Impact of Ischemia and Reperfusion Times on Myocardial Infarct Size in Mice *In Vivo*

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The murine *in vivo* model of acute myocardial infarction is increasingly used to study signal transduction pathways. However, methodological details of this model are rarely published, and durations of ischemia and reperfusion (REP) time vary considerably among different laboratories. In this study, we tested the hypothesis that infarct size (IS) is dependent on both duration of ischemia and REP time. Pentobarbital-anesthetized male C57BL/6 mice were intubated, mechanically ventilated, and instrumented for continuous monitoring of mean arterial blood pressure and heart rate. After left fourth thoracotomy, the left anterior descending coronary artery was ligated. Mice were randomly assigned to receive 30, 45, or 60 mins of coronary artery occlusion (CAO) and 120, 180, or 240 mins of REP, respectively. IS was determined with triphenyltetrazolium chloride and area at risk (AAR) with Evans blue, respectively. Arterial blood gas analysis and hemodynamics were not different among groups. Prolongation of CAO from 30 to 60 mins significantly (* $P < 0.05$) increased IS from 18% ± 5% to 69% ± 3%*, from 20% ± 2% to 69% ± 6%* and from 42% ± 10% to 75% ± 2%* after 120, 180, and 240 mins REP, respectively. Moreover, IS was increased from 18% ± 5% to 42% ± 10%* (30 mins CAO) and from 40% ± 3% to 72% ± 6%* (45 mins CAO) when REP time was prolonged from 120 to 240 mins. IS was not increased when REP was prolonged from 120 to 240 mins at 60 mins CAO (69% ± 3% vs. 75% ± 2%). In the present study, we describe important methodological aspects of the murine *in vivo* model of acute myocardial infarction and provide evidence that, in this model, IS depends both on duration of ischemia and on REP time. Exp Biol Med 233:84–93, 2008

Key words: method, mouse, myocardial infarction, reperfusion injury, preconditioning

Introduction

The mechanisms underlying myocardial ischemia/reperfusion (I/R) injury are under intense investigation. Models of acute myocardial infarction in rodents and large animals have served to elucidate the role of various signaling pathways involved in I/R injury. Mice are increasingly used to that end because they provide the striking possibility of overexpression or disruption of specific genes.

The little blood volume of mice and the small size of the mouse heart mandate blood-sparing microsurgical techniques. Exact identification and gentle manipulation of the coronary artery are inevitable prerequisites for a reproducible murine model of acute myocardial infarction. Because only a small number of published studies provide methodological details of this model (1–5), we aim to provide a step-by-step description of the murine *in vivo* model of myocardial infarction. In hitherto published studies, different durations of ischemia and reperfusion (REP) times are used that impede direct comparison of I/R injuries and infarct-sparing interventions. Duration of coronary artery occlusion (CAO) ranges from 20 (4) to 120 (6) mins, yielding infarct sizes from 10% to 65%.

Recently, we reported that myocardial infarct size (IS) increased with prolongation of CAO (5). CAO varied from 10 to 60 mins at a constant REP time of 120 mins. Conversely, at a 60-min CAO, an ischemic time considered to be a maximum ischemic stimulus (7), IS was increased with prolongation of REP time from 30 to 120 mins but not when REP was prolonged to 240 mins (5). Thus, we concluded that there was no influence of REP time on myocardial IS when a maximum ischemic stimulus of 60 mins was investigated.

To our knowledge, there is no systematic study that reports myocardial IS in relation to CAO of 30, 45, and 60

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mins duration when REP time is prolonged beyond 120 mins to 180 and 240 mins. We hypothesized that, at submaximum ischemic stimuli of 30 and 45 mins, CAO myocardial IS is dependent on REP time and that IS becomes independent of REP time when CAO is 60 mins.

Materials and Methods

Animals. Male C57BL/6 mice (8–12 weeks old, weighing 20–25 g) purchased from Charles River Laboratories (Sulzfeld, Germany) were used for all experiments. Animals were maintained under controlled conditions (22°C, 55%–65% humidity and 12:12-hr light:dark cycle) and were allowed free access to tap water and a standard laboratory chow.

All experimental procedures and protocols used in this investigation were reviewed and approved by the Animal Care and Use Committee of the Government of Lower Franconia, Bavaria, Germany. Furthermore, all conformed to the Guiding Principles in the Care and Use of Animals of the American Physiological Society and were in accordance with the *Guide for the Care and Use of Laboratory Animals* (8).

Anesthesia. Mice were anesthetized with an intraperitoneal (ip) injection of 60 mg/kg sodium pentobarbital (Merial, Hallbergmoos, Germany) followed by repeated ip injections of 15 mg/kg as needed. Depth of anesthesia was initially verified by loss of righting reflex and then by recurrent testing of hind paws withdrawal and corneal reflex throughout the experimental protocol. After placing mice on a servo-controlled heating pad (Föhr Medical Instruments, Seeheim, Germany) in a supine position, paws were taped onto the pad and rectal temperature was maintained at 37.0° ± 0.2°C.

Intubation and Ventilation. After fixation of the maxillar incisors onto the pad by using a plastic rubber band, the tongue was retracted with a forceps, and the trachea was intubated with a 22-gauge arterial cannula (BD Insyte-W, Heidelberg, Germany) under direct vision. Mice were ventilated with a rodent ventilator (SAR 830/AP, CWE Inc., Ardmore, PA) operated in pressure-controlled mode with a frequency of 130 breaths per minute, a maximal airway pressure of 30 cm H_2O, and a positive-end expiratory pressure of 1–3 cm H_2O. Air mixture was 50% air and 50% oxygen.

Analysis of Arterial Blood Gas Tensions. For analysis of arterial blood gas tensions, blood samples were drawn from the left ventricle with a heparinized syringe at the end of each experiment and immediately analyzed using the automated blood gas analyzing system ABL 735 (Radiometer Medical ApS, Bronshoj, Denmark). In additional experiments, blood gas analysis was performed to exclude hypoxia after induction of anesthesia but before endotracheal intubation (i.e., 10–15 mins after pentobarbital injection) and at the beginning of ischemia, that is, at completion of surgical procedure.

