



Protective Effect of Sufentanil against Myocardial Ischemia Reperfusion Injury in Rats

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ABSTRACT

The objective of this study was to investigate the action of sufentanil (Fen) pretreatment on inducible myocardial apoptosis by ischemia-reperfusion (I/R) in rats. 120 male rats (mean age = 3 months) were randomly assigned to six conditions: control group (group S), I/R group, normal saline (NS) I/R group, and low, medium and high dose Fen groups, where in high dose group: Fen1:2μg/kg; Fen2:4μg/kg; Fen3:6μg/kg. The measured items included heart rate (HR), mean arterial pressure (MAP), left ventricular developed pressure (LVDP), ±dp/dtmax, malondialdehyde (MDA), superoxide dismutase (SOD) activity, creatine phosphokinase MB (CK-MB) and cardiac troponin-I (cTnI). The total apoptotic cardiomyocytes, B cell lymphoma 2 (Bcl-2) and Bax protein and mRNA expression were detected in the myocardial ischemia (MI) region. The HR and the MAP of the Fen group exceeded that of the I/R group, while the LVDP and ±dp/dtmax were approximate to the basic values. The MDA concentrations and CK-MB values of the Fen group went down and the SOD activity went up when was compared with the I/R group. Whereas, cTnI concentrations of Fen1 and Fen2 groups sharply decreased (all P<0.05); the myocardial injury of the Fen group was less the I/R group. Whereas, the MI region and apoptosis indexes of Fen1 and Fen2 groups dropped significantly (all P<0.05); moreover, Bcl-2 protein and mRNA expression rose significantly in the Fen group by being compared to the I/R group, while Bax protein and mRNA expression were declined clearly (all P<0.05). Regulating Bcl-2 and Bax of Fen pretreatment can inhibit I/R-induced myocardial apoptosis of rats. As a result, Fen may be a potential drug to treat of I/R injury-related heart disease.

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Authors' Contribution

PY conceived the idea, supervised the study and wrote the manuscript. LB performed the experimental work. XS helped in collection of samples and literature review and performed all the statistical tests.

Key words

Sufentanil, Rats, Ischemia, Reperfusion, Cardiac muscles

INTRODUCTION

Myocardial infarction (MI) is a usual symptom in coronary artery diseases, partially featured with myocardial necrosis arising from persistent and severe myocardial ischemia (Martindale and Metzger, 2014). Ischemic heart disease is a major cause of mortality and morbidity worldwide (Myers *et al.*, 1981). Rapid reperfusion is essential for the treatment of myocardial ischemic events. It is necessary to restore the blood flow that endangers the myocardium after MI (Oeltgen *et al.*, 1987).

Ischemia-reperfusion injury (IRI) indicates a rapid increase of tissue injury after a certain period of reperfusion of ischemic tissue (Tao *et al.*, 2013). Recently, necrosis and apoptosis are considered to be two forms of injuries caused by reperfusion injury. It is considered to be a long-term problem in the treatment of myocardial ischemia (Oeltgen *et al.*, 1996). IRI is caused by ischemia and perfusion or re-supply of oxygen in tissues or organs. However, the pathogenesis of myocardial IRI is undefined. Previous studies have shown that the occurrence of myocardial IRI is closely associated with the decrease of ATP, production of a large number of oxygen free radicals (OFR), intracellular calcium overload and cell regulation mediated myocardial tubular and glomerular cell damages (Chien *et al.*, 1997). In spite of years of experience, clinicians still face the challenge of perioperative care in patients with heart disease. Most cardiac operations require the hypothermia, and the ischemic cardiac arrest also restricts the success in such operations. By the same token, warm-blooded animals are confronted with life challenges in cold and desolate environments. Lots of animals have successfully

