

Poloxamer-188 Reduces Muscular Edema After Tourniquet-Induced Ischemia-Reperfusion Injury in Rats

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Background: Skeletal muscle injury can result in significant edema, which can in turn lead to the development of acute extremity compartment syndrome (CS). Poloxamer-188 (P-188), a multiblock copolymer surfactant, has been shown to decrease edema by sealing damaged membranes in a number of tissues after a variety of injury modalities. The objective is to determine whether the administration of P-188 significantly reduces skeletal muscle edema associated with ischemia/reperfusion injury (I-R).

Methods: Male Sprague-Dawley rats underwent 180 minutes of tourniquet-induced ischemia. Five minutes before tourniquet release, rats received either a bolus of (1) P-188 (150 mg/kg; P-188 group) or (2) vehicle (Vehicle group) via a jugular catheter ($n = 10$ per group). After 240 minutes reperfusion, both groups received a second bolus of either P-188 (P-188) or vehicle (Vehicle) via a tail vein catheter. Sixteen hours later, rats were killed; muscle weights were determined, infarct size (2,3,5-triphenyltetrazolium chloride method), and blinded histologic analysis (hematoxylin and eosin) were performed on the gastrocnemius and tibialis anterior muscles, as well as indices of antioxidant status.

Results: P-188 resulted in significantly less edema (wet weight) and reduced an index of lipid peroxidation compared with Vehicle ($p < 0.05$). Wet:dry weight ratios were less in the P-188 group (indicating less edema). Muscle viability as indicated by 2,3,5-triphenyltetrazolium chloride staining or routine histology did not reveal statistically significant differences between groups.

Conclusion: P-188 significantly reduced ischemia-reperfusion-related muscle edema and lipid peroxidation but did not impact muscle viability. Excess edema can lead to acute extremity CS, which is associated with significant morbidity and mortality. P-188 may provide a potential adjunctive treatment for the reduction of CS.

Key Words: Muscle injury, Edema, Triblock copolymer, Compartment syndrome, Microvascular.

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Muscle injury, such as ischemia-reperfusion injury (I-R),¹ blunt trauma injury, electrocution,² burn, crush,³ and laceration, is associated with substantial edema. This can render the injured muscle susceptible to the development of acute extremity compartment syndrome (CS).⁴ CS is an emergent problem in combat-related injuries.⁵ Identifying treatments that can potentially reduce edema and therefore the development of CS are critical to reducing the overall morbidity of combat injuries.

I-R results in intra- and extracellular edema and cellular necrosis facilitated by disruptions in cell membranes.^{6,7} Poloxamer 188 (P-188), a triblock surfactant, has been shown to promote the resealing of disrupted plasma membranes resulting from I-R in cardiac muscle⁸ and testes.⁹ P-188 has an average molecular weight of 8,400 kDa and is composed of poly(oxyethylene) and poly(oxypropylene). The physical chemistry of P-188 has been well established in vitro using lipid monolayer models.¹⁰ This work supports the idea that P-188 seals the actual rupture in the cell membrane through interaction of the hydrophobic poly(oxypropylene) moiety, which inserts directly into the exposed hydrophobic core of the “broken” membrane.^{10,11} Once membrane integrity is restored, P-188 is physically squeezed out.¹⁰ Unbound P-188 is excreted intact via the kidneys and has no osmotic diuretic properties.¹²

Reactive oxygen species concentrations are known to increase markedly in response to I-R and are associated with the destruction of membranes by lipid peroxidation.¹³ P-188 has also been shown to possess antioxidant properties,¹⁴ suggesting that it may be protective against oxidative stress. In addition, P-188 has been shown to improve microcirculation via rheological effects.¹⁵

The objectives of this study were to determine whether the administration of P-188 after tourniquet-induced ischemia could reduce (1) the magnitude of edema; (2) the loss of muscle viability; and (3) the oxidative stress in skeletal muscle.

METHODS

Animal Care

All animal protocols were approved by the US Army Institute of Surgical Research Animal Care and Use Committee. This study adhered to National Institutes of Health guidelines for the care and use of laboratory animals (DHHS Publication, NIH, 86 to 23). Adult male Sprague-Dawley rats

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weighing $403 \text{ g} \pm 15 \text{ g}$ were obtained from colonies of Charles Rivers (Wilmington, MA). Animals were housed in a facility approved by the Association for the Assessment and Accreditation of Laboratory Animal Care. They were provided with food and water ad libitum before anesthesia induction and tourniquet application.

