

Ischemic Postconditioning Inhibits Apoptosis after Acute Myocardial Infarction in Pigs

Haimei Sun,¹ Tao Guo,² Liu Liu,³ Zhuo Yu,² Wangbing Xu,¹ Wenhui Chen,⁵ Lijuan Shen,⁴ Jiaping Wang,⁶ Xingkui Dou⁷

Departments of ¹ICU, ²Cardiology, and ³Plastic, The First Affiliated Hospital of Kunming Medical College; ⁴Department of Pathology, Kunming Medical College; ⁵Key Laboratory of Molecular Biology for Sinomedicine, Yunnan University of Traditional Chinese Medicine; Departments of ⁶Radiology and ⁷Anesthesiology, The Second Affiliated Hospital of Kunming Medical College, Kunming, P. R. China



Dr. Sun

ABSTRACT

Objectives: Recent studies have shown that ischemic postconditioning reduces myocardial ischemia-reperfusion (I/R) injury; however, the effects of inhibiting apoptosis on cardioprotection induced by ischemic postconditioning remain to be determined. The objective of this study was to investigate whether ischemic postconditioning attenuates myocardial I/R injury by reduced apoptosis in a closed-chest pig model of acute myocardial infarction.

Methods: Diannan small-ear pigs were randomly divided into 3 groups (5/group): (1) The sham group underwent a sham operation without ischemia; (2) the I/R group received 60 minutes of ischemia and 72 hours of reperfusion; and (3) the ischemic postconditioning (Postcond) group was treated the same as the I/R group except that the pigs received 8 cycles of 30 seconds of reperfusion and 30 seconds of ischemia at the onset of reperfusion. After 72 hours of reperfusion, infarct size was measured by 2,3,5-triphenyltetrazolium chloride staining. Apoptotic cells in the peri-infarct myocardium were evaluated with the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) method, and apoptosis-related molecules were studied with western blotting analysis.

Results: After 72 hours of reperfusion, mean (\pm SEM) infarct size was significantly smaller in the Postcond group than in the I/R group ($23.26\% \pm 3.13\%$ versus $10.89\% \pm 2.02\%$, $P < .05$). Apoptotic myocytes in the peri-infarct region were lower in the Postcond group than in the I/R group ($15.31\% \pm 4.58\%$ versus $33.83\% \pm 4.44\%$, $P < .05$). This decrease in the extent of apoptosis was accompanied by a significant decrease in Bax expression (0.306 ± 0.075 versus 0.433 ± 0.102 for the I/R group; $P < .05$) and a significant increase in Bcl-2 expression (1.801 ± 0.227 versus 1.267 ± 0.308 for the I/R group; $P < .05$).

Conclusions: In a clinically relevant closed-chest pig model of myocardial infarction, these data suggest the

following: (1) Ischemic postconditioning reduces infarct size following prolonged reperfusion, and (2) this cardioprotective effect is likely achieved via antiapoptotic mechanisms.

INTRODUCTION

Prolonged acute coronary artery occlusion leads to myocardial infarction. Although the ischemic myocardium can be salvaged by timely reperfusion of the occluded artery, reperfusion may lead to detrimental consequences, a phenomenon called *reperfusion injury*, which is now known to involve extension of the infarct size and progressive induction of apoptosis over the reperfusion time. Despite extensive research on the treatment of reperfusion injury over the past several decades, few cardioprotectants have been used successfully in clinical application [Verma 2002; Girn 2007].

Necrosis and apoptosis are 2 forms of cell death in the myocardium that have been associated with ischemia and reperfusion [Buja 1998; Takemura 2004; Abbate 2006]. In particular, previous studies have shown that myocardial apoptosis in the peri-infarct myocardium progressively develops from early into later phases of reperfusion, suggesting that apoptosis may participate in exacerbating myocardial injury [Zhao 2001]. The dynamic changes in apoptosis that occur during reperfusion may offer an opportunity for a new treatment to reduce myocardial injury through interrupting the apoptotic process.

