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Mouse Models for Myocardial Ischaemia/Reperfusion

E. Conci, O. Pachinger, B. Metzler

Abstract: The past few years have witnessed a remarkable advance in our understanding of the pathophysiology of coronary heart disease. Myocardial ischaemia usually occurs on the basis of coronary atherosclerosis. Although the functional consequences of depriving the myocardium of its blood supply have been appreciated for many years, the coronary heart disease is still the leading cause of morbidity and mortality in the western world. This has focused the attention of physicians on restoring blood flow to the ischaemic region in order to prevent tissue necrosis and regain organ function. Reperfusion of ischaemic tissues is often associated with microvascular dysfunction that is manifested as impaired endothelial-dependent dilatation in arterioles and leukocyte plugging in capillaries. The availability of a broad variety of knockout mice provides important clues about the progression of the ischaemia/reperfusion (I/R) injury. Therefore mouse models for I/R are of great importance for the development of new therapeutic strategies for humans.

Kurzfassung: Mausmodelle in der myokardialen Ischämie/Reperfusion. Die letzten Jahre wurden durch bemerkenswerte Fortschritte im Verständnis der Pathophysiologie der koronaren Herzkrankheit geprägt. Die myokardiale Ischämie ist hauptsächlich durch die Atherosklerose der Koronargefäße bedingt. Obwohl die funktionellen Folgen von verminderter Blutversorgung am Herzmuskel hinlänglich bekannt sind, ist die koronare Herzkrankheit immer noch die häufigste Todesursache in der westlichen Welt. Deshalb richten sich die therapeutischen Bemühungen auf die Wiederherstellung der Perfusion in den ischämischen Arealen, um die Entstehung einer Gewebsnekrose zu vermeiden und um die Funktion des Organs wiederherzustellen. Die Reperfusion von ischämischem Gewebe geht allerdings oft mit mikrovaskulärer Dysfunktion einher, die sich durch Beeinträchtigung der endothelabhängigen Vasodilatation in Arteriolen und Ansammlung von Leukozyten in den Kapillaren manifestiert. Die Verfügbarkeit einer großen Anzahl an Knockout-Mäusen ermöglicht wichtige Erkenntnisse über das Zustandekommen der Myokardschädigung durch Ischämie und Reperfusion. Deshalb sind Mausmodelle von großem Interesse für die Entwicklung neuer therapeutischer Strategien beim Menschen.
J Kardiol 2006; 13: 239–44.

■ Myocardial Ischaemia and Reperfusion

Transient myocardial ischaemia is defined as a state of myocardial impairment due to an imbalance between the level of coronary perfusion and myocardial energy demand. Clinical hallmark is the characteristic chest pain known as angina pectoris. However, episodes of silent myocardial ischaemia also occur, particularly in individuals with diabetes mellitus and cardiac transplants. Pathophysiologic manifestations of transient ischaemia include electromechanical uncoupling, impaired ventricular function, which may be manifest as hypokinesis or akinesis, and altered electrical activity, which may be relevant by an acute injury pattern on the electrocardiogram, arrhythmus and/or conduction disturbances. If coronary occlusion is removed within approximately 20 minutes after onset, tissue viability is preserved, and its only transient damage often results in the phenomenon of stunning, which exhibits temporary contractile failure of the myocardium, but it is not associated with development of necrosis.

Acute myocardial infarction is the irreversible injury and subsequent necrosis in a wavefront from subendocardium to subepicardium due to severe and prolonged reduction in coronary perfusion. The major determinants of myocardial infarct size are duration and severity of ischaemia, size of the myocardial area at risk, and magnitude of collateral blood flow available shortly after coronary occlusion. Infarct size also can be influenced by heart rate, wall tension and myocardial contractility. Systemic alterations in the adrenergic nervous system and local alterations in the adrenergic receptor-adenylate cyclase system also are operative in influencing the progression of myocardial ischaemic injury.