Electrocardiogram (ECG), Hemodynamic Monitoring, and Fluid Management. In mice, little data have been published concerning ECG changes during I/R injury in mice. Diminished R wave amplitudes and marked ST segment elevation during CAO were reported by Gehrmann *et al.* (9) and Takahashi *et al.* (10). Here, we used a three-lead needle-probe ECG to continuously monitor heart rate and ST-segment elevation. Persistence of ST-element elevation during REP was associated with large myocardial IS, irrespective of the duration of CAO (data not shown). Thus, by using continuous ECG recordings, successful CAO and REP can be monitored.

For measurement of arterial blood pressure and fluid management a saline-filled PE-10 catheter connected to a pressure transducer (Combitrans, B. Braun, Melsungen, Germany) was inserted into the right common carotid artery. After midline incision of the neck, the right submandibular salivary gland was moved laterally, and the common carotid artery was dissected without damaging surrounding veins or vagal nerve. A PE-10 catheter was inserted and secured by 6-0 silk ligatures.

Repetitive, mild flushing of the arterial catheter with 0.9% normal saline served to keep the line patent for invasive blood pressure measurements and to replace fluid loss from evaporation and renal elimination throughout the experimental protocol. Volume replacement was governed by mean arterial pressure and averaged 30 $ml \cdot kg^{-1} \cdot hr^{-1}$.

Surgical Procedure. All surgical instruments used for the surgical procedure described in this study are summarized in Appendix 1. All surgical procedures were performed with the aid of a stereo microscope (OPMI-9-FC, Zeiss, Jena, Germany) using a magnification of ×3.75–×10.

Fur hair was retracted with fluid paraffin (Merck, Darmstadt, Germany), and an anterolateral skin incision was placed from the left axilla to the xiphoid process. The pectoralis major muscle was dissected, cut at its sternal margin, and moved into the axillary pit. The pectoralis minor muscle was cut at its cranial margin and moved caudally. The muscle was later used as a muscle flap covering the heart during CAO. Muscles of the fourth intercostal space and the pleura parietalis were penetrated with tweezers at a point slightly medial to the margin of the left lung, thus avoiding damage to the lung or heart. After penetration of the pleura, the tweezers were carefully directed beyond the pleura toward the sternum without touching the heart, and the pleura and intercostal muscles were dissected with a battery driven cauterizer (FST, Heidelberg, Germany). Special care was exercised to avoid any bleeding. Using the same technique, the thoracotomy was extended to the mid axillary line. After cutting the third rib at its sternal margin and inserting a rib retractor (Noras, Würzburg, Germany), the intercostal space was widened until the whole heart was visible from base to apex. With two small artery forceps the pericardium was opened, and a pericardial cradle was formed to move the heart slightly anterior.

Identification of the left anterior descending (LAD) artery is probably the most crucial step of the surgical preparation because coronary arteries are located within the myocardium and do not run on the epicardial surface in mice. As the color of coronary arteries is almost identical to the color of surrounding myocardium, identification of LAD without manipulation is difficult. If it was not possible to identify the LAD using two additional tangentially directed light sources, soft pressure was applied to the apex with a 37°C warm sponge–armored forceps. That maneuver induced slight paleness of the myocardium and increased the tissue's contrast to the perfused and bright-red LAD. After definite identification of the LAD, a 6-0 silk suture with a tapered needle was passed beyond the LAD. The site of ligation of the LAD lies just caudal of the tip of the left auricle, about one-quarter of the line running from the atrioventricular crest to the apex of the left ventricle. When the needle is advanced beyond the vessel, mild pulling of the needle anteriorly while pushing the apex back with the sponge helps to ensure that the needle indeed passed beyond and around the LAD because this maneuver demarcates the LAD against the now-pale myocardium. However, care must be exercised to avoid occlusion of the LAD during the maneuver because that might have an influence on IS.

Potential pitfalls and remarks concerning problems occurring frequently during surgical procedures are presented in Appendix 2.

The murine *in vivo* model of acute myocardial infarction described in the current study can easily be modified to permit postoperative recovery and REP times longer than 24 hrs. For that purpose, several steps of the surgical procedure should be adapted:

Precautions to ensure sterile working conditions should be considered.

To avoid bleeding and neurologic disturbances in the postoperative period, invasive measurement of arterial blood pressure must not be performed. However, to exclude variations of arterial blood pressure that might have an impact on myocardial IS, we recommend monitoring mean arterial blood pressure noninvasively, for example, *via* the cuff-tail method.

To maintain the ability to move the left anterior limb in the postoperative period, pectoralis muscles must not be dissected; 5-0 prolene sutures might be advanced through the major and minor pectoralis muscles and serve to pull the muscles medially and laterally, respectively. That maneuver results in a thoracic area tall enough to perform the fourth thoracotomy.

The silicone tube should be removed, and the 6-0 silk LAD ligature should be cut approximately 4 cm from the LAD after CAO. The thoracotomy is then closed by a 5-0 prolene suture, and the ends of the 6–0 ligature are placed beyond the major pectoralis muscle. After closure of the skin with a 5-0 prolene suture and detachment of the ECG and limb tapes, intrathoracic air can be extruded by careful digital compression of the thorax. The overlaying pectoralis muscles will seal the thoracotomy and prevent pneumothorax during the postoperative period. When the mouse recovers from anesthesia and resumes sufficient reflexes and spontaneous breathing, the endotracheal tube is removed. If necessary, antibiotics or analgesics can be applied ip after the closure of the skin.

