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Therapeutic cold: An effective kind to modulate the oxidative damage resulting of a skeletal muscle contusion

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Abstract
Muscular contusions affect the function of the skeletal muscle system. This study investigated the oxidative damage as well as the main morphological changes related to a skeletal muscle contusion in the gastrocnemius muscle of rats and also the capacity of therapeutic cold to modulate these parameters. The therapeutic cold modulated the increase of oxidative stress markers and also modulated the reduction in the antioxidants levels in the injured muscle. In enzyme assays, therapeutic cold was also effective in normalizing the muscle Na^+/K^+ and Ca^{2+} ATPases, lactate dehydrogenase and myeloperoxidase activities. Similarly, the lesioned non-treated animals presented evident impairments in the mitochondrial functions and in the muscle morphology which were diminished by the cold treatment. The therapeutic cold was able to modulate the oxidative damage possibly by its capacity to limit the inflammatory response intensity, to attenuate the impairment of the mitochondrial function and also to preserve the skeletal muscle morphology.

Keywords: *Contusion, therapeutic cold, oxidative damage, mitochondria*

Introduction

Skeletal muscle lesions are responsible for the majority of the functional limitations of workers observed in sportive and occupational medicine [1]. One of the most common lesions which affect the function of the skeletal muscle system is the muscular contusion [2]. These lesions are characterized by the compression of the skeletal muscle cells due to an impacting weight under the muscle surface [2]. As a result, the contracting elements of the muscle structure could be damaged and become dysfunctional, leading to an impairment of some of the normal skeletal muscle functional properties such as elasticity, extensibility and contractility [1,2]. In response to a skeletal muscle contusion, the compression and consequently the rupture of some blood capillaries as well as the overflow of blood components in the injured region may occur [3]. Thus, inflammatory cells could be attracted to injured regions in order to promote the clearance, starting the rehabilitation and the restructuration of the tissues [4].

Currently, it is well established that an inflammatory response is needed to the structural and functional rehabilitation of the damaged tissues [3]. However, an excessive inflammatory response could be accompanied by an uncontrolled reactive species (RS) generation [5,6]. An imbalance between the antioxidant defense systems and the generated RS

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may determine the impairment of the normal cell functions [7]. Among the biological molecules that could be impaired are those that depend on the sulphydryl groups (SH) for their normal functioning. Some enzymes, such as the lactate dehydrogenase (LDH) [8–10] and the delta aminolevunilate dehydratase (δ-ALA-D) [11–13], as well as the non-enzymatic antioxidant three-peptide glutathione (GSH) may be affected in these conditions [14,15]. Besides, oxidant agents may interact with the thiol groups located at the active site of other important enzymes as the Na^+/K^+ and the Ca^{2+} ATPases, which are needed for the preservation of the adequate ionic gradient across the cellular membranes [16–19].

Many studies have indicated a central role of the oxidative damage in the development of several acute and chronic human disorders [20]. However, up to now, there are few data depicting the existence of such alterations in models of skeletal muscle tissue lesions, for example due to a skeletal muscle contusion [4]. The therapeutic cold has been considered one of the most efficient physical agents to treat different skeletal muscle lesions [21,22], but the biochemical mechanisms involved in its protective action are still unclear. In view of the potential oxidative damage induced by a contusion lesion, cold therapy, at least in part, is likely to have an important role in modulating this oxidative damage [23].

Thus, considering that data are scarce in the literature regarding the biochemical phenomena that underlie the therapeutic effects of cold in skeletal muscle lesions, we examined the possible role of the oxidative stress related to a skeletal muscle contusion induced in gastrocnemius muscle of rats. Subsequently, we also analysed the involvement of the inflammatory response intensity as well as the mitochondrial function impairment as possible mechanisms involved in the genesis of the oxidative damage in response to a skeletal muscle contusion. Besides, the benefits of the cold therapy under these parameters were investigated in order to improve the knowledge regarding its possible mechanism of action.

Materials and methods

Chemical reagents

The reagents thiobarbituric acid (TBA), dichlorofloureseine diacetate (DCFH-DA), methyltetrazolium (MTT), ethylene glycol tetraacetic acid (EGTA), Ellman's reagent (DTNB), N,N,N',N'-tetramethylbenzidine and ouabaine were supplied by Sigma–Aldrich Chemical Co. (St. Louis, MO). The other used reagents were obtained from local suppliers.

Animals

Adult male Wistar rats weighing 270–320 g from our own breeding colony were kept in cages of five animals each, with food and water *ad libitum* in a room with controlled temperature (22 ± 3°C) and on a 12-h light/dark cycle with lights on at 7:00 am. The animals were maintained and used in accordance with the guidelines of the Committee on Care and Use of Experimental Animal Resources of the Federal University of Santa Maria, Brazil. The animals were divided into four main groups:

1) Control non-treated and non-lesioned animals—animals not submitted to the standard skeletal muscle contusion;
2) Control cold treated and non-lesioned animals—animals not submitted to the standard skeletal muscle contusion and treated with the therapeutic cold;
3) Lesioned non-treated animals—animals submitted to the standard skeletal muscle contusion without any treatment; and
4) Lesioned and cold treated animals—animals submitted to the standard skeletal muscle contusion and treated with the therapeutic cold.

Skeletal muscle contusion

The skeletal muscle contusion was developed according to the method proposed by Crisco et al. [24], with few modifications. First, the animals were anaesthetized with ketamine (50 mg/kg; i.p.) and xilazine (10 mg/kg; i.p.). The fully anaesthetized animals were placed in a prone position and the right hind limb was placed to perform the skeletal muscle contusion. A mass of 100 g fell through a polyvinyl chloride tube used as a guide from a height of 100 cm onto the top of the impactor (radius of 6.0 mm) placed in direct contact with the skin covering the mid-belly of the right gastrocnemius muscle. After the contusion, the rats were allowed to recover from anaesthesia and returned to the cage. The animals of the lesioned and cold treated animals were also submitted to the first treatment section for 5 min immediately after the skeletal muscle contusion.

Therapeutic cold treatment

The treatment of the animals with the therapeutic cold was performed by the application of ice cubes directly under the contused muscle [23]. The treatment sections were developed twice a day for 5 min each section. The first application was performed immediately after and the second application 6 h after the skeletal muscle contusion.

The protocol of cold treatment used in this study was based in the previous data of our research group [23]. We observed that the cold treatment produced by the ice cubes application directly under the site of the lesion for 5 min immediately after the lesion and repeated 6 hours after the lesion is able to modulate significantly the oxidative damage induced by a strain muscle lesion [23].

Biochemical analysis

Biochemical analyses were performed in two distinct sets of time. The first set of biochemical analysis was carried out 30 min after the skeletal muscle contusion in order to check the immediate biochemical changes indicative of oxidative damage, as well as the effects of a single therapeutic cold treatment section under these conditions. The second set of biochemical analysis was carried out in the day following the skeletal muscle contusion in order to investigate the long-term biochemical changes indicative of the oxidative damage, as well as the effects of two therapeutic cold treatment sections under these conditions.