Myocardial reperfusion is the restoration of coronary blood flow, which either occurs spontaneously or is therapeutically induced, after a period of coronary occlusion. The effects of reperfusion include not only reversible, functional changes, but also irreversible injury. A wide spectrum of deleterious effects results from reperfusion *per se* when it is superimposed on already ischaemically altered myocardium. Reperfusion also has the potential to salvage ischaemic myocardium. The net effect depends upon the severity and duration of the ischaemic insult before reperfusion [1].

Myocardial ischaemia/reperfusion induces a profound inflammatory condition with activation of multiple cell types, including leucocytes and endothelial cells (Fig. 1). Reduced NO bioavailability signals important patho-physiological

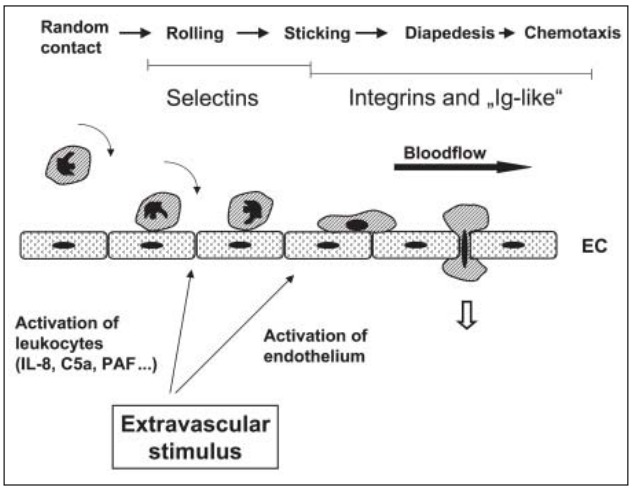

Figure 1: Scheme of involvement of the adhesion molecules in ischaemia/reperfusion. Under normal conditions, there are virtually no interactions between the endothelium and neutrophils. After coronary ischaemia/reperfusion, the blood vessel's ability to form NO is severely reduced. With decreased supplies of NO, the coronary vessel may constrict. Additional adhesion molecules were expressed on the endothelium and polymorphonuclear leukocytes (PMNs) begin with rolling as the first step in PMN sequestration. As the PMN slows, tighter interactions between CD18 (on PMN) and ICAM-1 (on endothelium) cause the second phase of the PMN-EC interaction called sticking. The final step, the diapedesis, involves the coordination of many factors.

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changes, such as leucocyte adherence and transmigration of mononuclear cells [2]. At least three major pathways may contribute to lethal reperfusion injury. The first mechanism involves massive calcium overload via Na^+/H^+ and Na^+/Ca^{2+} exchange during reperfusion, which leads to mitochondrial calcification and contraction band necrosis. A second mechanism leads to intracellular accumulation of osmotic active catabolites during ischaemia that may induce massive cell swelling and sarcolemmal rupture when reperfusion provides exposure of the injured cells to abundant extracellular fluid. A third way involves the consequences of a burst of oxygen-based free radical generation. Thus, myocardial reperfusion can cause accelerated progression of cell death in myocytes with prior severe ischaemic damage coupled with salvage of myocytes with less severe ischaemic injury. Some of the known risk factors for cardiovascular disease (hypercholesterolaemia, diabetes and hypertension) appear to exaggerate many of the microvascular alterations elicited by ischaemia and reperfusion.

Although much experimental evidence exists in support of the reperfusion component of injury, ischaemia without following reperfusion will cause the destruction of most of the ischaemic myocardium. This leads to an obvious paradox: the need for re-establishing blood flow at the expense of a profound inflammatory response [3].

■ Mouse Types Used in Cardiovascular Research

Ischaemia/reperfusion research with animal models has being carried out for a considerable time. Knowledge of the pathogenesis and therapeutic intervention of disease conditions and the use of animal models in the research have evolved almost simultaneously. Animal models are designed to be preliminary tools for better understanding of the pathogenesis and improvement in diagnosis, prevention and therapy of heart ischaemic diseases in humans. The evaluation of a risk factor as a single independent variable, with almost complete exclusion of other factors, can be performed in animals free of intercurrent diseases or abnormalities and with well known genetic characteristics. Furthermore, experiments using animals are the only way to develop and test new diagnostic, preventive and therapeutic procedures for both ethical and practical reasons. The investigator can choose the species, time and method, as well as obtain tissue, serum samples and other materials needed for measurements under optimal conditions, selective circumstances that are difficult, if not impossible, in studies with human subjects.