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adapted to this challenge through hibernation. There is an idea that the biological mechanisms of hibernation can be replicated in humans, to trigger a profound state to save energy at the whole human body or organ level (Veighey and MacAllister, 2015). Potential applications of induced hibernation include the transplantation and cardiac surgery. According to climatic conditions, hibernation occurs only in some certain animals, such as black bears, marmots and ground squirrels. In this procedure, the metabolic process of the human body is significantly slowed down. In fact, hibernant only takes away 10% of their normal functional consumption (Nock *et al.*, 1990). Hibernation is a mediated process by periodic changes in endogenous opioid compounds (Bolling *et al.*, 1997). It is also obvious that the hibernation is not only opioid-mediated, but also especially responsible for δ -opioid receptors (Bot *et al.*, 1998). In addition, when the serum of hibernating animals is injected into those who are active in summer, it will induce hibernating behaviors and physiological function. In contrast, opioid antagonists can reverse the dormancy (Shirakawa *et al.*, 2014). Previous researches have revealed that reperfusion can give rise to disorders of cardiomyocyte energy metabolism, changes in myocardial ultrastructure and apoptosis (Mohanty *et al.*, 2002). The ischemic preconditioning (IPC) strategy is applied to protect tissues from IRI. Furthermore, opioids have been proved that they can protect against IRI. Sufentanil (Fen), as an opioid analgesic, synthesized from phenylpiperidine derivatives is structurally related to pethidine. It acts primarily on μ receptors and is commonly used as an inducible adjuvant in neurosurgery (Yang *et al.*, 2009). It was suggested in several studies that Fen can be easily absorbed, and then reach the highest level of serum about two min. Moreover, there is almost no release of histamine and no cardiovascular effect. It is suggested that it is 75-100 times as effective as morphine (Ferrari *et al.*, 1998). However, Fen has a variety of side effects, including nausea, vomiting, bradycardia and respiratory inhibition, which are usually dose-dependent (Halestrap, 2010). What the important is that Fen can protect the heart from injury after ischemia in some reports (Coskun *et al.*, 2014). Nevertheless, the underlying mechanism of Fen and its relationship with cardiomyocyte apoptosis in IRI remain undefined. In this respect, a rat model was established to observe the effect of Fen pretreatment on myocardial apoptosis induced by IRI.

MATERIALS AND METHODS

Animals and grouping

120 male rats (weight: 200-250g; age: 3 months) were provided by our hospital. The experimental rats were

arranged in an appropriate experimental environment (room temperature: 25°C; humidity: 40-60%; lighting: 12 h/d), fasting 7 days in advance of the experiment and keeping an empty stomach for 12 h before a myocardial IRI model is built. Mice were randomly grouped into six ($n=20$), including control group (group S), I/R group, saline group (NS group), fentanyl low (2 μ g/kg of Fen1 group), medium (4 μ g/kg of Fen2 group) and high (6 μ g/kg of Fen3 group) dose groups. The experiment was conducted by strictly abiding to the guidelines of the National Health Research Institutes on the use of experimental animals. All traumatic operations were done with anesthesia and every effort was made to alleviate the pain.

Modeling of myocardial IRI

The rats fasted for 12 h before the establishment of myocardial IRI model. Electrocardiogram (ECG) examination was performed before operation in order to get the lead ECG and exclude the abnormal ECG in rats. The rats were intraperitoneally injected 1.5 mL/kg (30 g/l) pentobarbital sodium for anesthesia. Then, fixation, tracheal intubation and connection with ALC-V animal ventilator were carried out. The respiratory rate was set as 50-60/min. The rat ECG was recorded continuously. The skin of the chest was incised longitudinally along the median line of the left clavicle about 2 cm. The sternal is was clamped longitudinally with hemostatic forceps a few times, and then cut off with scissors. A small incision was made in the intercostal space between the 2nd and 3rd ribs on the left near the sternum, which was roughly parallel to the axilla. Thoracic incision and pericardial incision were carefully made to completely expose the rat's heart, and the left atrial appendage and pulmonary cone were exposed. The 5# needle and sutures were inserted vertically at the lower margin of the left atrial appendage and removed at the joint of the left atrial appendage and the pulmonary cone of rats. The left anterior descending branch (LAD) was ligated. ST elevation showed that the MI model was successfully established. After ligating the left ventricular apex and the inferior wall, blood pressure decreased and ECG changed. ECG showed local redness and ST segment depression after perfusing again. Chest was opened and sutures were not ligated in the group S. The chest was opened and the left ventricular branch sutures were ligated 30 min in the I/R group. Then the rats were re-perfused for 120 min. Before appearing ischemia in the NS group, normal saline was intravenously injected for 5 min, followed by a 5-minute interval (repeated 3 times). Then the ligation lasted 30 min and reperfusion lasted 120 min. Groups 1, 2 and 3: Fentanyl (2, 4 and 6 μ g/kg, respectively) was intravenously injected for 5 min before ischemia, followed by a 5-minute interval (repeated 3 times). Then

the ligation lasted 30 min and reperfusion lasted 120 min.

