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Original Article

Effects of morphine and sufentanil preconditioning against myocardial ischemic-reperfusion injury in rabbits

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Abstract: Objective: This study aims to explore the treatment method of myocardial ischemia-reperfusion injury. Methods: Myocardial Ischemia-reperfusion rabbit model was established in this study. They were divided into four groups: sham operation (S) group, IRI control (I/R) group and IRI with morphine (MF) group and sufentanil (SF). Myocardial infarct size was compared with HE staining method. TUNEL assay was used to detect cell apoptosis. Results: Myocardial infarct size of control group and morphine and sufetanil group was 36.0±3.6, 23.0±1.2 and 27.1±2.3, respectively. There were significant differences between them ($P < 0.01$). Apoptotic index of I/R, MF and SF groups was 26.9±2.2, 12.5±2.3, 15.8±2.0, with statistical significance ($P < 0.05$). The concentration of CK-MB in serum: there were no significant differences of CK-MB between each group at baseline. The concentration of CK-MB after reperfusion were higher than that of baseline, except for group S ($P < 0.05$); Compared with group S, after reperfusion, the CK-MB of other three groups were higher ($P < 0.05$); The concentration of CK-MB in group MF and SF were lower than group I/R ($P < 0.05$); In contrast to group MF, the concentration of CK-MB after reperfusion was higher in group SF ($P < 0.05$). Conclusion: Morphine and sufentanil can specifically protect the myocardial function.

Keywords: Myocardial ischemia-reperfusion, morphine, sufentanil

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].

Despite years of experience, clinicians are still challenged by the perioperative care of the cardiac patient. Most operations on the heart require the use of hypothermic, ischemic cardiac arrest, which limits the success of such operations. Similarly, warm-blooded animals are challenged by life in cold, inhospitable environments. Many animals have successfully adapted to this challenge via hibernation. A fascinating concept is that the biological mechanism of hibernation may be duplicated in humans, thereby inducing a profound state of energy conservation throughout the body or at the organ level. Conceivable applications for "induced hibernation" include transplantation and cardiac surgery.

Hibernation only occurs in certain animals, such as black bears, woodchucks, and ground squirrels, in response to climatic conditions. During this process, the metabolic processes of the body are dramatically slowed down. In fact, hibernating animals use only 10% of their normal, active energy expenditure [4]. Hibernation is a process mediated by cyclical variation in

endogenous opiate compounds [5–8]. It is also evident that hibernation is not only opiate-mediated, but that the δ-opiate receptor in particular is responsible [9]. Additionally, serum from hibernating animals, when injected into summer-active animals, induces hibernation behavior and physiology. Conversely, hibernation can be reversed by opiate antagonists [9].

Given that hibernation is a state of energy conservation and is reproducible with the administration of δ-opiates, potential implications for organ preservation arise. In fact, using hibernation triggers to extend organ viability has been done successfully in many models [10-14], including myocardial protection [15, 16]. Coincidentally, nonpeptide, opiate drugs are the mainstay of cardiac anesthetics. These drugs are used in this setting for their potent analgesic properties and cardiovascular stability. Coincidentally, nonpeptide, opiate drugs are the mainstay of cardiac anesthetics. These drugs are used in this setting for their potent analgesic properties and cardiovascular stability. To some degree, each opiate drug in common use is thought to possess an affinity for the d-opiate receptor [17-19]. In this study, we constructed the rabbit model of myocardial IRI in order to further explore whether opiate drugs such like with activity at the δ-receptor will confer protection against ischemic insult.

Methods

Experimental animals

A total of thirty-five specific pathogen-free New Zealand white rabbit (2-3 months old) weighing 2000-2500 g were obtained from the animal experimental center of Nanchang University School of Medicine. These rabbits were kept in standard laboratory conditions under natural light and dark cycles (approximately 12 h light/12 h dark) and maintained at humidity of 50±10% and an ambient temperature of 25±2°C. The rabbits 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.

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.

Experimental protocol

The animals were allowed to acclimatize for one week before the experiments and randomized into four main experimental groups. Hemodynamically unstable rats were excluded from the study.

Group S-Saline control group-Sham control (n=6).

Animals were administered 0.9% normal saline and then sacrificed. These rabbits underwent the entire surgical procedure except the left anterior descending (LAD) coronary artery occlusion.