Attracted by its well-defined genetic map, a number of investigators have begun to use the mouse as an experimental system for research on cardiovascular diseases. Hundreds of inbred lines have been established and both congenic and recombinant strains are available to facilitate genetic experimentation.

Recently, genetic models have been developed in which genes are either overexpressed, deleted or mutated. Such mouse models have considerable advantages, because they overcome the need to administer factors or their inhibitors, which can be problematic and often difficult to quantify. They also seem to tolerate prolonged monoclonal antibody treatments better.

Beside the use of conventional wild type mice the availability of a broad variety of genetically altered mice in the last years lead to a big rise in interest for these strains.

Knockout Mice

Knockout mice are produced by a laboratory technique called gene targeting. This is the replacement of a gene sequence from the mouse's own genome with a related one that has been modified to contain a mutation. The replacement occurs by homologous recombination, where two similar DNA sequences line up next to each other and exchange parts. Gene targeting is carried out in mouse embryonic stem cells (ES cells). The aim of this procedure is to get modified ES cells to contribute to the germ line, which gives rise to sperm. Some sperms are produced that carry the desired mutation, and if these fertilise a normal egg, mice develop with one copy of the mutated gene in every cell. Interbreeding such mice will produce some homozygous individuals in the next generation – mice inheriting the mutation from both parents and therefore carrying two copies of the mutant gene. These are knockout mice. Although they are extremely useful in studying gene function, producing and taking care of knockout mice in special facilities is very expensive. Other limitations are the developmental defects, so that many knockout mice die while they are still embryos. The goal of conventional knockout technology is to knock out both alleles so that the gene is entirely absent from all cells. The purpose of the more advanced types of knockouts, the so called conditional knockouts, in contrast, is to delete a gene in a particular organ, cell type, or stage of development.

Conditional Knockout Mice

The latest wave of mouse models has advanced beyond generalised gene knockouts to develop new strategies for precision engineering of endogenous genes within specific cell types. The most successful approach has been based on the generation of mice that harbour floxed alleles, which contain LoxP recognition sequences flanking a critical exon that is required for the expression or function of the gene of interest [4]. These mice are generated by homologous recombination of targeting vectors in embryonic stem cells that bring in the LoxP sites into the germ line. The floxed allele mice express the normal gene product, because the LoxP sites are located within the intron sequences that are spliced out during RNA processing. However, the intervening sequences between the LoxP sites can be excised by the expression of CRE recombinase, which is brought into the genetic background of the floxed allele mice via interbreeding. By controlling the expression of CRE recombinase to a specific tissue, for example to the ventricular chamber, it is possible to generate mice that harbour a ventricular-restricted mutation in a gene that is widely expressed, thereby allowing a direct examination of the role of a given gene within cardiac muscle.

Transgenic Mice

A transgenic mouse contains additional, artificially-introduced genetic material in every cell. This often confers a gain of function, for example the mouse may produce a new protein, but a loss of function may occur if the integrated DNA interrupts another gene. A transgenic mouse is a very useful system for studying mammalian gene function and regulation because analysis is carried out on the whole organism. DNA can be integrated by injecting it into the pronucleus of a fertilised ovum. The DNA can integrate anywhere in the genome, and multiple copies often integrate in a head-to-tail fashion. There is no need for homology between the injected DNA and the host genome.

■ Mouse Models for Myocardial Ischaemia and Reperfusion

In the present review we will describe different mouse models for myocardial ischaemia/reperfusion. With the help of a big variety of genetically manipulated mice the pathophysiological effects of the targeted genes can be assessed and should bring some new insights about ischaemia/reperfusion.

