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Effect of dexmedetomidine on tourniquet-induced skeletal muscle injury

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SUMMARY

OBJECTIVE: The aim of this study was to investigate whether dexmedetomidine could reduce tourniquet-induced skeletal muscle injury.

METHODS: C57BL6 male mice were randomly assigned to sham, ischemia/reperfusion, and dexmedetomidine groups. Mice in the ischemia/reperfusion and dexmedetomidine groups received normal saline solution and dexmedetomidine intraperitoneally, respectively. The sham group underwent the same procedure as the ischemia/reperfusion group, with the exception of tourniquet application. Subsequently, the ultrastructure of the gastrocnemius muscle was observed, and its contractile force was examined. In addition, Toll-like receptor 4 and nuclear factor-κB expression within muscles was detected by Western blot.

RESULTS: Dexmedetomidine alleviated myocyte damage and increased the contractility of skeletal muscles. Moreover, dexmedetomidine significantly inhibited the expression of Toll-like receptor 4/nuclear factor-κB in the gastrocnemius muscle.

CONCLUSION: Taken together, these results demonstrate that dexmedetomidine administration attenuated tourniquet-induced structural and functional impairment of the skeletal muscle, partly through inactivation of the Toll-like receptor 4/nuclear factor-κB pathway.

KEYWORDS: Dexmedetomidine. Tourniquets. Muscle, skeletal. Surgery.

INTRODUCTION

Tourniquet placement is a universal technique used to create a bloodless operating field[1]. However, tourniquet application leads to significant acute limb ischemia and reperfusion (I/R) injury, which is characterized by inflammation, tissue edema, muscle necrosis, and microvascular perfusion deficits[2,3]. Accordingly, these complications greatly limit tourniquet use[4]. Skeletal muscle injury plays a pivotal role in the tissue response to acute limb I/R[5]. Thus, improving the survival of skeletal myocytes in surgeries requiring tourniquet application would enhance patient recovery.

Toll-like receptors (TLRs), which are ubiquitous in nature, activate the innate immune system in response to pathogens, stressors, and/or cytokines[6]. Binding of a ligand to Toll-like receptor 4 (TLR4), a TLR subtype, leads to activation of nuclear factor (NF)-κB, thereby triggering the transcription of many pro-inflammatory genes[7].

Dexmedetomidine (Dex) is an agent with sedative, anxiolytic, and analgesic effects, which has been applied for surgical patients as an adjuvant anesthetic. Research has demonstrated that Dex elicits a protective effect against I/R injury in multiple organs through antioxidant and anti-inflammatory mechanisms[8,9]. However, to date, no study has evaluated the effects of Dex on tourniquet-induced I/R injury.

Therefore, we investigated whether Dex could attenuate the structural and functional impairment of skeletal muscle in a mouse skeletal I/R injury model and explored the involvement of the TLR4 pathway in the underlying molecular mechanism.

METHODS

Animal experiments were approved by the Animal Care and Use Committee of the Tianjin Medical University and performed in accordance with the National Institutes of Health guidelines for the Care and Use of Laboratory Animals. Two sets of C57BL6 male mice (aged 12–13 weeks) were subjected to 3 h of unilateral hind limb tourniquet ischemia, followed by 24 h of reperfusion (I/R), as previously described[1]. For formal experiments, mice were randomly assigned to sham, I/R, and Dex groups (n=16 mice/group) and anesthetized by an intraperitoneal injection of 80 mg/kg pentobarbital sodium. Dex was diluted in 0.9% NaCl. Mice in the I/R and Dex groups received normal saline solution and 9.6 μg/kg Dex intraperitoneally (i.p.), respectively, 30 min before ischemia. Mice in the Dex group were continuously infused with dexmedetomidine (6 μg/kg/h) throughout the 3-h ischemia using an electronic micropump (KD Scientific, Holliston, MA, USA), while mice in the I/R group received an equal amount of 0.9% saline instead. Mice in

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the sham group underwent the same procedure as those in the I/R group, except for the application of the orthodontic rubber band. All terminal experiments were performed on mice after 24 h of reperfusion.

Electron microscopy analysis of skeletal muscle

The ultrastructure of gastrocnemius muscles was observed by electron microscopy (EM) analysis. Skeletal muscle tissues were removed from mice in each group, cut into 1–3 mm pieces, and processed in accordance with a previous method described by Yang et al.[10]. Subsequently, sections were stained with uranyl acetate and lead citrate (Sigma-Aldrich, St. Louis, MO, USA) and observed using an H-7500/STEM EM (Hitachi, Tokyo, Japan) at 5,000× magnification.