Group I/R-Ischemia and reperfusion control group-I-R control (n=9).

The rabbits were administered 0.9% normal saline once daily for 4 weeks and in addition, underwent 30 min LAD coronary artery ligation followed by 120 min of reperfusion.

Group MF-Morphine treated group (n=6).

The total morphine dose of 1 mg/kg, firstly, used in the present study was selected and injected jugular vein at 5 min, 10 min and 15 min before 30 min LAD coronary artery ligation respectively. Finally, 120 min reperfusion was conducted following by the above procedure.

Group SF-Sufentanil treated group (n=6).

The total sufentanil dose of 1 μg/kg, firstly, used in the present study was selected and injected jugular vein at 5 min, 10 min and 15 min before 30 min LAD coronary artery ligation respectively. Finally, 120 min reperfusion was conducted following by the above procedure.

Establishment of rabbit myocardial IRI model

The model was established according to references [20]. Briefly, the rabbits were anesthetize 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 0-25 times/min and tidal volume of 10-12/kg ml. A 3 cm-long longitudi-

nal 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.

Recording of hemodynamic parameters

10 min after the model establishment was completed and became steady, the heart rate (HR) and mean atrial pressure (MAP) were recorded, which were regarded as the baseline level (T_0). And then the HR and MAP at the time point of 0 min (T_1) and after 30 min (T_2) of ischemia and after 30 (T_3), 60 (T_4), 120 min (T_5) of reperfusion after ischemia of each group were recorded, respectively.

Myocardial biochemical markers determination

At T_0 and T_5, the blood sample was centrifuged and serum was extracted to measure the levels of creative kinase-MB (CK-MB) using a high-sensitivity cardiac troponin T enzyme linked immunosorbent assay kit (USCN Life Science, Wuhan, CHN). Samples of myocardium from left ventricle were obtained using an auto-cardiac biopsy system (Bard MAGNUM REF/Cat. No. MG1522).

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 left ventricle weight (LV) and myocardiac area (MA) and infarction area (IA) were measured and area at risk (AAR) and infarction size (IS) were calculated.

AAR=MA/LV×100%, IS=IA/MA×100%

Observation of neutrophil infiltration with HE staining

After the experiment closed, the myocardial tissue were taken and were fixed for 24 h with 4% paraform in the surrounding area of the myocardial infarction in the I/R group, the MF group and the SM group. Through the conventional dehydration, wax infiltration and embedding, the issues were made into paraffin section with thickness of 5 mm to be stained with PE. And then the situation of neutrophil granulocyte infiltration was observed under optical microscope.

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 Dead End 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.

Statistical analysis

The statistical software SPSS 17.0 was adopted to conduct the statistical analysis. The measurement data were expressed by the mean ± standard deviation ($\bar{x} \pm s$). The comparison between every two groups was made with t detec-

Table 1. Effect of morphine and sufentanil on HR and MAP in rabbits subjected to I/R

Index	Group	Case	Baseline	After ischemia		After reperfusion		
				0 min	30 min	30 min	60 min	120 min
HR (times/min)	S	6	231±23	229±30	225±26	226±25	220±28	225±26
	I/R	9	241±17	240±19	187±30$^{*,\#}$	177±20$^{*,\#}$	170±16$^{*,\#}$	169±15$^{*,\#}$
	MF	9	232±10	198±22$^{*,\Delta}$	194±19*	201±24*	195±19*	189±18$^{*,\#}$
	SF	9	241±12	218±13*	198±15*	202±20*	198±16$^{*,\Delta}$	192±15$^{*,\#}$
MAP	S	6	105±9	105±8	99±5	100±4	100±3	99±6
(mmHg)	I/R	9	103±3	101±3	66±5$^{*,\#}$	65±4$^{*,\#}$	67±3$^{*,\#}$	66±4$^{*,\#}$
	MF	9	100±6	82±7$^{*,\#,\Delta}$	74±3$^{*,\#,\Delta}$	75±7$^{*,\#,\Delta}$	76±7$^{*,\#,\Delta}$	76±9$^{*,\#,\Delta}$
	SF	9	97±6	85±4$^{*,\#,\Delta}$	77±4$^{*,\#,\Delta}$	76±7$^{*,\#,\Delta}$	76±6$^{*,\#,\Delta}$	76±6$^{*,\#\Delta}$

Note: Compared with baseline level, *P<0.05; compared with group S, $^{\#}$P<0.05; compared with group I/R, $^{\Delta}$P<0.05.