Tissue preparation

Whole blood and blood components. Rats were euthanized and the whole blood was collected (cardiac puncture) in previously heparinized tubes and kept under refrigeration. Whole blood samples were precipitated with TCA 40% (1:1) and centrifuged (4000 × *g* at 4°C for 10 min) in order to obtain the supernatant fraction that was used for TBARS determination. Other heparinized blood samples were centrifuged at 1000 × *g* at 4°C for 10 min in order to obtain plasma and cellular blood fractions which were used for DCF-RS measurement. In addition, plasma aliquots were kept at –20°C for posterior creatine kinase activity measurement.

Skeletal muscle homogenates. For the determination of some of the oxidative damage markers and also the enzyme activity measurement, the right gastrocnemius muscle was removed, quickly homogenized in NaCl (150 mM), and kept in ice. After the homogenization, the skeletal muscle samples were centrifuged at 4000 × *g* at 4°C for 10 min to yield a low speed supernatant fraction (S1). For the MPO enzyme activity measurement, the muscle samples were homogenized in potassium phosphate buffer (20 mM, pH 7.4) containing EDTA (0.1 mM). After the homogenization, the skeletal muscle samples were centrifuged at 2000 × *g* at 4°C for 10 min to yield a low speed supernatant fraction (S1). Then, the S1 fraction was centrifuged again at 20 000 × *g* at 4°C for 15 min to yield a final pellet that was re-suspended in potassium phosphate buffer (50 mM, pH 6.0) containing hexadecyltrimethylammonium bromide (0.5%). The samples were finally freeze–thawed twice for the posterior enzymatic MPO assay. Besides, aliquots of skeletal muscle preparations were frozen (-20°C) for posterior analysis.

Isolation of skeletal muscle mitochondria. Rat skeletal muscle mitochondria were isolated as described by Tonkonogi and Salhin [25], with some modifications. First, the right gastrocnemius muscle was quickly removed and homogenized in a buffer containing mannitol (225 mM), sucrose (75 mM), EGTA (1 mM), bovine serum albumin (BSA) (0.1%) and HEPES (10 mM, pH 7.2). After the homogenization, the resulted suspension was centrifuged for 7 min at 2000 *g* in order to obtain a low speed supernatant fraction (S1). Then, S1 was re-centrifuged for 10 min at 12 000 *g*. The obtained pellet was re-suspended in a buffer containing mannitol (225 mM), sucrose (75 mM), EGTA (1 mM) and HEPES (10 mM, pH 7.2) and re-centrifuged at 12 000 *g* for 10 min. The supernatant was decanted and the final pellet re-suspended in a buffer containing KCl (65 mM), sucrose (100 mM), EGTA (0.05 mM), BSA (0.2%) and HEPES (10 mM, pH 7.2), to yield a protein concentration of 30–40 mg/mL.

Oxidative stress markers and cell viability determination

Thiobarbituric acid reactive substances (TBARS) levels. Analyses were performed in whole blood and in skeletal muscle S1 samples according to the method described by Ohkawa et al. [26]. Aliquots of 500 μL of supernatant fraction obtained after blood sample precipitation or 200 μL of skeletal muscle S1 were added to colour reaction. TBARS levels were measured at 532 nm using a standard curve of MDA and corrected by the protein content [26].

Oxidized diclorofluoresceine (DCF-RS) levels. DCF-RS levels were determined as an index of the peroxide production by the cellular components [27]. Aliquots of plasma (200 μL), cellular blood fraction (10 μL) or skeletal muscle S1 (50 μL) were added to a medium containing Tris-HCl buffer (0.01 mM; pH 7.4) and DCFH-DA (7 μM). After DCFH-DA addition, the medium was incubated in the dark for 1 h until fluorescence measurement procedure (excitation at 488 nm and emission at 525 nm and both slit widths used were at 5 nm). DCF-RS levels were determined using a standard curve of DCF and the results were corrected by the protein content [28].

Non-protein thiol (–SH) levels. Levels of non-protein –SH were determined in skeletal muscle S1 samples according to the method proposed by Ellman [29]

with some modifications. Briefly, the samples of the skeletal muscle S1 (0.5 mL) were precipitated with TCA (5%) (1 mL) and subsequently centrifuged at 4000 *g* for 10 min. After the centrifugation, the supernatant fraction (500 μL) was added to a reaction medium containing K^+-phosphate (0.25 mM and pH = 7.4) and DTNB (1 mM). Non-protein –SH levels were measured spectrophotometrically at 412 nm. Results were calculated in relation to a standard curve constructed with GSH at known concentrations and also corrected by the protein content [29].

Methyl-tetrazolium (MTT) reduction levels. MTT reduction levels were determined as an index of the dehydrogenase enzymes functions, which are involved in the cellular viability [30]. Aliquots of skeletal muscle S1 (500 μL) were added to a medium containing 0.5 mg/mL of MTT and were incubated in the dark for 1 h at 37°C. The MTT reduction reaction was stopped by the addition of 1 mL of dimethylsulphoxide (DMSO). The formed formazan levels were determined spectrophotometrically at 570 nm and the results were corrected by the protein content [31].

Enzymes activity determination

Creatine kinase (CK). The CK enzyme activity was measured spectrophotometrically in plasma samples as an index of the damage caused by the skeletal muscle contusion using diagnosis kits (CK-NAC Liquiform, Labtest, MG, Brazil).

Lactate dehydrogenase (LDH). The LDH enzyme activity was determined spectrophotometrically in skeletal muscle S1 samples as an index of the oxidative damage to this tissue using diagnosis kits (LDH Liquiform, Labtest, MG, Brazil).

Sodium potassium (Na^+/K^+) ATPase. The Na^+/K^+ ATPase enzyme activity was determined in skeletal muscle S1 samples according to the method proposed by Musbeck et al. [32], with some modifications. Briefly, the aliquots of skeletal muscle S1 (20 μL) were added to a reaction medium containing NaCl (115 mM), $MgCl_2$ (2.5 mM), KCl (18 mM) and Tris-HCl buffer (45 mM and pH 7.4), with or without the Na^+/K^+ ATPase enzyme inhibitor ouabaine (5 μM). The method for ATPase activity measurement was based on the determination of the inorganic phosphate (Pi) released to the reaction medium by the hydrolysis of the ATP according to the method proposed by Atkinson et al. [33]. The reaction was initiated with the addition of the substrate ATP (1.5 mM) to the reaction medium and was finished by the addition of the colour reagent (1 mL) containing ammonium molibdate (2%), triton-100X (5%) and H_2SO_4 1.8 M (10%) after 15 min of incubation at 37°C. The formed molibdate-Pi complexes were measured spectrophotometrically at 405 nm. Values were calculated in relation to a standard curve constructed with Pi at known concentrations and also corrected by the protein content.

Calcium (Ca^{2+}) ATPase. The Ca^{2+} ATPase enzyme activity was determined in skeletal muscle S1 samples according to the method proposed by Zaidi and Michaelis [34], with some modifications. Briefly, the aliquots of skeletal muscle S1 (20 μL) were added to a reaction medium containing $MgCl_2$ (1 mM), KCl (50 mM), EGTA (0.2 mM) and Tris-HCl buffer (25 mM and pH 7.4), with or without the $CaCl_2$ (150 μM) in order to ensure a final concentration of 1 μM of Ca^{2+} ions in the medium. The experimental procedures were similar to those used for the determination of the Na^+/K^+ ATPase enzyme activity, which were described above.