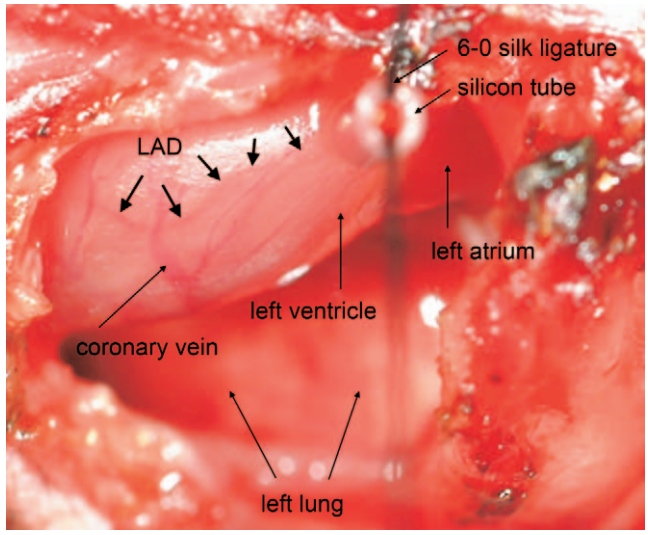

Figure 1. Anatomy of mouse heart and coronary vasculature. LAD (bold arrows) is located within the myocardium underneath coronary veins. The LAD is occluded by a 6-0 silk suture passed beyond the LAD and threaded through a 2-mm–long silicon tube. Arrows indicate left atrium, ventricle, coronary vein and left lung as depicted. Color figure available in the online version.

CAO and REP. CAO was achieved using the hanging-weight system as previously described (5). Both ends of the 6-0 silk ligature were moved through a 2-mm–long piece of silicon PE-10 tube. The silk suture was then directed over two horizontally mounted, movable, metal rods, and masses of 1 g each were attached to both ends of the suture. By elevation of the rods, the masses are suspended, and the occlusion of the LAD is instituted with a defined and constant pressure (Fig. 1). LAD occlusion was verified by paleness of the area at risk (AAR), the color of the LAD turning from bright-red to violet, indicating ceased blood flow, and ST-segment elevation in ECG (Fig. 2). REP is achieved by lowering the rods until the masses lie on the operating pad, and the tension of the ligature is relieved. REP was verified by the same three criteria used to verify occlusion, that is, change of LAD color from violet to bright red, return of red color in the AAR, and disappearance of the ST-segment elevation (Fig. 2). Mice were excluded from further analysis if all three criteria were not met at either the start of CAO or within 15 mins of REP, respectively.

During CAO, temperature and humidity of the heart surface were maintained by covering the heart with the pectoralis minor muscle flap and by sealing the thoracotomy with a 0.9% normal saline-wet sponge.

Experimental Protocols. After completion of instrumentation and surgery, mice were allowed a 15-min

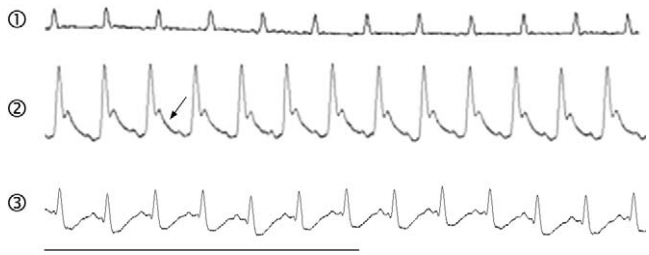

Figure 2. Representative recording of three-lead needle-probe ECG. Upper trace (1): ECG under baseline conditions. Middle trace (2): After 5 mins CAO, a marked ST elevation (arrow) develops. Lower trace (3): ST elevation disappears after 15 mins reperfusion and T wave inversion develops. Bar, 1 sec.

equilibration period. Mice were then randomly assigned to receive 30, 45, or 60 mins of CAO with either 120, 180, or 240 mins of REP time, respectively.

Measurement of Myocardial IS. IS and AAR were determined gravito-planimetrically as described elsewhere (1). After intravenous (iv) injection of 500 IU heparin, the LAD was reoccluded, and 1 ml Evans blue (0.1 g/ml; Sigma-Aldrich, Taufkirchen, Germany) was slowly injected into the carotid artery to delineate AAR. This causes dye to enter the nonischemic region (normal zone [NZ]) of the left ventricle and leaves the ischemic AAR unstained. After mice had been euthanized with a lethal dose of pentobarbital (150 mg/kg ip), the heart was rapidly removed, and excess blue dye and blood were rinsed off. The left ventricle was dissected, and both atria and the right ventricle were removed. The left ventricle was cooled at –20°C for 30 mins and cut into eight transverse slices of 1-mm thickness each. To cut slices exactly, an acrylic heart matrix (Aster Industries, McCandles, PA), with nine parallel, commercially available razor blades, was used. All slices were incubated at 37°C for 25 mins with 2% 2,3,5-triphenyltetrazolium chloride (Sigma Aldrich, Taufkirchen, Germany) dissolved in 0.1 *M* Na_2HPO_4/NaH_2PO_4 buffer adjusted to pH 7.4. Slices were fixed overnight in 3.5% formaldehyde (Fischar, Saarbrücken, Germany). Slices were weighed and placed between two cover slips. To avoid air bubbles, the space between both cover slips was filled with 0.9% normal saline. Both sides of each slice were digitally photographed using a high-resolution digital camera (Finepix S3 Pro, Fujifilm, Tokyo, Japan). The digital photographs were then analyzed with picture-analysis software (Adobe Photoshop CS 8.0.1; Adobe Systems Inc., San Jose, CA) run on a personal computer (Fujitsu Siemens, Augsburg, Germany), and size of infarcted area (pale), area at risk (red) and normal zone (blue) were outlined in each section by identification of their color appearance and color borders (Fig. 3). The areas were measured by planimetry (Adobe Photoshop CS 8.0.1). Areas were quantified on both sides of each slice and averaged by an investigator blinded to the treatment protocol. The resulting fractions of IS, AAR, and NZ of each slice were then multiplied by the weight of that slice. IS was calculated by the following formula: IS = weight of infarct area/weight of area at risk × 100 (IS = IA/AAR × 100). Weight of infarction was analyzed by the following formula: weight of infarction = $(IA_1 \times WT_1) + \ldots + (IA_8 \times WT_8)$, where IA is the percentage area of infarction by planimetry from subscripted numbers 1–8, representing slices, and WT is the weight of the same numbered slices. Weight of AAR was analyzed in a similar fashion: weight of AAR = $(AAR_1 \times WT_1) + \ldots + (AAR_8 \times WT_8)$. To measure reliability of this method of IS analysis, interobserver variability was tested. IS of animals receiving 45 mins CAO and 180 mins REP were assessed by two independent investigators both blinded to the experimental protocol. A total of 53 slices of 8 animals were evaluated. In 24 slices of 5 animals, IS was measured twice on two separate days by the same investigator to reveal intraobserver variability.