Table 2. Effect of morphine and sufentanil on CK-MB level in rabbits subjected to I/R

Group	Case	Baseline (ng/ml)	After reperfusion/ischemia (ng/ml)
S	6	0.270±0.035	0.293±0.033
I/R	9	0.266±0.044	0.906±0.046$^{*,\#}$
MF	9	0.286±0.033	0.510±0.065$^{*,\#,\Delta}$
SF	9	0.283±0.039	0.592±0.046$^{*,\#,\Delta,\blacktriangle}$

Note: Compared with baseline level, $^{*}P$<0.05; compared with group S, $^{\#}P$<0.05; compared with I/R, $^{\Delta}P$<0.05; compared with group MF, $^{\blacktriangle}P$<0.05.

Table 3. Effect of morphine and sufentanil on myocardial and infarction area in rabbits subjected to I/R

Group	Left ventricle weight (g)	Myocardiac area (%)	Infarction area (%)
I/R	3.28±0.17	37.0±2.4	36.0±3.6
MF	3.21±0.19	38.3±2.1	23.0±1.2*
SF	3.21±0.13	35.7±2.6	27.1±2.3$^{*,\#}$

Note: Compared with group I/R, $^{*}P$<0.01, compared with group MF, $^{\#}P$<0.05.

tion of two separate fully randomly designed, the comparison among multiple groups was made with one-factor analysis of variance while comparison between each two groups among the multiple groups was detected with LSD method, and the difference was regarded to have significance when 0.05.

Result

Analysis on the results of hemodynamic of the experimental rabbits in the four groups

Compared with the basic levels in these groups, the differences of HR and MAP had no statistical significance (P < 0.05). Compared with those in the sham group, both the HR and the MAP in the all groups declined relatively in the ischemia and after 30 min ischemia (P < 0.05). During reperfusion upon ischemia (T_3-T_5), compared with those in the sham group, both the HR and the MAP in I/R group and SF group and MF group, declined remarkably (P < 0.05). Compared with I/R group, HR of MF group declined significantly in the time point T_1 and HR of SF group descended remarkably in the time point T_4 (P < 0.05). Moreover, compared with I/R group, MAP declined significantly in the ischemia (T_1) and after 30 min ischemia (T_2) and after 30 (T_3), 60 (T_4), 120 min (T_5) of reperfusion after ischemia (P < 0.05), see **Table 1**.

Analysis on the results of myocardial biochemical markers of the experimental rabbits in the four groups

Compared with those on the baseline level in the groups, the differences of CK-MB had no statistical significance (P > 0.05). Compared with sham group through reperfusion for 6 h after ischemia, CK-MB in the I/R and MF and SF group remarkably decreased (P < 0.05). Compared with I/R group through reperfusion for 6 h after ischemia, CK-MB in the MF and SF group significantly decreased (P < 0.05). Compared with MF group through reperfusion, CK-MB in the SF group remarkably increased (P < 0.05), see **Table 2**.

Effects of morphine and sufentanil on myocardial infarction area

As shown in **Table 3** and **Figure 1**, the values of AAR of I/R and MF and SF were 37.0±2.4, 38.3±2.1, 35.7±2.6, respectively. The values of

