

Effects of icing or heat stress on the induction of fibrosis and/or regeneration of injured rat soleus muscle

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Abstract The effects of icing or heat stress on the regeneration of injured soleus muscle were investigated in male Wistar rats. Bupivacaine was injected into soleus muscles bilaterally to induce muscle injury. Icing (0 °C, 20 min) was carried out immediately after the injury. Heat stress (42 °C, 30 min) was applied every other day during 2–14 days after the bupivacaine injection. Injury-related increase in collagen deposition was promoted by icing. However, the level of collagen deposition in heat-stressed animals was maintained at control levels throughout the experimental period and was significantly lower than that in icing-treated animals at 15 and 28 days after bupivacaine injection. Furthermore, the recovery of muscle mass, protein content, and muscle fiber size of injured soleus toward control levels was partially facilitated by heat stress. These results suggest that, compared with icing, heat stress may be a beneficial treatment for successful muscle regeneration at least by reducing fibrosis.

Keywords Muscle regeneration · Fibrosis · Heat stress · Icing · Satellite cell · Heat shock protein 72

Introduction

Skeletal muscles are highly plastic tissues and have a remarkable capacity for regeneration from injury. Regeneration after skeletal muscle injury is a coordinated process that includes several interdependent phases: degeneration and inflammation, regeneration, and fibrosis [1]. After skeletal muscle injury, pro-inflammatory M1 macrophages, which express CD68, infiltrate into the injury site following a rapid infiltration of neutrophils [2]. These macrophages remove necrotic debris caused by the injury, secrete various pro-inflammatory cytokines, and stimulate proliferation of muscle satellite cells [2–4]. Following and partially overlapping the inflammatory reactions, muscle satellite cells, located between the plasma membrane and the basal lamina of the myofiber at quiescent state in normal condition [5], become activated, proliferate, differentiate, and either fuse with existing fiber to repair injured areas or with each other to form new myofibers [6–8]. It was reported that inhibition of macrophage infiltration *in vivo* [9, 10] or depletion of satellite cells in adult mouse muscle using a conditional knockout technique [11, 12] caused dramatic loss of regenerative response and severe fibrosis. Similar results were also obtained from osteopetrotic (*op/op*) mutant mice with inactivating mutation of macrophage colony-stimulating factor (M-CSF) gene, which results in the absence of a certain macrophage [13]. The basal level of satellite cells and myonuclei in slow soleus muscle further decreased following 10 days of hindlimb unloading, which caused muscle fiber atrophy. Although these parameters in wild-type and heterozygous mice were

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normalized after 10 days of ambulation recovery, none of them were recovered in *op/op* mice. These findings suggest that macrophages and muscle satellite cells are essential for successful skeletal muscle regeneration.

Icing is a widely accepted first-aid treatment, applied immediately after skeletal muscle injury. Application of icing immediately after muscle injury has been thought to be beneficial for a decrease in pain, swelling, degeneration, and inflammation [14–16]. Recently, however, Takagi et al. [17] reported that icing immediately after rat fast extensor digitorum longus (EDL) muscle crush injury delayed and impaired muscle regeneration, and caused excessive collagen deposition. They also indicated that icing immediately after the muscle injury could retard degeneration and macrophage infiltration, proliferation and differentiation of muscle satellite cells, and expression of growth factors, such as insulin-like growth factor-I and transforming growth factor- β (TGF- β), which are involved in proliferation and differentiation of satellite cells [6, 8, 18] and/or development of fibrosis [1, 19]. In addition, Ito et al. [20] also reported that mouse fast tibialis anterior (TA) muscle regeneration was partially inhibited by application of icing immediately after muscle injury induced by cardiotoxin injection. Although the mechanisms responsible for these phenomena remain unclear, these observations suggest that icing immediately after muscle injury is not the optimal treatment for skeletal muscle regeneration.

On the other hand, numerous studies have reported that heat stress is one of the beneficial stimuli on skeletal muscle. For example, application of heat stress to injured rat slow and/or fast skeletal muscles facilitates regeneration [21–23] and inhibits injury-related collagen deposition [23]. Furthermore, it has also been reported that heat stress attenuates skeletal muscle atrophy [24, 25], enhances the regrowth of atrophied muscle [26, 27], and induces hypertrophy [28–30]. These beneficial effects, caused by heat stress, are partially attributed to the upregulation of heat shock protein 72 (HSP72), which acts as molecular chaperones and plays an important role in maintaining cellular homeostasis and protecting cells and tissues from damage and dysfunction [31, 32]. It was also suggested that heat stress may influence degeneration and inflammatory reactions, such as macrophage infiltration into injury site after skeletal muscle injury [23], proliferative and differentiative potential of muscle satellite cells [21–23, 28, 30], and some intracellular signaling pathways involved in muscle protein synthesis [22, 28, 29, 33]. However, the precise mechanisms responsible for the positive effects of heat stress on skeletal muscle regeneration are not yet well understood.