Histologic Analysis. To selectively stain polymorphonuclear (PMN) leukocytes in the myocardium, mice were instrumented as described above and were assigned to receive 45 mins CAO and 120 or 240 mins REP, respectively. After REP, hearts were harvested and cut into four slices. Those slices were immediately embedded in paraffin, and sections of 1-μm thickness were cut from three different planes of each paraffin block. Thus, 12 sections were made from each heart. The tissue specimens were then stained using the Naphthol AS-D Chloroacetate Kit (Sigma-Aldrich, Taufkirchen, Germany, Catalog 91C). Naphthol AS-D Chloroacetate is hydrolyzed by an esterase specific for PMN leukocytes (11) and reacts with a diazonium salt to result in a stable red compound. Thus, PMN leukocytes appear red. After staining, sections were digitally photographed, and PMN leukocytes were counted.

Data Acquisition and Statistical Analysis. Hemodynamic parameters, ECG and body temperature were continuously recorded at a sampling rate of 1000 Hz and analyzed on a personal computer (Fujitsu Siemens, Augsburg, Germany) using hemodynamic data acquisition and analysis software (Notocord hem 3.5, Croissy sur Seine, France).

Statistical analysis of data within and among groups was performed with one-way and two-way analysis of variance (ANOVA) followed by *post hoc* Duncan test using Statmost software (Dataxiom Software Inc., Los Angeles, CA). Interrater correlation was calculated with Pearson's correlation, and analysis of PMN leukocytes was performed with Student's *t* test using the same statistical software. Changes were considered statistically significant if $P < 0.05$. All data are expressed as mean ± standard error of the mean (SEM).

Results

A total of 72 mice were used for this investigation. Nine mice were used to perform arterial blood gas analysis either before endotracheal intubation or after completion of the surgical procedure. Of 55 mice assigned to I/R protocols, 4 died during surgical procedures because of bradycardia or

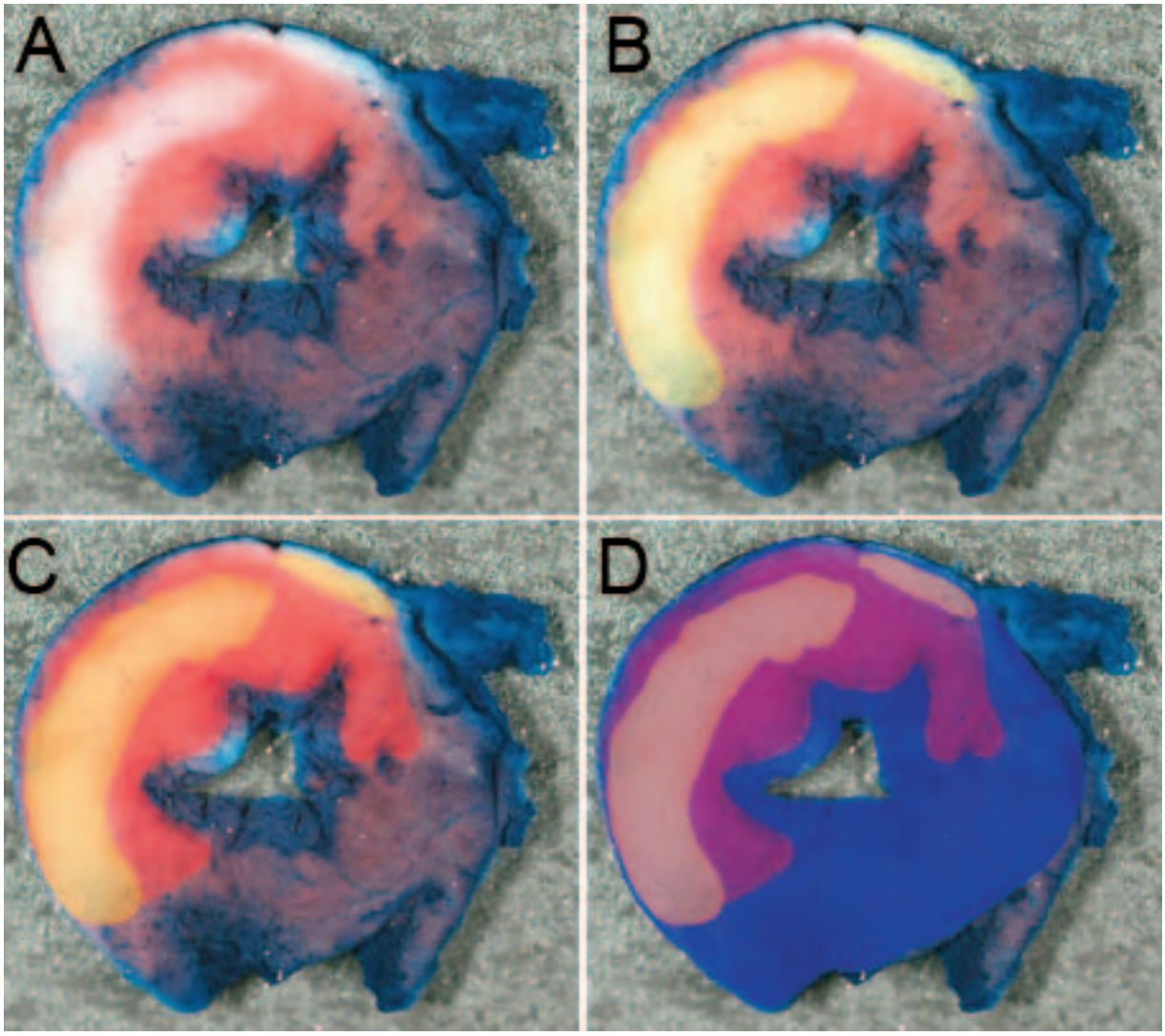

Figure 3. Planimetry of myocardial infarct size. (A) Digital photographs of a midventricular slice after Evans blue and triphenyl tetrazolium chloride (TTC) staining. Infarcted areas appear pale, viable myocardium within the area at risk is stained brick red. Note false color planimetry of (B) infarcted area (yellow), (C) area at risk (red), and (D) total area of the left ventricle (blue). Color figure available in the online version.

hypotension, and 11 were excluded from the study because of technical problems ($n = 5$) or lack of reversal of ST elevations during REP ($n = 6$). Eight mice were used for histologic staining of PMN leukocytes.