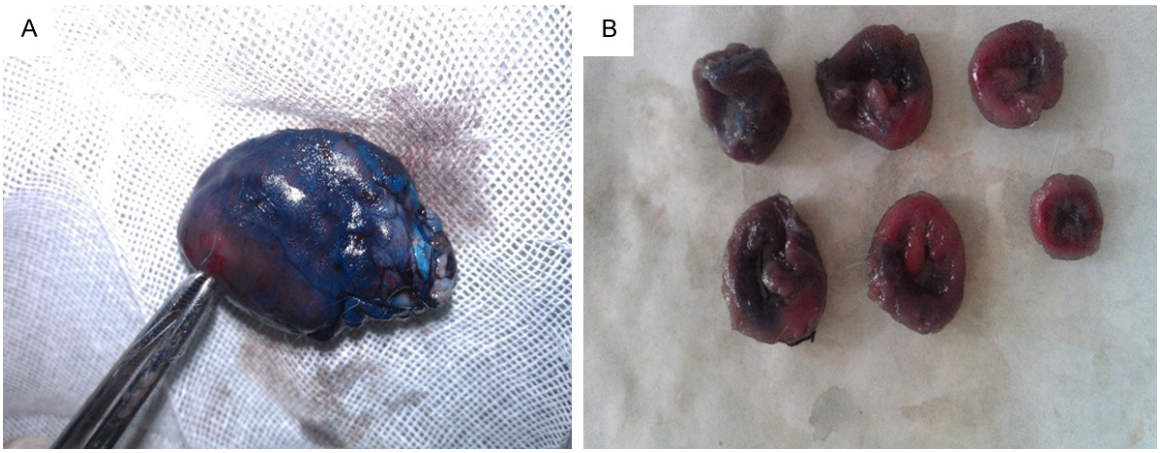

Figure 1. Myocardial Evan's blue and TTC staining results. The myocardial Evan's blue and TTC staining results in group MF and SF. Normal myocardium was stained blue by Evans blue (A), the myocardial ischemia was stained red by TTC, myocardial infarction was white (B).

