IN VIVO STUDIES OF CARDIOPROTECTION INDUCED BY VOLATILE ANAESTHETICS - EXPERIMENTAL MODEL IN RODENTS

Ordodi V. 2, G. Gheorghiu 2, C. Henția 2, Nicoleta Mirică 1, D. Bărglăzan 1, Andreea Răducan 1, C. Macarie 2, M. Păpurică 2, O. Bedreaș 2, F. Mic 3, V. Păunescu 3, D. Sândesc 2, Danina Muntean 1

“Victor Babes” University of Medicine and Pharmacy Timișoara
1 Department of Pathophysiology, 2 Department of Anaesthesiology and Intensive Care, 3 County Hospital of Timișoara, Romania
2 Eftimie Murgu Sq., 300041, Timișoara, Romania, daninamuntean@umft.ro

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Abstract: Myocardial ischemic/reperfusion (I/R) injury remains a major public health burden and cardioprotection defined as the totality of both non-pharmacological and pharmacological interventions aimed at preventing the reperfusion injury; still represents a stringent necessity. There is accumulating evidence that volatile anaesthetics induce myocardial preconditioning when given prior to ischemia and they also protect the heart at the very onset of reperfusion; an effect referred to as anaesthetic postconditioning. The aims of the present study were: (i) to develop and validate an experimental model of regional myocardial I/R injury in rat hearts in situ and (ii) to investigate in this model the protective effects of cardioprotection elicited by anesthetic pre- and postconditioning with Isoflurane and Sevoflurane. METHODS: Anaesthetized rats (n = 6-8/group) subjected to 30 min ischemia by coronary artery ligation and 2 h reperfusion were randomly assigned to one of the following groups: 1) Control group (Ctrl; no other intervention); 2) Ischemic preconditioning (I-PreC; preconditioning by three episodes of 5 min ischemia interspersed with three episodes of 10 min reperfusion); 3) Isoflurane preconditioning (Iso-PreC; preconditioning by three episodes of 5 min isoflurane 2.1% interspersed with three 10 min washout episodes); 4) Isoflurane postconditioning (Iso-PostC; 5 min postconditioning with isoflurane 2.1% started 3 min prior to and administered for 2 min within reperfusion); 5) Sevoflurane preconditioning (Sevo-PreC; preconditioning by two episodes of 5 min sevoflurane 2.5% interspersed with two 10 min washout episodes) and 6) Sevoflurane postconditioning (Sevo-PostC; 5 min postconditioning with sevoflurane 2.5% started 3 min prior to and administered for 2 min within reperfusion). RESULTS: Myocardial injury expressed as the percent of infarct to risk area ratio (I/R%) was significantly reduced in I-PreC (14.3 ± 6.8%) and Iso-PreC (17 ± 5.1%) and Sevo-PreC (19.5±8.5%) when compared to the Ctrl group (46.4±6.5%; mean ± SD; P<0.01); but no protection was found with the postconditioning protocols. CONCLUSIONS: We have validated an experimental model of regional ischemia that can be used in studies of in vivo cardioprotection. In this model anesthetic preconditioning but not postconditioning was associated with infarct size reduction.

INTRODUCTION

Myocardial ischemia/reperfusion (I/R) injury represents a major public health burden; mainly associated with acute coronary syndromes and cardiac surgery. Timely coronary reperfusion by either thrombolysis or primary coronary artery angioplasty has nowadays become the established routine therapy which effectively decreases infarct size and reduces mortality. Despite the unequivocal beneficial effects in stopping the progression of irreversible damage; reperfusion is considered a double-edged sword (Braunwald & Kloner; 1985; Skyschally et al; 2008) as it can itself induce severe myocardial lesions which paradoxically alleviate the
beneficial effects of revascularization. Accordingly; adjunct mechanical and/or pharmacological strategies aimed at limiting cardiomyocyte death beyond reperfusion therapies still represent a priority for both basic and clinical cardiovascular research.

**Ischemic preconditioning (IPreC) and postconditioning (IPostC)** are two mechanical strategies currently investigated; as major endogenous mechanisms of protection of the ischemic heart. IPreC was discovered more than two decades ago (Murry et al; 1986) and describes the ability of short periods of non-lethal ischemia alternated with reperfusion to protect the heart when applied **before** a subsequent prolonged lethal ischemia. The more recently described phenomenon of myocardial IPostC (Zhao et al; 2003); defined as a series of brief mechanical coronary artery occlusions and reperusions applied **at the very onset** of post-ischemic reperfusion; is able to elicit protection similar to IPreC. Anesthetic preconditioning (APreC) and postconditioning (APostC) refer to cardioprotection triggered by volatile anesthetics administered in either setting according to different protocols which is considered clinically more relevant than the ischemia induced one (Weber & Schlack; 2008).

The aims of the present study were: (i) to develop and validate an experimental model of regional myocardial I/R injury in rat hearts **in situ** and (ii) to investigate in this model the protective effects of cardioprotection elicited by anesthetic pre- and postconditioning as compared to cardioprotection induced by the most powerful cardioprotective mechanism against I/R injury; ischemic preconditioning.

