

Protective effect of active perfusion in porcine models of acute myocardial ischemia

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Abstract. Mortality rates associated with off-pump coronary artery bypass (CAB) are relatively high, as the majority of patients requiring CAB are at a high risk for cardiac events. The present study aimed to establish porcine models of acute myocardial ischemia, and evaluate the protective role of shunt and active perfusion. A total of 30 pigs were randomly assigned to five groups, as follows: i) Sham (control); ii) A1 (shunt; stenosis rate, 55%); iii) A2 (shunt; stenosis rate, 75%); iv) B1 (active perfusion; stenosis rate, 55%); and v) B2 (active perfusion; stenosis rate, 75%) groups. Aortic pressure (P_0), left anterior descending coronary pressure (P_1), and coronary effective perfusion pressure (P_1/P_0) were measured. The expression levels of tumor necrosis factor- α (TNF- α), cardiac troponin (cTnI), creatine kinase-myocardial band (CK-MB), interleukin (IL)-6, IL-10, B-cell lymphoma 2 (Bcl-2), and caspase-3 were detected using enzyme-linked immunosorbent assay or western blotting. The myocardial apoptosis rate was determined using the terminal deoxynucleotidyl transferase dUTP nick end labeling assay. Ischemia models with stenosis rates of 55 and 75% were successfully constructed following suturing of the descending artery. Compared with the control, the 55 and 75% stenosis groups demonstrated significantly decreased P_1/P_0 , increased expression levels of TNF- α , cTnI, CK-MB, IL-6, IL-10 and caspase-3, an increased rate of myocardial apoptosis, and a decreased expression level of anti-apoptotic protein, Bcl-2. At 30 min following successful establishment of the model (ST segment elevation to 1 mm), group B demonstrated significantly increased P_1/P_0 , decreased expression levels of TNF- α , cTnI, CK-MB, IL-6, IL-10 and caspase-3, a decreased rate of myocardial apoptosis, and an increased expression level of anti-apoptotic protein, Bcl-2. Furthermore, the current study indicated that active perfusion

was more efficacious in maintaining myocardial perfusion and alleviating ischemic injury when compared with traditional shunt perfusion.

Introduction

Coronary artery disease (CAD) is a leading cause of mortality and morbidity in developed and developing countries (1). An estimated 16.3 million Americans aged ≥ 20 years have been diagnosed with CAD, and the overall CAD prevalence is 7% in adults in the US (2). Typically, patients with CAD receive advice for managing the risk factors associated with the progression of atherosclerosis, as well as pharmacological treatment. In addition, some patients may undergo coronary revascularization, which falls into two main categories: Coronary artery bypass grafting (CABG) and catheter-based percutaneous coronary intervention (PCI) (3,4). A number of disadvantages are associated with PCI, including early restenosis and an inability to relieve the totally occluded arteries or vessels that occur with extensive atherosclerotic disease (5). CABG has the advantages of a greater durability (graft patency rates exceed 90% at 10 years with arterial conduits) and more complete revascularization regardless of the morphology of the obstructing atherosclerotic lesion (6).

Numerous surgeons and centers have adopted off-pump coronary artery bypass (OPCAB). An interest in off-pump techniques has been driven by an increased awareness of the deleterious effects of cardiopulmonary bypass and aortic manipulation (7,8). At present, OPCAB is the first choice for revascularization in patients with severe coronary artery disease; however, the mortality rate associated with this type of surgery is relatively high, since the majority of patients requiring CAB are at a high risk for cardiac events (9). The intracoronary shunts are designed to maintain myocardial perfusion by maintaining blood supply in the distal myocardium (10). However, as the shunt itself does not increase coronary blood flow, the distal flow remains limited by stenosis, markedly so in severe cases (11). The present study aimed to establish porcine models of myocardial ischemia with different levels of stenosis, and provide myocardial protection using active perfusion at a site distal to the anastomosis. By comparing this to the traditional method of shunt perfusion, the present study investigated the protective effect of this novel perfusion strategy in pigs with acute myocardial infarction.