Superoxide dismutase (SOD). The SOD enzyme activity was determined in skeletal muscle S1 according to the method proposed by Misra and Fridovich [35]. This method is based on the capacity of SOD in inhibiting auto-oxidation of adrenaline to adrenochrome. Briefly, different S1 aliquots (10–50 μL) were added to a medium containing glycine buffer (50 mM; pH 10.5) and adrenaline (1 mM). The kinetic analysis of SOD was started after adrenaline addition and the colour reaction was measured at 480 nm.

Catalase (CAT). The CAT enzyme activity was determined in skeletal muscle S1 according to the method proposed by Aebi [36]. Briefly, S1 aliquot (50 μL) was added to a medium containing potassium phosphate buffer (50 mM; pH 7.4) and H_2O_2 (1 mM). The kinetic analysis of CAT was started after H_2O_2 addition and the colour reaction was measured at 240 nm.

Myeloperoxidase (MPO). The MPO enzyme activity was determined in skeletal muscle S1 according to the method proposed by Grisham et al. [37], with some modifications. Briefly, a sample of the skeletal muscle preparation (20 μL) was added to a medium containing potassium phosphate buffer (50 mM; pH 6.0), hexadecyltrimethylammonium bromide (0.5%) and N,N,N',N'-tetramethylbenzidine (1.5 mM). The kinetic analysis of MPO was started after H_2O_2 (0.01%) addition and the colour reaction was measured at 655 nm at 37°C.

Indicators of the skeletal muscle mitochondria function. Mitochondrial DCF-RS level determination. The mitochondrial DCF-RS generation was assayed according

to Garcia-Ruiz et al. [38]. Briefly, the mitochondria samples (150 μg of protein per mL) were incubated in a medium containing KCl (65 mM), sucrose (100 mM), EGTA (0.05 mM), bovines serum albumin (BSA) (0.2%), HEPES (10 mM, pH 7.2) and the respiratory substrates glutamate (5 mM) and succinate (5 mM). The reaction was started with the DCFA-DA (1 μM) addition and the medium was kept at constant stirring during the assay period. The fluorescence analysis was performed at 488 nm for excitation and 525 nm for emission, with slit widths of 5 nm.

Mitochondrial membrane potential ($\Delta\Psi$) determination. The mitochondrial $\Delta\Psi$ determination was assayed according to Akerman and Wikstron [39]. Briefly, the mitochondria samples (150 μg protein/mL) were incubated in a medium containing KCl (65 mM), sucrose (100 mM), EGTA (0.05 mM), BSA (0.2%), HEPES (10 mM, pH 7.2), safranine O (10 μM) and the respiratory substrates glutamate (5 mM) and succinate (5 mM). The reaction was started with the mitochondria addition and the medium was kept at constant stirring during the assay period. The fluorescence analysis was performed at 495 nm for excitation and 586 nm for emission, with slit widths of 5 nm.

Mitochondrial swelling. The mitochondrial swelling was assayed according to Velho et al. [40]. Briefly, the mitochondria samples (150 μg of protein per mL) were incubated in a medium containing KCl (65 mM), sucrose (100 mM), EGTA (0.05 mM), BSA (0.2%), HEPES (10 mM, pH 7.2), $CaCl_2$ (0.2 mM), Pi (1 mM), as well as the respiratory substrates glutamate (5 mM) and succinate (5 mM). The reaction was started with the mitochondria addition and the medium was kept at constant stirring during the assay period. The fluorescence analysis was performed at 600 nm (slit 1.5 nm) for both excitation and emission wavelengths.

Protein determination. The protein content was determined according to Lowry et al. [41] using bovine serum albumin (BSA) as standard.

Histopathological analysis

One sample of the skeletal muscle tissue was used for the histopathological analysis in order to investigate microscopic changes in the normal tissue structure. We investigated the loss of skeletal muscle transverse striations and nucleus degeneration as well as the presence of necrotic skeletal muscle cells. Besides, the presence of neutrophils was examined as an index of the acute inflammatory infiltration extension. After being excised, the skeletal muscle was maintained in buffered formaldehyde solution (10%) until the microscopic preparation and colourization. The muscle samples were sectioned longitudinally along its proximal and distal origins. The histological slides were stained with hematoxylin and eosin and then submitted to the histopathological analysis.

Statistical analysis

Data were analysed by one-way and two-way ANOVA followed by Tukey test. Differences between groups were considered significant when $p < 0.05$.

Results

Effects of the cold treatment under markers of the oxidative damage and cell viability in the site of the lesion

Figures 1A–D depict the potential of the therapeutic cold in modulating the increased levels of some oxidative stress markers in the skeletal muscle tissue submitted to the contusion lesion. The increased DCF-RS and TBARS levels in the lesioned non-treated animals were significantly abolished by the therapeutic cold treatment (Figures 1A and B, respectively). Besides, the decreased MTT reduction levels in the lesioned non-treated animals were completely restored by the therapeutic cold treatment (Figure 1C).

Figures 2A and B show the role of the therapeutic cold treatment under the levels of some enzymatic and non-enzymatic antioxidant defense systems. Our data show that the cold treatment maintained the non protein –SH levels at control non-treated and non-lesioned animals values, which were significantly decreased in the lesioned non-treated animals (Figure 2A). Besides, the cold treatment counteracted the increased CAT enzyme activity depicted in the lesioned non-treated animals (Figure 2B). However, the SOD enzyme activity was not significantly changed by the cold treatment nor by the muscle contusion (data not shown).

Effects of the cold treatment under enzyme activities in the site of the lesion

Data presented in Figure 3 revealed that the therapeutic cold treatment was able to reduce the impairment in Na^+/K^+ ATPase and Ca^{2+} ATPase enzyme activities which were observed in the lesioned non-treated animals (Figures 3A and B, respectively). The LDH activity was altered 24 h after the lesion, and this alteration was modulated by the therapeutic cold treatment (Figure 3C). Furthermore, Figure 3D shows the power of the therapeutic cold to modulate the MPO enzyme