Detection of gastrocnemius muscle contractile force

The contractile force of gastrocnemius muscles was measured in all experimental groups. Under anesthesia (80 mg/kg pentobarbital sodium, i.p.), the left gastrocnemius muscles were quickly removed and rinsed with ice-cold modified Krebs-Henseleit (K-H) solution containing (in mmol/L): NaCl 118, KCl 4.7, $CaCl_2$ 1.8, $MgSO_4 \bullet 7H_2O$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 11, and HEPES 10 (pH 7.4±0.05, gassed with 95% O_2 and 5% CO_2). One end of the gastrocnemius muscle was fixed onto the bottom of a small chamber by a micropin. The other end was connected to a force transducer (AD Instruments, Dunedin, NZ, USA). Stimuli were delivered through a bipolar electrode placed in the chamber and connected to the stimulator. The gastrocnemius muscle was continuously perfused with K-H solution at a rate of 15 mL/min at 37°C for at least 1 h before experiments. Individual twitch contractions of the gastrocnemius muscle were induced by stimulation (5 V, 1 Hz, 1-ms pulse). PowerLab Data Acquisition Systems with LabChart 7 (AD Instruments) was used to record and analyze muscle contractions.

Protein expression of toll-like receptor 4 and nuclear factor-κB in gastrocnemius muscles

After 24 h of reperfusion, gastrocnemius muscles from six mice in the sham, I/R, and Dex groups were rapidly collected and stored at -80°C until analysis. Subsequently, tissues were dissected and lysed by RIPA Lysis Buffer (Santa Cruz, Dallas, TX, USA) for homogenization, and the resulting tissue homogenates were centrifuged at 12,000×g for 20 min at 4°C. Total protein concentrations in the centrifuged supernatants were determined with a bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA). Equal volumes of loading buffer were added into the protein samples, which were mixed and boiled for 10 min at 95°C, and then separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Protein samples were then transferred onto a polyvinylidene difluoride (PVDF) membrane at 200 mA for 3 h. After blocking the membrane with 5% non-fat milk for 1 h, membranes were cut into strips according to their molecular weight and probed overnight at 4°C with a rabbit anti-TLR4 antibody (Abcam, Cambridge, UK) or a rabbit anti-NF-κB antibody (Abcam). After washing membranes with Tris-buffered saline with Tween (TBST), they were incubated with a horseradish peroxidase-conjugated goat anti-rabbit IgG (Thermo Fisher Scientific) for 2 h at room temperature. β-Actin (Cell Signaling Technology, Beverly, MA, USA) served as the control. Finally, the PVDF membrane was washed with TBST three times for 15 min each. Specific immunoreactivity was visualized with an enhanced chemiluminescence substrate (Thermo Fisher Scientific), and specific bands were scanned for analysis using ImageJ (http://imagej.nih.gov).

Statistical analysis

Statistical analysis was performed using the SPSS version 17.0 software (SPSS Inc, IL, USA). Data were expressed as mean±standard deviation (SD). Continuous variables were compared using the Student's t-test. For multi-group comparisons, one-way analysis of variance (ANOVA) with the Bonferroni post hoc test was used to determine statistical significance. Statistical significance was considered at $p<0.05$.

RESULTS

Subcellular structural changes of gastrocnemius muscle cells

As shown in Figure 1, mice in the sham group displayed normal muscle cell morphology, whereas mice in the I/R group had incomplete membranes with disordered myofilaments and numerous severely swollen mitochondria with disrupted cristae that clustered under myolemma. In addition, swollen sarcoplasmic reticula were observed in gastrocnemius muscle cells of mice in the I/R group. Compared with the I/R group, subcellular characteristics of muscle cells in the Dex group displayed slight disruptions in the intercellular matrix, mild variation in form and size of mitochondria, and mild swelling in mitochondria with several vesicular cristae. Moreover, the number of mitochondria adjacent to the sarcolemma was higher in the Dex group compared with the I/R group.