Experimental Procedures and Monitoring

Rats were randomly assigned to P-188 (P-188 + vehicle) or Vehicle (vehicle only) groups ($n = 10$ per group). Rats were weighed and anesthetized using 1.5% to 2.5% isoflurane anesthesia and analgesia was administered (buprenorphine; 0.1 mg/kg intraperitoneally). Both hind limbs were shaved and animals were instrumented with a lubricated rectal temperature probe (Physiotemp Instrument, Clifton, NJ) inserted 5 cm beyond the rectal sphincter. Animals were then placed supine on a warm water flow temperature-regulated bed (EX-212; Euthanex, Allentown, PA) and core temperature was maintained at $37^\circ\text{C} \pm 1^\circ\text{C}$. Blood draining of the experimental leg was performed by elevation above the level of the heart for 5 minutes before tourniquet inflation. A pneumatic tourniquet was then applied to the proximal aspect of the elevated hind limb and inflated to a pressure of 250 mm Hg. All procedures have been detailed previously.¹⁶ Tourniquets were left in place for 180 minutes. A detailed description of the experiment is shown in Figure 1, which details all procedures including periods of anesthesia, recovery, catheterizations, and administration of drug treatments. Rats were returned to their cages and allowed ad libitum access to water and food during the periods between administering anesthesia. No resuscitation fluids were administered during the experimental period.

Administration of P-188

Sterile P-188 solution (SythRx, Bellaire, TX) contained 150 mg/mL highly purified P-188, 3.08 mg/mL sodium chloride, 2.38 mg/mL sodium citrate, and 0.366 mg/mL citric acid. The placebo solution contained the same ingredients with the exception of P-188. Doses consisted of 1.0 mL/kg body weight of P-188 solution or vehicle. Injections of P-188 or vehicle were administered via tail vein. Two injections were administered at (1) 5 minutes before tourniquet release and (2) 240 minutes after tourniquet release. Rats were lightly anesthetized (1.5% isoflurane) before the second injection. This administration schedule was designed to maximize the bioavailability of P-188.¹²

Tissue Harvesting

Rats were anesthetized as described previously. Rats were immediately killed with 1.0 mL intracardiac injection of euthanasia solution (Fatal Plus, Vortech Pharmaceutical Ltd, Dearborn, MI). The muscles were excised, trimmed of connective tissue, blotted dry with filter paper, and weighed. The gastrocnemius (Gastroc) and tibialis anterior (TA) muscles were excised. Whole muscle weights were obtained on a microbalance (MT-5; Mettler Toledo, Columbus, OH). The Medial Gastroc and TA were divided into three cross-sectional portions using a custom-designed tissue slicer and weighted. The proximal portion was frozen in liquid N_2 , the next portion (4 mm thick) was used for vital staining, and the distal portion of muscle, containing the muscle belly, was pinned at resting length to a tongue depressor and fixed for 24 hours in 10% buffered formalin solution (Fisher Scientific, Pittsburgh, PA).

Dry Weight and Wet Weight Determination

Dry weight was determined on the TA and Gastroc tissues. The fresh samples were weighed and then desiccated for 5 days in a drying oven set at 50°C before determination of dry weight.

Vital Staining

Assessment of muscle viability was determined using 2,3,5-triphenyltetrazolium chloride (TTC) as previously.¹⁷ This method is based on the reduction of TTC to water-insoluble red formazan by viable mitochondria. Viable myocytes stain red while nonviable myocytes remain unstained. The 4-mm-thick medial section was obtained from the Gastroc and TA muscles and incubated for 1 hour according to the method of Belkin et al.¹⁸ The reaction was stopped by placing samples in ice-cold phosphate-buffered saline, followed by fixation in buffered formalin. TTC staining was assessed using image analysis. Images were obtained using a Zeiss stereomicroscope (Stemi SV11) equipped with a Nikon CCD camera (DS-5 mol/L). Images of muscles were digitally isolated, gray-scaled, and thresholded using Adobe Photoshop, version 7.0 (Adobe Systems, San Jose, CA). The magnitude of injury was expressed as the percent of total area that remained unstained, i.e., the infarcted area.