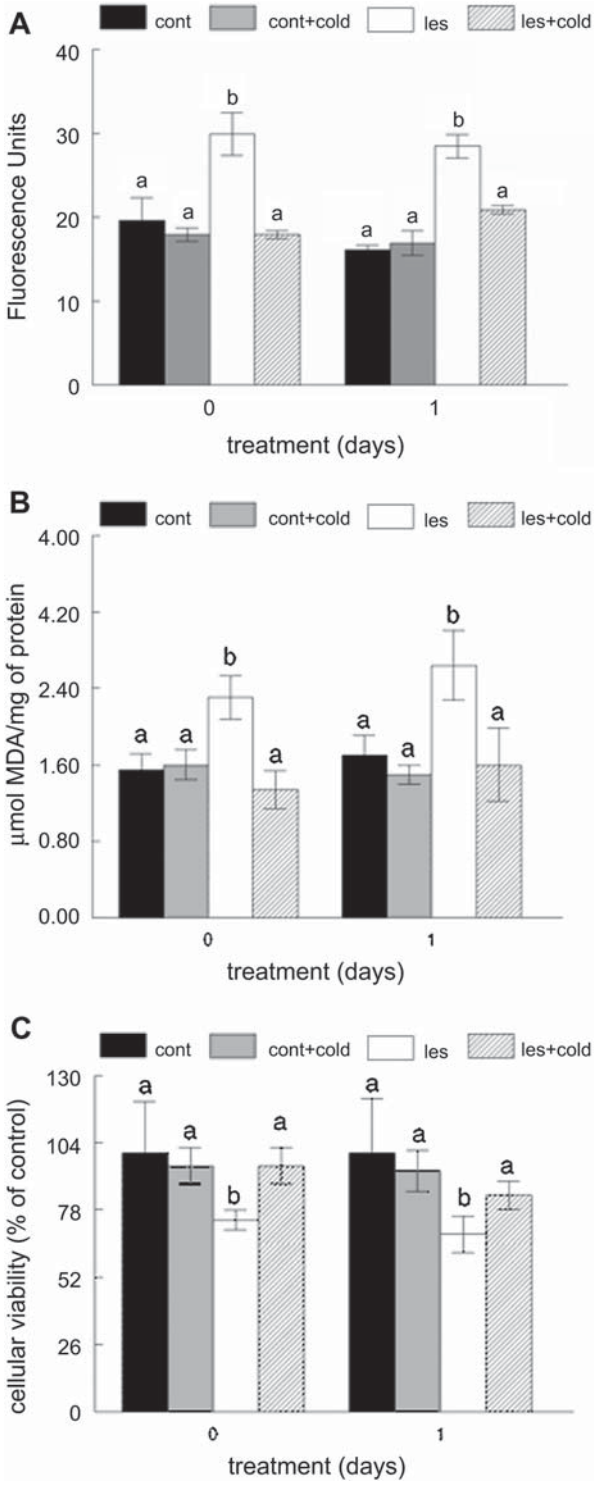

Figure 1. Effects of the cold treatment under oxidative stress markers and cell viability in the skeletal muscle tissue: (A) DCF-RS levels; (B) TBARS levels; (C) MTT reduction levels. In (A) the DCF-RS levels are expressed in fluorescence units/mg of protein; in (B) the TBARS levels are expressed in µmol of MDA/mg of protein; and in (C) the MTT reduction levels are expressed as a percentage of the control non-treated and non-lesioned animals value. Data are expressed as mean ± SE (n = 5–6) and were analysed by ANOVA, followed by Tukey test when appropriate. Differences were considered significant when $p \leq 0.05$. Significant differences are marked as [b]when compared to control non-treated and non-lesioned animals.

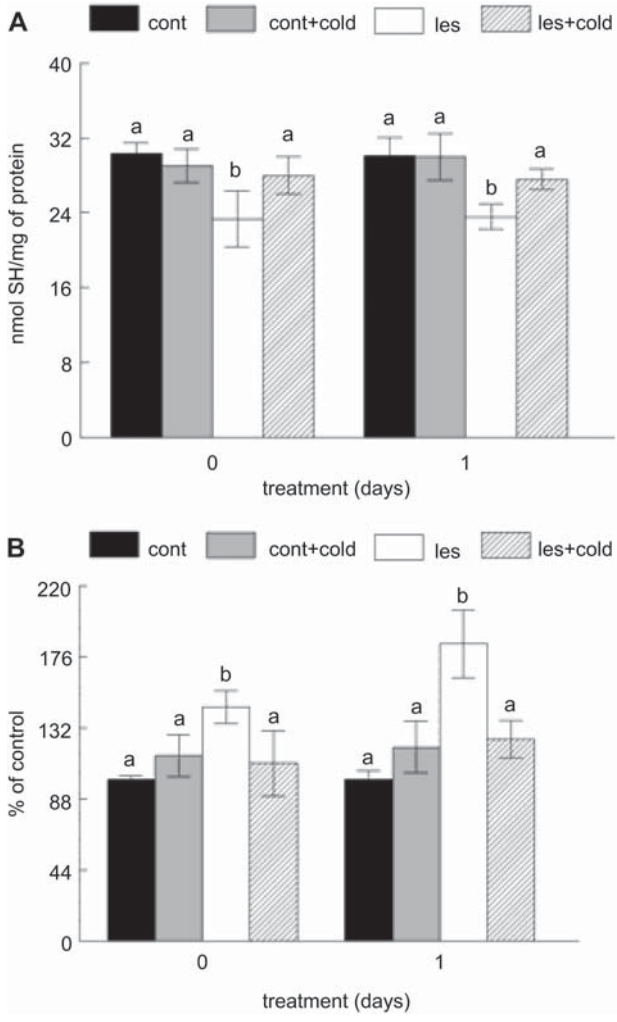

Figure 2. Effects of the cold treatment under antioxidant defense systems in the skeletal muscle tissue: (A) non-protein –SH levels; (B) CAT activity. In (A) the non-protein –SH levels are expressed in nmol of SH/mg of protein; in (B) the CAT activity is expressed as a percentage of the control non-treated and non-lesioned animals value (the control CAT activity was 135.7 ± 8.7 Units/mg of protein. Data are expressed as mean ± SE (n = 5–6) and were analysed by ANOVA, followed by Tukey test when appropriate. Differences were considered significant when $p \leq 0.05$. Significant differences are marked as [b]when compared to control non-treated and non-lesioned animals.

activity which was strongly increased only in the day following the skeletal muscle contusion.

Effects of the cold treatment under markers of the oxidative damage in the blood

Figures 4A–C show the capacity of the therapeutic cold treatment in modulating the increased levels of some oxidative stress markers in the whole blood and in blood components samples. The animals of the lesioned non-treated animals exhibited augmented DCF-RS levels both in plasma and in cellular blood fraction, which were significantly abolished by the cold treatment (Figures 4A and B, respectively). In the same way,

