Synergistic protective effect of ischemic preconditioning technique in combination of N-acetylcysteine on skeletal muscle ischemia reperfusion injury in rat; Histopathological study

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ABSTRACT

Many surgical procedures, such as limb revascularization and free-flap reconstruction, involve prolonged ischemia of skeletal muscle. Ischemic damage can lead to severe postoperative complications, including muscle dysfunction and necrosis. In order to prevent these complications of ischemia reperfusion injury, various free radical scavengers and methods have been tested experimentally. Either ischemic preconditioning or N-acetylcysteine alone attenuated these changes. This study, examined the effects of ischemic preconditioning in combination of N-acetylcysteine on skeletal muscle ischemia reperfusion injuries. Twenty four wistar male rats were divided randomly into four experimental groups: group A (ischemia reperfusion), group B (ischemia reperfusion + N-acetylcysteine), group C (ischemic preconditioning) and group D (ischemic preconditioning + N-acetylcysteine). After intramuscular ketamine and xylazine anesthesia, femoral artery was exposed. Animals in groups A and B were undergone 2h of ischemia by occlusion femoral artery and 24h of reperfusion and rats in groups C and D were undergone two 20-min cycles of ischemic preconditioning followed by 2h ischemia and 24h reperfusion. Rats that were treated with N-acetylcysteine (groups B and D) given IV at a dose of 150 mg/kg, immediately before reperfusion. After 24h of reperfusion, rats were euthanized and left gastrocnemius muscle harvested for histopathological analysis under optical microscopy. Inflammatory changes, neutrophil presence, interstitial edema, hemorrhage and necrotic fibers in the muscle were scored. The extent of muscle changes in the group D was significantly lower than other groups (p<0.05). Degree of muscle changes in B and C groups were lower than that in the A group (p<0.05). These results suggest that ischemic preconditioning and N-acetylcysteine act synergistically to protect skeletal muscle against ischemia reperfusion injuries.
INTRODUCTION

Many surgical procedures, such as limb revascularization and free-flap reconstruction, involve prolonged ischemia of skeletal muscle. The reestablishment of blood flow after ischemia paradoxically adds to the damage done by prolonged ischemia [1]. This phenomenon is known as ischemia reperfusion injury, and is characterized by perfusion disorders and interstitial edema resulting from capillary constriction and increased permeability [2].

A specific mechanism uniformly responsible for ischemia reperfusion injury is unlikely since energy degradation during ischemia, generation of reactive oxygen species during reperfusion, “no-reflow” phenomenon, and calcium overload reperfusion all have been reported to contribute to injury from ischemia reperfusion [3, 4].

Ischemic preconditioning is a new technique to improve tissue tolerance to prolonged ischemia by causing repeated brief episodes of vascular occlusion followed by reperfusion. This phenomenon was first described by Murry et al. [5], who showed that ischemic preconditioning induced myocardial tolerance to sustained ischemia. Moreover, exogenous or endogenous adenosine, a degradation product of adenosine triphosphate, has been shown to mimic ischemic preconditioning and is proposed to be one of the chemical mediators of ischemic preconditioning [6–7]. Many investigators have attempted to apply this technique for skeletal muscles [6, 8, 9, 7–14].

The results of the previous studies support the view that N-acetylcysteine can exert a protective effect against skeletal muscle injury caused by ischemia reperfusion in the rats. N-acetylcysteine is not solely an antioxidant, but also inhibits neutrophil infiltration and improves microcirculation and may be more beneficial than other antioxidants in case of ischemia reperfusion injury [15, 16].

MATERIALS AND METHODS

All animals of the present research were cared according to the norms of the Islamic Azad University Faculty of Veterinary Sciences laboratory of animal experimentations; this investigation was approved by the Committee of Ethics in Research with animals in Islamic Azad University.

Twenty four wistar male rats weighing 250–300 g (12-15 weeks old) were used in this study. All rats were kept at a constant room temperature under standard conditions with food and water ad libitum in individual plastic cages with soft bedding. Animals were divided randomly into four experimental groups of six rats each: group A (ischemia reperfusion), group B (ischemia reperfusion + N-acetylcysteine), group C (ischemic preconditioning) and group D (ischemic preconditioning + N-acetylcysteine).

1) Anesthesia
Anesthesia was induced using intramuscular ketamine (50 mg/kg) plus xylazine (10 mg/kg).

2) Surgical procedure
After induction of anesthesia, the left hind limb was completely clipped. After clipping, disinfecting and dropping (using a sterile technique), a skin incision was made on medial surface of the left hind limb. After isolated the femoral artery and vein from the surrounding structures, femoral artery was exposed and clamped with a mini bulldog forceps. All animals in groups A and B were undergone 2h of ischemia by occlusion femoral artery and 24h of reperfusion and rats in groups C and D were undergone two 20-min cycles of ischemic preconditioning followed by 2h ischemia and 24h reperfusion. Rats were maintained in a dorsal recumbency and kept anesthetized throughout the duration of the ischemic period. Additional doses were given as necessary to maintain anesthesia during the experiment. Body temperature was maintained with a heating pad under anesthesia. Following the ischemic period, the vascular forceps was removed and then surgical site was routinely closed and rats were returned to their cages with food and water ad libitum during the reperfusion period. After 24h of reperfusion, rats were euthanized and left gastrocnemius muscle harvested for histopathological analysis.
3) Drug administration
In all groups before clamped the femoral artery, 250 IU heparin was administered via the jugular vein to prevent clotting. In groups B and D, N-acetylcysteine (150 mg/kg) was injected intravenous immediately before reperfusion. After surgery, fluid losses were replaced by administration of 5ml of warm (37˚C) isotonic saline i.p. The analgesic nalbuphine hydrochloride (2 mg/kg) was used via subcutaneous during observation time.