Blood Gas Analysis and Hemodynamics. In four mice, blood gas analysis was performed after anesthesia, right before endotracheal intubation, and in five animals, it was done after completion of surgery, right before CAO. At the end of the experimental protocol, blood gas samples were obtained in 37 animals. Arterial pH, pO_2 and pCO_2 were kept within physiologic ranges at all time points (Table 1). There were no significant differences in hemodynamics among experimental groups at baseline (Table 2).

Electrocardiography. LAD occlusion resulted in a marked ST elevation that disappeared within 15 mins of REP (Fig. 2). REP was regularly accompanied by T wave inversion; in some mice Q waves were observed.

Influence of Duration of CAO and REP Time on IS. AAR was not significantly different among groups (Table 3). IS increased significantly with duration of CAO, independent of REP time (Fig. 4A). Prolongation of CAO from 30 to 60 mins increased IS at 120 mins REP, from 18% ± 5% to 69% ± 3%; at 180 mins REP, from 20% ± 2% to 69% ± 6%; and at 240 mins REP, from 42% ± 10% to 75% ± 2%.

After 60 mins CAO, duration of REP had no significant influence on myocardial IS. IS was 69% ± 3%, 69% ± 6%, and 75% ± 2% after 120, 180, and 240 mins REP, respectively (Fig. 4B). However, when the ischemic stimulus was lowered to 30 and 45 mins CAO, IS was increased by prolongation of REP time. After 30 mins CAO, IS was 18% ± 5% and 20% ± 2% (at 120 and 180 mins REP) and was increased to 42% ± 10% after 240 mins REP. Similarly, after 45 mins CAO, IS was 40% ± 3% and 46% ± 6% after

Table 1. Blood Gas Analysis[a]

	Intubation	Surgery	Reperfusion
	($n = 4$)	($n = 5$)	($n = 37$)
F_iO_2	0.21	0.5	0.5
pH	7.38 ± 0.01	7.43 ± 0.05	7.36 ± 0.08
pCO_2 (mmHg)	44.4 ± 3.23	29.22 ± 5.18	29.00 ± 2.87
pO_2 (mmHg)	85.78 ± 6.05	232.54 ± 52.4*	208.28 ± 10.36*

[a] Blood gas analysis was performed at intubation, after completion of surgery, and at the end of the experimental protocol (REP). Data are mean ± SEM.
* $P < 0.05$, different from intubation.

120 and 180 mins REP, respectively, and was significantly increased to 72% ± 6% after 240 mins REP.

Reliability of IS Measurement. The interobserver ratio (R^2) was 0.937 ($P < 0.05$) for planimetry of AAR and 0.927 ($P < 0.05$) for planimetry of IS. The intraobserver ratio (R^2) was 0.984 ($P < 0.05$) for AAR and 0.858 ($P < 0.05$) for IS.

Histologic Analysis. Eight hearts were stained for PMN leukocytes using naphthol AS-D chloroacetate-specific esterase staining. At 45 mins CAO and 120 mins REP, a total of 37 ± 2 ($n = 4$) PMN leukocytes was detectable in 12 slices harvested from each heart. Prolongation of REP duration to 240 mins resulted in a significant ($P < 0.05$) increase of PMN leukocytes to 67 ± 3 ($n = 4$) per heart (Fig. 5).

Table 2. Systemic Hemodynamic Parameters[a]

