

Hypertonic saline reduces skeletal muscle injury and associated remote organ injury following ischemia reperfusion injury

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Background and purpose Pharmacological modulation of skeletal muscle reperfusion injury after trauma-associated ischemia may improve limb salvage rates and prevent the associated systemic sequelae. Resuscitation with hypertonic saline restores the circulating volume and has favorable effects on tissue perfusion and blood pressure. We evaluated the effects of treatment with a bolus of hypertonic saline on skeletal muscle ischemia reperfusion (IR) injury and the associated end-organ injury.

Methods Adult male Sprague-Dawley rats ($n = 27$) were randomized into 3 groups: (1) a control group, (2) an IR group treated with normal saline, and (3) an IR group treated with hypertonic saline. Bilateral hind-limb ischemia was induced by application of a rubber band proximal to the level of the greater trochanters for 2.5 h. The treatment groups received either normal saline (4 mL/kg) or hypertonic saline (4 mL/kg) prior to tourniquet release. Following 12 h of reperfusion, the tibialis anterior muscle was dissected and muscle function was assessed electrophysiologically. The animals were then killed, and skeletal muscle and lung tissue were harvested for evaluation.

Results Hypertonic saline significantly attenuated skeletal muscle reperfusion injury, as shown by reduced myeloperoxidase content, wet-to-dry ratio, and electrical properties of skeletal muscle. There was a corresponding reduction in lung injury, as demonstrated by reduced myeloperoxidase content and reduced wet-to-dry ratio.

Interpretation Treatment with hypertonic saline attenuates skeletal muscle ischemia reperfusion injury and its associated systemic sequelae. ■

The local and systemic proinflammatory responses that accompany limb revascularization can adversely affect limb or patient survival (Yassin et al. 1996). Trauma is an important cause of an acutely ischemic limb, and the potential for limb salvage in this situation is greatly improved with modern methods of vascular surgery, fracture fixation, and soft tissue reconstruction. The muscle injury is characterized by permeability edema, impaired muscle function, and muscle necrosis (Ikebe et al. 2001). The associated systemic inflammatory response, of which lung is the most pertinent manifestation, represents the complex interplay between cytokine production and proinflammatory mediators—resulting in neutrophil recruitment and activation (Lechin and Varon 1994, Botha et al. 1995). Adhesion of neutrophils to the activated microvascular endothelium results in increased microvascular permeability and tissue injury from the release of reactive oxygen species and elastases (Welbourn et al. 1991, Weiser et al. 1996).

There has been renewed interest in hypertonic saline as a resuscitation fluid because of its favorable properties in the setting of trauma and hemor-

rhagic shock. It has been shown to be an effective means of restoring central hemodynamics, peripheral tissue perfusion, and oxygen delivery in states of hypovolemia (Younes et al. 1992). Furthermore, large randomized controlled human trials have established its safety and efficiency in this setting (Mattox et al. 1991, Wade et al. 1997). It also has beneficial immunomodulatory and anti-inflammatory properties, and has been shown to mediate dose-dependent inhibition of several neutrophil cytotoxic functions including expression of adhesion molecules, degranulation, and generation of reactive oxygen species (Junger et al. 1998, Ciesla et al. 2000). A treatment regime with a single bolus of hypertonic saline has not been thoroughly investigated in skeletal muscle ischemia reperfusion models. These observations led us to hypothesize that resuscitation with a bolus of hypertonic saline may protect against skeletal muscle ischemia reperfusion injury and its systemic sequelae.

Methods

Hindlimb ischemia and reperfusion model

We used adult male Sprague-Dawley rats (Biological Services Unit, University College Cork, Ireland) weighing 300–350 g in all experiments. A previously described rubber band model of tourniquet hindlimb ischemia and reperfusion was employed (Dillon et al. 2005). Briefly, under 60 mg/kg intraperitoneal (ip) thiopentone sodium anesthetic, bilateral rubber bands were applied above the greater trochanters to interrupt the arterial blood supply to the hindlimbs. Preliminary experiments employing several animals confirmed global ischemia and subsequent reperfusion with the aid of a laser Doppler blood flow monitor probe. The rubber bands were removed after 2.5 h, initiating hindlimb reperfusion. Animals used in this study were maintained in accordance with the guidelines of the Cruelty to Animals Act, 1876, of the Department of Health, Ireland, and those of the European Community Directive (86/609/EC).

Animal groups

The animals were randomized into 3 groups, with 9 per group. Animals in group A underwent anesthesia alone as a control for the anesthesia. Group

B animals were given an intravenous injection (4 mL/kg) of normal saline (0.9% NaCl) 15 min before tourniquet release, followed by 12 h of reperfusion. Group C underwent a similar volume of intravenous injection of hypertonic saline (7.5% NaCl, which has a Na load 8.3 times that of normal saline) at the same time point. Ischemia-reperfusion was induced by application of rubber bands above the greater trochanters bilaterally. Following 12 h of reperfusion, the animals were then killed for harvesting of tissue.