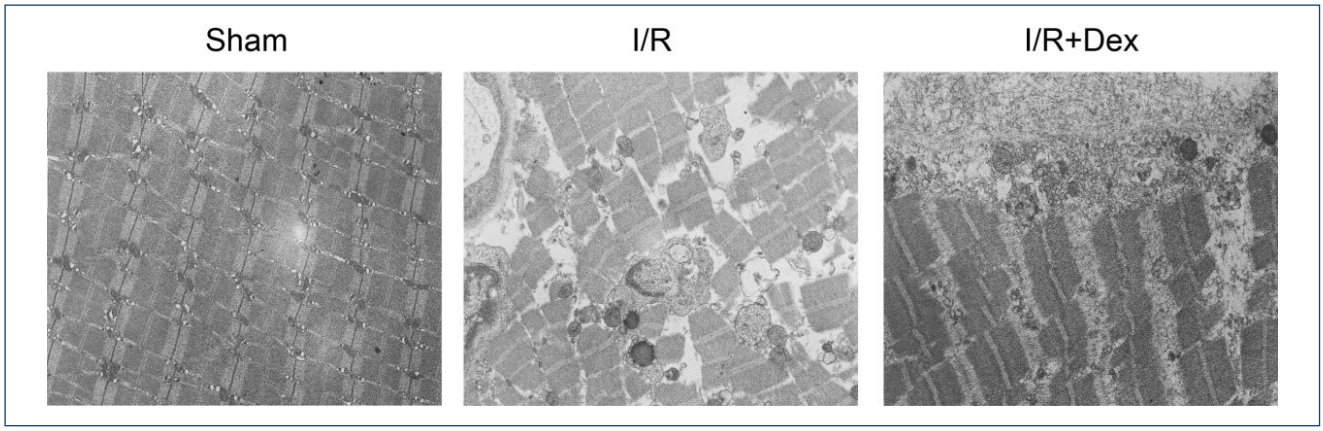

Figure 1. Electron microscopy images of gastrocnemius muscle tissues. Scale bar=20 μm, 5,000× magnification. I/R: ischemia-reperfusion; Dex: dexmedetomidine (n=5 per group).

Contractility of gastrocnemius muscles

As shown in Figure 2, compared with the sham group, gastrocnemius muscle contractility was significantly lower in the I/R group ($p<0.05$). In addition, Dex significantly increased the contractility of muscle tissues compared with the I/R group ($p<0.05$).

Expression of toll-like receptor 4 and nuclear factor-κB in gastrocnemius muscle tissues

As shown in Figure 3, the expression of TLR4 and NF-κB was maintained at a low level in the sham group. However, after 3 h of tourniquet-induced ischemia and 24 h of reperfusion, protein levels of TLR4 and NF-κB were significantly elevated in gastrocnemius muscle tissues ($p<0.05$). Dex pretreatment could observably decrease TLR4 and NF-κB overexpression compared with the I/R group ($p<0.05$).

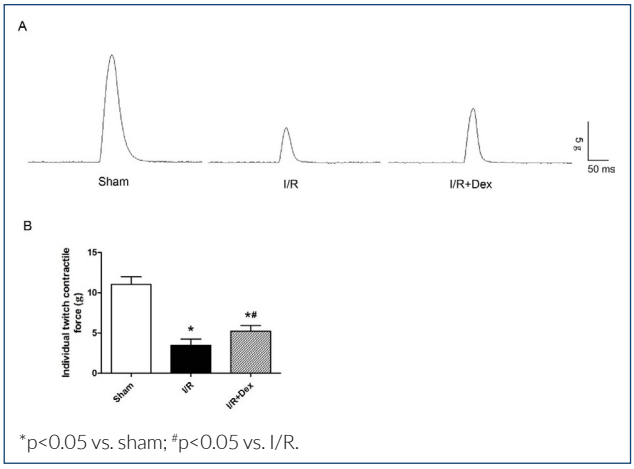

Figure 2. Gastrocnemius muscle contractile forces of experimental groups. **(A)** Individual twitch contraction of the gastrocnemius muscle. **(B)** Quantitative analysis of individual twitch contractile force in each group. I/R: ischemia-reperfusion; Dex: dexmedetomidine. Data are expressed as mean±standard deviation (n=6 per group).