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Materials and methods

Animals. A total of 30 male pigs (weight, 50–60 kg) were included in the present study. The animals were provided by the Animal Center of Binzhou Medical College (Binzhou, China), where they were maintained under a 12-h light/dark cycle. The present study was conducted in accordance with the 'Guiding Principles in the Care and Use of Laboratory Animals', as outlined by the Animal Center of Binzhou Medical College, and with approval from the Animal Care Committee of Binzhou Medical College.

Agents. The fentanyl and ketamine hydrochloride injections were purchased from Yichang Humanwell Pharmaceutical Co., Ltd. (Yichang, China). The malondialdehyde enzyme-linked immunosorbent assay (ELISA) kit was purchased from R&D Systems, Inc. (Minneapolis, MN, USA). The tumor necrosis factor- α (TNF- α), interleukin (IL)-6 and IL-10 ELISA kits were purchased from Jiancheng Bioengineering Research Institute (Nanjing, China). The cardiac troponin (cTnI), creatine kinase and myoglobin (Mb) ELISA kits were purchased from Beyotime Institute of Biotechnology (Haimen, China). The terminal deoxynucleotidyl-transferase dUTP nick end-labeling (TUNEL) kit was purchased from Roche Diagnostics GmbH (Mannheim, Germany). Atropine, propofol, heparin and lysis buffer were obtained from Sigma-Aldrich (St. Louis, MO, USA). Sodium dodecyl sulfate buffer, polyacrylamide gels, nitrocellulose membranes, Tris-buffered saline containing Tween-20 (TBST), enhanced chemiluminescence (ECL) solution, 4% paraformaldehyde, paraffin, Triton X-100, phosphate-buffered saline (PBS) and the Bicinchoninic Acid Protein Assay kit were purchased from Kangchen Bio-tech, Inc. (Shanghai, China). Potassium chloride (KCl) was obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

Instruments. The current study used an ALCBio ALC-V10B ventilator (Shanghai Alcott Biotech, Ltd., Shanghai, China), an invasive pressure monitor (BeneView T8; Agilent Technologies, Inc., Santa Clara, CA, USA), an invasive pressure sensor (PT161103; Shenzhen Mindray Bio-Medical Electronics Co., Ltd., Shenzhen, China), a blood pressure monitor (MC-6800; Shenzhen Mindray Bio-Medical Electronics Co., Ltd.), a fluorescence microscope (BX61; Olympus Corporation, Tokyo, Japan), and the SpectraMax® 190 Microplate Reader (Molecular Devices, LLC, Sunnyvale, CA, USA).

Active perfusion cannula. A scalp acupuncture hose was used to produce the cannula for active perfusion. The ends of the hose were heated and stretched to thin the wall, until the diameter of the hose reached ~1.5 mm. A ramp cutoff was made at the end of the hose, which was then connected to a T-tube. The ends of the T-tube were connected to a 16G syringe (inner diameter, 1.19 mm), for perfusion, and a blood pressure monitor.

Grouping of animals. Following 2 days of adaptive feeding, where the pigs were given free access to food and water and were housed apart and maintained under a 12-h light/dark cycle, the pigs were randomly assigned to five groups ($n=6$),

as follows: i) Sham (control); ii) A1 (shunt; stenosis rate, 55%); iii) A2 (shunt; stenosis rate, 75%); iv) B1 (active perfusion; stenosis rate, 55%); and v) B2 (active perfusion; stenosis rate, 75%).

Establishment of porcine models of acute stenosis. Sedation was provided by intramuscular injection of atropine (3 mg). Ketamine (0.1 mg) and fentanyl (0.3 g) were administered 5 min after atropine injection. The pigs were placed in the supine position with their limbs fixed on the operating table, and underwent endotracheal intubation using fentanyl and propofol (6 mg/kg/h) for anesthesia. Following preparation of the skin and monitoring by electrocardiogram (ECG), a midline incision was made in the chest (3 mg/kg heparin for heparinization), and a pressure sensor was placed in the ascending aorta for measuring aortic pressure (P_0). The proximal left anterior descending artery (~1 cm) was freed and transiently clipped to allow measurement of the lumen diameter. In the control group, the lumen was opened following occlusion, however, for the experimental group, the suture was positioned using a 7-0 Prolene thread. The suture was made at a length of one-third and one-half of the lumen diameter, thus the remaining size of the lumen was four-ninths and one-quarter of the original, thereby achieving a stenosis rate of 55 and 75%, respectively. A trocar was punctured in the distal third of the descending branch, and was then connected to the pressure sensor to monitor the left anterior descending coronary pressure (P_1), in order to calculate the effective perfusion pressure (P_1/P_0).

