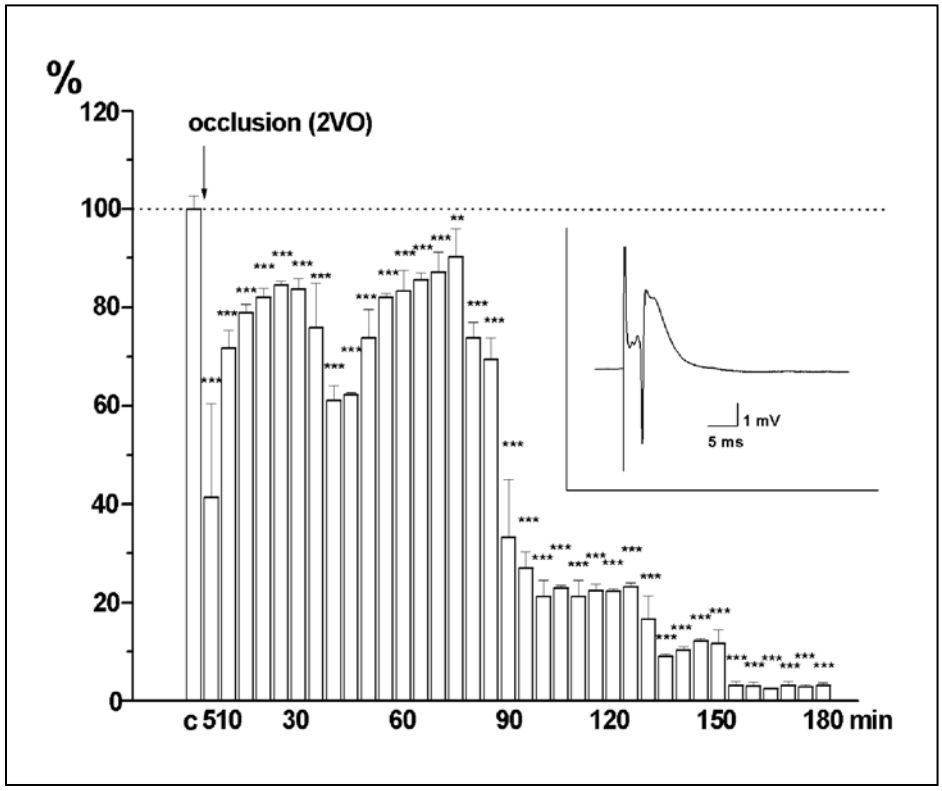


Figure 37. Changes in population spike amplitudes in rats in response to bilateral carotid occlusion (2VO). Insert: An example of the evoked spike. (The amplitudes were measured peak to peak.) Calibration: 1 mV; 5 ms. Each column represents the mean ± S.D. for 5 x 5 potentials. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ compared to the control value (first column, self-control, before induction of 2VO).

Through the use of brain slices, it is possible under *in vitro* circumstances to conduct electrophysiological experiments which would be inefficient or even impossible in anesthetized animals (*in vivo* circumstances). The detailed methodological description of making brain slices is beyond the scope of this chapter, but it should be mentioned that during *in vivo* experiments several compromises must be made. The two topmost ones relate to the age of the experimental animals and the thickness of the slices. The brain tissue of young rats (age 20-30 days) has an excellent ischemic tolerance relative to that of adult animals. In the

center of too thick slices (>400 μm) there is always an ischemic focus; O_2, glucose and end-metabolic substances travel in the tissue only by diffusion. However, in too thin slices, very few neurons have their arborization intact (<300 μm). Accordingly, it is difficult, but still possible, to produce appropriate brain slices from animals that have undergone global ischemia. In these slices, via high-frequency stimulation (HFS) of the Schaffer collaterals it is possible to produce a long-lasting augmentation of the population spike amplitudes in the CA1 region (Figure 2). This phenomenon is called long-term potentiation (LTP). The LTP (long-lasting augmentation of the probability of synaptic transmission) described first in this structure in the early 1970s is one of the most intensively studied and accepted models of the processes of learning and memory. There are two different ways to examine LTP in hippocampal slices that have undergone global ischemia. Data observed in sham-operated controls and in 2VO animals may be compared (the strength and duration of potentiation are studied), or the HFS elevation (the number of extra pulses) necessary for induction of the same level of LTP as observed in the controls can be studied.