Functional assessment of tibialis anterior muscle

After 12 h of reperfusion, and while each animal was still under intraperitoneal anesthesia, the tibialis anterior muscle was exposed. The animals were fixed to an external frame in a supine position. A 2-0 silk suture was tied around the distal tendon, which was then sectioned and attached to a force transducer (AD Instruments) to measure the isometric contractile force. The muscle temperature was maintained at 33–34°C using an overhead heating lamp. The in situ muscle was stimulated directly (0.1 msec duration, 10 volts) via 2 electrodes connected to a stimulator (AD Instruments). The length of resting muscle was adjusted to produce maximum twitch tension. The isometric twitch contractile properties were determined. Tetanic tension in response to a tetanic electrical stimulus (50 Hz) was then recorded for each muscle. Twitch and tetanic contractions were reported as N per g of muscle weight.

Wet-to-dry ratio

Sections of gastrocnemius and lung were excised and weighed (wet weight). The muscle and lung tissue was then dried at 60°C in a convection oven for 72 h and reweighed (dry weight). Wet-to-dry ratios were calculated and used as an index of edema formation.

Myeloperoxidase assay

The right ventricle was cannulated and the right pulmonary hilum was clamped. The pulmonary vasculature of the left lung was flushed with 50 mL PBS to clear the lung of intravascular neutrophils. The lung was then weighed and homogenized in buffer A (0.021% K₂HPO₄, 0.66% KH₂PO₄ and

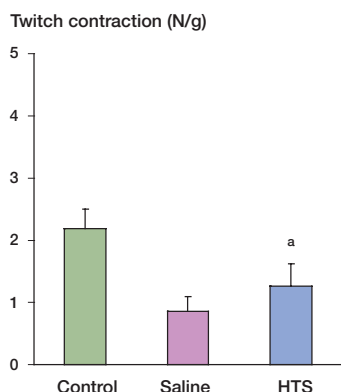


Figure 1. Effect of administration of hypertonic saline on mean peak twitch contraction in rats subjected to ischemia reperfusion injury. Data are expressed as mean (SD) for 9 rats in each group. ^a $p < 0.03$ for hypertonic saline (HTS) vs. normal saline.

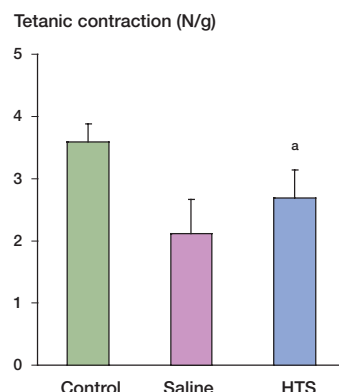


Figure 2. Effect of administration of hypertonic saline on mean tetanic contractions in rats subjected to ischemia reperfusion injury. Data are expressed as mean (SD) for 9 rats in each group. ^a $p < 0.03$ for hypertonic saline (HTS) vs. normal saline.

0.5% hexadecyltrimethyl ammonium bromide in distilled water). The homogenates were freeze-thawed 3 times and centrifuged at 2,000 rpm for 10 min. The supernatant was assayed spectrophotometrically for myeloperoxidase activity by adding 1 mL of supernatant to 2 mL of freshly prepared solution B. (Solution B was made by dissolving 0.0105 g K_2HPO_4 and 0.33 g KH_2PO_4 in 40 mL of distilled water and adding 5 mL of a 0.017% solution of dianisidine in methanol and 5 mL of 0.006% hydrogen peroxide in distilled water). The change in absorbance with time was then measured at 460 nm. 1 unit of myeloperoxidase was defined as that which degraded 1 micromole of hydrogen peroxide at 25°C, and was then calculated per gram of tissue. The procedure was repeated for fresh muscle samples.

Statistics

Statistical analysis was performed by one-way analysis of variance (ANOVA) with post hoc Tukey test analysis. Differences were considered significant at p -values < 0.05 .

Results

Functional assessment of tibialis anterior muscle

Muscle contractile function was measured after 12 h of reperfusion and expressed as the peak tension achieved for each muscle for both twitch and

tetanic contractions. Ischemia-reperfusion injury (IRI) impaired muscle twitch contraction compared with the control group. Resuscitation with hypertonic saline preserved twitch contraction after IRI (Figure 1). Muscle tetanic contraction was also significantly reduced by IRI when compared with control and again, this reduction was attenuated with hypertonic saline resuscitation (Figure 2).

