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Original Article

Renoprotective effects of propofol on the expression of iNOS protein in rats with ischemia reperfusion injury

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Abstract: Aims: This study aims to explore the pathogenesis of myocardial ischemia-reperfusion injury and the treatment method. Methods: Myocardial Ischemia-reperfusion rat model was established in this study. They were divided into three groups: sham operation (SO) group, IRI control (C) group and IRI with propofol (A) group (n = 9). Myocardial infarct size was compared with HE staining method. TUNEL assay was used to detect cell apoptosis. Changes in the expression of iNOS were detected using real-time PCR and Western blotting methods. Results: Myocardial infarct size of control group and propofol group was 53.03 ± 8.90% and 34.73 ± 7.20% respectively, there were significant differences between them ($P < 0.01$). Apoptotic index of two groups was 0.21 ± 0.02 and 0.31 ± 0.05, with statistical significance ($P < 0.05$). The expression levels of iNOS in propofol group reduced significantly ($P < 0.05$). Conclusion: The levels of iNOS increase in IRI rats, suggesting that the severity of myocardial failure may be correlated with iNOS; propofol can specifically inhibit iNOS and thus protect the myocardial function.

Keywords: Propofol, ischemia reperfusion injury, inducible nitric oxide synthase

Introduction

Ischemia-reperfusion injury (IRI) is the damage that caused by ischemia and perfusion or oxygen supplied again on the tissue or organ. However, pathogenesis of myocardial IRI has not yet fully understood. Previous studies showed that the occurrence of myocardial IRI was closely related with the myocardial tubular and glomerular cells injury mediated by the decrease of ATP, production of a large amount of oxygen free radical (OFR), over-load of intracellular calcium and regulation of cell apoptosis genes [1-3]. The inducible nitric oxide synthase (iNOS) played an important role in myocardial IRI, but its role remained controversial [4, 5]. Some studies showed that decreased expression of iNOS could relieve the IRI, others showed that increased expression of iNOS could relieve the IRI [6, 7].

In this study, we constructed the rat model of myocardial IRI in order to further explore the relationship between myocardial IRI and iNOS. We compared the expression of iNOS in sham operation group and IRI group with RT-PCR and immunohistochemical methods and preoperative application of propofol in IRI group to explore the relationship between iNOS and myocardial IRI and search treatment method.

Materials and methods

Experimental animals

A total of twenty-seven specific pathogen-free adult male Sprague-Dawley (SD) rats weighing 200-250 g were obtained from the animal experimental center of Fudan University School of Medicine. These rats were kept in a clean and quiet environment with a room temperature of 22 ± 1°C and relative humidity at 40-50%. The rats had free access to food and drinking water and allowed to acclimate to the environment prior to experimental initiation. Cages, food, and drinking water were changed regularly. Rats were divided into three groups: sham operation (SO) group, IRI control (C) group and IRI with propofol (A) group (n = 9). SO group: thoracotomy without ligation, equal volume of physiological saline was injected in jugular vein; C group: physiological saline was injected in

jugular vein at 5 min and 10 min before reperfusion respectively; A group: physiological saline and propofol (0.1 mg/kg) were injected in jugular vein at 5 min and 10 min before reperfusion respectively.

Housing and procedures involving experimental animals were in accordance with the Guide for the Care and Use of Laboratory Animals. All experimental procedures were approved by the Care of Experimental Animals Committee of our hospital.

Establishment of rat myocardial IRI model

The model was established according to references [8, 9]. Briefly, the rats were anesthetized with an intraperitoneal injection of pentobarbital (40 mg/kg), lay supine and fixed on the operation table. The tracheotomy was conducted and the respirator was connected with respiratory frequency of 80-100 times/min and tidal volume of 0.8-1.5 ml. A 2 cm-long longitudinal incision was made on the left thorax about 0.5 cm beside the midline after puncture and intubation in jugular vein and routine disinfection of skin. The muscles were isolated and the corresponding ribs were fixed and cut in order to expose heart fully. We found the left coronary artery and its accompanying great cardiac vein, ligated the left anterior descending coronary artery and great cardiac vein with No. 5/0 thread. Myocardial tissue below ligature look pale, ECG was recorded before and after surgery. ST elevated and fused with T-wave into single-phase curve represents the successful ligation. The chest was closed with forceps, the ligature was released after 30 min and the color of myocardial tissue recovered. The chest was closed layer by layer after a few minutes of observation. The specimens were collected after reperfusion for 120 min.