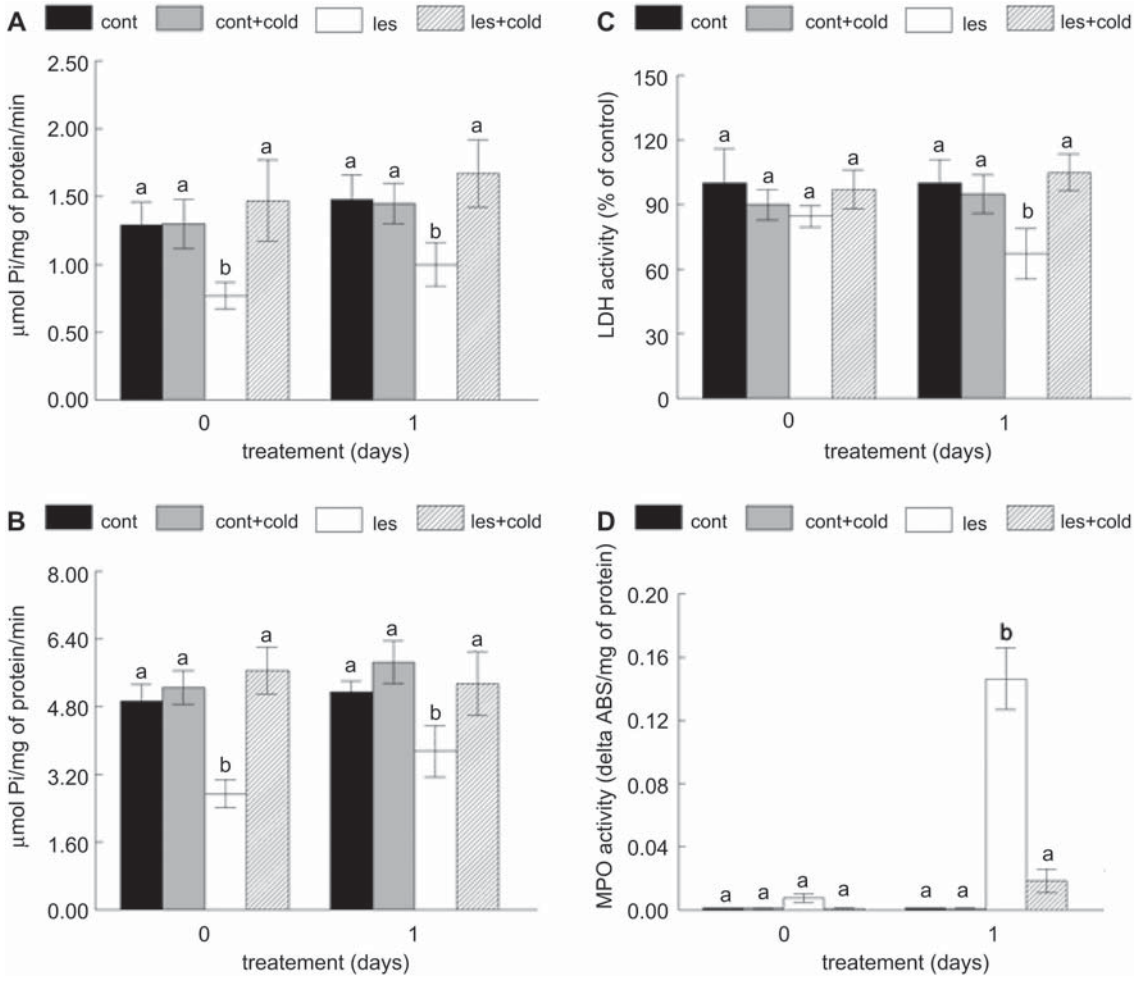

Figure 3. Effects of the cold treatment under enzymes activities in the skeletal muscle tissue: (A) Na^+/K^+ ATPase activity; (B) Ca^{2+} ATPase activity; (C) LDH activity; (D) MPO activity. In (A) and (B) the ATPases activities are expressed in µmol of Pi/mg of protein/minute of reaction; in (C) the LDH activity is expressed as a percentage of the control non-treated and non-lesioned animals value (the control LDH activity was 37.5 ± 3.2 Units/mg of protein); in (D) the MPO activity is expressed in absorbance variation unites (delta ABS) per mg of protein. Data are expressed as mean ± SE (n = 5–6) and were analysed by ANOVA, followed by Tukey test when appropriate. Differences were considered significant when $p \leq 0.05$. Significant differences are marked as [b] when compared to control non-treated and non-lesioned animals.

the increased TBARS levels in the whole blood were also reduced by the cold treatment (Figure 4C).

Effects of the cold treatment under CK enzyme activity

The cold treatment effectively modulated the CK enzyme activity which was highly increased in the lesioned non-treated animals (Figure 5).

Effects of the cold treatment under morphological changes in the site of the lesion

The histopathological analysis depicted the capacity of the therapeutic cold treatment to minimize the morphological changes induced by the muscle contusion (Figure 6). The effect of the cold was more evident at short-time (30 min after the lesion) since neither neutrophils infiltration nor loss of skeletal muscle transverse striation was observed (Figure 6C). At moderated-time (1 day after the lesion) the cold decreased the neutrophils infiltration, but localized sites of changes in skeletal muscle transverse striation were observed (Figure 6E). In general, the skeletal muscle contusion was accompanied by an accentuated neutrophils infiltration in the site of the lesion (Figures 6B and D). Besides, we observed some localized sites of necrosis in the muscle cells and also the impairment of the cell structures characterized by the loss of skeletal muscle transverse striations and nucleus degeneration mainly on the day after the lesion (Figure 6D).

Effects of the cold treatment under skeletal muscle mitochondria function

Mitochondrial DCF-RS generation. Figure 7A shows that the cold treatment was effective in diminishing the mitochondrial DCF-RS generation depicted by

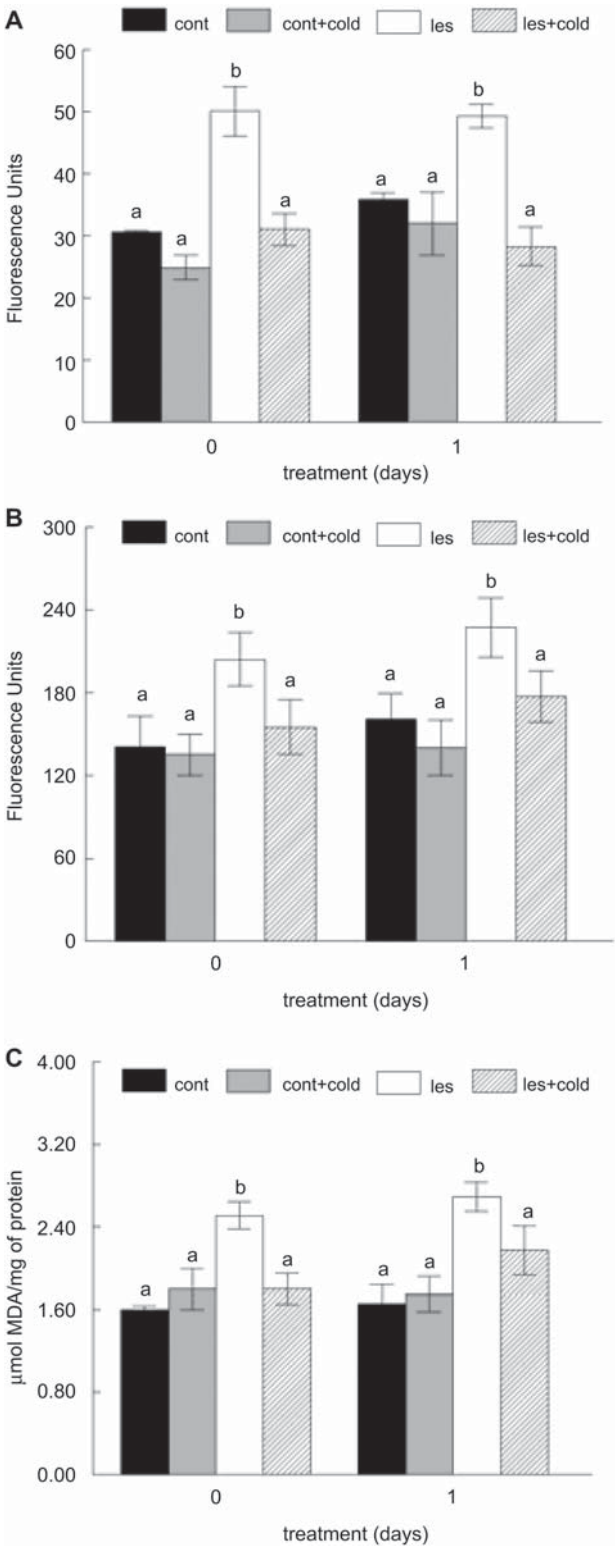

Figure 4. Effects of the cold treatment under oxidative stress markers in the whole blood and in blood components samples: (A) DCF-RS levels in plasma; (B) DCF-RS levels in cellular blood fractions; (C) TBARS levels in whole blood; (D) CK activity. In (A) and (B) the DCF-RS levels are expressed in fluorescence units/mg of protein; in (C) the TBARS levels are expressed in µmol of MDA/mg of protein; and in (D) the CK activity is expressed as a percentage of the control non-treated and non-lesioned animals value (the control CK activity was 540.3 ± 45.8 Units/L). Data are expressed as mean ± SE (n = 5–6) and were analysed by ANOVA, followed by Tukey test when appropriate. Differences were considered significant when $p \leq 0.05$. Significant differences are marked as [b] when compared to control non-treated and non-lesioned animals.

the skeletal muscle contusion. However, this effect was more pronounced 30 min after the lesion.