Histology

After fixation and paraffin embedding, muscles underwent cross-sectioning and hematoxylin and eosin staining. A modified system based on that used by Bhatt et al.¹⁹ was used

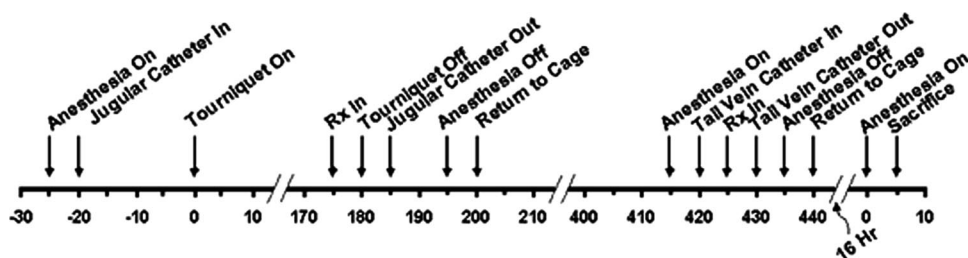


Figure 1. The timeline of experimental procedures. “Rx In” indicates the time that the P-188 or vehicle was administered. Note that rats were anesthetized twice; first during the period of ischemia (time, minute 25 through minute 95); and again during the time of the second administration of the drug treatment (minute 415 through minute 435).

TABLE 1. Histological Grading Criteria

Grade	Edema (% Tissue Edema)	Necrosis (% Tissue Necrosis)	Inflammation
0	Normal tissue	Normal tissue	Normal tissue
1	0–5	0–5	Minimal
2	5–20	5–20	Mild
3	20–40	20–40	Moderate
4	>40	>40	Severe

to grade ischemic and contralateral muscles for edema, degeneration/necrosis, and inflammation by the institute's staff veterinary pathologist, who was blinded to muscle and treatment. Histologic grading encompassed three categories—edema, necrosis, and inflammation. Each category was rated on a 5-point score (0–4), with 0 representing no apparent pathology and 4 signifying extreme pathology; see Table 1. This modified evaluation system was developed at this institute to provide a semiquantitative assessment of edema, degeneration, necrosis, and inflammation. This grading system has been validated in other muscle I-R studies from our laboratory and has been shown to consistently match wet to dry weights for assessment of edema and correlate functional deficit well with the degree of degeneration and necrosis.^{17,20}

Tissue Antioxidant Status

Tissues were homogenized in 50 mmol/L potassium phosphate buffer pH 7.4. Thiobarbituric acid reactive substances (TBARS), expressed as nanomoles of malondialdehyde per gram of tissue, were determined in the butanol phase as described by Natio et al.²¹ Total antioxidant capacity of tissue was determined spectrophotometrically by evaluating the iron reducing capacity of the tissue as described by the method of Benzie and Strain.²² Glutathione peroxidase, glutathione reductase, and superoxide dismutase (SOD) activities were determined spectrophotometrically as previously described by Dubick et al.²³ Reduced glutathione was determined spectrophotometrically using the enzymatic assay described by Anderson.²⁴

Myeloperoxidase (MPO) was used as an index of neutrophil infiltration into tissue. Activity was determined by a modification of the method of Thrush et al.²⁵ Briefly, tissues were homogenized in 50 mmol/L potassium phosphate buffer pH 6.0 containing 0.5% hexadecyltrimethylammonium bromide. The homogenates then underwent three freeze-thaw cycles and sonification, followed by incubation at 60°C in a water bath for 2 hours to extract MPO and reduce interfering substances. Samples were centrifuged at 10,000g for 30 minutes at 4°C. MPO activity was determined in the resultant supernatant using o-dianisidine as a substrate. Protein concentrations were determined with a commercial kit (BioRad Laboratories, Richmond, CA). All results were expressed per milligram protein to compensate for any edematous changes in tissue wet weight as a result of tourniquet placement.

Statistical Analysis

Comparisons were made between the injured muscles using a Student's *t* test. Semiquantitative analysis of pathology

scores were analyzed using Mann-Whitney rank sum test and are reported as median $\pm$ standard error of the median. All statistical comparisons were performed using commercially available software (SigmaStat 3.1; Systat Software, Chicago, IL). Differences were considered to be significant at $p < 0.05$. All data are presented as mean $\pm$ standard error of the mean.

RESULTS

Body Weight

All 20 rats survived the tourniquet application and treatment. Body weight decreased during the period of study from 398 g to 395 g in Vehicle, and 395 g to 386 g for P-188; these changes were not statistically different.

