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Methodology Article

Experimental Methodology of Inflammatory Factors Induced by Anaesthetics in Myocardial Ischemia

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Abstract: Current evidence indicates that anesthetics produce important effects to protect myocardium against ischemia-reperfusion (I/R) injury. The imbalance between pro- and anti-inflammatory cytokines might contribute to the mechanism of progression of I/R injury. To investigate experimental methodology of inflammatory factors in myocardial I/R with anesthetics preconditioning. This paper discusses the methods from the several aspects, including animal species, methods for model preparation, patterns of common anesthetics intervention, and the results of the intervention inflammatory factors including TNF-α, IL-1, IL-6, IL-8, IL-10 together with nuclear transcription factor NF-kB which regulates inflammation were explored in I/R models. The results showed that anesthetics exerted the protective effects on the injury of ischemia reperfusion injury through the regulation of inflammatory factors.

Keywords: Experimental Methodology, Anesthetics, Myocardial Ischemia-Reperfusion, Inflammatory Factor

1. Introdution

It is well-known that the non-specific innate immune reaction always take quickly response to the inflammatory or antigen-related stimulation. Since AMI elicits prompt tissue necrosis and inflammation, it is conceivable that innate immunity has an important role to play a rapidly immune-inflammatory response, especially at the early phase of AMI. Surprisingly, while the link between AMI and inflammatory reaction has been investigated in depth [1-7], the involvement of the innate immune system in inflammation after AMI has only been seldom reported [5, 7, 8]. Efficient inflammation is required for host defense against damage and tissue repair. However, excessive or chronic myocardial inflammation, which is reported to induce severe damage to the myocardium, is involved in a number of cardiac disorders, such as myocardial infarction (MI) [9], ischemia-reperfusion (I/R) injury [10], and so on. Acute myocardial infarction (MI) leads to molecular, structural, geometric and functional changes in the heart during a process known as ventricular remodeling. Myocardial infarction is followed by an inflammatory response in which pro- and anti-inflammatory cytokines play a crucial role, particularly in left ventricular remodeling. The increase of pro-inflammatory cytokines TNF-α and IL8 was accompanied by decreased anti-inflammatory IL-10. This imbalance between pro- and anti-inflammatory cytokines might contribute to the progression of left ventricular remodeling and may lead to heart failure [11].

All volatile anesthetics have cardiac depressant effects that decrease myocardial oxygen demand and may thus improve the myocardial oxygen balance during ischemia. Recent experimental evidence has clearly demonstrated that, in addition to these indirect effects, volatile anesthetic agents also directly protect from ischemic myocardial damage. Implementation of these effects during clinical anesthesia can provide an additional tool for treatment or prevention of ischemic cardiac dysfunction during the perioperative period

[12]. Pharmacological preconditioning with volatile anesthetics, or anesthetic-induced preconditioning (APC), is a phenomenon whereby a brief exposure to volatile anesthetic agents protects the heart from the potentially fatal consequences of a subsequent prolonged period of myocardial ischemia and reperfusion. Although not completely elucidated, the cellular and molecular mechanisms of APC appear to mimic those of ischemic preconditioning, the most powerful endogenous cardioprotective mechanism [13]. In recent years, there has been increased interest in mechanisms involved in the anaesthetic-induced cardioprotection. It is not thoroughly understood how volatile anaesthetics protect the myocardium from ischaemia or reperfusion injury, but the overall mechanism is likely to be multifactorial. A variety of intracellular signalling pathways have been implicated in the protective phenomenon, including adenosine [14], ALDH2 [15], nuclear factor-κB and upregulation of autophagy [16], endothelial glycocalyx [17], Hypoxia-inducible factor 1 [18], endogenous signal transduction proteins, reactive oxygen species, mitochondria, and ion channels [19, 20]. A more thorough understanding of the multiple signalling steps and the ultimate cytoprotective mechanisms underlying anaesthetic-induced preconditioning may lead to improvements in the management of ischaemia and/or reperfusion injury.