Mitochondrial $\Delta\psi$. Likewise to DCF-RS generation, the mitochondrial $\Delta\Psi$ in lesioned and cold treated animals was maintained similar to that observed in control non-treated and non-lesioned animals. The effect of cold treatment was also more pronounced at short-time (30 min after the lesion) (Figures 7B I–III). As illustrated in Figure 7B, the levels of fluorescence were more elevated in the mitochondrial samples of the lesioned non-treated animals, indicating that the contusion process promoted changes in the mitochondrial $\Delta\Psi$.

Mitochondrial swelling. Figure 7C (I–III) shows that the mitochondrial swelling was significantly diminished in response to the cold treatment (Figure 7C) in both sets of time analysed. The increase in mitochondrial swelling was more pronounced at short-time (30 min after the lesion) than in the long-time (24 h after the lesion) as depicted in Figure 7C parts II and III, respectively.

Discussion

The purpose of our study was to verify if the benefits of therapeutic cold could be associated with the modulation of the oxidative damage induced by a muscle contusion. In this way, the results of the present work clearly indicated that the skeletal muscle contusion increased the oxidative damage in both muscular and blood tissue and that the therapeutic cold was able to modulate these alterations. We believe that this similar variation in skeletal muscle and in blood could be related to the inflammatory response intensity that follows a common skeletal muscle lesion [3] such as strain [23] and muscle contusion [1].

Considering that an uncontrolled inflammatory response to a muscle damage determines an excessive RS generation [5,6], we suggest that the oxidative damage could extrapolate the site of the lesion and thus propagate to the blood. In agreement with this, our results showed a significant increase in the MPO enzyme activity in the site of the lesion in the day following the skeletal muscle contusion (Figure 3D). Besides, the presence of a pronounced neutrophils infiltration was also observed in the histopathological analysis of the contused skeletal muscle in this period (Figure 6D). Thus, we propose that the higher DCF-RS (Figures 1A and 4A and B) and TBARS

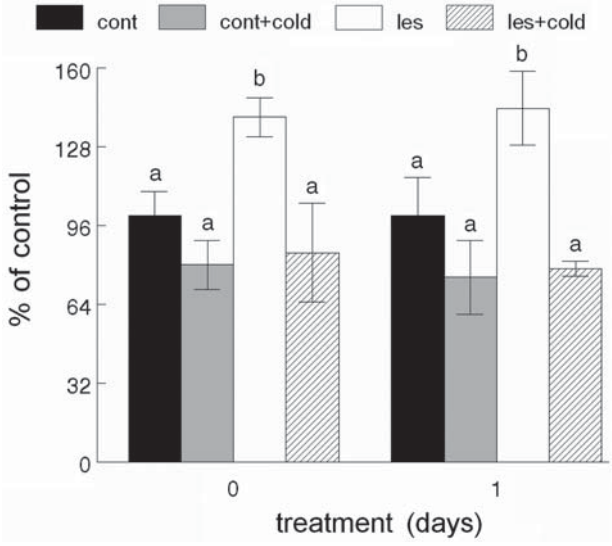

Figure 5. Effects of the cold treatment under CK activity in plasma: Figure shows the CK activity expressed as a percentage of the control non-treated and non-lesioned animals value (the control CK activity was 540.3 ± 45.8 Units/L). Data are expressed as mean ± SE (n = 5–6) and were analysed by ANOVA, followed by Tukey test when appropriate. Differences were considered significant when $p \leq 0.05$. Significant differences are marked as [b]when compared to control non-treated and non-lesioned animals.

(Figures 1B and 4C) levels observed in the site as well as in the blood of the lesioned non-treated animals could be related to the excessive RS production. Although we did not measure specifically the RS formation, it is well known that an excessive RS could supply not only the DCF-RS formation but also contribute to start a complex cascade of reactions, which culminates with the lipid peroxidation (increase in TBARS levels).

In addition, excessive RS are able to cause alterations in structure and function of enzymes and deficits in the antioxidant defense systems. In line with this, our results show the impairment of some important functional systems caused by the skeletal muscle contusion and possibly related to the excessive RS production. In fact, we observed a significant decrease in the LDH activity in the day following the skeletal muscle contusion (Figure 3C). The LDH is an enzyme which classically becomes functionally impaired due to the oxidation of critical –SH groups located in its active site [8–10]. Moreover, we observed a significant decrease in the Na^+/K^+ ATPase (Figure 2A) and Ca^{2+} ATPase (Figure 2B) activities in the contused skeletal muscle. These enzymes are also reported to depend on the –SH groups integrity to be functionally active [10,16]. Besides, the involvement of the –SH groups oxidation in the genesis of these functional impairments was improved in our results which show a significant decrease in the non-protein –SH levels (glutathione as major compound) in the site of the lesion (Figure 2A). It is interesting to note also that the lesion caused a significant increase in the CAT activity in the site of the lesion (Figure 2B). This response may be related to a compensatory response of tissue to a previous oxidative insult, as for example an increased H_2O_2 production. These corroborate with the highest production of RS and the lipid peroxidation in the lesioned non-treated animals when compared to the therapeutic cold group as demonstrated in the manuscript.

Regarding treatment, our results showed a significant capacity of the therapeutic cold to limit all parameters linked to oxidative stress. In fact, cold therapy was effective in reducing the increase of the DCF-RS (Figure 1A) and the TBARS (Figure 1B) levels. Furthermore, the therapeutic cold limited the oxidation of the non-protein –SH groups (Figure 2A) and, consequently, the functional impairment of the LDH (Figure 3C), Na^+/K^+ ATPase (Figure 3A) and Ca^{2+} ATPase (Figure 3B) enzyme activities, which were depicted by the skeletal muscle contusion. In this way, the increase in CAT activity observed in lesioned non-treated animals was effectively changed by the cold treatment (Figure 2B). It is important to observe that our results are in accordance with those observed in previous studies that show the benefits of the repeated and short-term cold exposure in the improvement of the antioxidant defense systems in humans and in rats [42–44]. Overall, we believe that the benefits of therapeutic cold are likely to be linked to its potential to modulate the intensity of the inflammatory response that follows the skeletal muscle contusion. This hypothesis is supported by our results which depict that the therapeutic cold treatment limited the significant increase in MPO enzyme activity in the day following the skeletal muscle contusion (Figure 3D). Besides, the histopathological assay revealed low levels of neutrophils infiltration in lesioned and cold-treated animals (Figures 6C and E). Since the cold treatment is well reported to modulate the intensity of the inflammatory response due to its ability to reduce the blood flow intensity to the treated areas [21,22], we understand that this could be an important factor to explain its capacity to limit the oxidative damage determined by the skeletal muscle contusion. The reduction in the blood flow in the cold-treated areas could also depict a decrease in the oxygen availability and consumption for these tissues. This condition could result in a decrease of the reactive oxygen species formation (ROS).