Muscle Wet Weight and Wet:Dry Weight Ratio

Ischemia resulted in substantial edema in all muscles of tourniquet hind limbs when the wet weight and wet:dry weight ratio were compared with the contralateral, no tourniquet hind limb (Fig. 2). In general, P-188 treatment resulted in significantly less edema compared with vehicle alone, indicated by 8% lower wet weights in the Gastroc (Fig. 2, A) and 13% lower wet weights in the TA (Fig. 2, B) when compared with the Vehicle group. The wet:dry weight ratio of the injured Gastroc was 8% less in the P-188 compared with Vehicle ($p < 0.05$; Fig. 2, C). The wet:dry weight ratio from the TA of the P-188 treated group was 5% less compared with the injured, Vehicle TA (Fig. 2, D). There was no significant difference between the uninjured contralateral muscles for either muscle.

Muscle Viability

Ischemia resulted in substantial loss of viability in both muscles and in both groups. The percent of the area occupied by infarcted cells in the Gastroc was $37\% \pm 3\%$ and $40\% \pm 5\%$ for Vehicle and P-188 groups, respectively. In the TA, the percent of area occupied by the infarcted cells was $43\% \pm 6\%$ and $35\% \pm 5\%$ for Vehicle and P-188 groups, respectively.

Muscle Histology

The predominant feature of the histologic examination of the Gastroc and TA after ischemia was extra- and intracellular edema. Polymorphonucleocytes were present at varying degrees in all ischemic muscles and extravascular red blood cells could be visualized in many sections, although there was no systematic difference between the two groups. Scores based on edema, degeneration/necrosis, and inflammation were significantly higher (indicating greater pathology) in ischemic muscles compared with the untreated contralateral muscles. However, there were no significant differences between the two ischemic groups for either the Gastroc or TA (Table 2). The muscles in the untreated contralateral leg were normal both macroscopically and microscopically, indicating no detectable damage induced systemically.

Oxidative Stress

As shown in Table 3, the muscle ischemia/reperfusion model used in this study resulted in an oxidative stress as noted by a 2.6-fold increase in TBARS and reductions in

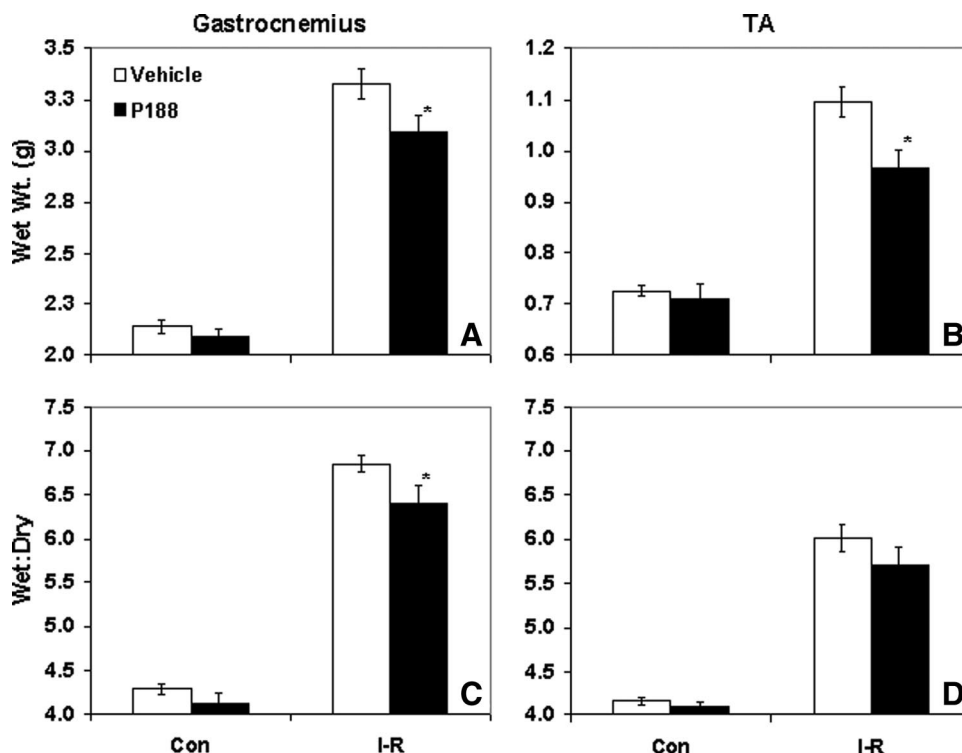


Figure 2. Muscle wet weights (A, B) and wet:dry weight ratios (C, D). Overall I-R resulted in a dramatic increase in muscle wet weight and wet:dry weight ratios. However, P-188 treated rats had a significant decrease in wet weight and wet:dry weight ratio when compared with the control group ($p < 0.05$).