Myocardial perfusion strategy. In the sham group, P_0 and left anterior descending coronary pressure (P_1) were monitored at 30 min following opening of the coronary artery. In group A, an incision was made on the coronary artery to insert a shunt when the ST segment elevation reached 1 mm. P_0 and P_1 were monitored following insertion of the shunt, and a blood sample (5 ml) was taken at 30 min post-perfusion. In group B, an active perfusion needle was pierced into the ascending aorta when ST segment elevation reached 1 mm, and P_0 was monitored using the T-tube. An incision was made on the coronary artery to insert the active perfusion tube distal to the vessel, and active perfusion was initiated by opening the tube. P_1 was monitored during active perfusion, and a blood sample (5 ml) was collected at 30 min post-perfusion.

Following blood sampling, the superior and inferior vena cava and the ascending aorta were clipped, and the heart was emptied via the aortic root. The pigs were sacrificed by intravenous injection with 10% KCl immediately following blood emptying, and the apical myocardium at the left ventricle was sampled (10 g) for further examination.

Determination of serum TNF- α , cTnI, Mb, creatine kinase-MB (CK-MB), IL-6 and IL-10 using ELISAs. The ELISA kits were used according to the manufacturer's protocols. Briefly, blood samples (5 ml) were placed in an ethylenediaminetetraacetic acid-containing Eppendorf tube (500 μ l) to allow thorough mixing for 20 min. The mixture was centrifuged at 2,500 \times g for 20 min to collect the supernatant. The concentration/optical density (OD) value standard curve was generated using the serially diluted standards provided with the kit. An automated microplate reader (SpectraMax® 190) was used to measure the

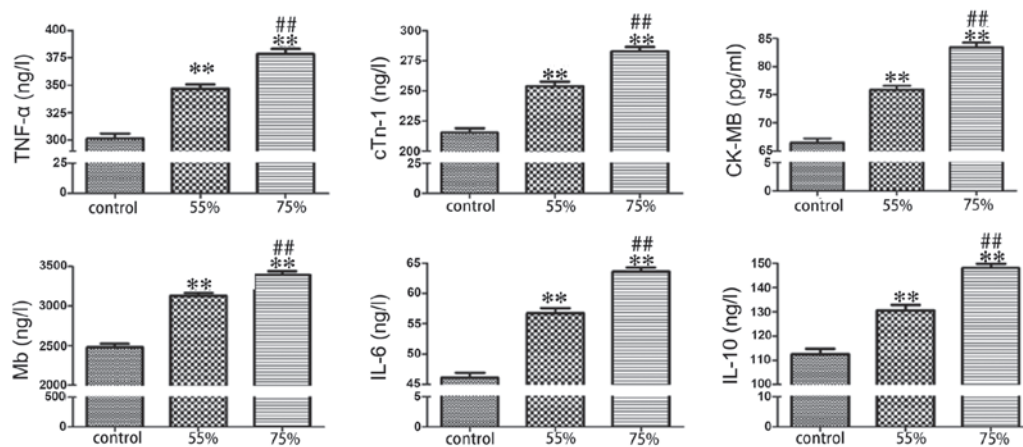


Figure 1. Serum level of TNF- α , cTnI, CK-MB, Mb, IL-6 and IL-10 30 min after stenosis formation. **P<0.01 vs. the control; ##P<0.01 vs. the 55% stenosis group. TNF- α , tumor necrosis factor- α ; cTnI, cardiac troponin; CK-MB, creatine kinase-myocardial band; Mb, myoglobin; IL, interleukin.

OD value of the centrifuged serum, and the concentration of target proteins was calculated according to their positions on the standard curve.