After 24h of reperfusion, rats were euthanized by overdose of intraperitoneal pentobarbital injection (300 mg/kg).

4) Histopathological analysis
Muscle samples were harvested at the end of the experiment into 10% buffered formalin. Using standard techniques, paraffin sections were obtained, stained with hematoxylin and eosin, and studied under optical microscopy by a pathologist who was blinded to the experiment and data. Histological changes were scored on a scale from 0 to 3 where 0 = absence (<5% of maximum pathology), 1 = mild (<10%), 2 = moderate (15–20%), and 3 = severe (20–25%) [17]. A total of four slides from each muscle sample were randomly screened, and the mean was accepted as the representative value of the sample. Severity of neutrophil infiltration was estimated and calculated for each experiment.

5) Statistical analysis:
Statistical analyses were carried out using SPSS statistical software (version 11.2). Results were expressed as the mean +/- standard deviation. The Mann-Whitney U-test was employed to analyze two groups consecutively. Values of P<0.05 were considered as statistically significant.

RESULTS
All of rats tolerated operation and survived until the final study period. Figure 1 to 4, illustrates representative photomicrograph of the muscle tissues from all groups that obtained 24h after reperfusion. Inflammatory changes, neutrophil presence, interstitial edema, hemorrhage and necrotic fibers in the muscle were scored. The extent of muscle changes in the group D was significantly lower than other groups (p<0.05). Degree of muscle changes in B and C groups were lower than that in the A group (p<0.05). Data belonging to scores of muscles histological changes from tissue samples after reperfusion are shown in Table 1.

Fig 1. Representative photomicrograph from skeletal muscle of the group A.

Fig 2. Representative photomicrograph from skeletal muscle of the group B.
Fig 3. Representative photomicrograph from skeletal muscle of the group C.  

Fig 4. Representative photomicrograph from skeletal muscle of the group D.  

TABLE 1- Scores of muscles histological changes.

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>6</td>
<td>2.66±0.51</td>
</tr>
<tr>
<td>B</td>
<td>6</td>
<td>1±0.63</td>
</tr>
<tr>
<td>C</td>
<td>6</td>
<td>1.16±0.41</td>
</tr>
<tr>
<td>D</td>
<td>6</td>
<td>0.66±0.51</td>
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DISCUSSION

Ischemia reperfusion injury may affect a number of organs and tissues during blood flow restoration after a prolonged ischemic insult and can be deleterious in various clinical settings due to complications caused by delayed vascular flow recovery. Ischemia reperfusion injury, if severe, can offset the benefits of procedures such as organ transplantation, free flap reconstruction, transluminal angioplasty, and coronary artery bypass surgery [18]. Following a period of ischemia, tissues adapt to anaerobic metabolism. Restoration of blood supply results in oxygen supply in excess of the requirements that lead to activation of macrophages in the vasculature and consequently generation of super oxide radicals, also referred to as reactive oxygen species, causing oxidative stress. The key event in the initial phase of reperfusion injury is activation of macrophages that are the primary source of extracellular reactive oxygen species. Reactive oxygen species are the key initiators of reperfusion injury, which leads to endothelial injury and further release of pro-inflammatory cytokines [19]. A number of studies have tested the protective effect of ischemic preconditioning on local [20] and systemic [21] Ischemia reperfusion injury induced inflammation.

Ischemic preconditioning is the most powerful innate mechanism protecting against Ischemia reperfusion injury. The effects of ischemic preconditioning have been observed in a number of mammalian species, including humans. The method consists of inducing brief periods of sub-lethal local tissue ischemia as a protection against subsequent lethal ischemia. The early phase of ischemic preconditioning, referred to as “classic preconditioning”, is observed immediately after brief ischemia and lasts approximately 3 h. A late phase (“second window”) of preconditioning has also been demonstrated 18-24 h after induction of brief ischemia [22].

In direct ischemic preconditioning, the early phase of protection is protein synthesis independent and this continues into a later phase of protection, which is protein synthesis dependent. Several pathways have been suggested for molecular mechanism of ischemic preconditioning. These include activation of G protein-coupled receptors, protein kinase C, mitogen-activated protein kinases, ATP sensitive K⁺ channels, heat shock protein, adenosine, metal ions, oxygen and nitrogen reactive species, oxidized and biological active lipids, cytokines, catecholamines, and nitric oxide synthase [23,24].
N-acetylcysteine is not simply an antioxidant drug. It acts as a glutathione precursor, as a chemical reductant of oxidized thiols, as a scavenger of radical oxygen species, as a vasodilator and also improves microcirculation by restoring the decreased activity of endothelium-derived relaxing factor and may have antiaggregan features [25,26]. There is growing evidence regarding its beneficial effects in ameliorating skeletal muscle ischemia-reperfusion injury [15,16].

CONCLUSION

This study like other previous studies confirmed that temporary occlusion of the femoral artery in rats leaded to severe histological changes and administration of the N-acetylcysteine treatment and used ischemic preconditioning technique significantly decreased skeletal muscle ischemia reperfusion. According to our histological findings, ischemic preconditioning and N-acetylcysteine act synergistically to protect skeletal muscle against ischemia reperfusion injuries. Different dosages, alternate time protocols should be investigated in future studies.

REFERENCES