Multiple cryotherapy applications attenuate oxidative stress following skeletal muscle injury

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ABSTRACT

Objectives: To investigate the effects of multiple cryotherapy applications after muscle injury on markers of oxidative stress.

Methods: Following cryolesion-induced skeletal muscle injury in rats, ice was applied at the injured site for 30 minutes, three times per day, on the day of injury, and for 2 days after injury. To determine the effect of the cryotherapy treatment on markers of oxidative stress, biochemical analyses were performed 3, 7, and 14 days after injury.

Results: Compared with non-treated animals, cryotherapy reduced dichlorofluorescein at 7 and 14 days post-injury and thiobarbituric acid reactive substances levels at 3 and 7 days post-injury ($P < 0.05$). Additionally, cryotherapy maintained methyl thiazol tetrazolium reduction levels compared to the control group at all analyzed time points ($P > 0.05$), whereas non-treated groups demonstrated lower levels than the control group ($P < 0.05$). Superoxide dismutase activity at 7 and 14 days post-injury and catalase activity at 3 days post-injury were lower in cryotherapy groups compared with non-treated groups ($P < 0.05$). Cryotherapy prevented the reduction of non-protein thiol levels and maintained within control group level, at 3 days post-injury ($P = 0.92$).

Discussion: Cryotherapy reduced the production of reactive oxygen species after muscle injury, resulting in an attenuated response of the antioxidant system. These findings suggest that using multiple cryotherapy applications is efficient to reduce oxidative stress.

KEYWORDS

Oxidative stress; antioxidants; cryotherapy; therapeutics; skeletal muscle; regeneration; physical therapy

Introduction

Skeletal muscle constitutes the largest tissue mass in the body and is highly susceptible to injury, especially in sporting activities [1]. Thereby, the medical care and indirect costs related to skeletal muscle injury result in large public health costs [2]. Regardless of the cause of the injury, muscle regeneration is an orchestrated process, which is distinguished in three stages: the destruction phase (lasts 0–72 hours); repair phase (lasts 3–4 weeks); and remodeling phase (lasts 3–6 months) [3]. During the destruction phase, the proliferation of inflammatory cells to injured tissue may amplify the injury by releasing reactive oxygen species (ROS) [4]. Furthermore, as a consequence of simultaneous vascular tissue damage, additional ROS production occurs after the restoration of perfusion and oxygen supply to the previously ischemic area [5,6]. ROS may modify lipids, proteins, and DNA, altering their function within the tissue. These molecular modifications are called oxidative damage and can affect cell homeostasis and survival [7,8]. Fortunately, there is a complex antioxidant system to combat the actions of ROS [9], and oxidative stress will occur

when the imbalance between ROS and antioxidants favors ROS [10].

Rehabilitation and sport medicine specialists have proposed using cryotherapy during muscle regeneration to minimize muscle injury [11–14], since a greater quantity of injured tissue requires a longer recovery time [5]. Many studies, although not all [15], have highlighted that cryotherapy may be beneficial to recovery, as it reduces inflammation within the affected muscles [16–20]. However, the mechanisms involved in the ability of cryotherapy to reduce oxidative damage are not completely understood. To date, only a few studies [6,9,21,22] have assessed the effects of cryotherapy after acute muscle injury with oxidative stress being a main variable of interest. Moreover, to the best of our knowledge, the effects of multiple cryotherapy applications performed within the destruction phase, as recommended in clinical practice [23,24], have not been assessed oxidative stress in a controlled experimental model. Thus, the aim of the present study was to examine the effect of multiple cryotherapy applications on oxidative stress during the 14 days following cryolesion of the *tibialis anterior*

(TA) of rats. Our hypothesis was that the cryotherapy protocol would minimize the oxidative stress within injured muscles.

Materials and methods

Animals

Three-month old male *Wistar* rats ($n = 35$; 325.1 ± 31.1 g) were used in this study. The animals were housed in plastic cages with a controlled temperature, 12-hour light–dark cycle, and free access to water and standard chow throughout the experiment. This study was conducted with University approval for the care and use of animal resources (number 059/2010) and in accordance with the Guide for the Care and Use of Laboratory Animals [25]. For the skeletal muscle injury and the cryotherapy treatment, the rats were anesthetized with an intraperitoneal injection of xylazine (12 mg/kg) and ketamine (95 mg/kg); and, at each group's final measurement time point, the animals were euthanized by anesthetic overdose.