			Reperfusion (mins)			
Parameters	BL	CAO	60	120	180	240
HR ($mins^{-1}$)						
30′CAO/120′REP	445 ± 11	474 ± 13	465 ± 16	453 ± 18	—	—
45′CAO/120′REP	404 ± 25	412 ± 11	409 ± 6	402 ± 19	—	—
60′CAO/120′REP	412 ± 10	518 ± 28	421 ± 22	437 ± 26	—	—
30′CAO/180′REP	419 ± 22	375 ± 13**	391 ± 13	382 ± 13	391 ± 13	—
45′CAO/180′REP	450 ± 8	461 ± 16	423 ± 24	419 ± 18	401 ± 22	—
60′CAO/180′REP	398 ± 10	432 ± 35****	422 ± 33	403 ± 10	433 ± 18	—
30′CAO/240′REP	431 ± 36	438 ± 41	443 ± 35	410 ± 17	399 ± 27	426 ± 44
45′CAO/240′REP	425 ± 15	480 ± 10	503 ± 16	476 ± 39	460 ± 32	428 ± 34
60′CAO/240′REP	454 ± 14	481 ± 33	416 ± 19	410 ± 5	430 ± 6	446 ± 10
MAP (mmHg)						
30′CAO/120′REP	62.8 ± 3.7	62.2 ± 1.5	61.0 ± 1.6	63.5 ± 4.8	—	—
45′CAO/120′REP	59.8 ± 3.1	62.5 ± 1.6	65.6 ± 1.1	59.2 ± 0.8	—	—
60′CAO/120′REP	65.6 ± 2.6	62.4 ± 1.4	64.7 ± 1.9	63.6 ± 3.1	—	—
30′CAO/180′REP	57.9 ± 1.6	58.4 ± 4.0	67.0 ± 2.8	56.9 ± 3.5	63.7 ± 2.6	—
45′CAO/180′REP	67.5 ± 3.0	62.0 ± 4.4	54.5 ± 2.5*	47.2 ± 2.7*	51.2 ± 6.4*	—
60′CAO/180′REP	59.7 ± 3.7	52.0 ± 5.0	54.9 ± 4.7	52.4 ± 4.4	56.2 ± 4.7	—
30′CAO/240′REP	65.6 ± 9.8	66.6 ± 9.5	68.4 ± 8.4	57.1 ± 8.1	52.8 ± 6.8	53.2 ± 8.3
45′CAO/240′REP	74.3 ± 2.1	65.8 ± 4.4	68.6 ± 4.8	77.2 ± 3.5***	69.1 ± 3.5	73.3 ± 5.7
60′CAO/240′REP	79.3 ± 6.8	77.7 ± 9.9	68.2 ± 7.4*	63.7 ± 7.0*	66.3 ± 7.6*	67.5 ± 8.4*
RPP ($mins^{-1}$·mmHg·10^{-3})						
30′CAO/120′REP	27.8 ± 1.0	29.5 ± 1.4	28.4 ± 1.4	28.8 ± 2.5	—	—
45′CAO/120′REP	24.1 ± 1.3	25.8 ± 2.4	26.8 ± 0.7	23.8 ± 1.4	—	—
60′CAO/120′REP	26.9 ± 0.8	32.3 ± 1.7*	27.3 ± 1.8	27.7 ± 1.7	—	—
30′CAO/180′REP	24.2 ± 0.9	21.8 ± 1.3	26.2 ± 1.9	21.9 ± 2.1	25.0 ± 1.8	—
45′CAO/180′REP	30.3 ± 1.4	29.0 ± 2.9	23.2 ± 2.0*	19.9 ± 1.7*	19.6 ± 1.8*	—
60′CAO/180′REP	23.1 ± 1.8	22.7 ± 3.0	23.5 ± 3.5	21.1 ± 1.8	24.4 ± 2.4	—
30′CAO/240′REP	29.3 ± 6.4	30.1 ± 6.2	30.9 ± 5.5	23.8 ± 4.2	21.5 ± 4.0	23.3 ± 5.0
45′CAO/240′REP	31.5 ± 1.2	31.7 ± 2.8	34.5 ± 2.7	36.5 ± 2.7***	32.1 ± 3.9	31.6 ± 4.1
60′CAO/240′REP	35.9 ± 3.3	38.3 ± 7.6	28.7 ± 4.4	26.1 ± 2.7*	28.4 ± 2.9	30.3 ± 4.2

[a] Data acquisition was performed at the end of baseline (BL), at the end of coronary artery occlusion (CAO), after 60 mins (60), 120 mins (120), 180 mins (180), and 240 mins (240) of REP. Data are mean ± SEM. Abbreviations: HR, heart rate; MAP, mean arterial blood pressure; RPP, rate-pressure product; 30′CAO/120′REP, 30 mins CAO and 120 mins REP; 45′CAO/120′REP, 45 mins CAO and 120 mins REP, etc.
* $P < 0.05$, different from baseline; ** $P < 0.05$, different from 30′CAO/120′REP; *** $P < 0.05$, different from 45′CAO/120′REP; **** $P < 0.05$, different from 60′CAO/120′REP.

Table 3. Area at Risk (% Left Ventricle)[a]

	Reperfusion (mins)		
CAO	120	180	240
30 mins	32.6 ± 5.6 ($n = 4$)	38.9 ± 12.6 ($n = 4$)	27.4 ± 6.4 ($n = 4$)
45 mins	31.2 ± 3.4 ($n = 3$)	30.9 ± 5.3 ($n = 8$)	30.8 ± 6.1 ($n = 4$)
60 mins	25.9 ± 3.2 ($n = 4$)	36.1 ± 3.6 ($n = 5$)	31.5 ± 6.1 ($n = 4$)

[a] Data are mean ± SEM. CAO, coronary artery occlusion.
* $P < 0.05$, different from 30 mins CAO/120 mins REP.

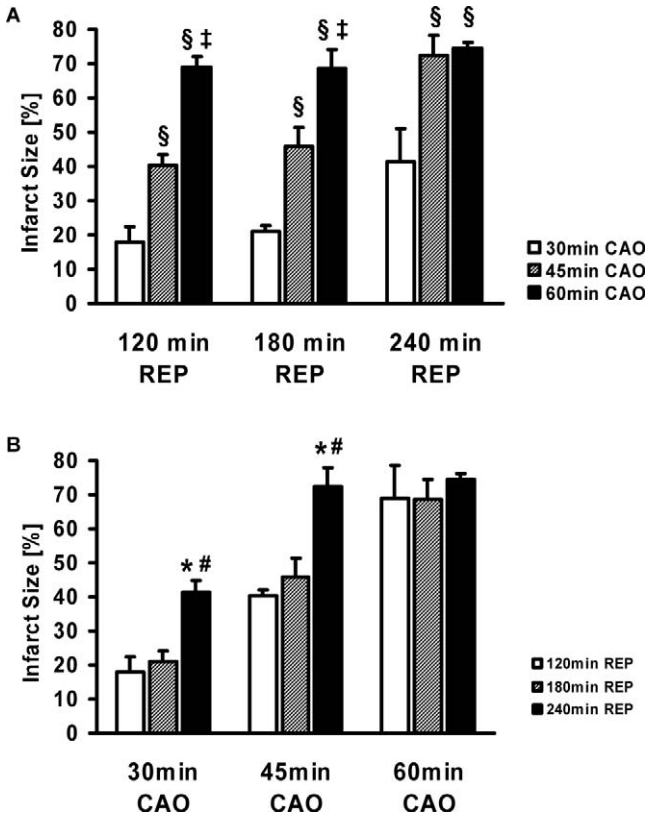

Figure 4. Infarct size as a percentage of the area at risk at various durations of ischemia and reperfusion. Myocardial IS increases with prolongation (A) of CAO or (B) REP. § Significantly ($P < 0.05$) different from 30 mins CAO. ‡ Significantly ($P < 0.05$) different from 45 mins CAO. * Significantly ($P < 0.05$) different from 120 mins REP. # Significantly ($P < 0.05$) different from 180 mins REP.