More than the oxidative insult, our results lead us to put forward that the skeletal muscle contusion determined a significant damage to the structure of the muscle cells, and then compromised their viability. We observed that the lesioned non-treated animals presented a significant increase in the plasma CK enzyme activity (Figure 5A). The CK is a cytosolic enzyme known to flow to the extra-cellular

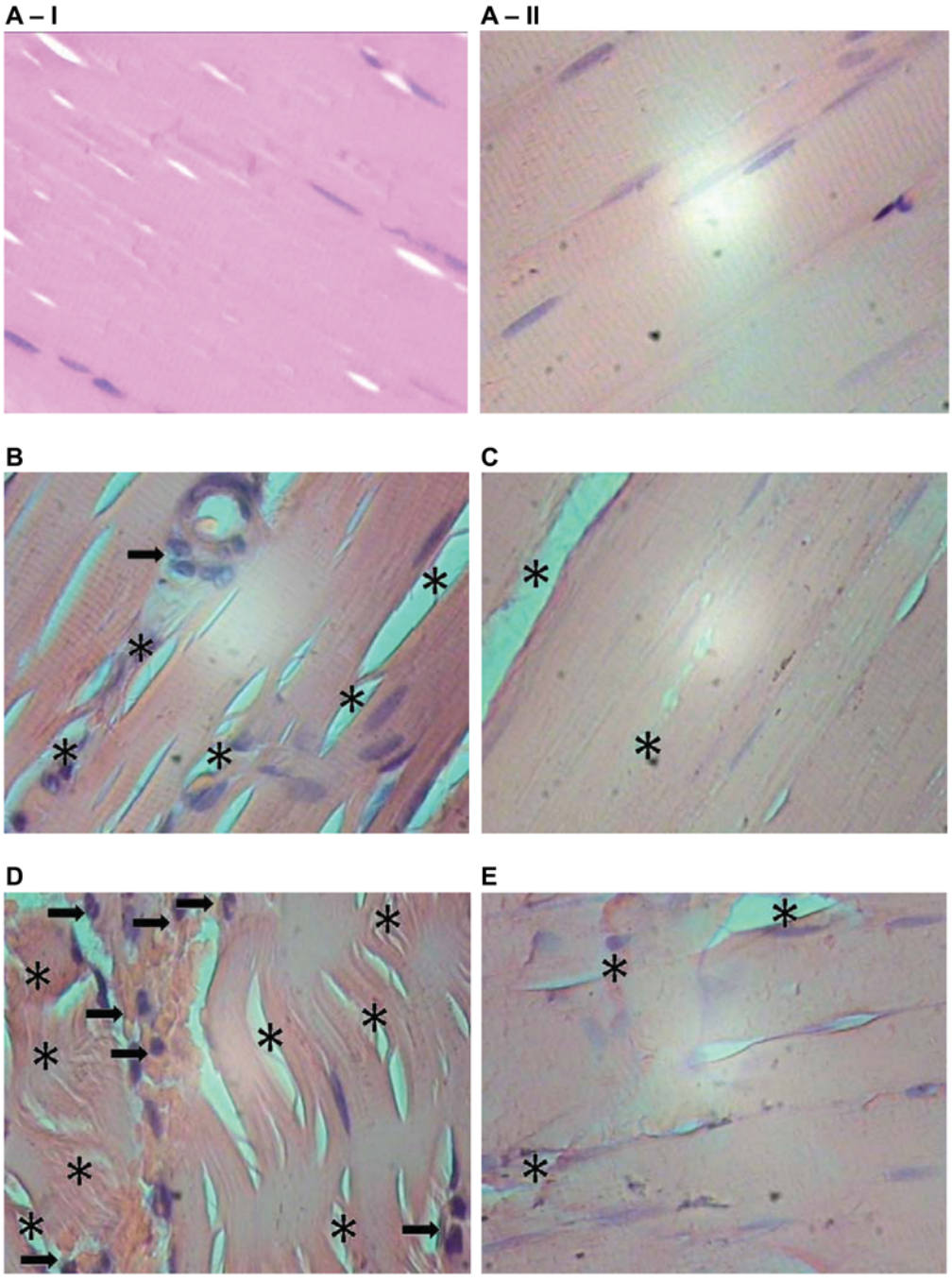

Figure 6. Histopathologic changes in skeletal muscle: The neutrophils infiltrations areas (arrow), as well as the impaired skeletal muscle cells striations areas (sharp) were identified: (A-I) control non-treated and non-lesioned muscle; (A-II) control cold treated and non-lesioned muscle; (B) lesioned and non-treated muscle after 30 min; (C) lesioned and cold treated muscle after 30 min; (D) lesioned and non-treated muscle after 24 h; (E) lesioned and cold treated muscle after 24 h. In all cases (A–E) the images were 400-times increased.

space when the cell structure is impaired [45,46]. Moreover, the impairment of the skeletal muscle cell structure becomes evident in the histopathological analysis of the lesioned non-treated animals (Figures 6B and C). Since the integrity of the cell membrane is important to the maintenance of the cell survival, the damage induced by the skeletal muscle contusion could also depict a reduction in the skeletal muscle cell viability [45,46]. This hypothesis is in accordance with our results that showed a significant decrease in muscle MTT reduction levels in the lesioned non-treated animals (Figure 1D). The MTT reduction depends on the adequate functionality of the oxidoreductase enzyme family, such as the dehydrogenase enzymes [30]. Since the majority of these enzymes are located in the mitochondria [30,47], their functional impairment could be related to the mitochondria functional impairment. The lesioned and cold treated animals, however, presented a preservation of the skeletal muscle cell structure. Our