Experimental groups

The effect of cryotherapy on markers of oxidative stress was analyzed at three experimental time points: 3, 7, and 14 days after muscle injury. The rats were randomly distributed into seven experimental groups with five animals each: control, animals with no interventions; TA injured, no cryotherapy treatment, and analyzed 3 days post-cryolesion (L3); TA injured, treated with cryotherapy, and analyzed 3 days post-cryolesion (L3 + C); TA injured, no cryotherapy treatment, and analyzed 7 days post-cryolesion (L7); TA injured, treated with cryotherapy, and analyzed 7 days post-cryolesion (L7 + C); TA injured, no cryotherapy treatment, and analyzed 14 days post-cryolesion (L14); and TA injured, treated with cryotherapy, and analyzed 14 days post-cryolesion (L14 + C). The control group was analyzed at the same time point as L3 and L3 + C (Figure 1).

Cryolesion

The TA was chosen because of its superficial position. Before inducing skeletal muscle injury, the animals were anesthetized and the skin of the right hind limb around the TA was trichotomized and cleaned with alcoholic iodine solution. A transverse incision (approximately 1 cm) over the muscle belly was made, exposing the TA. Muscle injury was induced on the non-treated and treated groups by cryolesion. This injury model has been shown to be a reproducible method of inducing muscle injury among animals [20,26–28]. Thereby, a rectangular iron bar (40×20 mm) was cooled using liquid nitrogen and applied

for 10 seconds on the widest part of TA belly. The freezing exposure was repeated twice more at the same site, with an interval of 30 seconds between them. After the final cryolesion application, the skin was sutured.

Cryotherapy

Under anesthesia, the animals were maintained in a horizontal position on a plastic table and the ankle of the right hind limb was fixed by tape. During cryotherapy applications, a plastic pack filled with crushed ice was fixed directly to the skin of the right TA using tape. Each cryotherapy treatment day comprised of three separate applications per day, each lasting for 30 minutes, with an interval of 2 hours between applications [26]. The cryotherapy treatment was performed immediately, 24, and 48 hours after injury, taking into account the destruction phase during muscle regeneration, in line with previous clinical recommendations [23,24] (Figure 1).

Muscle sample collection

At the time point that defined each experimental group, the respective animals were euthanized by anesthetic overdose and weighed. Then, the right TA was removed and weighed. For biochemical analyses, the belly of the TA was isolated and immediately frozen in isopentane, pre-cooled in liquid nitrogen, and stored in a freezer (Forma Scientific, Marietta, Ohio, U.S.A) at -80°C until analysis.

Biochemical analyses

Biochemical analyses were performed at three time points: 3, 7, and 14 days after TA injury. The TA fragment was homogenized in NaCl (150 mM) and centrifuged at $4000g$ for 10 minutes at 4°C to obtain a low speed supernatant fraction. The supernatant fraction was used to determine dichlorofluorescein reactive substances (DCF-RS) levels; thiobarbituric acid reactive substances (TBARS) levels; methyl thiazol tetrazolium (MTT) reduction levels; enzymatic activity of catalase (CAT) and superoxide dismutase (SOD); and non-protein thiol ($-SH$) levels.

DCF-RS levels

DCF-RS levels were determined as an index of peroxide production by cellular components [29], according to the method described by Carvalho et al. [9] The fluorescence analysis was performed with excitation at 488 nm and emission at 525 nm, and both slit widths used were 5 nm. DCF-RS levels were determined using a standard curve of oxidized dichlorofluorescein (DCF) [30]. The results were normalized by the protein content, which was determined according to

Figure 1. Experimental design. Vertical ticks indicate the time after muscle injury.

Lowry et al. [31] using bovine serum albumin (BSA) as the standard.

TBARS levels

TBARS levels were determined in skeletal muscle supernatant fraction samples as a measure of lipid peroxidation, according to the method described by Ohkawa et al. [32] TBARS levels were measured at 532 nm using a standard curve of malondialdehyde and the results were normalized by protein content.

MTT reduction levels

MTT reduction levels were determined as an index of cell viability [33], according to the method described by Mosmann [34]. The formed formazan levels were determined spectrophotometrically at 570 nm [34] and the results were normalized by protein content.

CAT and SOD activities

The CAT and SOD enzyme activities were analyzed as an index of the enzymatic antioxidant mechanisms. CAT enzyme activity was kinetically determined according to the method proposed by Aebi [35] and SOD enzyme activity was kinetically determined according to the method proposed by Misra and Fridovich [36].

Non-protein -SH levels

The non-protein -SH levels were determined as a non-enzymatic antioxidant mechanism, according to the method proposed by Ellman [37] with some modifications. Non-protein -SH levels were measured spectrophotometrically at 412 nm [37]. Results were calculated in relation to a standard curve constructed with glutathione (GSH) [37], and normalized by protein content.