Several studies, described above, suggested that icing immediately after muscle injury inhibits and heat stress

promotes the recovery of injured skeletal muscle. However, there are no reports simultaneously comparing the effects of icing with those of heat stress on skeletal muscle regeneration. Therefore, the present study was performed to investigate the effects of icing or heat stress on the regeneration of injured skeletal muscle.

Materials and methods

Animals

All experimental procedures were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* of the Physiological Society of Japan. This study was also approved by the Committee on Animal Care and Use in Yamaguchi University. Eight-week-old male Wistar rats (Kyudo, Japan) were used. Two or three rats were housed in a cage (28 × 45 cm and 20 cm height) in a climate-controlled room (24 ± 1 °C, 50–60 % humidity, and 12:12-h light–dark cycle). Standard solid diet (CE-2; CLEA Japan) and water were provided ad libitum.

Experimental design

After 1 week of acclimation, rats were randomly assigned to four groups ($n = 28$ –32/group): normal control (Con), bupivacaine-injected (BPVC), bupivacaine-injected plus icing (Ice), and bupivacaine-injected plus heat stress (Heat). Muscle injury was induced by intramuscular injection of bupivacaine into the soleus of both hindlimbs in all bupivacaine-injected animals under anesthesia (sodium pentobarbital, 50 mg/kg body weight, i.p.). Briefly, a skin incision was made at the lateral side of each lower leg after shaving and cleaning with 0.5 % chlorhexidine gluconate solution (Hibitane; Dainippon Sumitomo Pharma). The soleus muscles of both hindlimbs were surgically exposed, and then 0.3 ml of 0.5 % bupivacaine (Marcain; AstraZeneca) was injected bilaterally into the proximal, midbelly, and distal region of the muscles using a 27-gauge needle. These procedures were performed carefully to keep the nerve supply and blood flow intact. After the injection, the skin was sutured and swabbed with 0.5 % chlorhexidine gluconate solution.

In the area of sports medicine, it has been widely accepted that icing is applied immediately after induction of muscle injuries, and repeated heat treatments are performed a few days after during the recovery. Therefore, icing and heat stress were carried out according to the methods of Takagi et al. [17] and Oishi et al. [22], respectively, with slight modification in the present study. Immediately after the surgical procedure, icing was applied to anesthetized rats in the Ice group. Each

hindlimb of the rats were wrapped with ice packs containing crushed ice (0 °C) for 20 min without direct contacts between the ice packs and the abdomen of the rats. The muscle temperature of some rats in the Ice group ($n = 25$) was measured by inserting a needle probe (MT-29/5; Physitemp) into the left gastrocnemius muscle. The muscle temperature gradually decreased and reached to ~ 13 °C at the end of icing.

Application of heat stress to the rats in the Heat group was performed 2 days after the muscle injury and every other day up to a maximum of 2 weeks during the recovery periods. Under anesthesia with sodium pentobarbital (50 mg/kg body weight, i.p.), both legs of the animals were immersed into hot water maintained at 42 °C for 30 min, as described elsewhere [22, 33]. During the heat stress, the muscle temperature of some rats ($n = 26$) was measured by using the same procedures described above. The muscle temperature increased to ~ 41 °C about 10 min after the onset of heating and was maintained at 41–41.5 °C thereafter. To avoid the excessive increase of the body temperature in response to heating, cool air was applied to the body using a hair dryer during the application of heat stress to hindlimb muscles. In this study, rats in all other groups were not anesthetized, when heat stress was applied to the Heat group. Namely, Con rats were not anesthetized throughout the experimental period, and BPVC and Ice rats received anesthesia only once at the surgical procedure and subsequent icing treatment.

Rats in the BPVC, Ice, and Heat groups were killed under anesthesia 3, 7, 15, and 28 days after the bupivacaine injection ($n = 6$ –8/group at each time point). The soleus muscles were removed bilaterally, cleaned of excess fat and connective tissue, and weighted. The muscles were pinned on a cork at an optimum in vivo length, rapidly frozen in isopentane cooled with liquid nitrogen, and then stored at -80 °C until analyses. Muscles from weight-matched sedentary rats served as controls (Con) at each time point ($n = 7$ –12/time point). The age of these animals was 8–11, 9–11, 10–11, and 11–13 weeks old for the experimental groups at day 3, 7, 15, and 28 after induction of muscle injury, respectively. The matching weight was set at the mean body weight of all bupivacaine-treated animals in each recovery period.