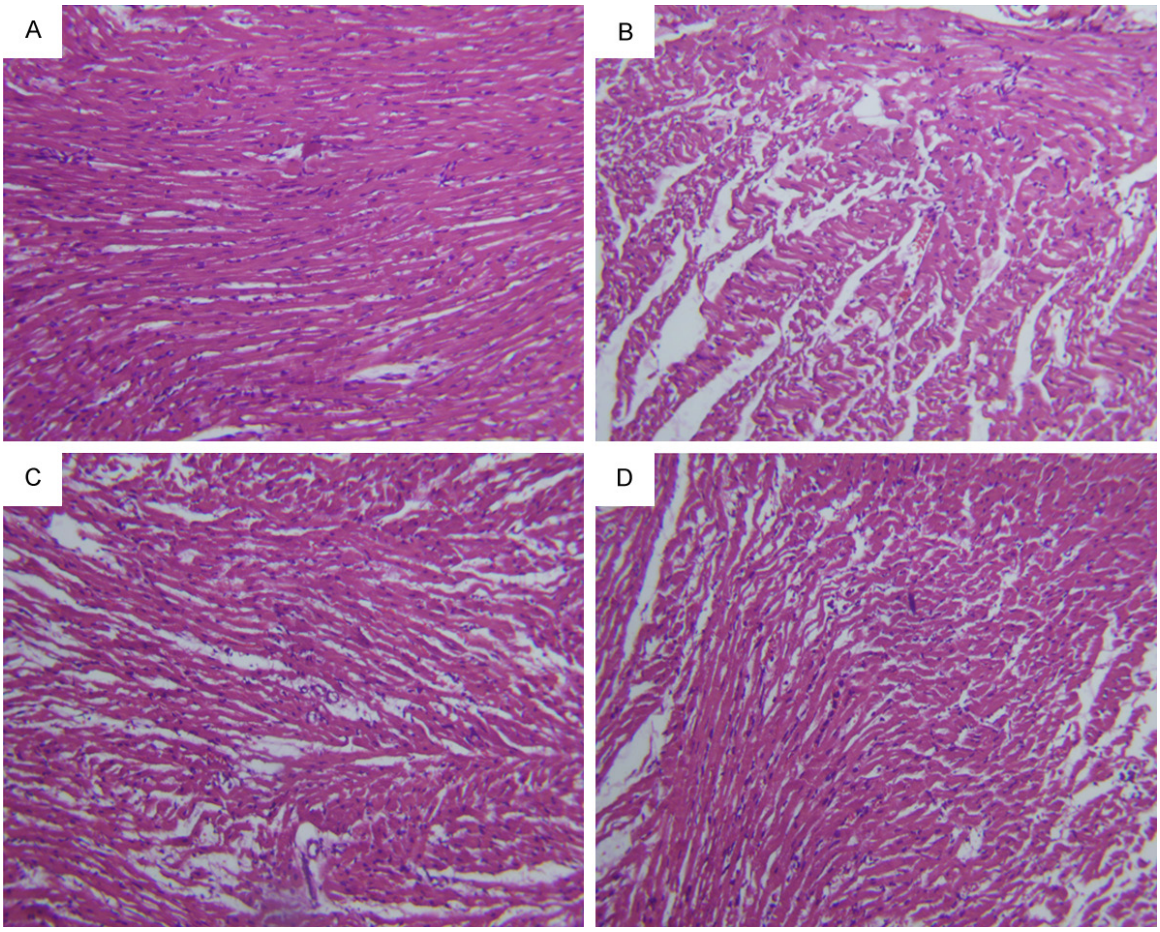

Figure 2. Effects of morphine and sufentanil on cell morphology and hematoxylin and eosin (HE) staining (×200). A-D. Represent staining results of S, I/R, MF, SF group, respectively.

left ventricle weight of I/R and MF and SF were 3.28±0.17 g, 3.21±0.19 g, 3.21±0.13 g. There were no significant differences in both above values, respectively ($P > 0.05$). However, com-