**MATERIAL AND METHODS**

The experimental procedures and protocols used in this study were conducted in accordance with the institutional and national Guide for the Care and Use of Laboratory Animals. The study was carried on adult rats fed ad libitum and housed at a 12 h light/dark cycle.

**Animal Preparation.** Male Sprague–Dawley rats (300-400 g) were anaesthetized with xylazine (5 mg/kg) and ketamine (75 mg/kg) intraperitoneally. The rats were intubated and ventilated with a custom made rodent pressure controlled ventilator (inspiratory oxygen concentration > 90%; respiration rate 80-85 strokes/min) to maintain blood PO$_2$; PCO$_2$ and pH in the normal physiological range (AVL 995 blood gas analyzer). The right jugular vein was cannulated for administration of additional anesthetics (xylazine 1/3 and ketamine 2/3) as appropriate. The left carotid artery was dissected free for the insertion of a fluid-filled catheter connected to a pressure transducer (APT 300 Hugo Sachs Elektronik-Harvard Apparatus GmbH) to monitor arterial blood pressure. A standard limb lead II electrocardiogram (ECG) was recorded from subcutaneous limb leads. Both ECG and mean arterial blood pressure (MABP) were continuously recorded (Digidata 1440A; Axoscope 10 software; Molecular Devices Ltd). Rectal temperature was recorded via a precalibrated steel thermistor probe and core temperature was maintained at 37-38°C with the aid of a heating pad. Left-sided lateral thoracotomy was performed in the 4th intercostal space. The pericardium was opened and the heart was gently exteriorized. A 5-0 (prolene) suture was placed under the left coronary artery located between the base of pulmonary artery and the left atrial appendage. The ends of the suture were threaded through a propylene tube to form a snare. The coronary artery was occluded by tightening the ends of the suture by a pair of clamps. Ischemia was confirmed by epicardial cyanosis downstream of the occlusion. Reperfusion of the artery was initiated by loosening the snare and was confirmed by visualizing epicardial hyperemia. In the group of
sham-operated animals the ligature was left untied. Any animal that had a sustained fall of MABP below 70 mmHg and dysrhythmias was discarded.

At the end of the surgical preparation; after placing the ligature; rats were allowed to stabilize up to 45 min according to the experimental protocols (see below). All animals underwent 30 minutes of ischemia and 120 minutes of reperfusion. Coronary occlusion was confirmed by the hemodynamic response (typical fall in MABP); the ECG aspect (ST elevation) and the appearance of ventricular arrhythmias after the first 8 - 10 minutes of occlusion (Fig. 1).

![Figure 1. Example of non-sustained ventricular tachycardia and systematized premature ventricular beats (bigeminy) recorded in min 11 of ischemia in one animal from the Ctrl group (lower panel of ECG recording). A hemodynamic response (severe drop of arterial pressure) accompanies dysrhythmia (upper panel of blood pressure recording).](image)

Infarct Size Studies. At the end of the 2 h-reperfusion period; the animals were euthanized by an overdose of anesthetic. The heart was retrogradely washed in situ with 50 mL saline administrated via the carotid artery after performing a small cut in the right atrium. From our experience this step is quite important in obtaining a clear cut delineation of both areas a risk and of infarction during the subsequent staining procedures. The suture around the left coronary artery was reoccluded and Evans blue dye (Sigma Chemicals) was injected into the left ventricle via the left carotid artery cannula and allowed to diffuse for approximately 1 minute. The Evans blue solution (2% w/v) stains the non-ischaemic (non-risk) zone of the myocardium deep blue whilst the ischemic (risk) region of the occluded left coronary artery territory remains uncolored (red). This step identifies the area at risk as the red area of myocardium left unstained by the blue dye. Then the heart was excised; the right ventricle carefully removed; the left ventricle weighed and placed in a -20°C freezer. After at least an hour at -20°C the heart was removed from the freezer and transversely sectioned in 2 mm slices from apex to base. These slices were incubated in triphenyltetrazolium chloride (TTC) (Sigma Chemicals) solution (1% in phosphate buffer; pH 7.4) at 37°C for 25 minutes. During incubation the dehydrogenases in the viable tissue react with the TTC to give formazan; a brick-red pigment. By contrast; the infarcted areas remain white as the dehydrogenases are washed out of these areas during the reperfusion period. This step identifies the area of necrosis as the pale area of myocardium left unstained by the TTC. Hearts which failed to survive the 120 min of reperfusion were excluded from the study since a two-hour reperfusion period is necessary to allow adequate washout of the enzymes. After removal from the TTC
solution the slices were immersed for 24 h in 10% formaldehyde solution in order to delineate the infarcted areas more clearly while being pressed between two glass plates which were wedged 2mm apart and clipped together. The slices were scanned and area at risk and area of necrosis were determined using the Image J software. The *ratio of the area of necrosis to area at risk* defines *infarct size* and was expressed as percentage.