Tissue edema in gastrocnemius muscle

For assessment of tissue edema, tissue wet-to-dry ratios were measured. The muscle wet-to-dry ratios in the hypertonic saline-treated group were significantly lower than those in the saline-treated group (Table).

Effect of IRI on muscle neutrophil infiltration

Myeloperoxidase activity was employed as a marker of skeletal muscle neutrophil infiltration. IRI treatment resulted in a significant increase in myeloperoxidase activity as compared to controls. This increase was attenuated with hypertonic saline resuscitation (Table).

Effect of ischemia-reperfusion on lung injury

IRI resulted in significant remote organ injury, as measured by lung wet-to-dry ratio and myeloperoxidase activity. These markers of lung injury were significantly better in the hypertonic saline-treated group than in the saline-treated group (Table).

Skeletal muscle and lung neutrophil infiltration (myeloperoxidase activity) and edema formation (wet-to-dry-ratio). Values are expressed as mean (SD)

	Skeletal muscle		Lung	
	Myeloperoxidase activity (units/g)	Wet-to-dry ratio	Myeloperoxidase activity (units/g)	Wet-to-dry ratio
Control	0.64 (0.08)	3.05 (0.10)	2.68 (0.54)	3.08 (0.12)
Normal saline	0.81 (0.07)	3.65 (0.26)	4.37 (0.23)	3.55 (0.24)
Hypertonic saline	0.73 (0.10)	3.37 (0.17)	3.83 (0.39)	3.29 (0.28)
P-value ^a	< 0.04	< 0.01	< 0.03	< 0.03

^a hypertonic saline vs. normal saline.

Discussion

Restoration of blood flow to an acutely ischemic limb initiates a cascade of cellular and biochemical events that result in muscle edema, necrosis, and impaired muscle function (Kearns et al. 2001). The associated lung injury is mediated by the adherence of activated neutrophils to the endothelium of the pulmonary microvasculature, resulting in spillage of protein-rich transudate into the alveolar spaces—hallmarks of the acute respiratory distress syndrome (ARDS) (Barry et al. 1997). While many agents including adenosine infusion and NF- κ B inhibitors have been shown to attenuate lower torso ischemia reperfusion injury, the pharmacodynamics or toxicity of these agents has limited their introduction into the clinical setting (Schroeder et al. 1996, Lille et al. 2001).

Our study shows that resuscitation with hypertonic saline reduces the tissue damage associated with the reperfusion injury that occurs following acute lower torso ischemia. Skeletal muscle injury, measured from leukosequestration and edema formation, was significantly lower with hypertonic saline treatment than with saline treatment. The functional injury induced by IRI was reflected in the maximum twitch and tetanic contraction forces. The preservation of the contractile properties in the hypertonic saline-treated group further emphasizes the suppression of the inflammatory response provoked by hypertonic resuscitation.

The associated systemic inflammatory response is believed to promote overexuberant microcirculatory activation, the release of reactive oxygen species and elastases causing microvascular damage, and subsequent tissue and organ injury (Yassin et

al. 2002). This systemic inflammatory response, as measured by pulmonary injury, was also dampened by hypertonic saline resuscitation. As already mentioned, an aberrant and unbridled neutrophil-endothelial interaction is believed to play a central role in ischemia reperfusion injury and the beneficial effects of administration of hypertonic saline can be partly explained by its ability to reduce neutrophil activation. Hypertonicity attenuates several receptor-mediated neutrophil cytotoxic responses including adhesion molecule expression, release of proteolytic enzymes, and production of reactive oxygen metabolites (Pascual et al. 2003).

Much of the previous work on skeletal muscle ischemia reperfusion injury has focused on the effects of pretreatment with various agents (Kearns et al. 2001). While this is important to further our understanding of this complex process and to elucidate the underlying mechanisms involved, this does not always correlate with the clinical setting where ischemia is often a sudden unpredictable event. In our experiments, hypertonic saline was given after the ischemic insult and a significant reduction in injury was observed, thereby suggesting that it could be used as a therapeutic intervention.

In conclusion, our study demonstrates that resuscitation with hypertonic saline attenuates skeletal muscle ischemia reperfusion injury and its associated systemic sequelae in a rodent model. We therefore feel that resuscitation with hypertonic saline may be a potential therapeutic intervention for skeletal muscle reperfusion in the clinical setting.

Contributions of authors

JPD, AJL, and CJS: formulation of idea, experimental design, experiments, and writing of manuscript. JRSC: experimental design, experiments, and processing of samples. JHW: formulation of research idea, experimental design, and experiments. AMcG and HPR: formulation of research idea, experimental design, and research supervisors.

No competing interests declared

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