TABLE 2. Comparison of Histological Categorization Based on a 5-Point Grading Score

Muscle	Group	Edema	Necrosis	Degeneration	Neutrophils	Macrophages
Gastrocnemius	Vehicle	2.25 ± 0.41	2.38 ± 0.50	2.50 ± 0.60	1.88 ± 0.44	1.75 ± 0.31
	P-188	2.33 ± 0.24	1.78 ± 0.22	2.11 ± 0.56	2.11 ± 0.39	1.78 ± 0.44
Tibialis anterior	Vehicle	2.50 ± 0.98	2.50 ± 0.50	3.38 ± 0.50	2.25 ± 0.37	1.25 ± 0.25
	P-188	2.11 ± 0.31	1.33 ± 0.41	2.89 ± 0.46	1.67 ± 0.24	0.56 ± 0.18*

* $p < 0.05$.

Values are depicted as median with standard error of the median.

TABLE 3. Antioxidant Status of Gastrocnemius

Parameter	Units	Control	Vehicle	P-188
TBARS	nmol/g	16.48 ± 2.03	45.08 ± 6.33	34.77 ± 6.73
FRAP	μmol/g	1.93 ± 0.35	2.59 ± 0.47	3.05 ± 0.53
GP	μ/g	6.08 ± 0.93	6.08 ± 1.10	5.88 ± 1.04
GR	μ/g	2.21 ± 0.32	1.67 ± 0.23	1.65 ± 0.18
SOD	μ/g	384.24 ± 55.14	224.69 ± 26.21	330.69 ± 53.43
MPO	μ/g	0.29 ± 0.05	0.22 ± 0.05	0.26 ± 0.07

TBARS, thiobarbituric acid reactive substances; FRAP, ferric ion reducing antioxidant power; GP, glutathione peroxidase; GR, glutathione reductase.

All values represent the mean ± SEM; n = 9/gp; * p value < 0.05 from control.

SOD activity (29%) and glutathione concentrations (53%) compared with controls. P-188 treatment reduced the elevated TBARS but not to control levels, whereas SOD activity was at control levels (Table 3). P-188, however, did not

improve reduced glutathione concentrations. MPO activity was barely detectable in these muscles and there were no significant differences among groups.

DISCUSSION

CS is an emergent problem in combat-related injuries. This may be due in part to the widespread use of tourniquets.^{5,26} Although we have shown that the effectiveness of early utilization of tourniquet application in combat is life-saving, they are associated with a fasciotomy rate of between 28% and 36%.²⁷ Thus, treatments that could reduce the need for fasciotomy may be useful. This study demonstrates that P-188 treatment before tourniquet release significantly reduces the magnitude of edema in skeletal muscle at 23 hours after 3 hours of tourniquet application. Although P-188 failed to positively impact any of the other variables examined (viability, oxidative stress, etc), the reduction in the magnitude of I-R-

induced edema alone is a critical finding in the context of providing a treatment for reducing or preventing CS.

Application of P-188 has been shown to seal breaches in cell membranes, thus restricting the transmembrane movement of intracellular markers.¹⁴ In addition, P-188 is a nonionic detergent that is capable of sealing damaged membranes.²⁸ The pathophysiology of ischemia and ischemia reperfusion injury is well documented. The microcirculatory changes that occur ultimately cause complete disjunction of adjacent endothelial cells resulting in extremely broad gaps in the capillary endothelial surface.¹ The nonionic and hydrophilic properties of P-188 may seal permeabilized membranes of damaged cells in skeletal muscle and lead to decreased edema. The administration of P-188 significantly reduced edema compared with the Vehicle group, although it did not eliminate edema. However, even a modest reduction in edema may have a large therapeutic effect.