Western blot analysis of caspase-3 and B-cell lymphoma 2 (Bcl-2) in myocardial tissue samples. The collected myocardial tissue samples were homogenized in a lysis buffer and centrifuged at 12,000 x g for 15 min. The lysates were collected and underwent protein quantification using the bicinchoninic acid assay. A total of 20 g protein was boiled with 1X sodium dodecyl sulfate (SDS) buffer for SDS-polyacrylamide gel electrophoresis (80 v for 30 min), and the electrophoresed proteins were transferred onto a nitrocellulose membrane. Western blotting was performed by blocking the membrane with TBST containing 5 g/l skimmed milk for 1.5 h. The membrane was incubated with primary antibodies against caspase-3, Bcl-2 and β -actin (1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA) at 4°C overnight. The membrane was washed with TBST three times (10 min each time), and re-incubated with horseradish peroxidase-conjugated goat anti-rabbit or goat anti-rat immunoglobulin G (1:5,000; Zhongshan Bio-tech Co., Ltd., Zhongshan, China) at room temperature for 2 h, followed by washing with TBST three times (10 min each time). Blots were developed using the ECL solution, and Quantity One 4.62 software was used to analyze the expression level of target proteins (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Measurement of myocardial tissue apoptosis using the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. The myocardial tissue samples were fixed with 4% paraformaldehyde for 24 h, and dehydrated and embedded in paraffin. The samples were sliced at a thickness of 0.6 mm, and were perforated for 5 min with Triton X-100, followed by washing with PBS three times (5 min each time). TUNEL was performed according to the manufacturer's protocols, and all surgical procedures were performed under dark room conditions. The sections were treated with 50 μ l TUNEL solution (containing 2 μ l terminal deoxynucleotidyl transferase (TdT) enzyme and 48 μ l fluorescent label) for 60 min at 37°C and were washed with PBS

Table I. Time required for ST segment elevation of 1 mm following establishment of the model.

Parameter	Stenosis	
	55%	75%
T _{ST} (min)	20.12±0.74	15.35±0.66 ^a

^aP<0.01 vs. the 55% stenosis group.

Table II. P₁ and P₁/P₀ ratio following establishment of the stenosis model (n=6; mean \pm standard deviation).

Pressure	Control	Stenosis	
		55%	75%
P ₀ (mmHg)	108±6.83	96±6.26	91±4.83
P ₁ (mmHg)	106±5.20	70±5.34 ^a	46±5.11 ^b
P ₁ /P ₀	0.98±0.03	0.72±0.04 ^a	0.51±0.03 ^{a,b}

^aP<0.01 vs. sham; ^bP<0.01 vs. the 55% stenosis group. P₀, aortic pressure; P₁, left anterior descending coronary pressure; P₁/P₀, coronary effective perfusion pressure.

(three times for 5 min) and embedded in paraffin. Green fluorescence was observed under a fluorescence microscope, and 10 fields were randomly selected for cell counting. The rate of apoptosis was calculated as follows: Apoptotic cell count / total cell count x100%. The experiment was repeated three times.

Statistical analysis. Statistical analysis was performed using SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA). Data were presented as the mean \pm standard deviation. Statistical differences between the 55% and 75% stenosis groups were assessed using a t one-way analysis of variance. P<0.05 was considered to indicate a statistically significant difference.

Table III. P_1 and P_1/P_0 ratio following shunt or active perfusion.

Pressure	A1	A2	B1	B2
P_0 (mmHg)	85±6.26	81±4.83	87±4.43	82±4.88
P_1 (mmHg)	59±5.34	40±5.11	69±4.81 ^a	65±5.31 ^b
P_1/P_0	0.70±0.04	0.51±0.04	0.81±0.04 ^a	0.80±0.03 ^b

Data are presented as the mean ± standard deviation (n=6). ^a $P<0.01$ vs. A1; ^b $P<0.01$ vs. A2. P_0 , aortic pressure; P_1 , left anterior descending coronary pressure; P_1/P_0 , coronary effective perfusion pressure; A1 (shunt; stenosis rate, 55%); A2 (shunt; stenosis rate, 75%); B1 (active perfusion; stenosis rate, 55%); B2 (active perfusion; stenosis rate, 75%).