Anesthetics and sedatives contribute to postoperative immunosuppression. Interleukin-8 (IL-8) is a chemotactic and activating factor that mediates neutrophil adhesion and margination and is essential for host defense. Anesthesia causes immune suppression and alters neutrophil function. Both midazolam and propofol decreased extracellular interleukin-8 accumulation, but intracellular levels and mRNA remained high. This suggests that propofol and midazolam alter interleukin-8 secretion from cells [21]. Propofol can inhibit the apoptosis of myocardium induced by ischemia-reperfusion injury by inhibiting the activation of NF-kB and down-regulating the levels of IL-8, which may be one of the action mechanisms of the effect of propofol [22]. Some factors which contribute to the effects of anesthetics on MI.

2. Materials and Methods

2.1. Animals

There are three different kinds of animals in the experiments involving:

2.1.1. Rat

Male adult Sprague Dawley rats were used in experiments with 200–350 g body weight. Adult male wistar rats with weighting 180-300 g were used in experiments. The animals were housed under standard laboratory conditions at 22-26°C, relative humidity of 55 ± 5% with a 12 hours light/dark cycle. The animals were raised in plastic cages, and allowed free access to food and water.

2.1.2. Rabbit

Male Japanese white rabbit with 2.5-3.0 kg body weight or male New Zealand white rabbits 2.0-2.5 kg were used in experiments. Male and female immature Japanese rabbits aged 2- 3 weeks were choosen in experiments.

2.1.3. Mice

Male C57BL/6 mice weighting 20- 25 g with 8 -10 Weeks of age participated the experiments.

2.2. Methods

2.2.1. Models for Rat

Rat was anesthetized with 2% pentobarbital sodium (50 mg/kg intraperitoneal), intubated through a tracheotomy and ventilated with 100% oxygen with a animal breathing machine. Electrocardiograph probes were applied to three legs to produce an ECG image. The left femoral artery was cannulated to monitor arterial blood pressure. To administer chemicals, a catheter filled with saline was placed in the right iliac vein. A thoracotomy was performed between the third and the fourth costal bones. An eyelid bracer was used to expose the thoracic cavity and a 5/0 silk suture was placed around the left anterior descending coronary artery. The ends of the suture were passed through a small piece of soft vinyl tubing to form a snare. Then, the snare was pulled and fixed by clamping the tubing with a small haemostat. Except for the sham Group, all the hearts were subjected to I/R. The time for ischemia (I) was 30 min and reperfusion (R) was 120 min, i.e. I/R 30 min/2 h [23], or I/R 30min/3 h [24].

2.2.2. Models for Rabbits

Male New Zealand white rabbits weighing between 2.5 and 3.0 kg were anesthetized with IV sodium pentobarbital (30 mg/kg) and acutely instrumented for the measurement of systemic hemodynamics as previously described. Baseline hemodynamics and arterial blood gas tensions were recorded 30 min after instrumentation was completed. All rabbits underwent a 30-min occlusion of the left anterior descending coronary artery (LAD) followed by 3 h of reperfusion. Briefly, a left thoracotomy was performed at the fourth intercostal space, and the heart was suspended in a pericardial cradle. A prominent branch of the LAD was identified, and a silk ligature was placed around this vessel approximately halfway between the base and apex for the production of coronary artery occlusion and reperfusion. Each rabbit was anticoagulated with 500 U of heparin immediately before LAD occlusion. Coronary artery occlusion was verified by the presence of epicardial cyanosis and regional dyskinesia in the ischemic zone, and reperfusion was confirmed by observing an epicardial hyperemic response [25]. The time for I/R also was 40 min/2h [26], or I/R 30min/3h [27], or I/R 30min/4h [28].

2.2.3. Models for Mice

3C57BL/6 mice were randomly divided into 3 groups (10 in each group). Myocardial I/R was induced by occlusion of left anterior descending (LAD) artery for 30min, followed by reperfusion for 6 h in I/R. Mice were anesthesiaed with 5%

pentobarbital sodium (40 mg·kg^{-1}) by intraperitoneal injection, and intubated through a tracheotomy and ventilated with a air respirator, the respiratory frequency was 130/min, and tidal volume maintained 1-2 ml. A thoracotomy was performed between the third and the fourth costal bones. Coronary artery ligation site was located on left anterior descending coronary artery (LAD) about 1 mm far from the inferior margin of left atrium [29].