Determination of hemodynamic parameters

In these experimental rats, the syringe was indwelling in the carotid artery and connected to a pressure sensor. The specific surgical procedures were described below: The right carotid artery was separated based on the tracheal intubation. The distal artery was ligated with 1.0# sutures. A 45-degree oblique incision was cut on the arterial wall below the ligating sutures about 1 cm by using an eye scissor. Inserted catheter into the artery and connected it to pressure sensor to monitor the change of arterial pressure patterns. Recorded hemodynamic parameters, including heart rate (HR), mean arterial pressure (MAP), left ventricular developed pressure (LVDP), left ventricular pressure rate ($\pm dp/dt_{max}$), each group's baseline condition (T0), immediately before ischemia (T1), ischemia for 30 min (T2), reperfusion for 30 min (T3), reperfusion for 60 min (T4) and reperfusion for 120 min (T5).

Determination of biochemical parameters

Sampled 2ml blood from the right carotid artery of rats of each group at T0 and T5, stood still 2 h, and centrifuged 10 min at 680 g under indoor temperature, then stored the upper layer of serum at -80°C. Malondialdehyde (MDA) was measured by thiobarbituric acid reactive substance assay. Superoxide dismutase (SOD) was detected by xanthine oxidase. Creatine phosphokinase-MB (CK-MB) was tested by immunosuppression. These operations were completed according to specifications of MDA, SOD and CK-MB kits. The plasma concentration of cardiac troponin-I (cTnI) was determined at T5. Put blood samples (2 ml) in clean test tubes with anticoagulants. These operation steps were done strictly according to the instructions of the kit. Colloidal gold immunochromatography and automated immunoassay analyzer were used for measurement.

Determination of histological and morphological changes

Histological and morphological changes were observed with hematoxylin-eosin staining. At the end of reperfusion, the heart was taken out to rinse with ice NS. The atria, right ventricle and connective tissue were removed. The left apex was taken out and cut into two myocardial sections with a thickness of 0.2 cm. Put myocardial sections in 5% of form aldehyde solution for 24 h, washed with phosphate buffered saline, and embedded them in paraffin. The embedded sections were cut into 5 cm at thickness and observed them after staining.

Determination of MI regions

The blue even 2, 3, 5-triphenyl tetrazolium chloride (TTC) method was adopted to detect the MI area. The

blood was drawn while reperfusion ends and LAD was sutured again, then, injected Avans Blue (5%, 2 ml) into the tail vein. When cardiomyocytes in the non-ischemic region were shown to be dark blue, the heart was dried with filter papers and quickly removed and weighed. The left ventricle (LV) was isolated, weighed, and then frozen for 1 h at -20°C. After that, cut the frozen LV into 6 myocardial sections (2 mm/section) along the long axis, and put the LV sections in 1% of TTC solution (pH 7.4), incubated in constant temperature water at 37°C, and fixed in 10% formaldehyde for 15 min. After that, taken photos of the slices and weighed. Because dehydrogenase was existed in living cells and TTC can be restored to a deep red color, the infarct size (IS) cannot be stained and presented at grayish white. The range of risk area (AAR) and IS was determined by applying Image-Pro Plus 5.0 for orthogonality. The weight of each myocardial section was adjusted to calculate the gross weight of the left ventricle. The results were expressed in percentage, and calculated the myocardial ischemic area in the form of AAR weight/ LV weight, and MI area as IS weight/AAR weight. $S = \sum[(A1 \times W1) + (A2 \times W2) + (A3 \times W3) + (A4 \times W4) + (A5 \times W5) + (A6 \times W6)] \times 100\%$, $AAR = \sum[(R1 \times W1) + (R2 \times W2) + (R3 \times W3) + (R4 \times W4) + (R5 \times W5) + (R6 \times W6)] \times 100\%$. $IS/AAR\%$ = each slice's infarct weight/hazardous area weight $\times 100\%$, of which A refers to the infarct area of the film, R refers to the risk area of the film in the left ventricle, and W refers to the weight of each part. The number is within a range of 1-6.

