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Effect of emulsified isoflurane on apoptosis of anoxia–reoxygenation neonatal rat cardiomyocytes

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ABSTRACT

Objective: To explore the effect of emulsified isoflurane (EI) on apoptosis of anoxia–reoxygenation neonatal rat cardiomyocytes and relevant protein expression. **Methods:** Cardiac muscle anoxia–reoxygenation damage model was established with culture in vitro neonatal rat cardiomyocytes. The cardiomyocytes were divided into control group, model group, fat emulsion group and EI group. The cardiomyocytes apoptosis rates and lactic dehydrogenase (LDH), superoxide dismutase (SOD) and malondialdehyde (MDA) index standardization were detected after relevant treatment. The expression of apoptosis–related proteins Bcl–2, Bax and Caspase–3 were detected with Western blot approach. **Results:** After hypoxia/reoxygenation (H/R) model was treated by EI, the cells apoptosis rate decreased and was dramatically below the fat emulsion group ($P < 0.05$). Cardiomyocytes biochemical index detection presented that, compared with the control group that the LDH activity and MDA content dramatically increased ($P < 0.05$), while the SOD activity notably decreased ($P < 0.05$); compared with the H/R group, the SOD activity of the fat emulsion group and EI group increased ($P < 0.05$); while the LDH activity and MDA content decreased ($P < 0.05$). And the change of the EI group was more remarkable than the fat emulsion group ($P < 0.05$). The Western blot analysis presented that, compared with the control group, the Bcl–2 protein expression of the other groups significantly decreased ($P < 0.05$), the expressions of Bax protein and Caspase–3 protein increased significantly ($P < 0.05$); compared with H/R group, cardiomyocytes Bcl–2 protein expression of EI group increased significantly ($P < 0.05$), the expressions of Bax protein and Caspase–3 protein decreased significantly ($P < 0.05$), and the change of EI group was more remarkable than the fat emulsion group ($P < 0.05$). **Conclusions:** EI can inhibit the apoptosis of anoxia–reoxygenation damage model cardiomyocytes, and may be related to the up–regulation of expression of Bcl–2 and down–regulation of expression of Caspase–3 protein.

1. Introduction

Hypoxia/reoxygenation damage model is a model simulating the myocardial ischemia–reperfusion injury *in vitro*, in which the effect of active substances on the changes of the morphological structure, physiological function and biochemical metabolism of the injured cardiomyocytes and

the mechanism of action of drugs can be explored, and which makes it the ideal model for screening the active substances of myocardial ischemia–reperfusion injury^[1,2]. Cardiomyocytes ischemia–reperfusion injury could lead to the apoptosis, which is one sort of non–inflammatory death of gene regulated autologous programmed cells caused by physiological or pathological stimulations in local environment. Thereinto, the Bcl–2 family, Caspase family, P53 protein and Survivin protein are important apoptosis–regulatory factors with interrelations and interactions, regulating the apoptosis.

Emulsified isoflurane (EI) is a new intravenous inhalation anesthetic which is made from 30% lipid emulsion carrier

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and isoflurane under certain circumstances^[3]. It can generate good anesthetic action and can be injected through the vein and exhaled through the lung. The induction and anaesthesia are fast. It has good safety and controllability and no toxic and side effect on important organs.

This research explored the effect of EI on H/R cardiomyocytes and the influence on the expression of apoptosis-related proteins with neonatal rat cardiomyocytes *in vitro*.

2. Materials and methods

2.1. Key instruments and reagents

BH75–HERA cell CO₂ incubator was purchased from GmbH Co., Germany; BD FACSCanto II flow cytometry (FCM) from BD Co., USA; 752 ultraviolet spectrophotometer from Nanjing Qilin Analytical Instrument Co. Ltd, and D–37520 high speed freezing centrifuge from Thermo Co., USA.

EI (with volume ratio of 8%) and 30% fat emulsion were purchased from Sino–Swed Pharmaceutical Corp. Ltd.; XTT reagent from Sino–American Biotechnology Co., Ltd.; collagenase II from Sigma Co., USA; SOD, LDH and MDA kits from Nanjing Jiancheng Bioengineering Institute.