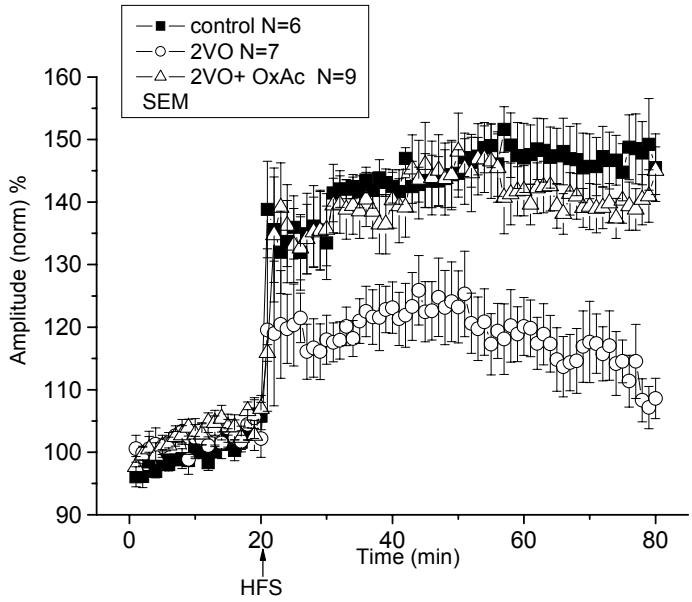


Figure 38. Long-term potentiation (LTP) is induced in region CA1 of the hippocampus by high-frequency stimulation (HFS) of the Schaffer collaterals (arrow, 500 pulses, 100 Hz) (black squares). The postischemic administration of oxaloacetate prevents the LTP impairment. The 2VO group underwent 30-min 2VO 3 days previously. The 2VO+OxAc group received the same ischemic insult followed by an i.v. injection of oxaloacetate (1 M, 1.5 ml). Data points are means ± SEM of normalized amplitudes of population spikes.

b) Histological methods

Fluoro Jade B (FJB) staining

FJB is an anionic fluorescein derivative that is useful for the histological staining of neurons undergoing degeneration. In response to excited light, the labeled neurons fluoresce, and this can be visualized with a fluorescence microscope. The advantage of this method is its rapidity because it does not involve an antigen-antibody reaction, and consequently not immunostaining. FJB staining precisely circumscribes the ischemic area. Since the CA1 region of the hippocampal formation is one of the most sensitive areas of the brain, in the event of global ischemia (4VO) this area will be stained rapidly.

NeuN immunostaining
This method is useful to demonstrate the intact and unharmed neurons. Through the use of vertebrate neuron-specific nuclear protein (NeuN), the intact neurons can be visualized. The immunohistochemical staining primarily occurs in the nucleus of the neurons, with lighter staining in the cytoplasm. Since FJB staining and NeuN immunostaining are consecutively applicable in one cutting, there is an opportunity to separate the ischemic and intact regions of the brain.

c) Behavioral studies

Long-term cerebral hypoperfusion results in serious brain damage, inducing impairments in learning and memory functions, and these can readily be studied under experimental circumstances.
This is especially so because the hippocampus is one of the most sensitive structures of the brain and is strongly involved in the learning and memory functions. To test the memory functions, sophisticated methods have been developed. One of the most frequently used method is the *Morris water maze test.* In this test, the rats are placed into a circular basin containing water 30-40 cm deep. A small invisible island (6-8 cm in diameter) is situated in the basin, 1 cm below the surface of the water. The animals initially find the island only by chance. In the course of the later tests, however, the animals learn the position of the island in relation to objects (lamps or paintings) around the basin. In the course of the tests, the time needed to find the island, or the swimming pathway, is determined with the aid of a special video-camera, and the results are evaluated by computer with special software (Noldus or Smart). The method is a very sensitive way to test the hippocampal memory functions. Other behavioral tests (open-field studies, Y and T mazes) can also be used in this kind of research.

2. Models of focal brain ischemia
In these models, the ischemia is limited to a small part of the brain (in most cases in the cortex), and thrombus-induced infarcts are studied.

Middle cerebral artery occlusion (MCAO)
In the course of the experiment, a silicone-coated 4-0 nylon monofilament is introduced into the internal carotid artery, while the external carotid artery is ligated. This procedure results in occlusion of the middle cerebral artery. A relatively large area of focal cerebral ischemia can be induced ipsilateral to the occlusion, which generally relates to the hippocampus and the striatum.