Myocardial apoptosis

Put the paraffin-embedded sections (5 cm in thickness) on the slide and measured the apoptosis index (AI) by applying terminal deoxynucleotidyl transferase mediated dUIP nick end labeling (TUNEL) assay. Under optical microscopy, the nuclei of TUNEL positive cells were shown as brown. And applied the image Pro Plus 4.5 image analysis software to calculate quantity of apoptotic cells, then, randomly chosen five non-overlapping high-power fields (40 \times) to calculate the quantity of apoptotic positive nuclei and the total number of nuclei. Formula for calculating AI of cardiomyocytes is: $AI = (\text{number of apoptotic nuclei of myocardial cells} / \text{total myocardial nuclei}) \times 100\%$, to get a mean AI.

Determination of protein expression

Rats were executed at T5. Their heart was removed with ice and washed with ice NS. The left ligated ventricular myocardial tissue was cut into small pieces and ground them in a glass grinding container. The protein lysate was added and stood still 30 min. The left ventricular homogenate was centrifuged continuously at

a speed of 300 g for 10 min under 4°C. Then extracted the supernatant and continuously centrifuged again at 300 g for 30 min under indoor temperature. After that, extracted the supernatant, and repacked into 20 µl, and then stored at -80°C for the next step. For purpose of analysis, thawed myocardial proteins, then homogenized and reheated. Next, the protein supernatant was transferred to the nitrocellulose membrane by applying the SDS-polyacrylamide electrophoresis and sealed it with 10% skim milk tris buffer saline and Tween 20 (TBS-liquid) for 1 h. Then, primary antibody (1:1000; rabbit-anti-mouse B cell lymphoma 2 (Bcl-2) and Bax) was added. After that, the whole system was incubated for 2 h under indoor temperature and flushed three times with TBST solution. The horseradish peroxidase labeled secondary antibody was put in. The whole system was hatched for 2 h under room temperature, flushed 3 times with TBST solution, and then developed through an electronic chemiluminescence kit. The gel imaging system was scanned and the gray value of each strip was analyzed via the Quality One software. The relative protein expression levels were worked out.

mRNA expression of Bcl-2 and Bax in cardiomyocytes

The necrotic myocardial tissue of the anterior wall of the left ventricle (about 100 mg) was loaded into a cryotube without contamination of RNA enzyme and stored in liquid nitrogen. The reverse transcription polymerase chain reaction (RT-PCR) was applied in the measurement of the expression of cardiomyocyte Bax and Bcl-2 mRNA. The total myocardial RNA was extracted by Trizol kit. A UV300 ultraviolet spectrophotometer was used in the detection of the RNA concentration. Then cDNA was synthesized. And the mRNA expression of Bax, Bcl-2 and internal reference β -actin was detected using a ABI7500 fluorescence quantitative PCR instrument. Bax upstream primer: 5'-GT-TACAGGGTTTCATCCAGG-3', downstream primer: 5'-CGTGTCCACGTCAGCAAT-3'. Amplified length was 178bp. Bcl-2: Upstream primer: 5'-TACGAGTGGGAT-ACTGGAGA-3', downstream primer: 5'-TCAGGCTG-GAAGGAGAAG-3'. Amplified length was 80 bp. β -actin upstream primers: 5'-CGTGCGTGACATTAAGAG-3', downstream primer: 5'-TTGCCGATAGTGATGACCT-3' With amplified length 132 bp. Cycle parameters: 30 cycles at 95°C for 30 s; 95°C for 30 s, 57°C for 30s and 72°C for 1 min and then extended to terminal to 72°C for 10 min.