2.2. Experimental animals and grouping

A total of 48 clean Wistar neonatal rats (not limited to one sex) were provided by the animal experimental center of The Third Xiangya Hospital Central–South University. Full-span pulsation cells that had been cultured for 5 d were selected and divided into four groups randomly, namely the control group, H/R group, fat emulsion group and EI group. Thereinto, the control group: 6 h cultivation in normal oxygen environment without drug treatment; H/R group: 3 h reoxygenation after 3 h anoxia without drug treatment; fat emulsion group: fat emulsion was added into the medium to reach a concentration equal to the fat emulsion concentration of the EI group, 2 h intervention, 3 h reoxygenation after 3 h anoxia; EI group: 2 h intervention with 8% EI (the final concentration was 0.28 mmol/L), then 3 h reoxygenation after 3 h anoxia.

2.3. Primary culture of cardiomyocytes

The method established in reference of Guo *et al*^[4] was improved by combining with practice. Wistar neonatal rats were selected 1–3 days after birth. The hearts were taken out in aseptic condition and washed for three times with low-temperature Hanks balanced salt solution or phosphate buffer carefully. The envelope and large vessels were removed on the aseptic operating platform and then cut into small blocks about 1 mm×1 mm×1 mm after washing 3 times. The blocks were put into the 50 mL capped

centrifuge tube with 0.125% collagenase II. Then the tube was put onto the thermostatic oscillator (37 °C, 280 r/min) for 8–10 min digestion, and the supernatant was drawn and abandoned after natural sedimentation. 5 mL collagenase II was added into the precipitated tissue blocks for 5–8 min digestion under circumstances mentioned above. Then the supernatant was collected after natural sedimentation and added into the centrifuge tube containing stop buffer of the same quantity [15% fetal bovine serum and 85% dulbecco's modified eagle medium (DMEM)] to terminate the digestion. The above mentioned process was repeated for 4–6 times until the tissue blocks were fully digested. The cell suspension collected was filtrated through 200 molybdenum nylon net. The supernate was abandoned after 10 min centrifugation (1 000 r/min) and added into nutrient solution (15% fetal bovine serum, 85% DMEM, 100 U/mL penicillin, 100 U/mL streptomycin and 0.1 mmol/L BrdU) for suspension and trypan blue counting. The cell number was adjusted to 1×10^6 /L. It was moved into the culture flask and cultured for 2 h in 37 °C, 5% CO₂ incubator to purify the cardiomyocytes (repeated adherence method) and then inoculated to the culture plate or culture flask for culture. The solution was replaced by DMEM (containing 15% fetal calf serum) and 0.1 mmol/L BrdU was added in to inhibit the development of the fibroblasts every other 24 h. Primary cardiomyocytes with 72 h culture were taken out for tests.

2.4. Modeling of cardiomyocytes H/R injury

In order to simulate the myocardial ischemia–reperfusion injury, hypoxia fluid [mmol/L] was prepared according to the method of Zhao *et al*^[5]: NaCl 98.5 mmol/L, KCl 10 mmol/L, NaH₂PO₄ 0.9 mmol/L, NaHCO₃ 6.0 mmol/L, CaCl₂ 1.8 mmol/L, MgSO₄ 1.2 mmol/L, CH₃CH(OH)COONa 40 mmol/L, HEPES 20 mmol/L; pH 6.8. Then it was saturated with gas mixture (95% N₂ and 5% CO₂). It contained K⁺, lactate and H⁺ in high concentration, with low oxygen concentration and no energy substrates like glucose; and reoxygenation fluid: NaCl 129.5 mmol/L, KCl 5.0 mmol/L, NaH₂PO₄ 0.9 mmol/L, NaHCO₃ 20 mmol/L, CaCl₂ 1.8 mmol/L, MgSO₄ 1.2 mmol/L, glucose 5.5 mmol/L, HEPES 20 mmol/L; pH 7.4; 10 mL fetal calf serum was added when using. Then it was saturated beforehand with gas mixture (95% O₂ and 5% CO₂) (1 L/min×30 min). The normal culture was replaced by hypoxia fluid, and then the primary cardiomyocytes were put into the hypoxia device and filled with gas mixture (95% N₂ and 5% CO₂). The cardiomyocytes culture flask together with the hypoxia device was put in the incubator for 3 h culture. The hypoxia model was established. The hypoxia fluid was tipped 3 h later and reoxygenation fluid was added. Then it was put in the incubator (95% air, 5% CO₂, 37 °C) for 3 h culture in normal oxygen environment. And the reoxygenation model was prepared.