2.2.4. Drug Intervention

I. Isoflurane

a. 1.0%Isoflurane was inhaled for 30 min [25]
b. 2.0%Isofluranewas inhaled for 2 h [26]
c. 2.0%Isofluranewas inhaled for 1 h [30]
d. 1.1%Isofluranewas inhaled for 30 min [31]

II. Sevoflurane

a. 2.5%sevoflurane was inhaled for 30 min [28]
b. 2.5% sevoflurane was inhaled for 10 min [32]
c. 2.5%sevoflurane was inhaled for 60 min [33]

III. Propofol

a. propofol 5 m g/kg, iv+ 20 mg/kg/ h 2 h [22]
b. propofol3, 6, 12 mg/k g, ivdrip, pre-ischemic 10 min+after reperfusion 2 h [34]
c. propofol 3, 6, 12mg/kg/h, ivdrip, pre-ischemic 10 min [35]
d. propofol 5 m g/kg/h 120 min [36]

IV. Butorphanol

a. butorphanol 40μg·kg^{-1}, im, at 30 min before ischemia [29]
b. butorphanol 50μg·kg^{-1}, iv, at 5 min before reperfusion [37]
c. butorphanol 50 μg/kg, iv, at the time of reperfusion [23]

V. Emulsified Isoflurane

a. 8% emulsified isoflurane, 8-10 ml was injected iv at 1 ml/s followed by continuous infusion at 6-8 ml/kg/h [38]
b. 8% emulsified isoflurane, 4 mL/kg/h, 30 min [39]

VI. Dexmedetomidine

a. 5 μ g/kg iv +5 μ g /kg/h for 1 h [40]
b. 10 μ g/kg iv +10 μ g /kg/h for 1 h [40]

Animals, Time for I/R, and Drug intervention were listed in Table1.

Table 1. *Animals, time for I/R, and drug intervention.*

Animals	Breed	Time for I/R	Drug intervention
Rats	SD, Wistar	30 min/2 h	Isoflurane, Propofol, Butorphanol, Dexmedetomidine, Sevoflurane
		30min/3 h	Emulsified isoflurane
Rabbits	Japanese white New Zealand	40 min/2h	Isoflurane
		30min/3h	Isoflurane
		30 min/2h	Isoflurane
Mice	C57BL/6	30min/4h	Sevoflurane
		30min/6h	Butorphanol

3. Results

Serum TNF- α and IL-6 concentrations were detected using an enzyme-labelled immunosorbent assay. After reperfusion for 120 min, 2 ml of blood was collected, centrifuged at 4°C (3000 r/min for 10 min) and transferred to a 1.5-ml EP tube. The next steps were performed according to the kit instructions. A coating plate was then placed in the special equipment (the length of the ultraviolet light wave was 450 nm). All optical density values were transferred to the final concentration. The serum TNF-α and IL-6 concentrations in B, B/N and B/G Groups were less than in the I/R Group

(all $P < 0.001$) [23]. TNF-α protein in myocardial tissue was measured by immunohistochemistry. TNF-α positive cells were also significantly reduced in emulsified isoflurane group compared with control group [24]. The plasma level of TNF-α was measured by ELISA, and isoflurane group had a lower level of TNF-α than I/R group [26]. A detection was made to the level of IL-8 and IL-10 at the time point of before ischemia (T1), 1 hour after reperfusion (T2), 2 hours after reperfusion (T3) and 4 hours after reperfusion (T4). The levels of IL-8 and IL-10 at T2 and T4 in both groups were higher than those at T1 ($P<0.05$) while no difference of statistical significance was found in the levels of IL-8 and IL-10 at T1 from those at T2 and T4 in each group; the levels of IL-8 at T2 and T4 in S group (sevoflurane postconditioning group) were much lower than those in I/R group (($P<0.05$) while the level of IL-10 in S group was much higher than that in I/R group [28]. Blood samples were taken from the abdominal aorta to determine the concentrations of serum TNF-α by ELISA [29], the NF-κB binding activity was detected by electrophoretic mobility shift assay (EMSA) [29]. The levels of serum TNF-α were significantly higher in group I /R than those in group sham (all $P<005$). The expressions of NF-κB binding activity were also significantly increased in group I /R ($P<0.05$). The levels of serum TNF-α in group B + I / R were significantly reduced ($P<0.05$). The expressions of NF-κB were also inhibited in group B + I / R ($P < 0.05$) [29]. Myocardial NF-KB was detected by immunohistochemistry, reduced activity of NF-kB play an important role in myocardial ischemia reperfusion injury [30].