Statistical analysis

Descriptive statistics (means and standard deviations) were used to report all data. The MTT reduction levels were calculated as a percentage of the control group. The Shapiro–Wilk and Levene tests showed that all data were normally and homogeneously distributed. Two-way analyses of variance (ANOVA) were used

to analyze time/intervention interactions for each variable. In the case of a significant difference, Tukey's post-hoc test was performed. For all analyses, a *P* value equal or less than 0.05 was accepted as statistically significant. Findings were analyzed with Statistical Package for Social Sciences (version 21.0).

Results

Oxidative markers and cell viability index

Compared to the control group, DCF-RS levels were greater in the non-treated groups at 7 days post-injury (L7: 7.5 fold, $P < 0.001$; L14: 9.0 fold, $P < 0.001$; Figure 2(a)). However, cryotherapy reduced the increase of DCF-RS levels at 7 and 14 days post-injury compared with non-treated groups (L7 + C: 0.1 fold, $P < 0.001$; L14 + C: 0.3 fold, $P < 0.001$; Figure 2(a)).

Compared to the control group, TBARS levels increased in non-treated groups at 3 and 7 days post-injury (L3: 2.8 fold, $P < 0.001$; L7: 2.3 fold, $P = 0.022$; Figure 2(b)), and returned to control levels by 14 days ($P = 0.94$; Figure 2(b)). Interestingly, compared to the non-treated groups, the cryotherapy groups presented a reduced increase of TBARS levels, both at 3 and 7 days post-injury (L3 + C: 0.4 fold, $P = 0.002$; L7 + C: 0.5 fold, $P = 0.032$; Figure 2(b)).

The non-treated L3, presented a decrease in MTT reduction levels (L3: 0.5 fold, $P = 0.023$; Figure 2(c)), compared with control group. There were no differences between cryotherapy groups and control at any analyzed time points ($P > 0.05$). However, there were differences between treated and non-treated groups at all-time points (L3 + C: 2.2 fold, $P = 0.002$; L7 + C: 1.7 fold, $P = 0.044$; L14 + C: 1.7 fold, $P = 0.035$; Figure 2(c)).

Enzymatic and non-enzymatic antioxidant mechanisms

Compared to the control group, SOD enzyme activity increased in the non-treated groups at all analyzed time points (L3: 2.2 fold, $P = 0.005$; L7: 2.8 fold, $P < 0.001$; L14: 2.8 fold, $P < 0.001$; Figure 3(a)). Nevertheless,

Figure 2. Effects of multiple cryotherapy applications performed during the destruction phase following cryolesion in the *tibialis anterior* (TA) muscle of rats on markers of reactive species production, oxidative damage, and cell viability, respectively: (a) dichlorofluorescein reactive substances (DCF-RS) levels; (b) thiobarbituric acid reactive substances (TBARS) levels; (c) methyl thiazol tetrazolium (MTT) reduction levels. Data are expressed as mean $\pm$ SD. *Differences when compared to control group ($P < 0.05$); #differences between the treated and non-treated groups at the same time point ($P < 0.05$); †different from the value at 3 days after muscle injury ($P < 0.05$).

at 7 and 14 days post-injury, cryotherapy reduced the increase of SOD enzyme activity in the treated groups compared to the non-treated groups (L7 + C: 0.5 fold, $P = 0.001$; L14 + C: 0.6 fold, $P = 0.008$; Figure 3(a)).

Figure 3. Effects of multiple cryotherapy applications performed within the destruction phase, following cryolesion in the *tibialis anterior* (TA) muscle of rats on antioxidant enzymatic and non-enzymatic detoxification mechanisms: (a) enzymatic activity of superoxide dismutase (SOD); (b) enzymatic activity of catalase (CAT); (c) non-protein thiol (–SH) levels. Data are expressed as mean $\pm$ SD. *Differences when compared to control group ($P < 0.05$); #differences between the treated and non-treated groups at the same time point ($P < 0.05$); †different from the value at 3 days after muscle injury ($P < 0.05$).

Only at 3 days post-injury, CAT enzyme activity of the non-treated groups was greater compared to control (L3: 3.4 fold, $P < 0.001$; Figure 3(b)). Interestingly, cryotherapy reduced the increase of CAT enzyme activity at the same time point compared to the non-treated group (L3 + C: 0.2 fold, $P < 0.001$; Figure 3(b)). The non-treated L3 group displayed

lesser non-protein –SH levels (L3: 0.5 fold, $P=0.015$; Figure 3(c)) compared with control. On the other hand, at the same time point, cryotherapy maintained non-protein –SH levels compared to control (L3 + C: $P=0.920$; Figure 3(c)), while also not being significantly different from the non-treated group (L3 + C: 2.1 fold, $P=0.174$, Figure 3(c)).