2.5. Detection of cardiomyocytes apoptosis rate

FCM was used for the detection. The adherence cardiomyocytes were digested by 0.25% trypsin and collected into the test tube for 10min centrifugation (1 000 rpm) after they were separated. The cell suspension was made into single-cell suspension after double suction with 1ml needle, and hung overnight in 70% cooled alcohol (4 °C). 10 min centrifugation first was conducted when in detection. Alcohol was scoured away with PBS. The cell deposition was stained for 30 min in 4 °C away from light with PI. Then it was put on the device for detection. The hypodiploid peak before Period G represented the apoptosis peak which reflected the percentage of apoptotic cells.

2.6. Detection of cardiomyocytes biochemical indexes

Cell culture supernatant was fetched and its lactic dehydrogenase (LDH) was detected with semiautomatic biochemistry analyzer; SOD activity was detected with xanthine oxidase method; MDA content was detected by barhituric acid method. Each method should be strictly conducted according to the kti operation instructions.

2.7. Western blot detection of apoptosis-related protein expression

Defined amount of protein extraction RIPA and PMSF were added into the cardiomyocytes of 72 h primary culture after 3 times lavation with PBS. After a short period of hatch, the cells were scraped off by cell scraper. The cell debris and lysate were moved into 1.5 mL EP tube followed by 30 min ice incubation, 3 times swirl oscillation and 5min 4 °C 12 000 rpm centrifugation. The supernatant was moved into clean EP tubes. The protein concentration of each group was detected by coomassie brilliant blue protein quantification kit. They were stored in -80 °C refrigerator. 40 μg protein sample was fetched for SDS-PAGE electrophoresis and moved in the nitrocellulose membrane. Non-specific binding sites were blocked by block buffer containing 5% skimmed milk. Rabbit-anti-mouse antibody Bcl-2 (or bax or caspase-3) was added for incubation. Sheep-anti-rabbit second antibody marked by horseradish peroxidase was added after lavation for 2 h incubation. ECL chemiluminescence reagent was added after the membrane was washed with PBST buffer for three times. Then they were exposed to light for imaging. The integrated optical density of each strip was detected by Quantity One 4.62 software. The corresponding protein expression index was indicated in densitometric analysis value of the objective stripe/densitometric analysis value of GAPDH stripe.

2.8. Statistical analysis

The statistical analysis of all data was made by MATLAB7.0

software. The measurement data was indicated with (mean ±SD). Variance analysis was adopted for the comparison of multiple groups. And $P<0.05$ difference was taken of statistical significance.

3. Results

3.1. Effect of EI on cardiomyocytes apoptosis rate

It can be seen from Table 1 that there existed cardiomyocytes apoptosis in the control group, H/R group, fat emulsion group and EI group, and apoptosis differences between any two groups ($P<0.05$). Any of the apoptosis rates of H/R group, fat emulsion group and EI group was significantly higher than that of the control group ($P<0.05$). Compared with the H/R group, the apoptosis rates of the fat emulsion group and EI group decreased notably ($P<0.05$). And compared with the fat emulsion group, the apoptosis rate of EI group decreased significantly ($P<0.05$).

Table 1

Apoptosis rates of neonatal rat cardiomyocytes.

Groups	Apoptosis rate (%)
Control group	3.99±1.21
H/R group	41.25±4.66*
Fat emulsion group	28.31±5.87* [△]
EI group	18.96±3.74* ^{△▲}

Note: compared with control group, * $P<0.05$; compared with H/R group, [△] $P<0.05$; compared with fat emulsion group, [▲] $P<0.05$.

