Emulsified Isoflurane Produces Cardioprotection Against Severe Burn-Induced Cardiac Shock
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ABSTRACT

Background: Cardiac shock, occurring at the early stage of severe burns, causes cardiac insufficiency. Cardiac dysfunction after severe burn is associated with myocardial injury. Emulsified isoflurane (Elso) has been demonstrated to have cardioprotective effect against ischemia and reperfusion injury. Whether burn-induced cardiac dysfunction is influenced by Elso is currently unknown. We tested the hypothesis that Elso decreases myocardial damage in rat model with severe burn.

Methods: In an in vivo study, sprague dawley rats, weighting 250-300 g, were randomized into four groups: 1) sham-burn group (sham, saline), 2) burn group (CON, saline, 30% total body surface area [TBSA] full-thickness burn), 3) burn+30% intralipid (IL, vehicle for Elso) group, and 4) burn+8% Elso of 1.5 ml/kg (Elso) group. Lactated ringers solution was immediately injected intraperitoneally to all rats after burn injury according to Parkland formula (4.0 ml/kg/1% TBSA), followed by continuous intravenous infusion of isovolumetric saline, intralipid and Elso for 30 minutes in each group. Hemodynamic parameters were constantly monitored throughout the experiment. 3 hours post-burn, hearts were excised for determination of enzyme activity and apoptosis.

Results: Under baseline conditions, no significant differences in systemic hemodynamics were observed among the groups. Heart rate decreased after burn injury in the CON, Elso and IL groups. Compared with the sham group, burn injury was accompanied by a reduction of systolic function in left ventricular systolic pressure (LVSP) and maximum rate of increase in left ventricular pressure (dP/dtmax), and an increase in maximum rate of decrease in left ventricular pressure (-dP/dtmax). 2 to 3 hours after burn, Elso attenuated decreases in LVSP and dP/dtmax. Malondialdehyde (MDA) was reduced from 2.55 ± 0.29 mmol/mg prot in CON group and 2.21 ± 0.23 in IL group to 1.79 ± 0.28 in Elso group (P<0.01 vs. CON and P<0.05 vs. IL). Elso also increased the level of superoxide dismutase (SOD) (162.5 ± 10.5 U/mg prot), compared with CON group (140.4 ± 12.1, P<0.01). However, there was no difference regarding the changes of apoptotic protein (Bcl-2 and Bax) expression among groups. Meanwhile, no apoptotic cells were detected throughout the experiment in any groups.

Conclusions: Elso could protect hearts against severe burns during the early stage postburn, which may be independent from the involvement of apoptosis.
Cardiac shock happens at the early stage of severe burns, and is defined as a major factor that affects patients’ outcome. It can cause cardiac depression and dysfunction post burn. This burn-related cardiac dysfunction is associated with myocardial injury.

Treatment of anesthetics such as isoflurane has been proved to have strong cardioprotective effect against myocardial ischemia and reperfusion injury in various animal models by limiting infarct size and improving cardiac function. Emulsified isoflurane (Elso) is a recently developed formulation that can be administered intravenously rather than as an inhalant, as was the traditional practice for this anesthetic. It has been extensively studied and showed also a strong character of protecting hearts against local myocardial ischemia or ischemia/reperfusion injury. However, whether burn-induced cardiac dysfunction can be attenuated by Elso is currently unknown.

Furthermore, the potential mechanisms contribute to Elso-induced protective phenomenon against severe burn-related cardiac shock has never been studied. We previously showed in the rat myocardial ischemia/reperfusion model that Elso could sufficiently inhibit apoptosis, however, whether its inhibitory effect could also be applied to protect hearts after severe burn remains unclear.

This investigation with an in vivo rat model aimed to determine: 1) whether administering Elso after burn injury could protect heart; and 2) whether Elso could inhibit myocardial apoptosis after severe burn injury.

**METHODS**

All experimental protocols and procedures were approved by the Institutional Animal Care and Use Committee of Sichuan University (Sichuan, China). Adult sprague dawley (SD) rats of both sexes (250-300 g) were used. All animals were kept under controlled conditions and a 12-hour day and night cycle. The purity of Elso (8% Vol/Vol) was rechecked by gas chromatography at the beginning of the experiment (Agilent 4890 D; Tegent Technology Ltd., Shanghai, China).