Discussion

This study was conducted in order to evaluate the effects of multiple cryotherapy applications on classical markers of oxidative stress in the TA of rats obtained 3, 7, and 14 days after cryolesion-induced muscle injury. In agreement with our initial hypothesis, multiple cryotherapy applications, performed within the destruction phase after injury, alleviated markers of oxidative stress.

It is well established that the inflammatory response leads to elevated production of ROS, as consequence of an increased number of macrophages and neutrophils [9,14,38]. To combat the production of ROS, evidence has demonstrated that cryotherapy may be effective, as it reduces ROS in injured tissue by reducing the magnitude of inflammatory response [6,9,21]. Additionally, a recent study from our laboratory [20] demonstrated that multiple applications of cryotherapy reduced the quantity of macrophages in the injured area. One potential explanation for the reduction of macrophages in the injured area might be related to the blood-flow reducing effects of cryotherapy [14,17,39] or the capacity of cryotherapy to reduce leukocyte adhesion in the microvasculature [16,40]. The present study showed that multiple cryotherapy applications during the destruction phase of muscle regeneration attenuated the increase of DCF-RS and TBARS levels compared to the levels seen in the non-treated rats. Thus, cryotherapy appeared to influence the production of ROS and reduce the biomolecular impairments associated with ROS [41,42]. Therefore, it is possible that post-injury cryotherapy may reduce the magnitude of a muscle injury that has already occurred [6,9,21]. In addition, as the treated rats displayed greater MTT reduction levels than non-treated rats (Figure 2(c)), cryotherapy may serve as a potential therapeutic strategy to maintain cell viability after injury. Although the treated groups presented mean MTT reduction values that were slightly greater than control group, these values were not significant and we believe that such slight increases in MTT reduction levels presented by treated groups might be due an elevated cell' metabolic rate [43] than non-treated groups. Nevertheless, this idea is hypothetical and should be explicitly examined in future research.

Similar effects of cryotherapy were observed by Puntel et al. [21], who associated MTT reduction levels with specific indicators of mitochondrial

function. From this vantage point, the efficacy of cryotherapy could be explained by a reduction in the rate of chemical reactions and, therefore, the demand for ATP, reducing cellular collapse [19]. However, it is not possible to determine if cryotherapy prevents or delays the death of cells that were primarily injured but not initially destroyed. Therefore, cryotherapy continues to be credited for reducing secondary injury [5]. Similar to previous studies [6,9,21], the present study showed that multiple cryotherapy applications maintained SOD and CAT activity and non-protein –SH levels within control levels. Taking into account the oxidative markers, cell viability, and antioxidant system, these results show that cryotherapy maintained homeostatic mechanisms by reducing ROS production and avoiding an increase in antioxidant system activity after injury.

The post-injury biochemical responses indicate that the cryolesion model adopted in the present study was effective at inducing muscle injury. When collectively comparing the results of control group with the non-treated groups, the results suggest that an early increase of TBARS and a decrease of MTT reduction levels could have been associated with ROS that were not detectable in the DCF-RS assay [28], whereas at later time points, a greater activation of antioxidant mechanisms neutralized a significant generation of hydroperoxides. Interestingly, SOD activity showed a progressive increase over the duration of this study, differing from previous studies showing that SOD activity has not significantly changed after injury [6,9,21,22]. The increase of SOD activity may be related to a greater muscle injury caused by cryolesion compared to other models of injury such as skeletal strain [9], contusion [21], ischemia–reperfusion [6], and exercise-induced muscle damage [22]. Factually, previous studies had associated cryolesion's low temperature to an alteration in quaternary structures of enzymes [28,44].

Although the present study indicates that cryotherapy may attenuate the oxidative stress following muscle injury, limitations are present. For example, tissue and core body temperature were not measured during cryotherapy applications and biochemical markers of muscular damage were not quantified. Nonetheless, it is also important to emphasize that studies investigating the effects of cryotherapy on oxidative stress are scarce in the literature and show heterogeneity between cryotherapy protocols.

Finally, only one clinical trial has been conducted with the purpose to investigate the effect of cryotherapy on oxidative stress biomarkers after muscle injury [22]. Similar to previous animal trials [9,21], a reduction in TBARS plasma concentration after a submaximal physical exertion was observed after 5 minutes of cold water immersion [22]. Although this study contributes to the elucidation of cryotherapy's effects on

oxidative damage in humans, our study is the first study that has determined the effects of a multi-application cryotherapy protocol on markers of oxidative stress. Nevertheless, further studies should be conducted, especially in humans, in order to address the effects of a cryotherapy protocol on muscle regeneration.

Conclusion

In conclusion, multiple cryotherapy applications, performed within the destruction phase after TA injury of rats, reduced oxidative stress. In terms of practical applications, cryotherapy can play an important role during the destruction phase of the regeneration process by limiting the severity of muscle skeletal injury.

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Disclosure statement

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