Ischemic postconditioning is a novel strategy for attaining cardioprotection [Zhao 2006]. Zhao and colleagues first documented that ischemic postconditioning reduces myocardial injury [Zhao 2003a, 2003b]. Most previous studies have reported that ischemic postconditioning inhibits local inflammatory responses in the area at risk, attenuates neutrophil and endothelial cell-cell interactions, and reduces infarct size in vivo and ex vivo in small-animal models after a short period of reperfusion; however, little information is available about whether cardioprotection with postconditioning persists after prolonged reperfusion [Zhao 2003a; Kin 2004; Yang 2005; Crisostomo 2006; Iliodromitis 2006; Mykytenko 2007]. Data suggest that reperfusion injury begins during the first minutes of reflow [Kin 2004] and may continue for several days [Zhao 2000; Piper 2004]. Although previous studies have found that postconditioning reduces infarct size after a prolonged period

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Correspondence: Tao Guo, MD, Cardiology, The First Affiliated Hospital of Kunming Medical College, 295 Xin-Chang Rd, Kunming 650032, P. R. China; +8613888988986 (e-mail: guotao20@botmail.com).

of reperfusion, they did not examine whether protection by postconditioning is linked to the inhibition of apoptosis, which is potentially involved in long-term reperfusion-mediated myocardial injury [Argaud 2005; Mykytenko 2007]. In addition, to the best of our knowledge, little work relevant to cardioprotection with postconditioning has yet been performed in a large animal closed-chest model of myocardial infarction [Zhao 2003b; Kin 2004; Couvreur 2006; Iliodromitis 2006; Schwartz 2006; Mykytenko 2007]. In the present study, we used a closed-chest pig model of myocardial infarction to investigate infarct size, apoptosis, and apoptosis-related molecules in the peri-infarct myocardium. The objective of this study was to evaluate the hypothesis that ischemic postconditioning reduces myocardial ischemia-reperfusion (I/R) injury by inhibiting apoptosis after prolonged reperfusion (72 hours).

METHODS

Experimental Animals and Closed-Chest Model of Myocardial Infarction

Fifteen healthy Diannan small-ear pigs (19-24.5 kg; Laboratory Animal Center, Kunming Medical College, Kunming, China) were included in the experiment. The project was approved by our experimental animal committee, and the procedures were performed according to the Guide for the Care and Use of Laboratory Animals [ILAR 1996].

All animals were fasted for 6 hours before the experiments were conducted. All interventions were performed with the animal under general anesthesia, which was introduced with intravenously administered pentobarbital sodium (20-30 mg/kg body weight initially and 2-5 mg/kg as needed). An intravenous catheter was introduced into an ear vein for the administration of pentobarbital sodium for the subsequent experiments. A 6F arterial sheath (B. Braun Melsungen, Melsungen, Germany) was placed in the right femoral artery in all animals after local surgical preparation. Before coronary catheterization, 8000 IU heparin was administered by intravenous injection, and an additional 2000 IU was injected per hour to prevent clotting during the experiment. Digital angiography (FD10 models; Philips Healthcare, Best, the Netherlands) was used in all cases. Pigs were placed in the supine position, and a 6F guiding catheter (B. Braun Melsungen) was advanced through the introducer sheath into the ostium of the left main coronary artery. A baseline coronary angiogram was obtained to delineate the vessel and to plan the location of the occlusion. Then, a guidewire was advanced through the guiding catheter into the left anterior descending coronary artery (LAD). A percutaneous transluminal coronary angioplasty balloon catheter (B. Braun Melsungen) was introduced and directed to the distal position of the first diagonal artery and proximal to the second diagonal artery of the LAD. Balloon catheters with a 2- to 3-mm balloon were used, depending on the size of the individual vessels. The balloon was carefully inflated to 3 to 4 atmospheres; complete occlusion of the vessel was confirmed by angiography and by the presence of typical changes in the electrocardiogram, such as elevation of the T wave as a first sign and, later, ST elevation. The occlusion time was 60 minutes.

Experimental Protocol

After preparation was finished, each animal was randomly assigned to one of the following 3 groups (n = 5 per group): (1) the sham group, in which animals underwent an experimental protocol identical to that of the other groups but without ischemia; (2) the I/R group, in which animals underwent 60 minutes of LAD occlusion followed by 72 hours of reperfusion without additional intervention; and (3) the ischemic postconditioning group, in which animals were treated the same as the I/R group except that the pigs underwent 8 cycles of 30 seconds of reperfusion and 30 seconds of ischemia before reperfusion.

Lidocaine (1-2 mg/kg) was administered intravenously to suppress ventricular arrhythmias during coronary artery occlusion. If a heart developed ventricular fibrillation or sustained ventricular tachycardia, we immediately used non-synchronized direct current defibrillation (Heartstart XL M4735; Philips). We chose 150 J and pressed the paddles against the anterior chest wall above the sternum on the right side and below the sternum on the left. To reduce resistance and avoid skin burning, we applied ultrasound gel to the paddles.