**General Preparation**

All rats were anesthetized with a single intraperitoneal injection of 1% sodium pentobarbital (5 ml/kg) and allowed to stabilize for 15 minutes before baseline parameters measurement. Rats were then randomized into four treatment groups: 1) sham-burn group (sham, saline), 2) burn group (CON, saline), 3) burn + 30% intralipid (IL, vehicle for Elso) group, and (4) burn + 8% Elso of 1.5 ml/kg (Elso) group (N=7-8, each group). All rats except for sham suffered a 30% total body surface area (TBSA) full-thickness burn by immersing the back of the trunk for 18 seconds in 98°C water. Lactated ringers solution was immediately injected intraperitoneally to all rats after burn injury according to Parkland formula (4.0 ml/kg/1%TBSA) (1-3), followed by continuous intravenous infusion of isovolumetric saline, intralipid and Elso for 30 minutes in each group. A 24-G catheter (Spacelabs Medical, Inc., Redmond, WA, USA) filled with heparinized saline was inserted into the caudal vein for saline and drug infusion. Sham group was treated in the same manner as the trauma group except for in 37°C water. 3 hours post-burn, hearts were excised for determination of enzyme activity and apoptosis (Figure 1).

**Measurements of Hemodynamics**

Hemodynamic parameters, such as heart rate (HR), left ventricular systolic pressure (LVSP), left ventricular end diastolic pressure (LVEDP), and maximum rate of increase/decrease in left ventricular pressure (±dP/dtmax), were continuously monitored prior to- or post-burn injury in our study. A heparin-saline solution filled 20-G catheter (Spacelabs Medical, Inc., Redmond, WA, USA) was inserted from the right carotid artery to the left ventricle for hemodynamic measurement. Pressure signals were amplified and digitally converted and stored in the computer throughout the experiment by the connected calibrated pressure transducer with physiologic recorder (Biolap 420F, Taimeng, Chengdu, China).

**Preparation of Emulsified Isoflurane**

Elso (8% Vol/Vol) is produced by Huarui Pharmaceutical Co., Chengdu, China. Intralipid (Huarui Pharmacy, Ltd., Chengdu, China) in this study was used as vehicle. Elso purity was rechecked by gas chromatography before the experiment (Agilent 4890 D; Tegent Technology Ltd., Shanghai, China).
Measurement of Enzyme Activity

Rats were sacrificed at the end of the experiment. Serum levels of superoxide dismutase (SOD) and malondialdehyde (MDA) were determined spectrophotometrically according to manufacturer's instruction (Nanjing Jiancheng Biological Product, Nanjing, China) using a UV spectrophotometer (Model UV-2401PC; Shimadzu Co., Kyoto, Japan). All samples and standards were measured in duplicate.

Tissue Collection

At the end of the experiment, heart was dissected and sliced into 2-mm-thick sections parallel to the atrioventricular groove. The atria and the right ventricle were removed. Transverse sections of the heart were rinsed in saline, blotted onto filter paper, and dried, then immersed overnight in 10% phosphate buffered formalin at room temperature before sliced into 5 μm thick sections after been fixed in formaldehyde, dehydrated, and embedded in paraffin, consecutively. Left ventricular walls and the septum were included in each slice for immunohistochemical analysis and apoptosis measurement. All analyses and measurements of pro- and anti-apoptotic proteins and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) assay were performed by a single researcher blinded to the treatment group.

Immunohistochemical Procedure and Evaluation

Immunohistochemical method was used to evaluate the expression of Bcl-2, Bax proteins. Heart sections were deparaffinized in xylene and isopropanol. Anti-Bcl-2, anti-Bax antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used as the primary antibodies, and goat anti-rabbit IgG (Bcl-2) or goat anti-mouse IgG (Bax) antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was added as the second antibody. Tissues were stained with fresh 3, 3-diaminobenzene (Beijing Zhongshan Golden Bridge Biotechnology Co., Beijing, China) solution and counterstained with hematoxylin for microscopy. A phosphate buffer solution was used as negative control. Brown staining in the cytoplasm of cells was evaluated as positive expression. Ten visual sights were randomly selected. Image analysis was conducted with Image-pro plus (Media Cybernetics Inc., Carlsbad, CA, USA). Statistical value was calculated by the ratio of optical density of area positively stained to mean optical density, i.e. positive expressive index (PEI).

Determination of Myocardial Apoptosis

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay was used to analyze myocardial apoptosis according to the manufacturer’s protocol (Roche Diagnostics, USA). More than 10 random different fields from each heart were chosen. The apoptotic index (AI) was calculated as a percent of the number of positively TUNEL-stained cardiomyocyte apoptotic nuclei/total cardiomyocyte nuclei population. Images were obtained using a CAST system (Olympus A/S, Denmark) and analyzed with Image-pro plus (Media Cybernetics Inc., Carlsbad, CA, USA). Assays were performed in a blinded manner.