3.2. Changes of LDH, SOD activity and MDA content of cardiomyocytes

The comparisons of LDH, SOD activity and MDA content of neonatal rat cardiomyocytes among the four groups were of statistical significance ($P<0.05$). Compared with control group, LDH activity and MDA content of other groups increased notably ($P<0.05$), while SOD activity was obviously lower ($P<0.05$); compared with H/R group, SOD activity of fat emulsion group and EI group were higher ($P<0.05$), while LDH activity and MDA content lower ($P<0.05$); compared with fat emulsion group, SOD activity of EI group was higher ($P<0.05$), while LDH activity and MDA content were lower ($P<0.05$) (Table 2).

Table 2

Changes of LDH, SOD activity and MDA content of cardiomyocytes.

Groups	LDH (U/L)	SOD (U/mg)	MDA (nmol/mg)
Control group	1 293.77±71.52	308.11±4.67	1.62±0.87
H/R group	3 018.94±62.31*	186.39±4.11*	9.56±0.77*
Fat emulsion group	2 701.66±57.13* [△]	196.74±3.68* [△]	8.62±0.36* [△]
EI group	1 997.34±97.42* ^{△▲}	263.41±8.53* ^{△▲}	3.51±0.69* ^{△▲}

Note: compared with control group, * $P<0.05$; compared with H/R group, [△] $P<0.05$; compared with fat emulsion group, [▲] $P<0.05$.

3.3. Expressions of cardiomyocytes Bcl-2, Bax and Caspase-3 protein

From the Western bolt detection of the expressions of neonatal rat cardiomyocytes Bcl-2, Bax and Caspase-3 protein of the four groups, it found that compared with control group, the Bcl-2 protein expression of H/R group, fat emulsion group and EI group was notably lower ($P<0.05$) and Bax and Caspase-3 protein expressions were higher; compared with H/R group, there was no statistical significance in the expression difference of Bcl-2, Bax and Caspase-3 protein of fat emulsion group ($P<0.05$), while Bcl-2 protein expression of cardiomyocytes of EI group was higher and Bax and Caspase-3 protein expressions were lower ($P<0.05$); compared with fat emulsion group, Bcl-2 protein expression of EI group was higher while Bax and Caspase-3 protein expressions were lower ($P<0.05$) (Figure 1 and Table 3).

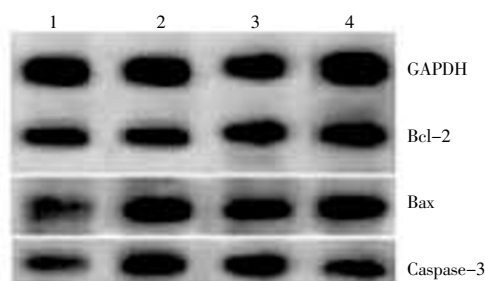


Figure 1. Western bolt detection of Bcl-2, Bax and Caspase-3 protein. Lane 1: control group; Lane 2: H/R group; Lane 3: fat emulsion group; Lane 4: EI group.

Table 3

Expressions of Bcl-2, Bax and Caspase-3 protein (relative to GAPDH).

Groups	Bcl-2	Bax	Caspase-3
Control group	1.33±0.15	0.36±0.27	0.25±0.048
H/R group	0.24±0.38*	1.03±0.15*	0.67±0.045*
Fat emulsion group	0.41±0.22*	0.80±0.09*	0.62±0.019*
EI group	0.91±0.14* ^{△▲}	0.51±0.24* ^{△▲}	0.31±0.022* ^{△▲}

Note: compared with control group, * $P<0.05$; compared with H/R group, [△] $P<0.05$; compared with fat emulsion group, [▲] $P<0.05$.