Open Chest Ischaemia/Reperfusion Model

In 1995 Michael et al. [5] first described the open chest *in vivo* mouse model. Mice are anaesthetised with phenobarbital (50 mg/kg, i.p.), fixed in the supine position by taping the extremities and the upper jaw. A midline skin incision from the xiphoid process to the submentum is made. After separating the salivary glands, the muscles overlying the trachea are retracted, and a tracheotomy is performed. A polyethylene tube (No. 90) is carefully inserted into the trachea (about 5 mm from the larynx), taped in place to prevent dislodgement and connected via a loose junction to a rodent ventilator (model 687; Harvard Apparatus). After ventilation (tidal volume 1,2 ml/min., rate 110 strokes/min.) is started by supplementation of 100 % oxygen the chest is opened by a lateral cut along the left side of the sternum. An adequate tidal volume results in an adequate inflation of the lungs without overexpression. The whole procedure is aided by a microscope (Olympus SZH 10). With an electrocoagulator, intercostal blood vessels are coagulated. The chest walls are then retracted using a 6-0 silk suture for better visualisation of the heart. After removing the pericardial sac and slightly retracting the left auricle, the left descending artery (LAD) becomes clearly visible. A 1 mm section of a PE-10 tubing is placed on top of the LAD to secure its ligation without damaging the artery. The LAD ligation is done with an 8-0 silk and becomes evident by discoloration of the left ventricle (Fig. 2). To avoid cooling of the animal, it is important to use a heating pad placed under the mouse. After a defined time of LAD occlusion, the ligature can be removed by cutting the knot on top of this PE-10 tube and reperfusion can be visually confirmed.

Minimal Invasive Ischaemia/Reperfusion Model

The advantage of this *in vivo* model with an implantable device for artery occlusion is to overcome the high level of background inflammation due to surgical trauma associated with the open chest model, permitting a more accurate and interpretable response about the involvement of different cytokines and other mediators in the ischaemia/reperfusion injury of the myocardium [6].

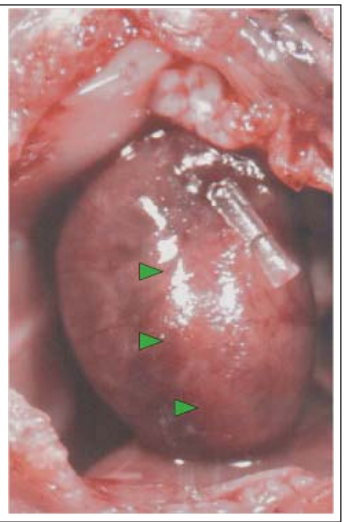

Figure 2: Open mouse chest with occluded left descending coronary artery. Photograph of open chest mouse with occluded left descending coronary artery by the help of a PE-10 plastic tubing. The LAD ligation was done with an 8-0 silk and was evident by discoloration of the left ventricle. Arrows indicate the area at risk.

After performing a thoracotomy as described in the open chest model, an 8-0 Surgipro monofilament polypropylene suture with the U-shaped tapered needle is passed under the LAD. The two ends of the suture are threaded through a 0.5 mm piece of PE-10 tubing, forming a loose snare around the LAD, and are then threaded through the end of a size 3 Kalt suture needle (Fine Science Tools) and exteriorised through each side of the chest wall. The chest is closed, and the ends of the exteriorised 8-0 suture are then tucked under the skin. The animal is removed from the respirator and is allowed to breathe 100 % O_2 via a nasal cone until full recovery of consciousness.

Ischaemia Without Reperfusion

Similar to the *in vivo* open chest model for myocardial ischaemia/reperfusion the mouse thorax is opened and prepared to make the anterior heart surface visible. Because reperfusion is not demanded, the LAD ligation is done with an 8-0 silk and will not be removed later. Afterwards, the retraction sutures are removed and the chest wall and skin are closed with sutures, and the animal is allowed to recover. According to the experimental protocol mice can be sacrificed after induction of a transmural myocardial infarction with any desired time of ischaemia. This protocol seems to be particularly interesting for heart failure studies.