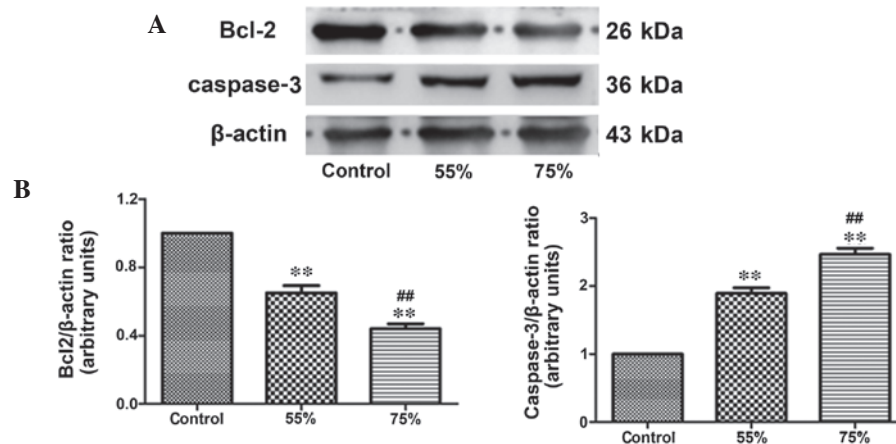


Figure 2. Myocardial expression levels of Bcl-2 and caspase-3 30 min after stenosis formation. (A) Western blots of Bcl-2 and caspase-3; (B) Grey value statistics of Bcl-2 and caspase-3. ** $P<0.01$ vs. the control; ## $P<0.01$ vs. the 55% stenosis group. Bcl-2, B-cell lymphoma 2.

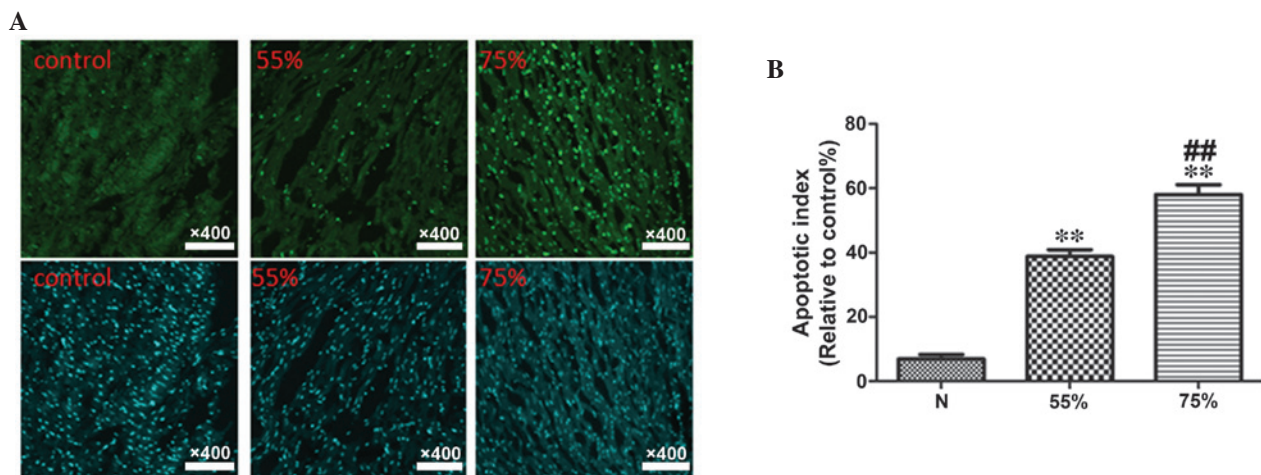


Figure 3. Myocardial apoptosis rate 30 min after stenosis formation. (A) The apoptosis of myocardial cells was assessed by performing a TUNEL assay. TUNEL staining was performed to stain the nuclei of the apoptotic cells (green), and DAPI was used to stain all of the nuclei (blue; magnification, $\times 400$; 1 cm=100 μ m). (B) Cellular apoptosis is expressed as the apoptotic index, which was calculated using the following formula: Apoptotic index (%) = the number of positively stained apoptotic cells/the total number of cells counted \times 100%. ** $P<0.01$ vs. the control; ## $P<0.01$ vs. the 55% stenosis group. DAPI, 4',6-diamidino-2-phenylindole; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labelling; N, control.