It has been shown in large animal models that when fluid is infused into the anterior compartment, there is a curvilinear relationship between fluid volume and intracompartmental pressure (ICP).^{29,30} More specifically, these studies have shown that above 33 mm Hg, infusion of even small amounts of fluid (1–2 mL) results in a prodigious increase in ICP. The threshold ICP for the clinical diagnosis of CS is generally accepted to be 30 mm to 40 mm Hg.³¹ Thus, reducing the fluid volume of the compartment could have significant clinical benefits. In fact, these observations have been exploited clinically. In small clinical studies, it has been shown that ICP can be significantly reduced when small amounts of fluid are removed from the anterior compartment of patients (closed tibial fractures) with elevated ICP using an ultrafiltration catheter system.³² These studies have, in turn, lead to a currently ongoing multicenter clinical trial.³³ Taken together, these observations lend credence to the possible therapeutic benefit of P-188 for the treatment of trauma patients at risk for the development of CS.

Poloxamer 188 has been shown to display a number of other potentially beneficial properties that made it an attractive candidate for the treatment of I-R. It was originally used as a rheological agent to decrease whole blood viscosity particularly in patients with sickle cell disease. In this capacity, P-188 has been observed to improve mesenteric microcirculation in minutes and counter the no-reflow phenomenon and improve microvascular circulation.¹⁵ This alternative mechanism has explained P-188's efficacy in prolonging survival in hypotensive resuscitation¹⁵ and in addition to its properties of breaching sealed membranes may explain our results.

Oxidative stress plays a major role in I-R.^{1,34} Reactive oxygen species produce injury to the cell membrane through peroxidation of phospholipids and oxidative deamination of proteins.³⁵ One of the beneficial properties that have been ascribed to P-188 has been its ability to scavenge highly reactive oxygen species.¹⁴ In this study, P-188 demonstrate some reduction in oxidative stress as indicated by a reduction in the elevated TBARS (index of lipid peroxidation) and a return of SOD activity to control levels in muscle subjected to ischemia/reperfusion. However, P-188 treatment was unable

to restore reduced glutathione levels in these muscles. The results of this study also indicate that as MPO activity was similar among groups, the oxidant stress induced by tourniquet application was not a result of infiltration of neutrophils into the muscle but arises from the muscle tissue itself in response to ischemia and reperfusion.

P-188 administration did not reduce myocyte injury as indicated by either nitroblue tetrazolamine staining (Fig. 2) or routine histology (Table 2). Based on P-188's lack of impact on reducing lipid peroxidation, this is not surprising. It also emphasizes the lack of a direct relationship between edema and myocyte damage in the absence of CS. However, if P-188 is effective in reducing edema and preventing increased ICP, it would indirectly reduce myocyte injury by preventing CS.

The magnitude of I-R is a function of the duration of ischemia.¹ In this study, we used a 3-hour ischemic period, based on unpublished observations from our laboratory; this duration results in a dramatic loss of function, with near complete recovery by 6 weeks. The dose of P-188 (150 mg/kg) was based on published reports showing that this dose was well tolerated, i.e., not toxic.³⁶ However, larger doses have been used with no apparent toxic effects.³⁷ It is possible that greater benefits may have been observed with higher doses of P-188.

Unlike humans, the relatively compliant fascia of most commonly used laboratory animals seems to preclude them from developing CS in response to I-R or direct trauma. The exception is the canine, which has been shown to develop CS in response to prolonged ischemia³⁸ or as a result of trauma. For this reason, CS must be induced artificially, usually by direct infusion of fluid, such as plasma,^{29,38,39} directly into the anterior compartment to raise compartment pressure to a clinically relevant level. This technique is not useful in models testing therapeutic agents designed to reduce vascular permeability. Our rodent model of tourniquet-induced ischemia better simulates the progression to CS and is a valid means of testing therapeutic agents designed to reduce edema, by extension this would be expected to reduce the chances of developing CS.

In our investigation, we used muscle wet weight and the wet weight:dry weight ratios as indicators of edema. The ratio of the injured:uninjured contralateral leg show significant differences between the TA and the Gastroc between P-188 and Vehicle groups. Although the wet weight:dry weight ratios closely match this pattern, the difference between P-188 and Vehicle for the TA is not statistically different. A probable explanation for this is that a portion of the excess fluid is lost when the piece of muscle is cut for wet weight:dry weight determination; this would cause the wet weight to be underestimated.

CONCLUSION

This study demonstrated that P-188 provided significant protection from muscle edema and to some degree on oxidant stress. However, this protection did not extend to muscle viability. Future studies that look at dose-dependent relationships based on the amount of P-188 injected would be beneficial. In addition, future work will involve the adminis-

tration of other agents in conjunction with P-188 designed to reduce edema and protect tissues from I-R.

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