The expression of NF-κB were examined by immunohistochemical technique, Isoflurane preconditioning may provide obviously myocardial protection against ischemia-reperfusion injury in immature rabbit heart by decreasing the expression of NF-κB [31]. The levels of proinflammatory cytokines TNF-α and IL-1 were assessed in heart homogenates by rat enzyme-linked immunosorbent assay kits according to the manufacturer's instructions (R&D

System, Minneapolis, MN). A 1:2 dilution of samples in calibrator diluent was used for cytokine determination. Quantitation of cytokines was normalized to wet tissue weight. The results show that myocardial TNF-α was greatly increased at the end of reperfusion in IR group compared with the control group (P <0.01). APC significantly decreased the production of TNF-α. IR also significantly increased the IL-1 level, and it, too, was attenuated by APC [32]. Expression of NF-κB was determined by Western blotting. Compared with sham control group, expression of NF-κB in the nuclei markedly increased in I/R group, compared with I/R group, propofol 6 and 12 mg/kg significantly attenuated expression of NF-κB in the nuclei [34]. The concentrations of TNF-α, IL-6, in serum and myocardium were detected by ELISA. The translocation of NF-κB in the cardiomyocytes was determined by immunohistochemistry, and the expression levels of NF-κB were detected by Western Blot. The concentrations of TNF-α, IL-6 in serum and myocardium, the expression levels of TNF-α, IL-6 mRNA in myocardium of group IV (diabetes group I/R) were significantly higher than those of group II (non-diabetes group, I/R). As compared with those in group IV (diabetes group I/R), administration of propofol at 6, 12mg/kg/h significantly inhibited the NF-κB to translocate into nucleus and decreased the expression of NF-κB in the nuclei, and reduced the concentrations of TNF-α, IL-6 in serum and myocardium, respectively [35].

The expression of TNF-α, NF-κB were determined by WB. The myocardial expressions of TNF-α and NF-kB protein were significantly higher in Groups B (I/R) and C (propofol+ I/R) than in control Group A (sham operation). The myocardial expressions of TLR-4 and TNF-α and NF-kB protein were down-regulated in Group C compared with Group B. Intravenous injection of propofol can protect against myocardial damage, Propofol can suppress the increase in myocardial TNF-α and NF-kB protein expressions induced by I/R [36]. Plasma TNF- α and IL-6 were measured after 120 mins of reperfusion respectively. Plasma TNF-α and IL-6 concentration in Group C (butorphanol postconditioning group) were lower than those in Group B (I/R), Butorphanol postconditioning has cardio-protective effects and can restrain the inflammatory factor releasing from injured hearts [37]. Blood samples were taken from carotid artery at T6 (60 min of reperfusion) for determination of IL-6 and IL-10 concentrations. IL-6 concentration were significantly lower while serum IL-10 concentration was significantly higher in emulsified isoflurane preconditioning group than in group I/R [38]. Myocardial TNF-α protein was measured by immunohistochemical method. TNF-α positive cells were also significantly reduced in emulsified isoflurane group compared with control group [39]. Serum TNF- α, IL-6 concentration were analyzed. Serum TNF- α, IL-6 were lower in group Dex1 (regular dosage of dexmeditomidine group) and Dex2 (large dosage of dexmeditomidine group) group than in group I/R, and there was no difference between group Dex1 and group Dex2 [40]. The experimental results to confirm the conclusion were listed in Table 2, Table 3, Figure 1, Table 4.