Discussion

The murine model of myocardial ischemia and REP was first described by Michael *et al*. (1) in 1995. Because of its difficulty and capriciousness, the murine *in vivo* myocardial infarction model is limited to a number of laboratories. This study describes methodological details of this model and investigated the impact of ischemia and REP duration on IS to foster propagation of this valuable model.

The method to occlude the coronary artery by a hanging weight system as recently published (5) provides a definite and easy method for representative CAO. In our opinion, this method is advantageous compared with tying a knot on the LAD because repetitive tightening and loosening of the knot, as needed with ischemic preconditioning protocols, can result in rupture of the myocardium or damage to the coronary artery. Moreover, it is critical that the knot is tightened enough; otherwise, coronary artery flow might ensue because of high-frequency movement of the heart. This is prevented by the hanging weight method as demonstrated in this investigation. With this method a definite and continuous pull of 1 g at both ends of the suture around the LAD throughout the CAO period is ensured. Thus, this method of CAO is highly reliable and reproducible. In fact, we confirm the reliability of the described murine model because all experiments of this study have been performed in a different laboratory and were conducted by a different experimenter compared with our previously published study (5). ISs were similar when equal durations of ischemia and REP were chosen by two different investigators in two different labs.

Analysis of IS by planimetry is hampered by sometimes blurred borders between infarcted and noninfarcted tissue. In preconditioned tissue, the border of the infarcted area and AAR appears as a pink-colored zone that cannot always be precisely assigned to either the infarcted (pale) or noninfarcted (red) zone. Nonetheless, the intra- and interobserver variability of > 0.85 that was found in the present study confirms that planimetric assessment of myocardial IS is accurate. Because of the measured high interobserver reliability of > 0.92 in the present study, IS measurement can be simplified to a single measurement by one investigator. As a result, a duplicate measurement by two independent investigators is not needed to obtain reproducible results as has been suggested before (2).

To our knowledge, this is the first systematic study to investigate the mutual impact of duration of ischemia and REP time on myocardial IS in mice *in vivo*. Because of a high variety of experimental protocols, reported data from different laboratories cannot be compared directly. In fact, IS after 20 mins CAO was reported to be 21% (12) or 33% (13, 14) at 30 mins CAO. IS depends on the duration of CAO in mice (1,6). Recently, a sigmoid relationship between CAO duration and myocardial IS was demonstrated (7). ISs were 7%, 37%, 58%, and 65% after 20, 30, 45, and 60 mins CAO, respectively (7). The data reported in the present study are in line with these and our previously published (5) results: prolongation of CAO from 30 to 45 and 60 mins resulted in IS of 18%, 40%, and 69% at 120 mins REP; 20%, 46%, and 69% at 180 mins REP; and 42%, 72%, and 75% at 240 mins REP, respectively.

Furthermore, we investigated the influence of REP time exceeding 120 mins after submaximum (30 and 45 mins) and maximum (60 mins) myocardial ischemia. Prolongation of REP time from 120 to 240 mins increased IS from 18% to 42% (30 mins CAO) and from 40% to 72% (45 mins CAO) but not after 60 mins CAO (69% vs. 75%). Thus, at 60 mins

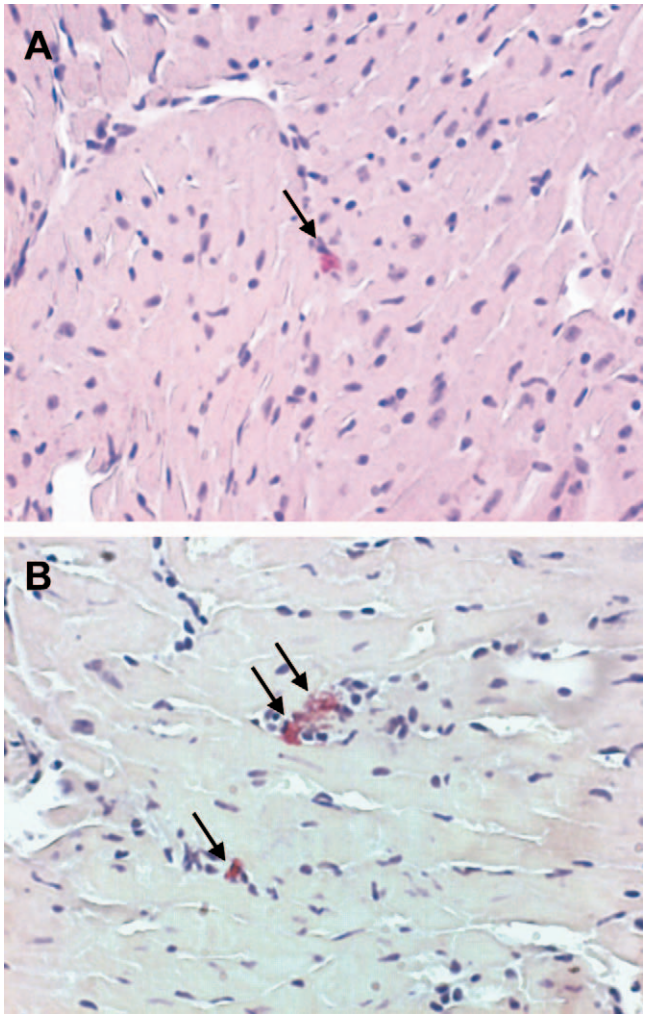

Figure 5. Staining of polymorphonuclear neutrophils. Representative slices of mid ventricular sections after incubation with naphthol AS-D chloroacetate (×200). PMN leukocytes are stained bright red (arrows). After 45 mins CAO and (B) 240 mins REP, significantly more PMN leukocytes are detectable in the myocardium compared with (A) 120 mins REP. Color figure available in the online version.

CAO, REP time has no impact on myocardial IS. However, when duration of CAO was lowered to submaximum levels (30 and 45 mins), duration of REP had a significant impact on myocardial IS.