Determination of the area of myocardial infarction

The area heart myocardial infarction was determined in 6 rats of each group. The chest was re-opened after reperfusion for 120 min and anterior descending branch was ligated in situ. Injection of 1 ml of 1% Evan's blue was conducted from the apex of left ventricle, blue tissues were non-ischemic area and uncolored tissues were ischemic area. Heart was removed quickly and the blood in heart was washed out with cold physiological saline. The left ventricle below ligature was cut into 5-6 slices with 1-2 mm thickness of each slice, they were put into 1% TTC solution and incubated at 37°C for 3-5 min in order to differentiate the ischemia area and infarct area. Then they were fixed with 10% formaldehyde for more than 10 minutes and photos were taken. The area of left ventricle (LV), area at risk (AAR) and infarction size (IS) were measured with the Leica QWin V3 computer image analysis system, AAR/LV and IS/AAR were calculated.

Myocardial apoptosis was detected with TUNEL method

Myocardial tissues perpendicular to the long axis of heart midline were sliced with the thickness of 1-2 mm after reperfusion for 120 min. They were fixed in 4% neutral formaldehyde and routine paraffin embedded. Slices with 4 μm-thick were stained using DeadEnd Fluorometric TUNEL System (Promega) following the protocol of manufacture. The TUNEL-positive cells were visualized and counted and the percentage of apoptotic cells to total cell number was calculated with the Leica QWin V3 computer image analysis system, myocardial apoptosis index (AI) was the average value.

Detection of expression levels of iNOS gene with RT-PCR and Western-blotting methods

The total RNA of tissues was extracted with an RNA Isolation Kit (TaKaRa Biotech (Dalian) Co., Ltd., Dalian, China) according to the protocol. The process of reverse transcription was conducted using PrimeScript 1st Strand cDNA Synthesis Kit (TaKaRa Biotech (Dalian) Co., Ltd., Dalian, China). The primers for PCR were as follows: iNOS F: 5'-GTGTTCCACCAGGAGATGTTG -3', iNOS R: 5'-CTCCTGCCCACTGAGTTCGTC -3'; β-actin F: 5'-TCAGGTCATCACTATCGGCAAT-3', R: 5'-AAAGAAAGGGTGTAAAACGCA-3'. The procedure for the PCR reaction as following: predegeneration for 5 min at 95°C; degeneration for 30 sec at 95°C, annealing for 30 sec at 56°C, extension for 30 sec at 72°C for 30 cycles, and amplification extension for 10 min at 72°C. The PCR product was analyzed by 1% agarose gel electrophoresis (5 V/cm × 20 min), photos were taken.

The tissues were lysed in lysis buffer and total protein was extracted. Samples were separated by SDS-PAGE and transferred to PVDF membranes. The membranes were blocked with 5%