Table 2. *The serum TNF-α and IL-6 concentrations in rats.*

Group	TNF-α (ng/L)	IL-6 (ng/L)
sham	40.45±2.48	51.15±2.27
I/R	137.67±7.18$^{\Delta\Delta\Delta}$	176.98±3.70$^{\Delta\Delta\Delta}$
Butorphanol	88.21±3.64***	125.50±1.80***

Values are means±standard deviations, $^{\Delta\Delta\Delta}$P<0.001 vs sham,***P<0.001 vs I/R (PS. The data were from reference 23).

Table 3. *The expression of TNF-α in mice by ELISA.*

Group	TNF-α (ng/ml)
sham	183±34
I/R	796±91$^{\Delta}$
Butorphanol	334±65*

Values are means ± standard deviations, ΔP<0.05 vs sham, *P<0.05 vs I/R (PS. The data were from reference 29).

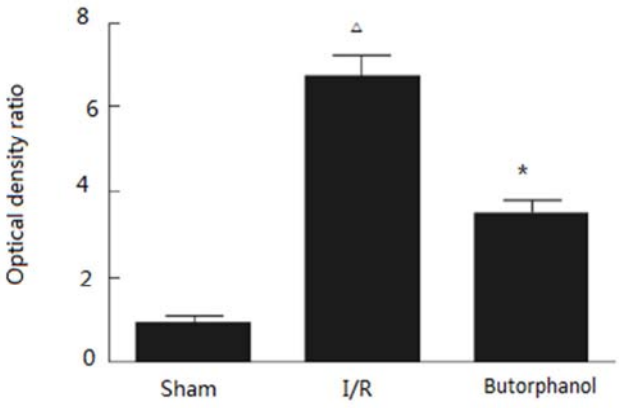

Figure 1. *The NF-κB binding activity in mouse myocardium, Values are means±standard deviations, $^{\Delta}$P<0.05 vs sham, *P<0.05 vs I/R (PS. The author amendmented the figure to explain the purpose according to the reference 29).*

Table 4. *The expression of NF-κB in immature rabbit myocardial cells using immunohistochemical technique.*

Group	NF-κB (Intensity Image)
sham	141.45±8.04
I/R	112.13±7.25$^{\Delta\Delta\Delta}$
Isoflurane	131.31±8.66**

Values are means±standard deviations, ΔP<0.001 vs sham,**P<0.01 vs I/R (PS. The data were from reference 31, the data were tested using U-test with knowning means, standard deviations, and samples capacities by the authors).

4. Discussion

To investigate the myocardial ischemia-reperfusion with anesthetics preconditioning (APC) would present beneficial effects on autonomic and cardiac function indexes after the acute phase of a myocardial ischemia-reperfusion. Anesthetics modify regional left ventricular dysfunction following ischemia/reperfusion but their effects on global function in this setting are less clear. Current evidence indicates that the imbalance between pro- and anti-inflammatory cytokines might contribute to the mechanism of progression of I/R injury. The experimental methodology of inflammatory factors in myocardial ischemia is of great significance. This paper discusses the methods from the several aspects,

including animal species, methods for model preparation, patterns of common anesthetics intervention, and the results of the intervention inflammatory factors including TNF-α, IL-1, IL-6, IL-8, IL-10 together with nuclear transcription factor NF-kB which regulates inflammation were explored in I/R models. The results showed that anesthetics exerted the protective effects on the injury of ischemia reperfusion injury through the regulation of inflammatory factors.

This paper can also provide reference for myocardial ischemia in new drug development. The experimental methodology of model in myocardial ischemia including specific methods, anesthesia, and the drug interactions between anesthetic and tested new molecular entity (NME), natural drug monomer, and biologics should be clearly worth considering.

5. Conclusion

This article discusses the methods from the several aspects, including animal species, methods for model preparation, patterns of common anesthetics intervention, and the results of the intervention inflammatory factors. Current evidence indicates that anesthetics produce important effects to protect myocardium against I/R injury. The appropriate methods of inflammatory factors induced by anaesthetics in myocardial ischemia will be important in future.

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