After the balloon and arterial sheath were removed, 4×10^6 U penicillin was administered intravenously before the animal was taken back to the Animal Services Center. All pigs were maintained under standard laboratory conditions of temperature, humidity, and a 12-hour day/12-hour night cycle. Animals were allowed free access to food and water.

Measurement of Infarct Size

After 72 hours of reperfusion, pigs were anesthetized with pentobarbital sodium. To assess infarction size, we excised the hearts and cut them into transverse slices 4 to 5 mm thick. Each heart slice was then incubated for 15 minutes at 37°C in a 1% solution of 2,3,5-triphenyltetrazolium chloride (TTC). This method reliably identifies necrotic myocardium, which appears pale, and viable myocardium, which stains brick red [Khalil 2006]. We then scanned the slices from both sides with a flatbed scanner for further planimetry analysis of the necrotic area. The size of the infarcted tissue was measured with the public domain NIH Image program (developed at the US National Institutes of Health and available on the Internet at <http://rsb.info.nih.gov/nih-image/>) and was expressed as a percentage of the left ventricular surface area [Krombach 2005]. To calculate infarct size, we used the mean value of the scans of the 2 sides of each slice.

Assessment of Apoptosis in Peri-infarct Myocardium

Apoptosis was analyzed in 1 left ventricular section obtained from the sample that showed the maximal infarct size. After the 72 hours of reperfusion, a tissue sample extending 0.5 to 1.0 mm from the infarcted zone was obtained from the myocardium. This sample was considered to represent the peri-infarct myocardium, as described previously [Palojoki 2001].

Apoptotic cardiomyocytes were detected with a terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay, as described previously [Zhao 2001]. The TUNEL technique was used to assess DNA

damage. The positively labeled nuclei (brown color) were distinguished from the negative unstained nuclei (blue color), which were counterstained with hematoxylin. The TUNEL-positive nuclei were visually quantified in 5 random visual microscope fields under high-power magnification ($\times 400$) by an investigator who was blinded to the studies. Apoptotic index was calculated as the percentage of TUNEL-positive cell with the following formula: (number of TUNEL-positively stained apoptotic myocytes/total number of myocytes counts $\times 100$) [Gao 2004].

Western Blot Analysis

Bcl-2 and Bax proteins were detected with western blot analysis. In brief, tissue samples from peri-infarct zones were homogenized in ice-cold lysis buffer. The protein content was measured with a Coomassie brilliant blue protein assay. Equal amounts of protein (40 μg) were loaded per lane of an 8% polyacrylamide gel for separation by electrophoresis at 100 to 200 V. Proteins were then transferred to nitrocellulose membranes at 10 V for 50 to 70 minutes. The membranes had been blocked with 5% nonfat dry milk in 1 \times Tris-borate-EDTA buffer containing 0.1% polysorbate 20 (Tween 20) at 25°C with gentle shaking for 2 hours. The nitrocellulose membrane was then incubated overnight at 4°C with a rabbit antibody (1:200 dilution) specific for Bcl-2 or Bax and then treated with a horseradish peroxidase-conjugated secondary antibody for 2 hours at 25°C. Antibody labeling was detected with a chemiluminescence assay kit. After stripping, the same nitrocellulose membrane was incubated with anti- β -actin antibody (1:800) as an internal control. Western blotting results were quantified by relative density, which was calculated according to the following formula: Relative Density = (Density of Tested Band)/(Density of the β -Actin Band of the Same Sample).

Statistical Analyses

All data are expressed as the mean \pm SEM. We used 1-way analysis of variance along with the Student-Newman-Keuls test for multiple comparisons. Differences were considered statistically significant for P levels $< .05$.

RESULTS

Ischemic Postconditioning Attenuated Infarct Size

TTC staining showed that sham-operated pigs had no infarctions. Compared with the I/R group, the ischemic postconditioning group had a significantly reduced mean (\pm SEM) infarct size (from 23.26% \pm 3.13% to 10.89% \pm 2.02%, $P < .05$) (Figure 1).