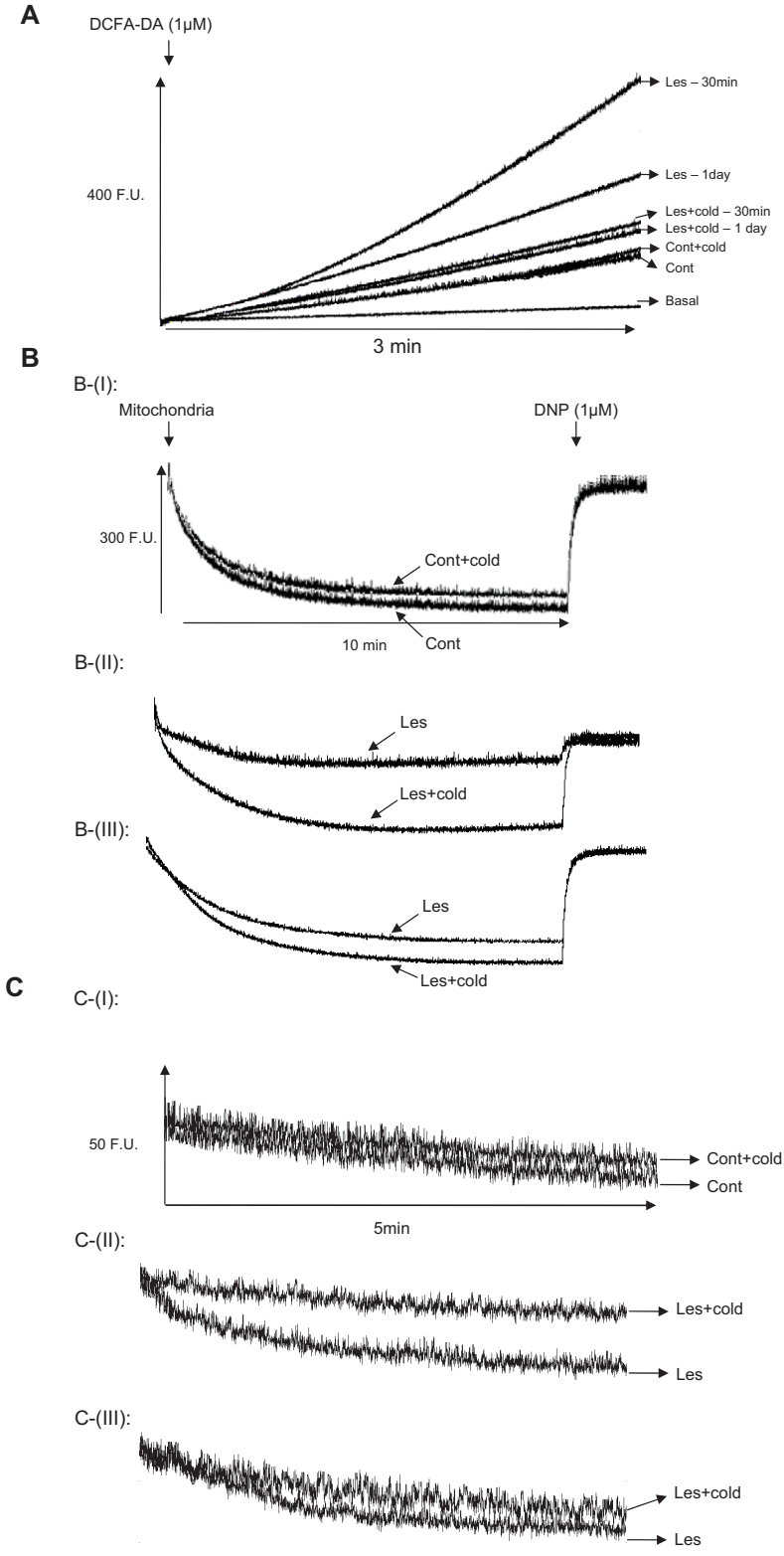

Figure 7. Indicators of the rat skeletal muscle mitochondria functioning: (A) mitochondrial DCF-RS generation; (B) mitochondrial $\Delta\psi$; (C) mitochondrial swelling. The values are presented in fluorescence units (F.U.) as described in the Materials and methods. In (B–D) panel (I) indicates control non-treated and non-lesioned animals mitochondria, panel (II) indicates the mitochondria 30 min after lesion and panel (III) indicates the mitochondria 1 day after lesion. Similar results were obtained with at least three different independent mitochondrial preparations.

results support this hypothesis since the lesioned and cold-treated animals showed a plama CK activity close to the control non-treated and non-lesioned animals (Figure 5A) and did not reveal considerable differences in the histopathological analysis from the control non-treated and non-lesioned animals (Figures 6C and E). Besides, the lesioned and cold-treated animals presented the muscle MTT reduction levels similar to

the control non-treated and non-lesioned animals (Figure 1C). We believe that this result could also be due to the capacity of the cold to limit the mitochondrial functioning impairment.

Interestingly, while the inflammatory response seems to increase significantly only in the day following the skeletal muscle contusion (Figures 3D and 6C), the oxidative damage and the impairment in the skeletal muscle cell structure was more pronounced a short-time (30 min) after the lesion. In order to understand these results we investigated the involvement of the mitochondrial function in the genesis of these short-time alterations. Moreover, the significant decrease in muscle MTT reduction levels observed in lesioned non-treated animals lead us to suppose the possible involvement of the mitochondrial dysfunction in the genesis of the short-time oxidative damage.

We observed that the mitochondrial DCF-RS generation as well as the mitochondrial swelling were increased in the lesioned non-treated animals and that these effects were higher in the initial moments after the skeletal muscle contusion (Figure 7A). Besides, the mitochondrial $\Delta\Psi$ was decreased at this moment (Figure 7A). According to these results we are able to suppose that the impairment of the mitochondrial membrane integrity and the high mitochondrial RS generation in the lesioned non-treated animals group in comparison to the control non-treated and non-lesioned animals and the lesioned and cold treated animals. Thus, taken together these results corroborate the previous data regarding the lost of skeletal muscle cells integrity (plama CK activity) and the RS formation (DCF-RS and TBARS levels) in analysis developed with skeletal muscle S1. Moreover, we observed that the mitochondrial functioning impairment could be a primary issue responsible for the oxidative damage in the early stages after a skeletal muscle contusion while the inflammatory response following a contusion injury is a secondary issue responsible for the oxidative damage. Regarding the effects of the cold treatment we observed that the mitochondrial swelling, mitochondrial DCF-RS formation and mitochondrial $\Delta\Psi$ of the lesioned and cold-treated animals were similar to those observed in control non-treated and non-lesioned animals. Thus, we believe that the benefits of the cold treatment could be related also to its capacity to modulate the mitochondrial functioning impairment depicted by the skeletal muscle contusion.

It is important to highlight that other mechanisms, beyond the inflammatory response and the mitochondrial impairment, could be involved in the genesis of the oxidative damage that follows a skeletal muscle contusion. Since the skeletal muscle contusion is characterized as a traumatological lesion [2] it is possible to hypothesize that factors such as the ischemia/reperfusion injury could be involved in the oxidative damage genesis. The ischemia/reperfusion injury is an event well known to depict an increase in the reactive oxygen species (ROS) in the injured tissue [48,49]. Furthermore, the mitochondrial functioning is directly involved in the oxidative stress resulting from a ischemia/reperfusion injury [50]. In this context, we believe that our results, which point to the impairment in the mitochondrial functioning as a result of the skeletal muscle contusion, could be related to the changes in oxygen availability to the damaged tissue in response to the ischemia/reperfusion injury. However, more studies are necessary to improve the knowledge regarding the involvement of the ischemia/reperfusion insult in the genesis of the oxidative damage that follows a skeletal muscle contusion.

Concluding, our results depict that the skeletal muscle contusion was followed by significant oxidative damage in the skeletal muscle and in the blood tissues. These oxidative impairments were accompanied by morphological changes in the skeletal muscle cell structure and related to the mitochondrial functioning impairment in the early stage and to the inflammatory response intensity in the late stage after the lesion. The absence of significant differences in the results obtained in the different moments after the lesion could be related with the different mechanisms involved in the genesis of the oxidative damage after the skeletal muscle contusion. Besides, the cold treatment was able to modulate the oxidative damage that follows the contusion injury, possibly by its capacity to limit the inflammatory response and the mitochondrial dysfunction. Furthermore, the benefits of the therapeutic cold could also be linked to its ability to preserve the muscle cell structure against the damage induced by the skeletal muscle contusion. Finally, our results contribute to improve the knowledge regarding the benefits and the mechanisms related with the use of the therapeutic cold as a kind to treat skeletal muscle contusions.

Declaration of interest

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