Statistical methods

As for the analysis of the data, Graph Pad Prism 7 statistical software was applied. consequently, the data reported included mean and standard deviation. The t test was utilized to study differences between groups. One-way ANOVA was introduced to investigate parameters among

multiple groups. $P < 0.05$ indicated there was a significant difference.

RESULTS

The difference in HR, MAP, LVDP and $\pm dp/dt_{max}$ was not statistically significant in each group at T0 as shown in Table I (all $P > 0.05$). The HR and MAP of I/R, NS, Fen1, Fen2 and Fen3 groups decreased at other time points by being compared with the group S (all $P < 0.05$). The HR and the MAP of other groups at T1, T2, T3, T4 and T5 were also significantly different from that at T0 (all $P < 0.05$). During the I/R, LVDP and $+ dp/dt_{max}$ (two indicators of left ventricular systolic function) of I/R group and Fen3 group at T1 were lower (all $P < 0.05$). Moreover, LVDP was relatively low at T2 and $+ dp/dt_{max}$ was lower at T4 and T5 in the Fen3 group (all $P < 0.05$). The LVDP and the $+ dp/dt_{max}$ at T2, T3, T4 and T5 were significantly different from that at T0 in the I/R group (all $P < 0.05$). The $+ dp/dt_{max}$ at T4 and T5 obviously decreased (all $P < 0.05$) in the Fen3 group. The LVDP and $+ dp/dt_{max}$ in the Fen 1 and Fen 2 groups notably reduced by being compared with that in the Fen1 and Fen2 groups. The $- dp/dt_{max}$ (left ventricular diastolic function indexes) at T3, T4 and T5 in the I/R, Fen1 and Fen3 groups and at T3 in the Fen2 group was obviously lower than that at T0 in the corresponding groups (all $P < 0.05$). The $- dp/dt_{max}$ declined at T2, T3 and T5 in the I/R and Fen3 groups, at T3 and T5 in the -Fen1 group, at T3 in the Fen2 group, compared to group S (all $P < 0.05$). The $- dp/dt_{max}$ in the Fen2 and Fen1 groups were approximate to the group S while being compared to the Fen3 group (Table I).

The SOD and MDA indexes at T0 hadn't significant difference in the two groups (both $P > 0.05$). The SOD values decreased while the MDA values increased in other groups after reperfusion, except group S when they were compared with the baseline values of SOD and MDA (all $P < 0.05$). The SOD values in other 5 groups went up while the MDA went down after reperfusion while being compared to the group S (all $P < 0.05$). The MDA concentration after reperfusion in the myocardial tissue significantly reduced while the SOD activity elevated in the Fen group when it was compared with the I/R group (all $P < 0.05$). However, the difference in SOD values and MDA concentrations was not significant between NS group and I/R group (all $P > 0.05$; Table II).

The serum CK-MB was similar at baseline ($P > 0.05$ in all groups). Other groups' CK-MB values were higher than the baseline value of CK-MB after reperfusion except for the group S (all $P < 0.05$). The CK-MB values of other groups were higher than those of the group S after re-perfusing (all $P < 0.05$). The CK-MB values in Fen1 and Fen2 groups were below that in

Table I. Effect of sufentanil (Fen) on ischemia and reperfusion indexes of rats.