Detection of TUNEL-Positive Cells in the Peri-infarct Myocardium

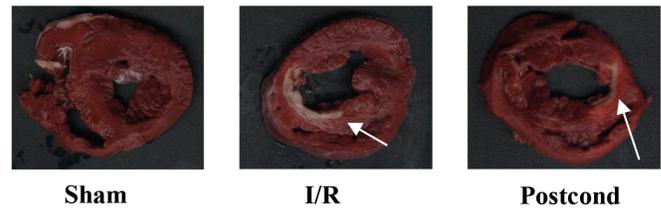
TUNEL staining of nuclei is accepted to be both specific and sensitive to apoptotic cell death. In the present study, TUNEL-positive and -negative nuclei stained brown and blue, respectively. In the TUNEL assay, a large number of TUNEL-positive cells were observed in the peri-infarct myocardium of pigs subjected to I/R injury, whereas

TUNEL-positive cells were not detected in the sham group. The percentage of TUNEL-positive cells in the peri-infarct myocardial tissue samples from the ischemic postconditioning group (apoptotic index, 15.31% \pm 4.58%) was significantly lower than in the I/R group (apoptotic index, 33.83% \pm 4.44%; $P < .05$) (Figure 2).

Expression of Bcl-2 and Bax Proteins in the Peri-infarct Zone

Compared with the sham group, the I/R group showed significantly increased expression of Bcl-2 (0.433 \pm 0.102 versus 0.151 \pm 0.019, $P < .05$) and Bax (1.267 \pm 0.308 versus 1.068 \pm 0.010, $P < .05$). Compared with the I/R group, the ischemic postconditioning treatment showed decreased Bax levels (0.306 \pm 0.075 versus 0.433 \pm 0.102, $P < .05$) and increased Bcl-2 levels (1.801 \pm 0.227 versus 1.267 \pm 0.308, $P < .05$) (Figure 3). Consistent with the decreased number of TUNEL-positive cells after ischemia and reperfusion, the ischemic postconditioning treatment was associated with increased Bcl-2 expression and decreased Bax expression, compared with the I/R group.

A



B

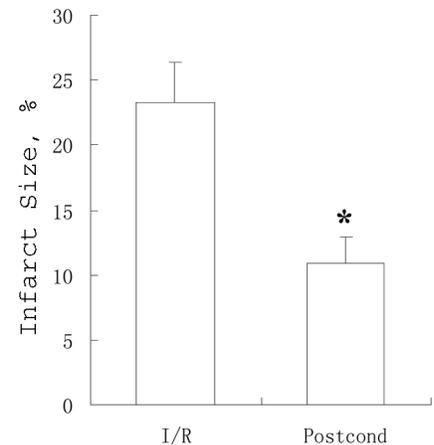


Figure 1. 2,3,5-Triphenyltetrazolium chloride staining of myocardium after 72 hours of reperfusion. The infarct is pale (arrows), whereas viable myocardium is stained brick red (A). Quantification of infarct size after 72 hours of reperfusion. The sham operation group (sham group) did not have any infarctions. There was a significant decrease in infarct size in the ischemic postconditioning (Postcond) group compared with the ischemia-reperfusion (I/R) group (B). All values are expressed as the mean percentage \pm SEM ($n = 5$). * $P < .05$, versus the I/R group.

DISCUSSION

The main finding in the present study of a closed-chest pig model of acute myocardial infarction was that ischemic postconditioning decreased infarct size and inhibited apoptosis. These results were maintained after 72 hours of reperfusion. The significant cardioprotection observed in the present study is consistent with the reductions in infarct size previously reported for studies in animal models [Zhao 2003a, 2003b; Kin 2004; Yang 2005; Crisostomo 2006; Iliodromitis 2006; Mykytenko 2007] and humans [Staat 2005]. These results suggest that postconditioning provides long-term cardioprotection via antiapoptotic mechanisms.

The phenomenon of reperfusion inducing or accelerating myocardial apoptosis has been well confirmed in different animal species and humans [Buja 1998; Zhao 2001; Takemura 2004; Abbate 2006; Abbate 2008]. Recent studies have found that during extended reperfusion the number of apoptotic cells in the peri-infarct myocardium progressively increases after a fixed ischemic event, a finding suggesting a role for apoptosis in the development of myocardial infarction [Zhao 2001]. It is conceivable, therefore, that apoptotic cells detected in the peri-infarct myocardium may undergo secondary necrosis and that the extension of the infarction may be elicited in part