4. Discussion

Myocardial ischemia-reperfusion injury is a common pathological and physiological phenomenon during the perioperative period of ischemic heart disease and cardiac operation, and it has been a hot spot in this field to alleviate or eliminate this injury[6]. Cardiomyocytes H/R injury model can simulate myocardial ischemia-reperfusion injury despite the influence of nerve and body fluid for direct observation of the effect of pharmaceuticals on cells and has been widely used in cardiovascular studies[7].

Once the cardiomyocytes injury activates the apoptosis program, the cardiomyocytes will go into the irreversible death process, which makes quite a number of irreparable cardiomyocytes go into death process too, decreasing the number of cardiomyocytes and the myocardial function and exacerbating myocardial ischemia-reperfusion injury[8].

Myocardial anoxia is the basic pathological process of ischemic heart disease, as well as an important initial factor of reasons like shock after severe trauma leading to heart failure[9]. The apoptosis phenomenon exists in the H/R process, so inhibition of cardiomyocytes apoptosis could become a new way to treat ischemic heart disease[10]. Isoflurane induction preprocessing can inhibit the apoptosis of ischemia-reperfusion cardiomyocytes, and this inhibition is in relation to the up-regulation of the expression of antiapoptotic gene Bcl-2 family and the down-regulation of the expression of apoptotic gene Bax[11]. Hochhauser *et al*[12] have confirmed through transgenic mouse experiments that high expression of Bax gene could promote the cardiomyocytes apoptosis, and there was a positive correlation between the expression degree and the apoptosis degree.

It was found in this experiment that Bcl-2 expression of normal cardiomyocytes was high, while Bax expression was low with little apoptosis; Bcl-2 expression of H/R group was high and Bax low with notable increase of apoptosis; emulsified isoflurane could enhance Bcl-2 expression and reduce Bax expression. The ratio of Bcl-2/Bax was significantly increased. And the apoptosis was reduced. Compared with H/R group, the effect of fat emulsion on Bcl-2 and Bax expression was of no statistical significance. And the anti apoptosis effect of fat emulsion was relatively weak. Bcl-2/Bax had an important gene regulation effect on inhibiting apoptosis in the cardiomyocytes ischemia-reperfusion injury mechanism. Appropriate intervention of Bcl-2/Bax gene expression would have a positive effect on the prevention and treatment of ischemic myocardial injury. Emulsified isoflurane had a protection effect on ischemia-reperfusion injury of primary cultured cardiomyocytes, which could be in relation to its inhibition of Caspase-3 protein expression.

LDH is a glycolytic enzyme, and the change of LDH leakage of cardiomyocytes is an important indicator for reflecting cardiomyocytes necrosis[13]. MDA is an important product of the lipid peroxidation, whose content can reflect the degree of lipid peroxidation of the myocardial membrane structure[14], and indirectly reflect the generation of oxygen radical and the degree of the cellular damage. SOD is a specific enzyme eliminating oxygen radicals *in vivo*, whose activity is a reflection of the antioxidant function of the organism[15]. In the experiment, compared with the control group, the LDH activity and MDA content increased and the SOD activity reduced ($P<0.05$), which

indicated that cardiomyocytes of H/R group were damaged during the ischemia–reperfusion process and proved that the preparation of the ischemia–reperfusion model of neonatal rat cardiomyocytes was successful^[16]. With the EI intervention, however, the LDH activity and MDA content notably decreased compared with H/R group, and the SOD activity increased significantly ($P < 0.05$). This indicated that EI could alleviate the H/R injury of cardiomyocytes with an obvious cardiac muscle protection effect, whose mechanism of action may be in relation to antioxidation. EI had the protective effect on neonatal rat H/R injury of cardiomyocytes and the mechanism may be in relation to antioxidation. As the carrier of emulsified isoflurane, fat emulsion could increase the ATP supply of heart, enhance the utility of fatty acid substrate and have a certain effect on the cardiac muscle protection^[17–20]. From the research results it can be seen that both emulsified isoflurane and fat emulsion have protection and apoptosis inhibition effect on cardiomyocytes of the ischemia–reperfusion model, however, the anti–apoptosis effect of fat emulsion is relatively weak.

Conflict of interest statement

We declare that we have no conflict of interest.

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