Results

Successful establishment of the model. Pigs in all groups survived the experimental period. Following formation of the stenosis, the ST segment, observed on an ECG, elevated gradually, and elevation

of 1 mm indicated successful construction of the model. The time for establishment of the model shortened with the increase in severity of the stenosis (Table I). Groups with 75% stenosis exhibited a significantly shortened duration for establishment of the model compared with groups with 55% stenosis ($P<0.05$).

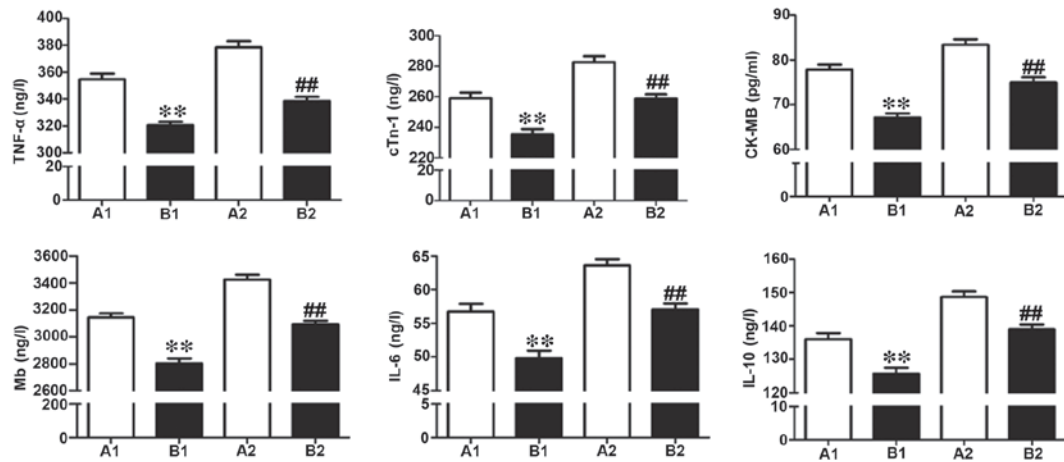


Figure 4. Serum level of TNF-α, cTnI, CK-MB, Mb, IL-6 and IL-10 30 min after shunt or active perfusion. **P<0.01 vs. A1; ##P<0.01 vs. A2. TNF-α, tumor necrosis factor-α; cTnI, cardiac troponin; CK-MB, creatine kinase-myocardial band; Mb, myoglobin; IL, interleukin.

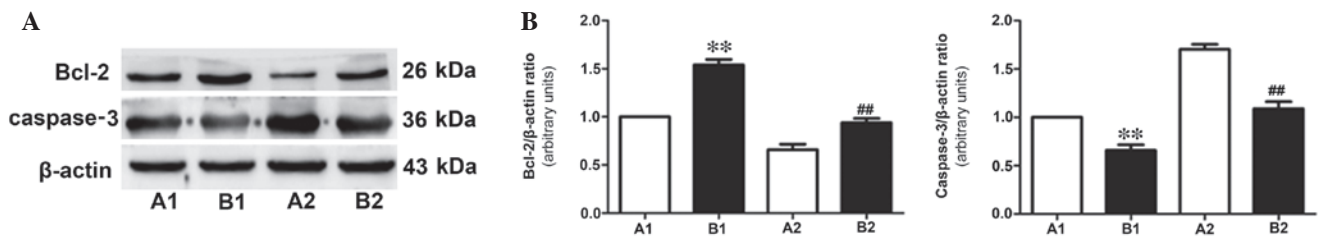


Figure 5. Myocardial expression levels of Bcl-2 and caspase-3 30 min after shunt or active perfusion. (A) The protein expression levels of Bcl-2 and caspase-3 were determined by an immunoblot, in which β-actin was the protein loading control. (B) The ratios of Bcl-2/β-actin and caspase-3/β-actin. **P<0.01 vs. A1; ##P<0.01 vs. A2.