Statistical Analysis

Data were analyzed with SPSS 16.0 software for Windows (SPSS Inc., Chicago, IL, USA). The values were given as means ± standard deviation (SD). Homogeneity of variance was tested by
### RESULTS

**Hemodynamics**

Under baseline conditions, no significant differences in systemic hemodynamics were observed among the groups. Repeated measures ANOVA confirmed a statistically significant difference among sham, CON, IL, and Elso groups in HR (P=0.000), LVSP (P=0.034), dp/dtmax (P=0.002) and -dp/dtmax (P=0.014). There were significant interactions between group assignment and time for HR (P=0.000), LVSP (P=0.002), dp/dtmax (P=0.004) and -dp/dtmax (P=0.000). HR decreased after burn injury in the CON, Elso and IL groups (P=0.000 vs sham). Compared with the sham group, burn injury was accompanied by a reduction of systolic function in LVSP (CON: P=0.034) and dp/dtmax (CON: P=0.012; IL: P=0.004), and an increase in -dp/dtmax (CON: P=0.031; IL: P=0.034), which indicated the depression of diastolic function. No significant group-related differences were found in hemodynamics throughout the experiment among the CON, IL, and Elso groups. 2 to 3 hours post burn injury, Elso attenuated decreases in LVSP (P=0.022 vs CON) and dp/dtmax (P<0.05 vs CON and IL) (Table).

**Serum Enzyme Activity**

3 hours post burn injury, MDA was reduced from 2.55±0.29 mmol/mg prot in CON group and 2.21±0.23 in IL group to 1.79±0.28 in Elso group (P<0.01 vs. CON and P<0.05 vs. IL). Meanwhile, Elso also increased the level of SOD (162.5±10.5 U/mg prot), compared with CON group (140.4±12.1, P<0.01). No statistical significance was defined between CON and IL groups (P>0.05) (Figure 2).
Expression of Bcl-2 and Bax Proteins
Bcl-2 and Bax immunoreactivity was detected in the left ventricular myocardium in each group (Figure 3). The cardiomyocytes contained very low levels of Bcl-2 protein, as 0.13 ± 0.05% in sham, 0.11 ± 0.03% in CON, 0.10 ± 0.03% in IL and 0.10 ± 0.04% in Elso group. No difference was found among groups in this area (P = 0.7579). Similarly, the expression of Bax protein was limited in four groups. Compared with the sham-operated group (0.12 ± 0.04%), neither burn injury (0.12 ± 0.03%), nor burn injury in combination with IL (0.13 ± 0.05%) or Elso (0.13 ± 0.04%) treatment did change Bax protein expression (P = 0.9785). Meanwhile, Bcl-2/Bax ratio was unaltered among groups (P = 0.9355).

Myocardial Apoptosis
TUNEL positive cardiomyocytes were not detected in the sham-operated group, nor in either group after 3 hours post burn injury (Figure 4). Administration of Elso did not alter the ratio of apoptotic cardiomyocytes to total number of cardiomyocytes. Meanwhile, treatment with intralipid had no effect on cardiomyocyte apoptosis.

**DISCUSSION**

We showed in our study that Elso provided cardioprotection against severe burn-induced cardiac dysfunction in the rat heart in vivo. The main findings were as follows: first, in a model of third-degree burn injury over 30% of TBSA, treatment with Elso (8% Vol/Vol) 1.5 ml/kg post burn, increased cardiac function. Second, Elso attenuated the increase in serum MDA and SOD concentrations. Third, this protective phenomenon is independent from the mechanism involving apoptosis, nor the involvement of modulation of the expression of pro- and anti-apoptotic proteins, specifically Bax, and Bcl-2.

Severe burn injury is accompanied by multiple organs dysfunction, which results in high mortality and mobility (1). Cardiac shock, occurring at the early stage of severe burns, is usually associated with myocardial injury (2). This burn induced cardiac depression includes changes of myocytes histology, alternation of myocardial performance and reduced cardic function. It has been well characterized and defined as a major factor that affects patient outcomes (3). Anesthetic-induced cardioprotection, including preconditioning and postconditioning, are effective and potent strategies available for myocardial protection. Specifically, isoflurane has been repeated shown to exert strong cardioprotective effect against myocardial ischemia and reperfusion injury (4). Elso, an innovative formation of isoflurane, that enables an intravenous (rather than the traditional inhalation) route of administration for this anesthetic (5, 6), was shown to have similar cardioprotective effect as isoflurane does. For example, we and other researches have demonstrated that intravenous infusion of Elso could produce acute and delayed preconditioning or postconditioning against myocardial infarction in animals (7-12). The current results also confirmed these previous findings demonstrating that in a rat model of severe burn injury, Elso could also protect hearts by decreasing serum levels of SOD and MDA and attenuating decreases in LVSP and dP/dtmax 2 to 3 hours post burn injury.