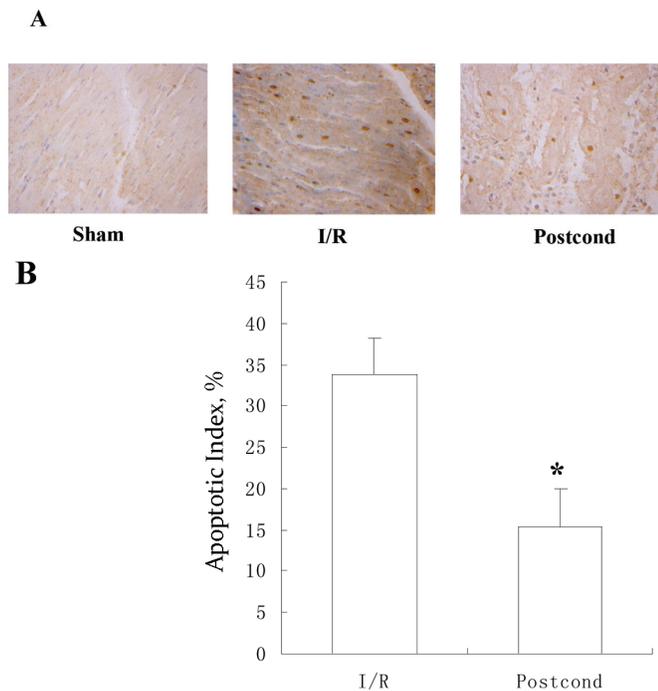


Figure 2. Detection of apoptotic myocytes in the peri-infarct region by TUNEL staining at 72 h after reperfusion. A, Brown staining indicates terminal deoxynucleotidyl transferase-mediated dUTP fluorescence nick end labeling (TUNEL)-positive cells (original magnification $\times 400$). B, Quantitative analysis showed that the ischemic postconditioning (Postcond) treatment reduced apoptotic rates significantly compared with the ischemia-reperfusion (I/R) group. Sham indicates the sham operation group. All values are expressed as the mean percentage \pm SEM (n = 5). *P < .05, versus the I/R group.

