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# Effect of time-dependent cryotherapy on redox balance of quadriceps injuries

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ABSTRACT

Muscle trauma represents a high number of injuries in professional sport and recreation and may occur through several mechanisms. This study aims at analyzing time-dependent effects of cryotherapy on the redox balance in lesioned quadriceps muscles in F1 mice. Twenty male F1 mice were divided into five groups: (a) animals were not subjected to muscle lesioning or treatment (CTR); (b) quadriceps muscle was lesioned without treatment (L); (c) quadriceps muscle was lesioned and treated with cryotherapy for 5 min (LC5); (d) quadriceps muscle was lesioned and treated with cryotherapy for 20 min (LC20); and quadriceps muscle was lesioned and treated with cryotherapy for 40 min (LC40). The mice were euthanized; the quadriceps muscles were collected and subjected to analyses for levels of protein, hydroperoxides, nitrite, catalase (CAT) activity, oxidized glutathione (GSSG) and reduced glutathione (GSH). Protein levels were reduced in L (−39%; $p < 0.05$), LC5 (−54%; $p < 0.05$), LC20 (−40%; $p < 0.05$) and LC40 (−50%; $p < 0.05$) compared to CTR. There was an increase in lipid peroxidation in L (158%; $p < 0.05$), LC5 (300%; $p < 0.01$), LC20 (292%; $p < 0.01$) and LC40 (362%; $p < 0.01$) compared to CTR. We observed a significant increase in CAT activity in L (164%; $p < 0.05$) and LC5 (193%; $p < 0.01$) compared to CTR; a significant reduction in GSH in L (−60%; $p < 0.05$) and LC20 (−61%; $p < 0.05$) compared to CTR; and a significant increase in GSSG in LC5 (171%; $p < 0.05$) compared to CTR. In addition, GSH/GSSG was reduced in L (−89%; $p < 0.01$), LC5 (−95%; $p < 0.01$), LC20 (−59%; $p < 0.05$), and LC40 (−82%; $p < 0.01$) compared to CTR. This study showed that the cryotherapy does not improve the oxidative stress in lesioned muscles.



## 1. Introduction

Muscle trauma represents a high number of injuries in professional sport and recreation and may occur through several mechanisms [1,2]. The biological processes that occur after acute muscle injury are inflammation, swelling, repair, and remodeling, and these events are interrelated and time dependent [3].

The increase in oxygen ($O_2$) consumption during exercise induces the formation of reactive oxygen species (ROS) [4]. High levels of ROS are responsible for biomolecular damage such as peroxidation of membrane lipids [5], the formation of protein carbonyls, and even damage to intracellular DNA [6], which ultimately interferes with and changes intracellular metabolism and may even cause cell death [7]. Several studies have observed a correlation of increased ROS production with the onset of muscle fatigue [8,9] and acute muscle injury [10].

During ROS production, reactive intermediates such as the superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide ($H_2O_2$), the hydroperoxyl (•OOH) radical, and the hydroxyl (•OH) radical are formed [11]. Reactive nitrogen species (RNS) such as peroxynitrite ($ONOO^-$) are mostly formed from the reaction of $O_2^{\bullet -}$ with nitric oxide (NO), which in turn is produced through the conversion of L-arginine to L-citrulline by nitric oxide synthase [12–14].

The body has effective enzymatic and non-enzymatic antioxidant systems to counterbalance the production of ROS and RNS

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[15–18]. The activation of inflammatory cells results in the formation of $O_2^{\bullet-}$, which is rapidly converted to $H_2O_2$ by the enzyme superoxide dismutase (SOD). The enzyme catalase reacts very efficiently with the $H_2O_2$ produced endogenously by the cells to form water and molecular oxygen [19]. The biochemical function of glutathione peroxidase (GPx) consists of reducing hydroperoxides to alcohols and $H_2O_2$ to $H_2O$ by using reduced glutathione (GSH) as a substrate, thus generating oxidized glutathione (GSSG). A decrease in the GSH/GSSG ratio indicates the presence of oxidative stress [20]. Redox imbalance occurs as a result of either an increase or a decrease in the activity of antioxidant enzymes against oxidative stimuli, by influencing either the substrates directly or the synthesis of antioxidant enzymes [21]. The oxidative stress occurs when the level of oxidants is higher than the level of antioxidants, which is characterized by the oxidative damage to proteins, lipids, and/or DNA [22].