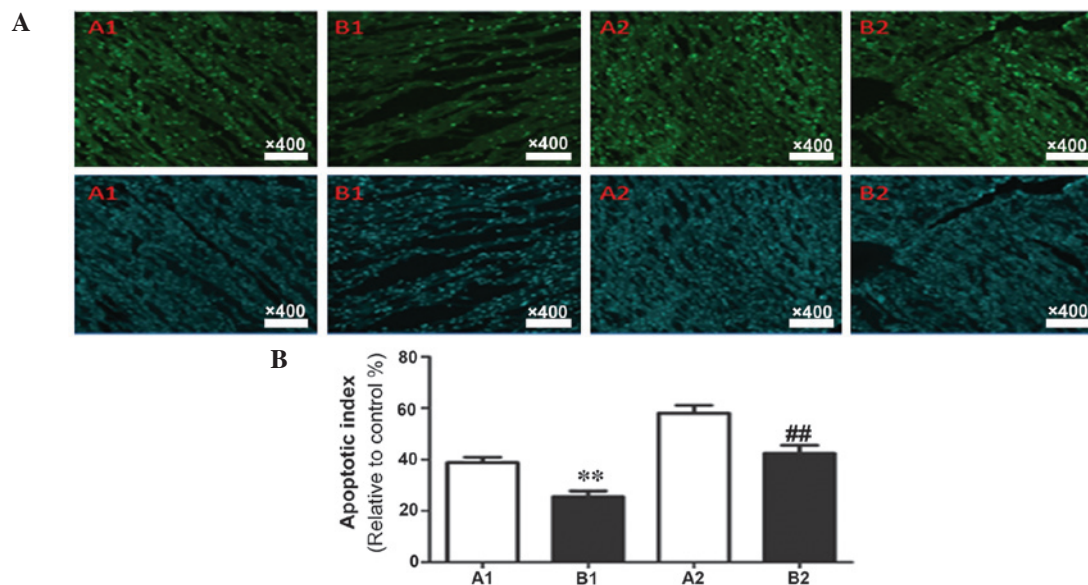


Figure 6. Myocardial apoptosis rate 30 min after shunt or active perfusion. (A) The apoptosis of myocardial cells was assessed by performing a TUNEL assay. TUNEL staining was performed to stain the nuclei of the apoptotic cells (green), and DAPI was used to stain all of the nuclei (blue; magnification, x400; 1 cm=100 μm). (B) Cellular apoptosis is expressed as the apoptotic index, which was calculated using the following formula: Apoptotic index (%) = the number of positively stained apoptotic cells/the total number of cells counted x 100%. **P<0.01 vs. A1; ##P<0.01 vs. A2. DAPI, 4',6-diamidino-2-phenylindole; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.

Models with stenosis developed myocardial ischemia due to the significant decrease in P_1 , and pigs with a higher rate of

stenosis demonstrated lower P_1 . As presented in Table II, the P_1 and P_1/P_0 ratio in all experimental groups were significantly

lower than the control (sham group; $P < 0.01$). In addition, the absolute P_1 value and the P_1/P_0 ratio in pigs with 75% stenosis were significantly lower compared with the 55% stenosis groups ($P < 0.01$).

Following formation of the stenosis, ELISA was used to determine the expression level of serum TNF- α , cTnI, CK-MB, Mb, IL-6 and IL-10 (Fig. 1). Western blotting was used to detect the expression level of the apoptotic protein, caspase-3 and the anti-apoptotic protein, Bcl-2 (Fig. 2). In addition, TUNEL staining demonstrated the rate of apoptosis in myocardial tissue samples (Fig. 3). Compared with the sham group, the stenosis groups exhibited significantly increased levels of ischemic and apoptotic indicators, while the level of anti-apoptotic protein, Bcl-2 was decreased ($P < 0.01$). In addition, the elevation of ischemic and apoptotic indicators, and the decline of Bcl-2 in the 75% stenosis groups were significant compared with the 55% stenosis groups ($P < 0.01$; Figs. 1-3).