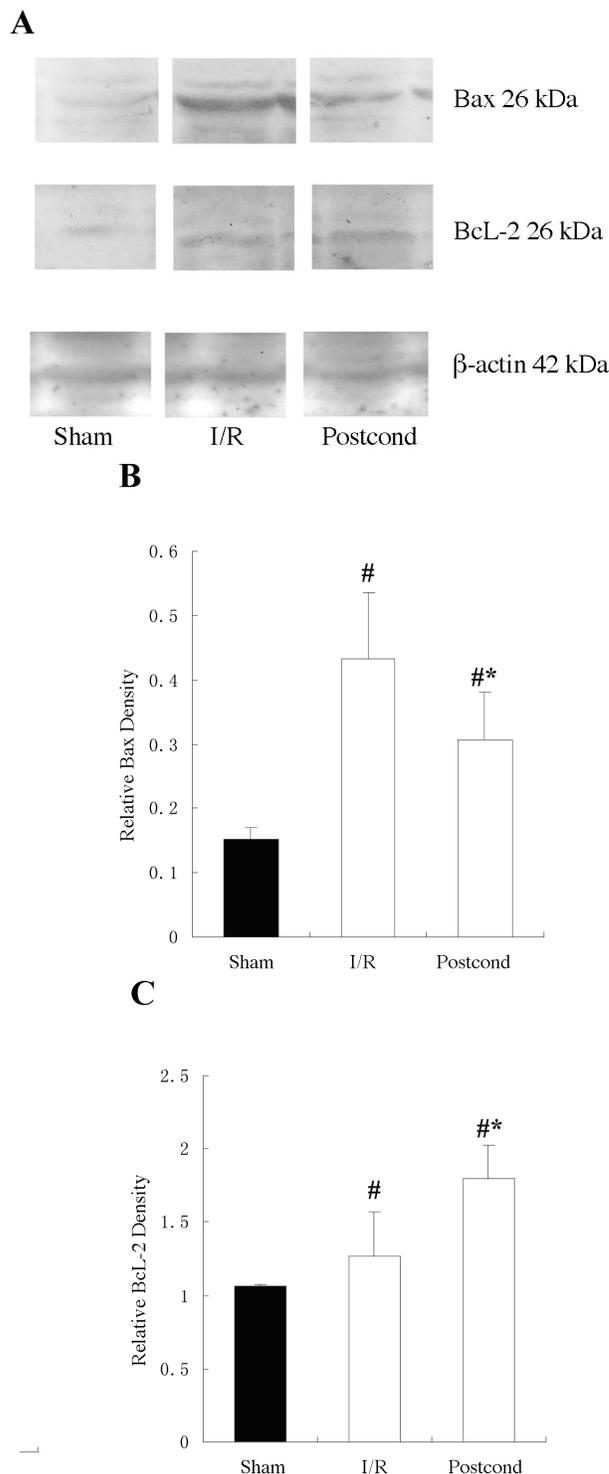


Figure 3. Bcl-2 and Bax expression at 72 hours after reperfusion. A, Representative blots showing the effect of ischemic postconditioning (Postcond) treatment on Bax and Bcl-2 expression. B, Bax band densities relative to the mean of the control. C, Bcl-2 band densities relative to the mean of the control. Sham indicates sham operation group; I/R, ischemia-reperfusion group. All values are expressed as the mean \pm SEM (n = 5). #P < .05, versus sham group. *P < .05, versus I/R group.

from the delayed apoptosis occurring during reperfusion. If apoptosis modulates the end stage of reperfusion injury, the ideal preventive or therapeutic approach would therefore target apoptosis occurring after I/R.

Although some studies have found that postconditioning reduces infarct size after prolonged reperfusion, they did not investigate whether this cardioprotection by postconditioning was related to apoptosis inhibition [Argaud 2005; Mykytenko 2007]. Our study has shown that ischemic postconditioning at the onset of reperfusion produced significant reductions in infarct size and apoptosis in the peri-infarct myocardium after 72 hours of reperfusion, which were demonstrated with TTC staining and the TUNEL assay. To further clarify the mechanism of ischemic postconditioning protection, we investigated the expression of key apoptosis-related molecules. Our study showed that ischemic postconditioning increased the level of the antiapoptotic Bcl-2 protein and inhibited the expression of the proapoptotic Bax protein in the peri-infarct myocardium. Our study has provided direct evidence that extension of infarct size can be limited by ischemic postconditioning inhibition of apoptosis. Moreover, ischemic postconditioning triggered cardioprotection, possibly by regulating the Bcl-2 family to maintain myocardial stabilization. Whether other endogenous protective molecules besides the Bcl-2 family of proteins are involved in the protection afforded by ischemic postconditioning in myocardial infarction requires further study.

There is increasing evidence from experiments with rat, rabbit, and canine models that postconditioning limits infarct size both in situ and ex vivo, but the effects on larger animals are not yet clear. Schwartz and Lagranha [Schwartz 2006] have reported that postconditioning was ineffective in pigs, a large-animal species that should be most relevant to humans, whereas Iliodromitis and colleagues [Iliodromitis 2006] reported good protection in experiments with open-chest pigs but also found that such protection required more than 4 I/R cycles.

Whether a large animal such as the pig can be postconditioned is a very important question, because it is directly associated with the likelihood that human hearts can be protected effectively and reliably with postconditioning. In our study, we used 8 I/R cycles in our experiments, which demonstrated that an effective postconditioning protocol could limit infarct size in pigs. Whether ischemic postconditioning played a role in the number and duration of occlusions was not fully elucidated in this study, and the I/R protocol we used may not afford the maximal protective effect against myocardial I/R injury. Thus, determining the optimal number of intervals and cycles may also require additional investigation.

Furthermore, many studies describing reductions in postischemic injury via postconditioning have now been reported for small-animal models, such as rats, rabbits, and canines. Ischemia is often created in these studies by ligating a coronary artery after surgical opening of the chest and exposure of the heart, but the experience with closed-chest investigations with larger animals is not as clear [Zhao 2003a, 2003b; Kin 2004, 2005; Iliodromitis 2006; Schwartz 2006; Mykytenko 2007]. In the present study, we successfully performed balloon occlusion of the LAD in a closed-chest pig model of

myocardial infarction. Compared with open-chest surgery, the present technique avoids extensive surgery, allows control of both the location and time of the occlusion, and is the closest to the process of clinical practice.

In conclusion, our study has demonstrated that ischemic postconditioning consistently reduces infarct size and attenuates apoptosis in the peri-infarct myocardium in the closed-chest pig model of myocardial infarction. This prolonged cardioprotective effect is likely achieved via antiapoptotic mechanisms.

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