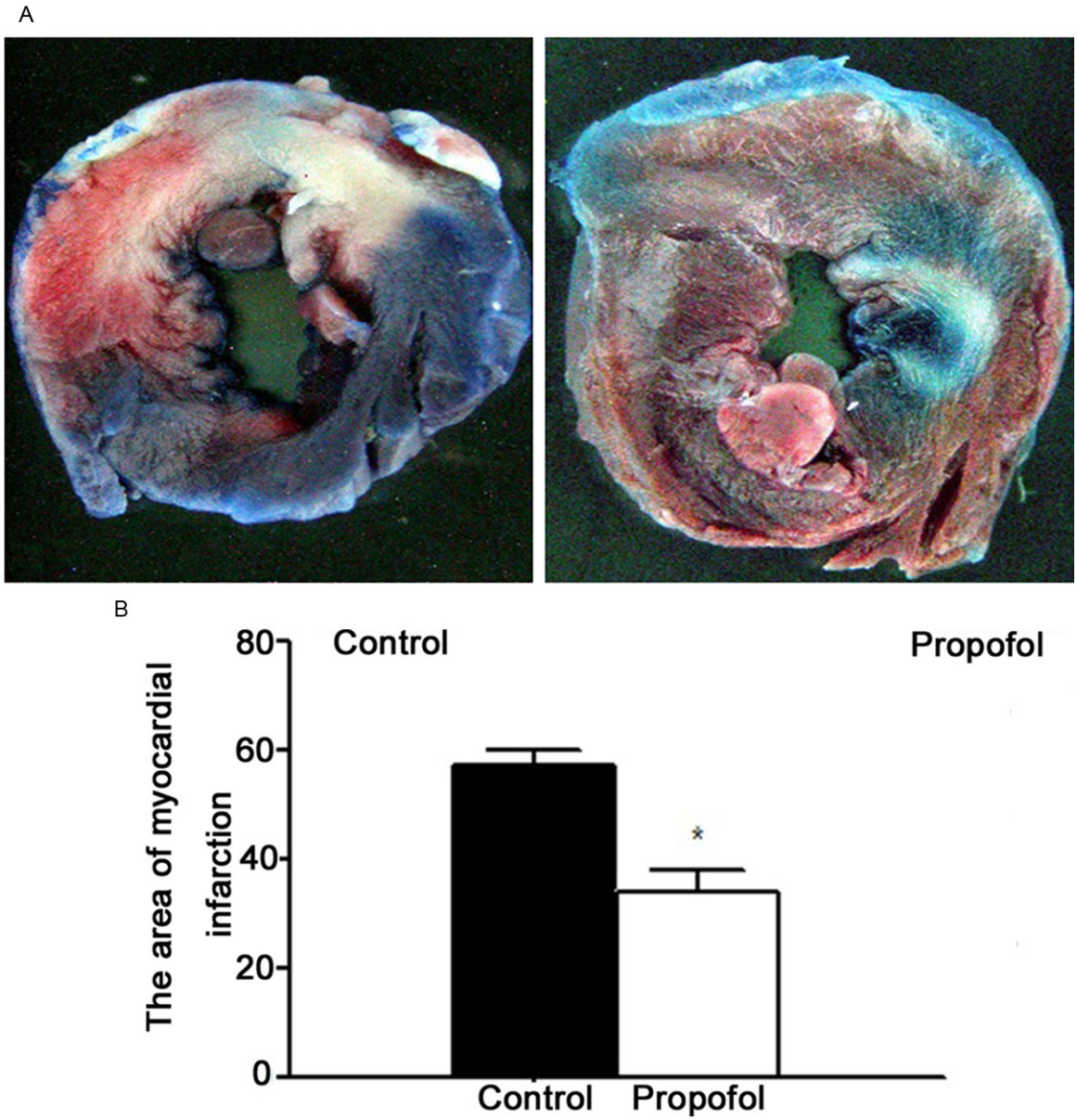

Figure 1. Myocardial Evan's blue and TTC staining results. A: The myocardial Evan's blue and TTC staining results in group C and A. Normal myocardium was stained blue by Evans blue, the myocardial ischemia was stained red by TTC, myocardial infarction was white; B: Comparison of infarction area, *P < 0.05 vs. control.

Table 1. Comparison of apoptotic index (AI) between control and propofol group

Group	AI
Control	0.31 ± 0.05
propofol	0.21 ± 0.02*

*P < 0.05 vs control.

skimmed milk in TBST (10 mM Tris–HCl, pH 8.0, 150 mM NaCl, and 0.1% Tween-20) at 37°C for 1 h and then incubated with the primary antibodies at 4°C overnight. Following incubation, membranes were incubated with secondary antibody conjugated to horseradish peroxidase for 2 h at room temperature. Photos were taken.

Statistic analysis

The data were expressed as mean ± SD. All analyses were performed with SPSS (version 13; SPSS Inc., Chicago, IL, USA). Comparison between two groups using paired t-test. P < 0.05 was considered statistically significant.

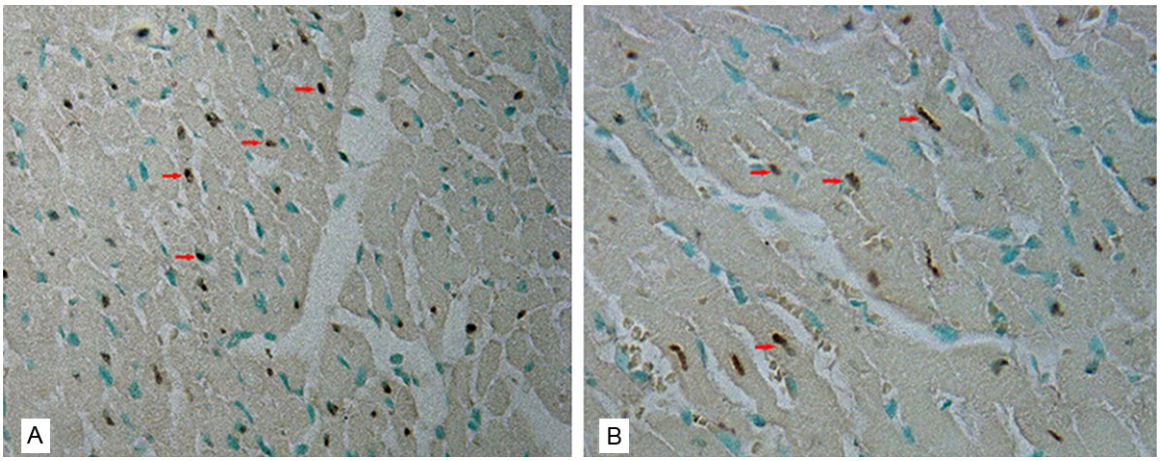

Figure 2. TUNEL staining results (400×). A: Control group; B: Propofol group. The red arrow means apoptotic nucleus.