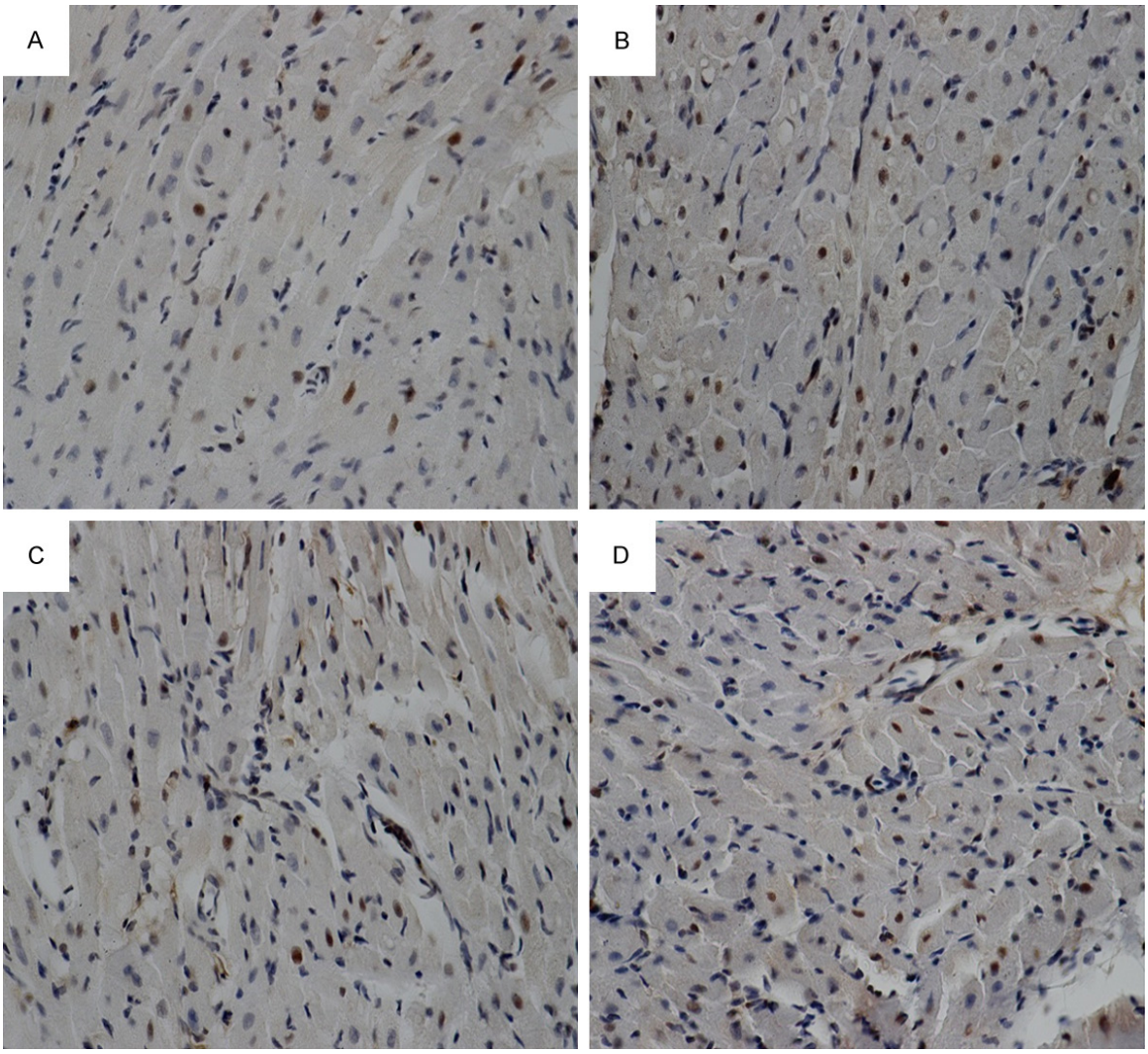

Figure 3. Effects of morphine and sufentanil suppression on cardiomyocyte apoptosis (×400). A-D. Represent results of S, I/R, MF, SF group, respectively.

Table 4. Apoptotic index (AI) of groups MF and SF and I/R

Group	N	Apoptotic index (%)
S	3	3.2±1.4
I/R	3	26.9±2.2*
MF	3	12.5±2.3*,#
SF	3	15.8±2.0*,#,Δ

Note: Compared with group S, *P<0.01; compared with group I/R, #P<0.01; compared with group MF, ΔP<0.01.

pared with I/R group the value of IS of MF and SF groups decreased significantly (P < 0.01) and compared with MF the value of IS of SF increased remarkably (P < 0.05). It indicated that morphine and sufentanil could reduce myocardial injury and the infarction area.

Morphine and sufentanil impaired myocardial structure turbulence induced by I/R injury

The changes in the morphological structures of myocardial tissues were evaluated by HE coloration. Optical micrographs of rabbits myocardial structures are shown in **Figure 2**. The myocardial structures of the control group (**Figure 2A**) are as follows: muscle fibers were neatly arranged; interstitial substance contained no edema; muscle membrane was not damaged, and muscle fibers showed no fracture, degeneration, and necrosis. By contrast, the myocardial structures of the I/R group (**Figure 2B**) are as follows: Muscle fibers were irregularly arranged; interstitial substance exhibited edema; muscle membrane was damaged; and muscle

fibers showed fracture, degeneration, and necrosis. Compared with the I/R group, the group pretreated with morphine or sufentanil (**Figure 2C**, **2D**) showed significantly reduced I/R-induced myocardial structure turbulence.

Effects of morphine and sufentanil on myocardial cell apoptosis

As seen in the **Figure 3** and **Table 4**, the numbers of positive apoptotic cells in group SF and MF was lower than that of group I/R, the apoptotic index (AI) of groups MF and SF and I/R were 12.5±2.3, 15.8±2.0 and 26.9±2.2 respectively, there was significant difference between the three groups ($P < 0.05$). It suggested that the apoptosis of myocardial cells was significantly reduced in MF and SF groups.

Discussion

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 [20]. Animal model used in this study is relatively mature, successful preparation of model is the key to successful experiment. We studied whether morphine and sufentanil 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 and IS in all samples was significantly different, which proved that the ligation position is correct and the model was successfully established. The area of infarction in MF and SF groups decreased significantly. Apoptosis occurred after myocardial ischemia and reperfusion. There was significant difference in AI between groups MF or SF and I/R. These results suggested that morphine or sufentanil 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 [22].

The oxidant/antioxidant balance in healthy tissue is maintained with antioxidant superiority. Various aggressive factors that may lead to tissue damage result in impairment of the oxidant/antioxidant balance in favor of oxidants [23]. This is known as oxidative stress [24]. Parameters such as creatine kinase isoenzyme MB (CK-MB) are used to assess cardiotoxicity in the literature. CK-MB levels in heart tissue have been reported to increase in parallel to the rise in oxidant parameters [25]. In the research based on the fact that both of CK-MB level of SF and MF decreased remarkably compared with I/R group, it was safely conclude that morphine or sufentanil could decrease cardiotoxic effect of Myocardial IRI.

Conclusively, this study further confirmed the protective effect of morphine or sufentanil on myocardial IRI. It is expected to become a new target for clinical prevention and treatment of IRI.

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Disclosure of conflict of interest

None.

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