The mechanisms responsible for the beneficial effects of Elso-induced cardioprotection against severe burn injury have not yet to be firmly established. Apoptosis links to the pathogenesis of myocardial injury, which is determined by apoptotic regulating genes and triggering signals molecules. Bcl-2 family plays vital role in apoptosis. It is composed of anti-apop-
Tonic (Bcl-2, Bcl-x, Bcl-xL, Bcl-w, Mcl-1 and A1), and pro-apoptotic proteins (Bax, Bak, Bok), all of which possess Bcl-2 homology regions which form a hydrophobic groove (13). They regulate the caspase family activation, and modulate the release of cytochrome c and calcium. Bcl-2, the 26-kDa anti-apoptotic protein that localizes in the outer mitochondrial membrane, was considered to be the most important gene that prolongs cell survival. In contrast, pro-apoptotic Bax is a homologous protein that has opposing effects on regulating apoptosis. EIso was shown to increase Bcl-2 expression and decrease Bax expression, facilitating Bcl-2/Bax ration upregulation as a stress response to myocardial ischemia and reperfusion injury (7, 8).

![Figure 3. Immunoreactivity of Bcl-2 and Bax Protein in the Cytoplasm of the Myocytes of Hearts.](image)

Brown staining in the cytoplasm was evaluated as positive expression. CON: control group; IL: 30% intralipid group; EIso: emulsified isoflurane 1.5 ml/kg group (magnification 400x). Each group N=5. Data were presented as mean ± SD. PEI=positive expressive index.

![Figure 4. Apoptosis Determined by Measurement of Terminal Transferase dUTP Nick End Labeling (TUNEL) Positive Cardiomyocyte Nuclei in the Myocardium Obtained from Rats Post Burn Injury.](image)

Rats were receiving saline (sham, CON), 30% intralipid (IL) or 1.5 ml/kg emulsified isoflurane (Elso) after burn (magnification 400x).
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8, 10). However, limited numbers of previous studies have shown that apoptosis is associated with severe burn-induced cardiac shock event and it is remaining controversial due to the experimental design protocol and the severity of burn injury. It has been reported that 3 hours post burn, caspase-3 activity could increase but other significant morphologic alterations characteristic of apoptosis can be found at 6 hours post-burn (14). Xiao et al. (15) have demonstrated that severe burn injury could trigger myocardial autophagy as early as 3 hours post burn, which is associated with post-burn cardiac dysfunction. However, substantial levels of myocardial apoptosis were not detected until 6 hours post burn. In our study, using the same burn protocol as 30% TBSA burn injury, we found that Elso induced cardioprotection occurred independent from apoptosis at least 3 hours post burn injury, which was in consistent with this previous report, showing no apoptosis occurred throughout 6 hours post burn injury. In contrast, other researcher used a 40% TBSA burn injury model, and found that apoptotic cells could be seen as early as 1 hour post burn and the increase of apoptosis is time dependent (16). The discrepancy may be attributed to the severity of burn injury and the TBSA involved in the burn injury.

Concentrations of SOD and MDA in serum are indicators of oxidative stress. Their elevation is associated with cell injury. We found in our study that, Elso led to a significant decrease in SOD and MDA after severe burn injury, indicating amelioration of cardiomyocytes injury.

We acknowledged several limitations of this study. Firstly, we did not measure the long term outcomes regarding Elso induced cardioprotection against severe burn injury. Therefore, whether this anesthetic could produce long term protection remains unknown. Secondly, we only measured apoptosis 3 hours post burn, it is not clear that after longer time, such as 6 hours post burn, whether apoptotic cells could be detected and whether Elso could inhibit apoptosis. The relative contribution of the mechanisms of Elso induced cardioprotection against severe burn-induced cardiac shock remains to be elucidated.

In conclusion, the current results supported that intravenous administration of Elso (8% Vol/ Vol) 1.5 ml/kg after a 30% TBSA full-thickness burn injury, afforded an effective protection against severe burn-induced myocardial injury in rats, which may occur independently from myocardial apoptosis.

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References