Cryotherapy is one of the simplest and oldest treatments for acute muscle injury; it anesthetizes the area to which it is applied and decreases local blood flow and metabolism [23]. Cold application causes discomfort in the first few minutes, followed by paralysis and anesthesia, and a reduction in muscle spasm, edema, and metabolism [24]. The initial feeling of skin discomfort (3 min) caused by cold water is reversed with a fall in body temperature, causing a burning sensation that lasts for 2–7 min, followed by paresthesia and analgesia for 5–12 min and then profound vasodilation in the skin for 12–15 min which is not accompanied by an increase in metabolism [24–26]. Skin temperature must reach 14.4 °C for analgesia and 13.8 °C to reduce local blood flow to obtain therapeutic benefits from the application of cold. Under extremely cold conditions (−20 °C to −70 °C), the tissue will be destroyed. However, the surface temperature only decreases to 1°C-10 °C when ice is used directly on the skin for general cooling of tissues [24–26].

Edema, experimentally induced in animals, has shown a variable response to the application of cooling [27]. A randomized controlled trial reported a significant reduction in the volume of injured limbs in rats after immersion in cold water (12.8 °C–15.6 °C) and its authors concluded that immediate cooling after injury was effective in restricting the development of edema [28]. In addition, numerous clinical studies provide empirical evidence that the use of ice reduces edema [29]. The ideal way to treat muscle injury has not been established; there are various different therapies that use physical or chemical approaches. Physiological responses to the primary lesion can lead to the formation of a secondary lesion via enzymatic and hypoxic mechanisms that affect the cells of the peripheral region as well as the area of the initial injury. Secondary injury from posttraumatic hypoxia can be caused by several factors such as bleeding from injured vessels, hemostasis, decreased blood flow due to increasing blood viscosity and increased extravascular pressure. Moreover, the edema caused by damage to the cellular membrane may occlude small vessels, increasing the ischemic area [30]. Secondary injury can also be caused by enzymes released from lysosomes or from dead cells that are necrotizing [31]. Thus, in the early hours after the primary injury, it is common to observe an increase in the total injured area, which results from the secondary injury. Some studies have shown an increase in edema after cryotherapy which may be due to an effect named vasodilatation induced by cold (VDIC), which occurs 15 min from the start of cryotherapy, or possibly due to an expansion resulting from thermal injury to the lymphatic system [32]. Cryotherapy also acts on circulation, causing vasoconstriction and decreasing hematoma. The decrease in local circulation reduces edema, inflammation, hemorrhage, and hematoma [25]. Although there are frequent reports that the decrease in tissue temperature induced by cryotherapy is able to reduce pain, nerve conduction, metabolism, muscle spasm and injury from hypoxia and the release of inflammatory mediators [33,34]; there is no information on the effect of cryotherapy on the redox balance of the injured muscle tissue. Therefore, this study aimed at analyzing time-dependent effects of cryotherapy on the redox balance in lesioned quadriceps muscles in F1 mice.

## 2. Methods

This project was approved by the Ethics Commission on Animal Use under protocol number 036/2012. The ethical principles for animal experimentation established were respected.

### 2.1. Animals

Twenty male F1 mice from animal house were used. During the experimental protocol, the animals were kept in collective cages, with a maximum of three animals per cage. The mice were maintained at 25 °C with a photoperiod of 12-h dark/light and received standard food and water *ad libitum*.

### 2.2. Experimental groups

Animals were divided into five groups consisting of four animals each: a control group in which the animals were not subjected to either muscle lesioning or treatment (CTR); a lesioned group in which the quadriceps muscle was lesioned without treatment (L); a group in which the quadriceps muscle was lesioned and then treated with cryotherapy by cold-water immersion for 5 min (LC 5); a group in which the quadriceps muscle was lesioned and then treated with cryotherapy by cold-water immersion for 20 min (LC 20); and a group in which the quadriceps muscle was lesioned and then treated with cryotherapy by cold-water immersion for 40 min.

### 2.3. Inductor apparatus used to induce experimental muscle lesions

Experimental lesions were induced using an adaptation of a device originally proposed by Stratton et al. [35] and made by Oliveira [32], which causes lesions in rats by direct impact. The equipment consists of two metal rods with a diameter of 10 mm, stabilized by a superior perpendicular bar. These rods are attached to a plastic base, which is attached to a rectangular metal base with an area of 12.25 $cm^2$, which served to position the pelvic limb of the animal and support the free fall of the weight used to induce the lesion.

### 2.4. Induction of muscle lesions

The animals were anesthetized with a combination of ketamine (80 mg/kg) and xylazine (15 mg/kg) at a dose of 0.6 mL of the mixture per 100 g body weight to cause the muscle lesion via direct impact [36]. Each animal was subjected to a single trauma. The middle third of the quadriceps of the right hindlimb of each animal was positioned on the metal base of the device, with the animal in ventral recumbency and the joints of the hip, knee, and ankle in dorsiflexion at 90°.

The weight set was controlled by a guide and a transparent acrylic yarn to ensure accuracy of its delivery and was released by means of a pulley attached to the top of the rod [32]. According to the previously described method, a 200-g load was released from a height of 10 cm directly onto the quadriceps muscle of the immobilized mouse in order to produce an impact force of approximately 98 N (10 kg) [32].

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