Ischaemia/Reperfusion With Ischaemic Preconditioning

Miller et al. [7] have been among the first who demonstrated the early phase of ischaemic preconditioning in the myocardium in the *in vivo* mouse model. We also used this protocol and showed its highly effectiveness in inducing a significant limitation of the myocardial damage [8]. After dissecting the pericardium an 8-0 silk suture with a U-shaped needle is passed under the LAD. The LAD ligation becomes evident by discoloration of the left ventricle. For the preconditioning

protocol the knot on top of the 1 mm plastic tube is tighten carefully and loosen after the given time. Great attention should be paid in order to avoid damage of the LAD.

For preconditioning, the mouse undergoes three cycles of 5 min. artery occlusion followed by 5 min. reperfusion, respectively. Ten minutes later, the animal undergoes 30 min. of coronary occlusion of the artery, followed by 2 h of reperfusion. By this protocol it is possible to demonstrate a reduction of the infarct size of about 50 %. The responsible mechanism is still not fully elucidated. Various possibly involved signalling pathways have already been described. Among the most important seem to be reactive oxygen species, isoforms of protein kinase C (PKC) and adenosine.

Langendorf Model (*Ex Vivo*)

Animals are usually injected with heparin (1000 U/kg, i. p.) 20 min. prior to the experimental protocol. Once the animal is anaesthetised, the heart is removed and placed in a weigh bath with some Ca^{2+}-containing buffer. Holding the aorta with two forceps, the heart is lifted from the buffer and placed on a perfusion cannula that should have buffer running through at a slow rate. After clamping the aorta to the perfusion needle, buffer flow rate may be increased. If the procedure is performed successfully, the heart will begin to beat rhythmically and the drops will become clear.

Once the heart is successfully hung, it is perfused (at 37 °C) with Krebs-Henseleit bicarbonate (KHB) buffer containing (in mM): 118 NaCl, 4.7 KCl, 1.25 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 25 $NaHCO_3$, 10 N-[2-hydro-ethyl]-piperazine-N'-[2-ethanesulfonic acid] (HEPES) and 11.1 glucose, equilibrated with 5 % CO_2 – 95 % O_2.

■ Measurements of Myocardial Tissue Damage

Measurement of Cardiac Enzymes

Blood concentration of cardiac enzymes can be used as an index of cardiac cellular damage [9]. Heparin-blood is collected when mice are sacrificed for organ removal, and it is usually taken by puncture of the Vena cava inferior or of the Vena portae. Blood concentration of troponin T is measured by using a quantitative rapid assay kit (Roche Diagnostics, Mannheim, Germany).

Creatine kinase and lactate dehydrogenase isoenzyme 1 (LDH-1) may be electrophoretically analysed using commercially available kits (Paragon, Fullerton, CA, USA). Gels are scanned with a densitometer and the relative activity of each single isoenzyme fraction is calculated against total enzyme activities.

Measurement of the Infarction Area With Transmission Microscope

After reperfusion, hearts are removed and the portion of tissue below the ligation site is fixed in 4 % paraformaldehyde at 4 °C overnight and embedded in paraffin. Sections of 5 µm are cut from the cross area and stained with hematoxilin and eosin (HE) for histological evaluation of tissue damage.

The measurement of the scar area may be done by reviewing these sections with a B×60 microscope (Zeiss, Jena, Germany) equipped with a Sony 3CCD camera and a television monitor. A transmission scanning microscope (Bio-Rad), equipped with a 488 nm argon ion laser and Plan Neofluar 10×/0.3 oculars connected with the program START LSM 510 may be used to scan the images. The scar is defined as the region between the last living myocytes and the cardiac membrane, and is measured in square micrometers [10]. Despite of the real three-dimensional extension of myocardial infarction, this two-dimensional approach has some practical advantages: it allows to distinguish damaged tissue already after a short time frame of ischaemia and to make an accurate measurement of the infarction area despite of its small size. This technique is quite easy to perform and is less time consuming than Evans blue staining.