Index/ group	Base line (T0)	After ischemia ischemia (T1)	Ischemia 30min(T3)	After reperfusion perfusion 10 min (T3)	Perfusion 60 min (T4)	Perfusion 120 min (T5)
HR (Time/min)						
S	351.21±21.21	345.34±31.35	341.23±05	347.46±23.56	350.34±23.54	352.36±43.51
I/R	352.31±14.34	353.13±23.54	316.35±26.21	324.35±23.61	324.37±23.53	327.64±34.62
NS	352.98±31.23	354.23±24.86	318.23±23.12	325.35±23.51	324.37±25.36	327.74±23.68
Fen1	357.00±31.02	329.34±36.37	331.47±27.84	329.34±32.57	327.36±42.12	329.82±32.64
Fen2	361.45±31.23	330.34±36.14	319.36±24.15	327.37±25.34	330.84±36.84	330.25±43.65
Fen3	360.26±21.32	340.36±23.76	320.25±25.68	329.45±32.86	328.63±36.73	327.47±35.95
MAP (mmHg)						
S	102.35±34.65	106.03±23.21	103.56±34.64	103.94±23.83	106.98±23.64	109.35±23.54
I/R	103.46±23.54	102.46±45.32	94.04±53.02	82.35±35.21	82.31±36.73	78.34±37.85
NS	98.00±32.65	93.45±34.25	93.25±36.82	81.46±43.32	82.95±36.54	80.93±36.42
Fen1	97.03±24.07	94.93±32.45	87.92±35.42	81.36±24.76	84.53±23.46	83.93±35.65
Fen2	102.46±20.03	87.24±24.46	84.36±42.54	83.02±53.43	81.00±37.32	79.46±35.21
Fen3	100.43±35.65	88.93±23.54	92.36±24.54	84.92±43.92	83.51±25.97	80.42±37.53
LVDP (kPa)						
S	13.90±1.40	14.57±32.54	13.35±23.12	12.36±2.31	12.35±2.41	12.03±3.57
I/R	12.34±1.43	13.58±1.57	12.31±35.63	10.36±2.36	9.36±2.43	9.30±2.53
NS	12.46±3.21	12.54±2.47	11.70±1.57	16.86±1.43	11.36±2.41	10.82±3.27
Fen1	13.53±2.64	12.64±2.46	13.26±1.65	11.48±3.53	10.36±2.51	10.23±2.64
Fen2	12.58±3.41	12.30±3.46	14.36±1.53	10.31±3.51	9.73±3.54	11.37±2.43
Fen3	11.36±2.65	11.68±3.25	13.25±1.53	11.36±2.16	12.46±2.46	9.82±3.27
Left ventricular pressure rate +dp/dtmax (kPa s⁻¹)						
S	453.34±32.54	453.34±23.54	454.34±23.54	424.34±21.54	436.34±23.54	454.34±23.54
I/R	425.37±23.54	443.45±27.43	437.45±27.43	437.45±14.43	437.37±27.43	435.45±27.43
NS	437.37±63.37	416.36±32.41	416.36±27.41	416.36±32.41	416.26±27.41	416.26±27.41
Fen1	423.47±23.54	403.65±53.18	403.65±25.18	436.25±25.18	403.37±25.18	435.65±25.18
Fen2	436.73±37.54	421.13±26.58	421.53±26.58	437.53±26.58	421.53±26.58	432.53±26.58
Fen3	453.89±23.54	413.32±24.74	413.47±24.74	413.47±16.74	413.30±24.74	412.47±24.74
Left ventricular pressure rate -dp/dtmax (kPa s⁻¹)						
S	345.36±24.63	336.36±24.63	345.36±24.63	363.54±34.23	335.54±34.23	363.54±25.23
I/R	330.46±24.62	330.46±24.62	329.46±24.62	243.73±24.34	243.45±24.14	214.73±24.34
NS	325.57±13.64	325.36±13.64	315.57±13.64	323.5±25.56	314.5±25.56	323.5±24.56
Fen1	332.57±25.74	337.57±25.74	337.57±25.74	312.46±25.31	312.46±25.31	313.56±25.31
Fen2	364.62±53.54	364.37±53.54	348.62±53.54	257.34±24.47	225.34±24.47	256.74±24.47
Fen3	331.46±34.23	331.38±34.23	333.66±34.23	354.36±23.65	335.36±23.65	355.96±23.65

S, control group; I/R, ischemia-reperfusion group; NS, normal saline I/R group; Fen1, 2µg/kg Fen group; Fen2, 4µg/kg Fen group; Fen3, 6µg/kg Fen group; HR, heart rate; MAP, mean arterial pressure; LVDP, left ventricular developed pressure.

the I/R group (both $P < 0.05$) after reperfusion (Table II). The difference in CK-MB values was not significant after reperfusion among Fen3, NS and I/R groups (all $P > 0.05$). The serum cTnI concentration dramatically increased in I/R, Fen and NS groups when it was compared to the group

S (all $P < 0.05$). The cTnI concentration in the Fen1 and Fen2 groups was markedly lower than that in the I/R group ($P < 0.05$). There was not significant difference at the cTnI concentration among Fen3, NS and I/R groups ($P = 0.355$; Table II).