The finding that myocardial infarction increases with prolongation of REP time is controversial, but a series of studies published previously (6, 15) is in accordance with this finding. Our results support the concept of REP injury indicating that REP of ischemic tissue introduces additional lethal injury that is not present at the end of ischemia. Mechanisms underlying REP injury have been investigated intensively during the past decade and include release of oxygen-derived free radicals, endothelial and microvascular dysfunction, metabolic dysfunction, osmotic overload, contractile dysfunction, dysrhythmias, necrotic and apoptotic cellular death, and an inflammatory reaction involving influx of PMN leukocytes and other populations of immune cells (15–17). Incipient inflammatory reaction by infiltration of PMN leukocytes into the infarcted area might have increased IS at 240 mins REP compared with 120 mins REP. Indeed, the number of PMN leukocytes was significantly increased at 240 mins REP compared with 120 mins REP at 45 mins CAO as evidenced by naphthol AS-D chloroacetate esterase staining (Fig. 5). Thus, incipient inflammatory processes as evidenced by PMN leukocyte accumulation might, at least in part, be responsible for the observed increase in IS within the first 4 hrs of REP.

There is evidence that duration of myocardial ischemia is related to the rate of necrosis and that duration of REP is related to the amount of apoptosis (18). Although the exact relative contribution of apoptosis and necrosis to I/R injury and their respective time course remain to be determined (19), it has been suggested that apoptosis commences during ischemia and is completed during REP (20–23). Sixty minutes of CAO in mice might result mainly in necrotic cell death, whereas 30 and 45 mins CAO result not only in necrotic, but also apoptotic, cell death. Thus, the enlargement of IS with increasing duration of REP after 30 and 45 mins of CAO as seen in this study may, at least in part, be due to increased apoptotic cell death. However, this intriguing suggestion needs further investigation to be verified.

The area of the left ventricle at risk for infarction is an important determinant of the extent of myocardial infarction (24), potentially limiting the results of the current investigation. However, no differences in the size of the AAR accounting for the current findings were observed among experimental groups.

In conclusion, when CAO times below 60 mins are chosen, myocardial IS is dependent on both ischemia and REP time in the murine *in vivo* model of acute myocardial infarction.

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Appendix 1: Specification of Surgical Instruments

Instrument	Specification	Purpose	FST product number[a]
Graefe forceps	Curved, serrated, blunt tip, 10 cm	All dissecting procedures	11151–10
Iris scissors	Straight blades, 11 cm	Skin incisions, post mortem dissection of the heart	14090–11
Spring scissors	Straight blades, 8 cm	Incision of carotid artery Post mortem dissection of the heart	15000–10
Dumont forceps	Standard tip, Inox, 11 cm 0.10 × 0.06 mm	Inserting and advancing PE-10 catheter[b]	11251–20
Battery operated small vessel cauterizer	Angled tip	Thoracotomy	18000–00, 18000–02
Rib retractor	2 × 3 sharp teeth, 3 cm, maximum spread 1.8 cm	During dissection of the heart	17003–03
Bulldog Type Serrefine	Straight, 35 mm	Fixing the pericardium to form a cradle	18050–35
Spring needle holder	With lock, 14 cm	6-0 coronary artery ligature	12565–14
Halsted-Mosquito	Curved tip, 12.5 cm	Laryngoscopy, Ligature of carotid artery	13009–12
Halsted-Mosquito	Angled tip, 12.5 cm	Ligature of carotid artery, fixing sponge for heart manipulation	13008–12

[a] All surgical instruments used in the current study were purchased from FST (Heidelberg, Germany). A detailed description of each instrument can be found *via* the product number at www.finescience.com.
[b] A self-made, cut, curved, and smoothed G12 cannula mounted on a 1-ml syringe was used to open the lumen of the carotid artery to ease the insertion of the PE-10 catheter.

Appendix 2: Troubleshooting

Key method	Potential pitfall	Prevention/troubleshooting
Endotracheal intubation	I can't visualize the glottis.	Make sure that your eyes are in line with the pharyngeal axis. Make sure that transtracheal illumination is bright enough. Make sure the tongue is retracted far enough. Slightly withdraw or advance tip of the Halestep Mosquito. Incline the Mosquito and slightly lift its tip.
	I can visualize but not intubate the glottis.	Make sure that the tip of the cannula's mandrin does not exceed the cannula's tip. Use the curvature of the epiglottis as a tunnel to guide the tip of the endotracheal tube. Advance the tube with its tip directed to the anterior wall of the trachea.
Intubation of carotid artery	After dissection and ligature of the carotid artery, the vessel is collapsed and empty of blood.	Tighten first the knot of the distal ligature, then tense the loop of the proximal ligature.
	The vessel tears off during cannulation of its lumen.	Lessen tension on the vessel. Minimize the size of the cut of the vessel.
	I can insert but not advance the PE-10 catheter.	Enlarge the cut of the vessel and make sure not to dissect the carotid wall. Pull the catheter's tip over a flame to give it a tapered shape. Lessen the tension of the proximal ligature's loop.
	After insertion of the PE-10 catheter no blood pressure signal occurs.	Make sure that no air is captured in the catheter. Make sure that the catheter's tip is not obstructed by the vessel's wall and carefully withdraw the catheter.
Thoracotomy	The lung or the heart is injured after thoracotomy.	Choose the point just medial to the lung's medial margin for primary access to the thoracic cave and then advance the tweezers tangentially right beyond the pleura.
	Bleeding occurs during thoracotomy.	Avoid touching the axillary vein when dissecting the pectoralis muscle. Avoid touching the intercostal vessels in performing the thoracotomy right above the cranial margin of the rib. First visualize, then cauterize the Arteria mammaria interna.
	I can't identify the fourth ICR.	4th ICR is identified by a small vessel nourishing the minor pectoralis muscle originating in the 4th ICR and by the gap between the upper and lower lobe of the left lung visible through the 4th ICR.
Ligature of LAD	I can't identify the LAD.	Use additional light sources directed tangentially to the heart. Make sure that arterial blood pressure is $>$60 mmHg and that arterial blood is well saturated. Identify the small vein originating at the caudal margin of the right atrium's auricle. The LAD most often runs parallel and laterally to this vein. Carefully apply soft pressure to the apex with a wet, warm sponge. This causes paleness of the myocardium and leaves the LAD bright red.
	Bleeding occurs after ligature.	Make sure not to penetrate the myocardium when advancing the needle.