Zingarelli Damage Scoring System

For quantitative histological evaluation of tissue damage the previously published scoring system by Zingarelli et al. [11] can be used. The following criteria are considered: score 0, no damage; score 1 (mild), interstitial oedema and focal necrosis; score 2 (moderate), diffuse myocardial cell swelling and necrosis; score 3 (severe), necrosis with the presence of contraction bands and neutrophil infiltrate; and score 4 (highly severe), widespread necrosis with the presence of contraction bands, neutrophil infiltrate and haemorrhage. At least ten tissue samples/group should be used for a satisfying statistical analysis.

Assessment of Area at Risk and Infarction Size With Evans Blue and TTC-Staining

After ligation of the coronary artery 1 % Evans blue is perfused into the aorta and coronary arteries with distribution throughout the ventricular wall. The left ventricle of each heart is excised and weighed. Sections of the ventricle below the site of ligature have uniformly blue areas, surrounding a smaller colourless one, which is the tissue supplied by the ligated vessel. After incubation in 1.5 % triphenyltetrazoliumchloride (TTC), viable myocardium stains brick red and the infarct appears pale white (Fig. 3). The area of infarction is determined by computerised planimetry using an image analysis software program (OPTIMAS, Bioscan, Redmond, WA). The size of infarction is determined by the following equations: weight of infarction = $(A_1 \times WT_1) + (A_2 \times WT_2) + (A_3 \times WT_3) + \ldots$, where A is the percent area of infarction by planimetry from subscribed numbers representing sections, and WT is weight of the same numbered sections. Percentage of infarcted LV is (WT of infarction/WT of LV) × 100. Area at risk as percentage of LV is calculated by (WT of LV – WT of LV stained blue)/WT of LV. The weight of LV stained blue is calculated in a similar fashion: sum of products of the percent area of each slice × the weight of the representative slice.

This technique shows good results after at least a few hours of ischaemia. Due to the small size of murine heart and to unintentional diffusion of the staining colour, this measurement is

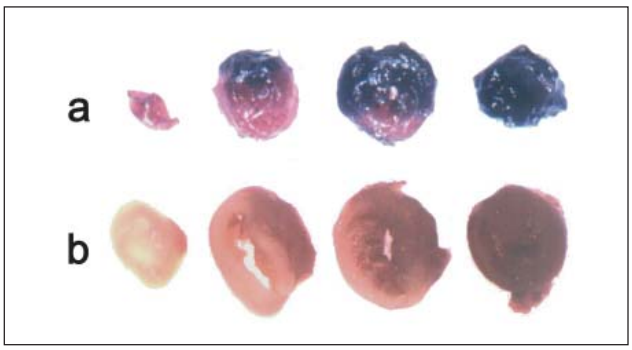

Figure 3: Cross sections of the TTC-stained heart. **a)** Areas at risk are delineated by Evans blue dye in a representative heart with occlusion of the left descending coronary artery. All areas that stain blue were therefore not at risk. **b)** Areas that appear pale white are infarcted. Areas that remain red were not infarcted but were within the area at risk. Transverse sections are from the apex to the base (from left to right side).

relatively inaccurate, so that it seems to be much more suitable for bigger animal hearts like those of a pig or a dog.

Transthoracal and Transesophageal Echocardiography

Transthoracic echocardiography may be performed using a Sonos 5500 ultrasound machine (Hewlett Packard Co.) with a 12 MHz phased array transducer and a frame rate of 41/s. The transducer is used at a depth setting of 2 cm to optimise resolution. Mice are anaesthetised and placed on a heating pad in a shallow left lateral position and a standard lead II electrocardiogram is recorded for heart rate (HR) measurement. After a 2-dimensional (2 D) image is obtained in parasternal short axis view at the level close to papillary muscles, a 2 D guided M-mode trace crossing the anterior and posterior wall of the LV is recorded at a sweep speed of 100 mm/s. It is possible to measure digitally following parameters: LV internal end-systolic and end-diastolic diameters (LVESd, LVEDd), external LV diastolic diameter (ExLVDd), anterior and posterior wall thickness of systole and diastole (Aws th, Awd th, Pws th and Pwd th). Scherrer-Crosbie et al. described a three-dimensional echocardiographic assessment of left ventricular wall motion abnormalities after infarction in the mouse heart [12]. Also a transesophageal echocardiographic approach is described [13].