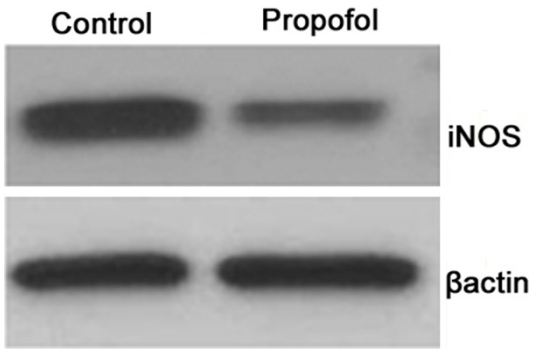

Figure 3. Western-blotting results of iNOS protein.

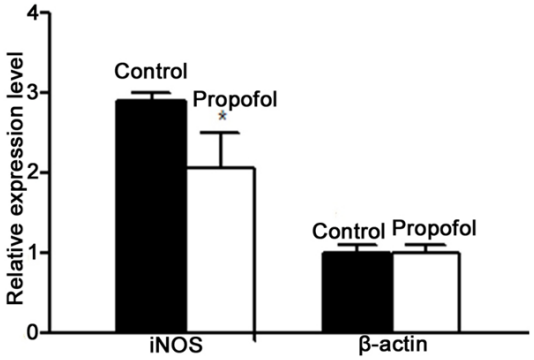

Figure 4. RT-PCR results of expression level of iNOS. *$P < 0.05$ vs. control.

Results

Effects of propofol on myocardial infarction area

The AAR/LV of C and A group were 50.45 ± 9.10% and 45.07 ± 7.06% respectively, there was no significant difference ($P = 0.28$). The IS/AAR of C and A group were 53.03 ± 8.90% and 34.73 ± 7.20% respectively, there was significant difference between the two groups ($P < 0.01$). It indicated that propofol could reduce myocardial injury and the infarction area (**Figure 1**).

Effects of propofol on myocardial cell apoptosis

The numbers of positive apoptotic cells in group A was lower than that of group C, the apoptotic index (AI) of groups A and C were 0.21 ± 0.02 and 0.31 ± 0.05 respectively, there was significant difference between the two groups ($P < 0.05$). It suggested that the apoptosis of myocardial cells was significantly reduced in propofol group (**Table 1**; **Figure 2**).

Changes of expression levels of iNOS gene

Western-blotting results showed that the level of iNOS protein decreased in propofol group (**Figure 3**). RT-PCR results were shown in **Figure 4**, it showed the expression level of iNOS gene in propofol group was lower than that of control group ($P < 0.05$).

Disscussion

Myocardial IRI is a major cause of acute heart failure. Incomplete repair after injury and hyperplasia of fibrous tissue can cause persistent myocardial damage and gradually progress to chronic cardiac failure, which is serious harm to human health [9]. At present, it is not clear about its pathogenesis and without specific treatment [10, 11]. In this study we found that

the myocardial IRI can increase the level of iNOS and aggravate myocardial injury, while application of propofol can reduce myocardial injury, which indicated that myocardial IRI was closely related to the increase of iNOS and propofol had the therapeutic effect on myocardial IRI [12].

Animal model used in this study is relatively mature, successful preparation of model is the key to successful experiment. This model is more close to the real clinical situation. We studied whether propofol can inhibit myocardial IRI in order to protect the heart when it was given during reperfusion from two aspects of myocardial infarction area and cellular apoptosis. The determination of the area of myocardial infarction with TTC and Evan's blue double staining method showed that AAR/LV in all samples was > 30%, which proved that the ligation position is correct and the model was successfully established. The area of infarction in propofol group decreased significantly. Apoptosis occurred after myocardial ischemia and reperfusion. There was significant difference in AI between group A and C. These results suggested that propofol can reduce infarct area after ischemia reperfusion and inhibit myocardial apoptosis, it had a protective effect on myocardial tissues. It was consistent with previous researches [13, 14].

In a word, this study further confirmed the protective effect of propofol on myocardial IRI. It is expected to become a new target for clinical prevention and treatment of IRI.

Disclosure of conflict of interest

None.

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