Table II. Effect of sufentanil (Fen) on SOD, MDA, CK- mB in rats with myocardial IRI.

Indexes		Study groups					
		S	I/R	NS	Fen1	Fen2	Fen3
SOD	Baseline	162.34±35.72	153.25±37.52	155.37±25.63	157.34±64.23	164.47±36.74	156.36±43.61
	Reperfusion	173.24±42.51	77.48±35.21	79.37±35.62	106.35±53.21	127.46±35.64	102.42±36.72
MDA	Baseline	3.61±1.32	4.25±2.31	4.52±2.51	4.64±3.24	4.26±1.42	4.74±2.41
	Reperfusion	4.02±1.42	10.13±3.52	10.32±3.62	6.21±3.41	5.72±3.62	8.36±3.61
CK-MB	Baseline	0.43±0.12	0.32±0.31	0.35±0.14	0.36±0.15	0.36±0.16	0.41±0.15
	Reperfusion	0.45±0.21	1.01±0.54	0.83±0.25	0.72±0.36	0.62±0.14	1.23±1.14
cTnI		7.34±2.71	72.31±35.26	68.26±46.27	44.93±23.15	35.35±24.14	67.43±45.24

SOD, superoxide dismutase; MDA, malondialdehyde; CK-MB, creatine phosphokinase MB; cTnI, cardiac troponin-I; for details of group, please see Table I.

DISCUSSION

This study assessed the impacts of various concentrations of Fen pretreatment on I/R-induced myocardial apoptosis of rats. The I/R model for rats was developed as I/R model in the present study. The cardiac function results reveal that the HR and the MAP of other groups decreased at other time points except baseline time when they were compared to the group S. The LVDP and the $\pm dp/dt_{max}$ rather low during the I/R process in the I/R group, indicating that the process may affect the cardiac function. Therefore, the model is successfully established. It was also found that the LVDP and the $+dp/dt_{max}$ were lower in the Fen3 groups than that in the Fen1 and Fen1 groups, while the $-dp/dt_{max}$ in the Fen2 and Fen1 groups were close to the group S, which indicates that Fen can relieve IRI-induced cardiac dysfunction. Besides, the low and medium concentrations of fentanyl work better. Fen, as an effective synthetic opioid analgesic, has been applied in the treatment of breakthrough pain with advantages of high efficacy and short action time (Shirakawa *et al.*, 2014). It has been reported that the opioid system plays a variety of important roles in maintaining cardiac function by affecting cardiac rhythm and even developmental process (Myers *et al.*, 1981). IReports said that fentanyl can prevent infarction by mediating δ opioid receptor and protein kinase C (Martindale and Metzger, 2014). To investigate the protective effect of Fen on the heart, other relevant indexes were also measured. One of our results shows that the MDA level of myocardial tissue after reperfusion clearly fell off while the SOD activity went up in the Fen group by being compared to that of the I/R group, which explains that fentanyl has an antioxidation procedure in cardiomyocytes during the I/R. MDA can destroy biofilm with lipid peroxidation, while SOD's regulating the balance of body oxidation may reduce lipid peroxidation (Mohanty *et al.*, 2002). Reduced SOD activity does not eliminate