**Experimental protocols.** Animals (n = 6-8/group) were randomly assigned to one of the following groups (Fig. 2): 1) Control group (Ctrl; no other intervention); 2) Ischemic preconditioning (I-PreC; preconditioning by three episodes of 5 min ischemia interspersed with three episodes of 10 min reperfusion); 3) Isoflurane preconditioning (Iso-PreC; preconditioning by three episodes of 5 min isoflurane 2.1% interspersed with three 10 min washout episodes); 4) Isoflurane postconditioning (Iso-PostC; 5 min postconditioning with isoflurane 2.1% started 3 min prior to and administered for 2 min within reperfusion); 5) Sevoflurane preconditioning (Sevo-PreC; preconditioning by two episodes of 5 min sevoflurane 2.5% interspersed with two 10 min washout episodes) and 6) Sevoflurane postconditioning (Sevo-PostC; 5 min postconditioning with sevoflurane 2.5% started 3 min prior to and administered for 2 min within reperfusion).

![Figure 2. Outline of the experimental protocol.](image)

**Statistical Analysis.** Data are presented as means ± SD. Difference in the relationship between infarct size and area at risk were evaluated by one-way ANOVA and post hoc Tukey’s test (Graphpad Prism v 4.0 software). A *p* value < 0.05 was considered to be statistically significant.

**RESULTS AND DISCUSSIONS**

A total of 51 animals were instrumented to obtain 44 successful experiments. Two rats were excluded because of technical problems during instrumentation. Three animals died from intractable malignant arrhythmias during the prolonged ischemia and two rats failed to
complete the reperfusion period. No significant differences in hemodynamic parameters (MABP and HR) were observed among the groups at various time points (data not shown).

Figure 3. Myocardial infarct size displayed as percentage of the left ventricular area at risk in:
- **Ctrl** group (n=7);
- **I-PreC** (n=8);
- **Iso-PreC** (n=8);
- **Iso-PostC** (n=7);
- **Sevo-PreC** (n=8) and
- **Sevo-PostC** (n=6).

*P* < 0.01 for I-PreC; Iso-PreC; Sevo-PreC vs. Ctrl.

However; decreases in mean arterial blood pressure were observed during the second hour of reperfusion mainly in animals subjected to the anesthetic postconditioning protocols. Data concerning infarct size determination are summarized in Fig. 3.

The infarct size was 46.4 ± 6.5% in **Ctrl** group (n=7) and 14.3 ± 6.8% in **I-PreC** group (n=8; *p*<0.001) as expected since ischemic preconditioning is associated with a major antinecrotic effect in every tested species. Also the current results confirm previous findings in the in vivo rat hearts as regarding the protective effects of anesthetic preconditioning with both Isoflurane and Sevoflurane as infarcted to risk ratio in Iso-PreC (n=8) and Sevo-PreC (n=8) groups were 17.0 ± 5.2% and 19.5±8.5%; respectively (*p*<0.001 vs. **Ctrl**; *p* NS vs. **I-PreC**).

However; in our hands the 2 protocols of postconditioning with either Isoflurane or Sevoflurane were not able to elicit cardioprotection. The infarct size was significantly larger; 37.9±8.2% and 41.8±6.0% in Iso-PostC group (n=7); and Sevo-PostC group (n=6); respectively (*p* NS vs. **Ctrl**). These data are intriguing since postconditioning with Sevoflurane recapitulated a similar protocol/dose used by Obal et al. (2005) in the same experimental setting (in vivo rat hearts) whereas the protocol used with Isoflurane was reproduced in the in situ rabbit heart (Chiari et al.; 2005) and both papers reported cardioprotective effects. Isoflurane postconditioning; albeit given for a longer period during the postischemic reperfusion (15 min and 10 min); was also reported to protect isolated perfused rat hearts (Feng et al; 2005; Deyhimy et al.; 2007; respectively). However; there are conflicting results concerning the possibility to enhance myocardial protection by combining Sevoflurane pre- and postconditioning according to the experimental model used: additive cardioprotection in the in vivo rat hearts (Obal et al.; 2005) but no additional protection in isolated hearts (Deyhimy et al; 2007). The reason for the divergent results may be related to the different anesthetics used for the surgical preparation; mainly racemic ketamine in our study (vs. alpha-chloralose in the study by Obal et al.; 2005); which was reported to block cardioprotection triggered by experimental ischemic preconditioning in rabbits (Mullenheim; 2001). Even if IPC-related cardioprotection was not blunted in the present study; possible interferences with other anesthetics cannot be excluded. We are currently investigating the effects of different anesthetic regimens in the presence of anesthetic pre-and postconditioning protocols.
CONCLUSIONS

In conclusion; we have validated an experimental model that can be readily used in studies of in vivo cardioprotection. In this model anesthetic preconditioning; but not postconditioning; with either Isoflurane or Sevoflurane elicited a reduction in infarct size comparable to the one induced by ischemic preconditioning; the most powerful mechanism of endogenous protection.

Lethal reperfusion injury was proved to be a clinical reality; accordingly; both mechanistic and pharmacological research approaches in the field of reperfusion injury appear to be necessary and clinically relevant. However; before transferring the results obtained in a peculiar animal model to the clinical more complicated situation; a cautious outlook is warranted. Further elucidation of the mechanisms responsible for the anesthetic-related cardioprotection will certainly contribute to our understanding of their use in targeted clinical situations.

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