Magnetic Resonance Imaging (MRI) Assessment of Murine Heart Function

The group around Weiss described an *in vivo* MRI assessment technique of the murine heart [14]. High-resolution images may be obtained to confirm position, define regions of metabolic interest or quantify ventricular function. It is possible to determine left ventricular volumes at end diastole and end systole using a suitable software package. The left ventricular ejection fraction may be calculated from the relative difference in end-diastolic and end-systolic cavity volumes.

Single Photon Emission Computed Tomography (SPECT)

The importance of myocardial imaging in small animals has led to the development of SPECT systems designed specifi-

cally for this application, which give good agreement between *in vivo* imaging data and *post mortem* autoradiographic and staining studies. The true size of myocardial perfusion defects can be detected with very high accuracy. This has important implications for the study of cardiac function in mice, and may be useful in phenotyping transgenic mouse models of heart disease.

■ Electrocardiogram and *In Vivo* Left Ventricular Pressure-Volume Measurements

Electrocardiographic Measurements

Continuous ECG recordings can be made with a two-lead ECG Haemodynamic Data Acquisition System apparatus (Instrument Services, Maastricht, The Netherlands) to detect arrhythmias. The first lead is positioned in the left lower leg, the second lead in the right upper leg. Recordings are made every 5 seconds in trend save mode with a sample interval of 1 ms and are started at the beginning of the surgical procedure [15].

In Vivo Left Ventricular Pressure-Volume Measurements

Left ventricular function can be assessed by simultaneous measurement of left ventricular pressure and volume. This can be done with a Sigma SA (CDLeycom, Zoetermeer, The Netherlands) single segment data acquisition module operating on a constant excitation current of 30 μA and with Cirlab software (LUMC, Leiden, The Netherlands) for offline data analysis. A 1.4 Fr Millar pressure-conductance catheter (SPR-719, Millar Instruments, Houston, TX, USA) is used for the LV pressure-volume measurements. The time-varying ventricular volume V(t) may be estimated from $V(t) = \rho L^2 [G(t) - G^P]$ where ρ (rho) is the mouse specific blood resistance, L indicates the distance between the sensing electrodes and G(t) the instantaneous conductance.

■ Conclusion

Due to the fact that coronary heart disease is still the number one killer in the western world, scientists and doctors are encouraged to find new therapeutic strategies. Due to ethical considerations experimental ischaemia/reperfusion (I/R) studies almost exclusively can be performed in laboratory animals. The availability of a broad variety of genetically altered mice in the last years led to a remarkable rise in the interest for experimental mouse models.

This review describes the different mouse I/R models and the techniques for quantification of I/R damage. Beside the conventional *in vivo* mouse model for I/R, the open chest mouse model, also newer ones, such as the closed chest I/R model are described. Further the novel quantification of I/R damage by help of a transmission microscope is explained. Also known techniques established in clinical practice in humans, such as echocardiography and MRI, are described for mice. The advantages and disadvantages of the different approaches also were discussed. The given company names for the different technical devices should serve just as a proposal. We know that there are a number of equal technical devices manufactured by different companies. This review should help the interested scientists to choose the most suitable mouse I/R model to clarify their specific questions.

With the new gained insights in the ischaemia/reperfusion damage by the use of mouse models new pathways will be revealed. This findings could generate new hypotheses that can be tested in human subjects and may lead to the development of new therapeutic strategies in future.

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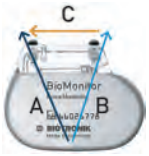

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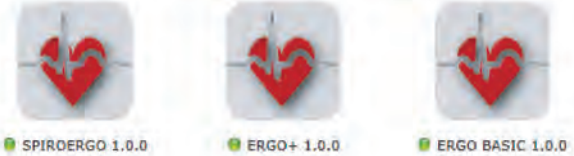

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