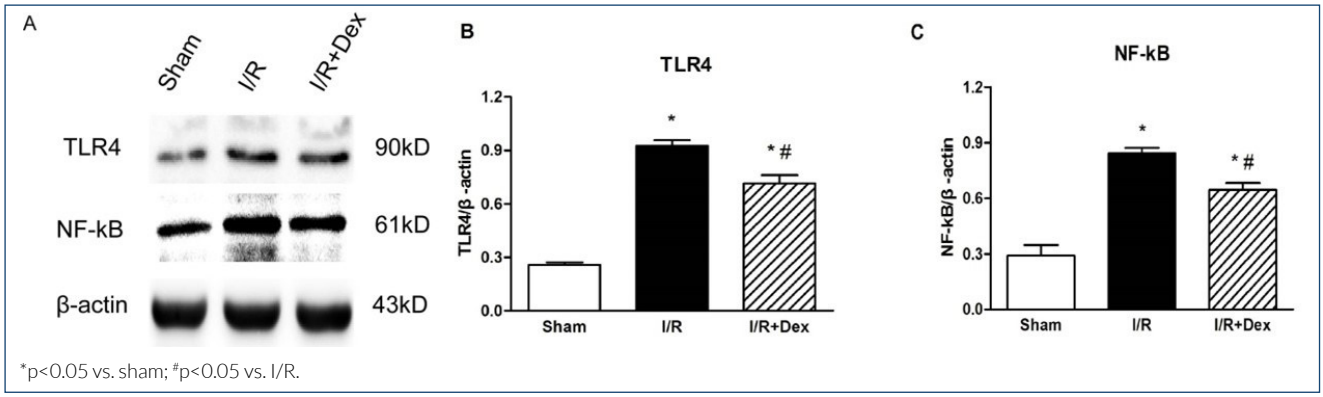

Figure 3. Expression of toll-like receptor 4 and nuclear factor-κB in gastrocnemius muscle tissues detected by western blot. **(A)** Representative band images of toll-like receptor 4 and nuclear factor-κB. **(B,C)** Summary data for expression of toll-like receptor 4 and nuclear factor-κB. I/R: ischemia-reperfusion; Dex: dexmedetomidine. Data are expressed as mean±standard deviation (n=6 per group).

DISCUSSION

Although tourniquets play an important role in vascular and orthopedic surgeries, several local and systemic complications are associated with the use of this device[11]. Prolonged limb tourniquet application and the subsequent restoration of blood flow result in local inflammation in ischemic skeletal muscle, leading to a complex cytokine cascade associated with secondary remote organ damage[12].

Accumulating evidence in animal models demonstrates that Dex has a remarkable protective effect against I/R-induced organ injury[8,9]. Given the prominent role of skeletal muscle injury in tissue responses to limb I/R[5], we hypothesized that Dex could mitigate the severity of skeletal muscle damage. Our EM analysis shows that tourniquet-induced I/R caused morphological damage and leukocyte infiltration into skeletal muscles. However, Dex preconditioning in the setting of limb I/R could reduce both gastrocnemius muscle injury and inflammatory cell infiltration.

Activation of TLRs can trigger the release of a series of inflammatory factors; therefore, blocking TLR activation may be an ideal approach to attenuate tissue damage[7]. TLR4 can activate NF-κB signaling, initiating the transcription of many pro-inflammatory genes[13,14]. Consistent with a previous report, we observed significantly higher expression of TLR4 and NF-κB in the I/R group compared with the sham group[15], indicating an important role for TLR4/NF-κB pathway activation in the pathophysiology of skeletal muscle I/R injury. Moreover, levels of TLR4 and NF-κB in skeletal muscle were significantly lower in mice treated with Dex compared with untreated mice after I/R. Collectively, these results demonstrate that Dex pretreatment inhibited the activation of TLR4 and NF-κB induced by I/R injury. It should be noted that other mechanisms, such as reactive oxygen species (ROS) and miRNA, are involved in skeletal muscle and cerebral I/R injury[16,17]. ROS have crucial roles in both the protective mechanisms and pathogeneses of ischemic preconditioning and postconditioning, whether being come to age or ripening[18]. Indeed, excessive ROS production during the reperfusion phase paves the way for tissue injury. Of the many targets for treatment of I/R injury, attenuation of ROS represents a major mechanism in the early minutes of reperfusion. Future studies are needed to clarify the roles of ROS and miRNA in the protective effect of Dex against I/R injury.

There are some limitations to this study. First, we only investigated a single intraperitoneal dose of Dex. Second, we did not explore the prolonged benefits of Dex, which warrants further investigation.

CONCLUSION

Our findings demonstrate that Dex administration, partly through inactivation of the TLR4/NF-κB pathway, attenuated tourniquet-induced structural and functional impairment of skeletal muscle.

AUTHORS' CONTRIBUTIONS

WC: Conceptualization, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. **ZW:** Data curation, Formal Analysis, Software. **JZ:** Investigation, Validation. **WR:** Project administration, Supervision, Writing – review & editing.

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