Photothrombotic lesion
With this procedure we are able to induce a relatively small, limited area of focal cerebral ischemia in the cortex, one of the most frequently used stroke models. The basis of this method is the (fluorescein derivative Rose Bengal), a potent photosensitizing dye. The illuminated dye coagulates the blood, causing thrombotic plugs and releasing free radicals, usually resulting in non-necrotic, rather than apoptotic cell death. The advantage of this method is that, without incision of the skull, we can induce a reproducible ischemic area. After the intravenous administration of Rose Bengal, an ischemic lesion is formed by irradiating the given brain area through the skull; this leads to a thrombic plug, which occludes the blood vessel. Thus, the area of the brain supplied by that blood vessel will be hypoxic and evolve an ischemic area, which is divided into two discernible areas: the lesioned

cortical area (core) containing dead cells, and the affected cells in the penumbra. These two areas are distinguishable with histological techniques.

Studies of focal ischemia-induced changes in brain functions

a) Electrophysiological studies

Cortical focal ischemia induces characteristic changes in the electrophysiological activity of the brain. The most characteristic is the *cortical hyperexcitability*, which can be studied appropriately via cortical evoked potentials. The most often used are the somatosensory stimulation- evoked potentials, which can be recorded from both the primary somatosensory and the motor cortices. After the ischemic attack, a perilesion (penumbra) region will develop around the core of the ischemic center. In this penumbra region, the cortex becomes highly excitable, resulting in enhanced amplitudes of the evoked potentials. The increased excitability lasts for hours. The method is suitable for testing the different kinds of neuroprotective interventions[77].

b) Histological studies

To demonstrate a larger ischemic area, a useful model is 2,3,5-Triphenyltatrazolium chloride (TTC) staining, which is suitable for macroscopic evaluation (lesion extension). The basis of this technique is dehydrogenase, which can be found in most types of tissue. The active enzyme reduces the colorless TTC, converting it into red triphenylformazan, which stains the tissues red. Where dehydrogenase is inactive, as in dead cells in the ischemic areas, it is unable to reduce TTC and the tissue remains white.
Similarly to TTC, conventional Nissl staining is a useful method for the evaluation of ischemic areas. Cresyl violet may be used to demonstrate Nissl substance. The rationale of the technique is a simple acid-base reaction, in which the cationic dyes bind to the anionic RNA of the Nissl substance.
Middle cerebral artery occlusion (MCAO) and photothrombotic cortical lesions can be examined with ease by means of FJB staining and NeuN immunostaining.

3. Traumatic brain injury models

Focal cold lesion
This method is suitable for the demonstration of vasogenic brain edema and necrotic processes. Cold injuries can be produced by applying a copper cylinder coated with nickel (diameter 2 mm) cooled to -78 ^{o}C (by filling it with an acetone/dry ice two-phase system). The cooling period is usually less than 1 min.

Traumatic head injury
Similarly to focal cold lesions, this method is suitable for examinations of necrotic processes. Under experimental circumstances, by means of a construction developed for the purpose, a cranial impact of 0.5 J is delivered by a free-falling silicone-coated rod.

***In situ* end labeling or terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) technique**
This technique is a model frequently used to demonstrate apoptotic processes. In the period of apoptotic cascade, the DNA strand of the neurons is fragmented. A suitable reagent is able to

recognize the 3-OH ends of the fragmented DNA. With the aid of the conventional diaminobenzidine (DAB) – horseradish peroxidase reaction, the apoptotic neurons are colored brown and can readily be examined under a microscope.

XIV. References

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XV. Abbreviations

4VO	the rat model of four-vessel occlusion
ARDS	acute (or adult) respiratory distress syndrome
BAL	bronchoalveolar lavage
COPD	chronic obstructive pulmonary disease
DW	dry weight
EB	Evans blue
FJB	Fluoro Jade B staining
FRC	functional residual capacity
HFS	high-frequency stimulation
IL-13	interleukin-13
IVM	intravital videomicroscopy
KO	gene knockout animals
LTP	long-term potentiation
MHC	major histocompatibility complex
OA	ovalbumin
OPS	orthogonal polarization spectral imaging
P	pressure
pCO_2	partial pressure of carbon dioxide in the blood
pH	measure of hydrogen ion concentration in the blood
PMN	polymorphonuclear leucocytes
pO_2	partial pressure of oxygen in the blood
TLC	total lung capacity
TTC	2,3,5-Triphenyltatrazolium chloride staining
V	volume
WW	wet weight
Z_0	basal thoracic impedance