Comparison of the two perfusion strategies. As the time of ischemia lengthened, the P_1/P_0 indicated further decline. Compared with group A, group B demonstrated significantly elevated P_1 and P_1/P_0 ratio ($P < 0.05$), indicating a significant decrease in P_1/P_0 in the artery distal to coronary stenosis ($P < 0.01$). The P_1/P_0 in the active perfusion groups (B1 and B2) was higher than in the corresponding shunt groups (A1 and A2; $P < 0.05$), and the difference was more significant in groups with the higher stenosis rate. The P_1/P_0 provided by shunt decreased significantly as the severity of stenosis increased ($P < 0.05$), indicating that blood supply at the distal artery is limited by coronary stenosis. However, as active perfusion was not affected by coronary stenosis, no significant difference in blood supply was detected in the models with different levels of stenosis ($P > 0.05$; Table III).

Comparison of myocardial injury indicators in the two perfusion groups. Coronary perfusion was performed at 30 min following formation of the stenosis, and ELISA was used to detect the level of myocardial injury indicators, including TNF- α , cTnI, CK-MB, Mb, IL-6 and IL-10. Compared to group A, group B demonstrated significantly decreased expression levels of all myocardial injury indicators, indicating the myocardial protective function of active perfusion (Fig. 4).

Comparison of apoptosis-associated proteins, caspase-3 and Bcl-2 in the two perfusion groups. Following 30 min of coronary perfusion, western blotting was performed to measure the expression levels of anti-apoptotic protein, Bcl-2 and apoptotic protein, caspase-3. As presented in Fig. 5, in group B, the expression level of caspase-3 was downregulated ($P < 0.05$) while Bcl-2 was upregulated compared with group A, indicating that active perfusion alleviated myocardial ischemia more successfully than conventional shunt perfusion.

Determination of myocardial apoptosis rate using TUNEL. The results of TUNEL staining and the data statistics are presented in Fig. 6. Compared with the corresponding subgroups in group A, group B demonstrated a significantly decreased myocardial apoptosis rate ($P < 0.05$).

Discussion

Acute myocardial dysfunction predominantly manifests as ischemic injury (12), and it is particularly prominent under ischemic, function-inhibitory or necrotic conditions (4). Currently, myocardial protections implemented in coronary artery bypass grafting (CABG) include improvement in myocardial perfusion and stress response, as well as alleviation of reperfusion injury. During CABG, the combined use of antegrade, retrograde and bypass perfusion may improve the uneven distribution of cardioplegic fluid (13), and during OPCAB, a shunt alleviates myocardial ischemia by maintaining continuous blood flow at the anastomosis. Ischemic preconditioning includes intraoperative and preoperative preconditioning (14). Therapeutic agents, such as β -1,3/1,6 glucan (15), levosimendan (16), and diazoxide are commonly used in preconditioning, while other studies have suggested that preconditioning with hyperbaric oxygen may also reduce myocardial injury during ischemia-reperfusion (17,18). Therapeutic agents, including atrial natriuretic peptide (19), nesiritide, carvedilol, sodium-hydrogen exchange inhibitor, erythropoietin, reamberin, L-arginine, large doses of insulin, and C1 esterase inhibitor are often administered to alleviate reperfusion injury (20,21). Despite these measures, myocardial protection during CAB is unsatisfactory, as myocardial blood supply or preservation fluid perfusion has always been affected and limited by proximal coronary artery stenosis (22).

In conclusion, in the present study, surgical methods were used to establish porcine models of myocardial ischemia with controllable degrees of stenosis at the descending coronary artery, to more effectively simulate the pathological anatomy of coronary heart disease. Active perfusion of the coronary artery was performed distal to stenosis via cannulation across the anastomosis, thus solving the fundamental problem of ischemic cardiomyopathy and providing a more effective method for myocardial protection in clinical CAB. In further studies, hemodynamic methods are required to investigate the distribution and variation of pressure at the coronary artery proximal and distal to stenosis, following formation of the stenosis. On the basis of this, we expect to modify active perfusion strategies, including altering the location of active perfusion at the coronary artery distal to stenosis and the size of the cannula for active perfusion.

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