excess oxygen radicals. This may lead to the formation of a large amount of MDA (Yang *et al.*, 2009). It was also discovered that CK-MB values and cTnI concentrations after reperfusion are lower in the Fen1 and Fen2 groups than the I/R group. It is well known that cTnI is highly expressed in the myocardium, at the same not, it is also a preferred biomarker in MI identification (Ferrari *et al.*, 1998). Previous studies have shown that cTnI and CK-MB are released after cardiac surgery (Halestrap, 2010). Based on this, it is believed that Fen may help alleviate IRI. It was also reported in one clinical study that the application of opioid may contribute to reducing the release of CK-MB and cTnI (Coskun *et al.*, 2014). The study also displays a distinct uptrend of MI area and a downtrend of apoptotic cardiomyocytes in the Fen1 and Fen2 groups as being compared with the I/R group. It is suggested that low and medium doses of fentanyl prevent from I/R-induced cardiomyocyte apoptosis. Fen, a fentanil analogue, can limit the size of the MI and protect the heart in a dose-dependent manner. The phosphorylation plays a role of mediation with connexin 43 (Veighey and MacAllister, 2015). It shows in our study that Fen down-regulates Bax protein and mRNA expression and up-regulates Bcl-2 protein and mRNA expression to resist IRI and grow the ratio of Bcl-2 to Bax. Among vertebrates, apoptosis occurs primarily by regulating Bcl-2, which involves alterations in the integrity of the outer mitochondrial membrane (OMM) (Nock *et al.*, 1990; Bolling *et al.*, 1997). Bax, as one of the pro-apoptotic effector proteins, can destroy the Bcl-2 of OMM. It is known that it can give rise to the permeabilization of outer mitochondrial membrane, resulting in activation of caspases and cysteine proteases and thus leading to cell destruction (Bot *et al.*, 1998; Shirakawa *et al.*, 2014). In other words, Fen protects IRI by reducing apoptosis via increasing Bcl-2 expression and reducing Bax expression. MI is the main cause of acute heart failure. Incomplete repair after injury and proliferation of fibrous tissue can cause

sustained myocardial injury and it gradually develops into chronic heart failure, leading to serious damages to human health (Oeltgen *et al.*, 1987). The animal model used in this study is relatively mature. The successful preparation of the model is the key to successful experiments. We have investigated whether Fen could inhibit MI from the two aspects: MI area and apoptosis, thereby protecting the heart during reperfusion. Some studies have used the TTC and Evan blue double staining for the determination of MI area. The difference in AAR and IS of all samples is significant. This proves that the ligation position is correct and the model is successfully established. Some scholars have believed that the infarct area decreases significantly in the MF and SF groups. The apoptosis occurs after myocardial ischemia reperfusion. The difference in AI is significant between the MF or SF group and I/R group. These results suggest that Fen can reduce the infarct area after ischemia-reperfusion and inhibit the myocardial apoptosis. It can protect the myocardial tissue. It is consistent with the results of previous studies (Mohanty *et al.*, 2002). The oxidant/antioxidant balance in healthy tissues is sustained by antioxidant dominance. Aggressive factors may give rise to tissue damage, thus causing impaired oxidant/antioxidant balance, which is beneficial to oxidants (Yang *et al.*, 2009). This is called oxidative stress (Ferrari *et al.*, 1998). Creatine kinase MB (CK-MB) and other parameters are introduced to assess cardiotoxicity in the literature. CK-MB levels in cardiac tissues have been reported to raise in parallel with increased oxidant parameters (Oeltgen *et al.*, 1996). Studies based on the fact that CK-MB levels of both SF and MF are dramatically declined have safely concluded that Fen reduces the cardiac toxicity in MI when being compared to the I/R group. According to these findings, it is suggested that ischemic preconditioning with Fen can prevent the cardiac reperfusion injury. RIPc and morphine may cut down the reperfusion injury during the initial percutaneous coronary intervention. Although Fen has been shown to protect against IRI, the optimal dose may be atopic worth discussing. It was found that, despite the IRI of Fen3 group is less than that of the I/R group, and its protective effect is lower than that of Fen1 and Fen2 groups. Based on this result, it is speculated that the accumulated fentanyl may trigger a large number of opioid receptors, thereby leading to antagonism. Nevertheless, this research is a preliminary. In order to confirm our findings, there is a need for more clinical studies.

CONCLUSIONS

To sum up, Fen pretreatment may play an antioxidant role in cardiomyocytes during the I/R. It may bring down the I/R injury-induced myocardial apoptosis by

down-regulating Bax expression and up-regulating Bcl-2 expression. As a result, Fen may be a potential drug to treat of IRI-related heart disease.

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IRB approval

Ethics committee approval was obtained from the Institutional Ethics Committee of "The Third Hospital of Hebei Medical University" to the commencement of the study.

Ethical approval

The study was carried out in compliance with guidelines issued by ethical review board and institutional biosafety committee of The Third Hospital of Hebei Medical University.

Statement of conflict of interest

The authors have declared no conflict of interest.

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