Previous studies reported that a large number of inflammatory cells were observed in injured rat soleus muscles at early phases of regeneration after bupivacaine injection [34, 35]. In addition, it was also reported that the soleus muscle weight decreased until 1 week after bupivacaine injection, and then gradually increased during the recovery periods [34]. Therefore, the relative wet weight and protein content of soleus muscles were not determined at the 3rd day after bupivacaine injection in the present study, because it is possible that the mass of soleus muscles

at this time point may not necessarily reflect the recovery of injured muscle.

Muscle preparation

A portion of the left soleus muscles was minced and homogenized in 10 volumes of ice-cold homogenization buffer (20 mM Tris-HCl, 25 mM KCl, 5 mM EDTA, 5 mM EGTA, 1 mM dithiothreitol, and 1 % Triton X-100, pH 7.4) with phosphatase inhibitors (PhosSTOP; Roche) and protease inhibitors (Protease Inhibitor Cocktail for use with mammalian cell and tissue extracts; Nacalai Tesque). The homogenates were centrifuged at 3000 rpm for 10 min at 4 °C, and the supernatants were isolated as the cytosolic fraction (soluble protein). The protein concentration of the supernatants was determined by a protein assay kit (Bio-Rad) using bovine serum albumin as the standard. Myofibrillar proteins were obtained from the residual pellets according to the procedure described by Solaro et al. [36]. The myofibrillar protein concentration was measured by the biuret method. Total, myofibrillar, and soluble protein contents in whole muscle were then calculated. Midbelly region of the frozen right soleus muscles was mounted perpendicularly on a cork using frozen section compound (FSC22; Leica Microsystems) for histochemical and immunohistochemical analyses.

Histochemical and immunohistochemical analyses

Three to five muscle samples per group at each time point were randomly selected and used for histochemical and immunohistochemical analyses. Cross sections (8 μ m thickness) of the midbelly region of right soleus muscles were cut in a cryostat (CM1100; Leica Microsystems) at -20 °C and mounted on the slide glasses.

For collagen staining, Masson's trichrome stain was performed according to the procedures described by Kim et al. [37] with slight modification using Masson's trichrome stain kit (Sigma-Aldrich). Briefly, cross sections were air-dried for 30 min at room temperature and fixed overnight at 25 °C with Bouin's solution. The sections were washed in running tap water for 10–20 min and then rinsed in distilled water for 3 min. Sections were stained with Mayer's hematoxylin (Wako) for 20 min and then washed in running tap water for 5 min. After washing in distilled water, the sections were stained with 1 % Biebrich scarlet-acid fuchsin for 15 min and then washed in distilled water for 5 min. Following differentiation in 2.5 % phosphomolybdic-phosphotungstic acid solution for 15 min, the sections were stained with 2.4 % aniline blue solution for 12 min. The section were washed in 1 % acetic acid solution and then rinsed in distilled water. Finally, the sections were dehydrated in 100 % ethanol, cleared in

xylene, and then mounted in mounting medium (NEW MX; Matsunami).

For immunohistochemistry, cross sections were air-dried for 30 min at room temperature and fixed with 4 % paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 15 min. The sections were then rinsed in 0.1 M phosphate-buffered saline (PBS, pH 7.4) and post-fixed in 100 % methanol for 15 min at -20°C . After washing in 0.1 M PBS, the sections were blocked in 1 % blocking reagent (Roche) for 30 min at room temperature. The sections were reacted overnight at 8°C with rabbit polyclonal anti-laminin (1:1000; Dako, Z0097) and mouse monoclonal anti-Pax7 (1:500; undiluted tissue culture supernatant of hybridoma cells obtained from the Developmental Studies Hybridoma Bank, Iowa, IA, USA) antibodies diluted in 1 % blocking reagent containing 0.5 % Triton X-100. After washing in 0.1 M PBS, the sections were reacted with secondary FITC-conjugated anti-rabbit IgG (1:500; Sigma-Aldrich, F0382) and Cy3-conjugated anti-mouse IgG (1:1000; Jackson ImmunoResearch, 115-165-166) antibodies diluted in 1 % blocking reagent containing 0.5 % Triton X-100 for 60 min at room temperature. Finally, the sections were washed in 0.1 M PBS, rinsed in distilled water, and mounted in Fluoroshield with 4',6-diamidino-2-phenylindole (DAPI) (Immuno-BioScience) for nuclear staining.

To determine the cross-sectional area of individual muscle fibers, laminin and desmin were stained by using rabbit polyclonal anti-laminin (1:3000) and mouse monoclonal anti-desmin (1:50; Leica Biosystems, NCL-DES-DERII) antibodies ($n = 3/\text{group}$ at each time point). This staining was performed using the same procedure described above, except for the thickness ($10\ \mu\text{m}$) of cross sections, the incubation time (2 h) of secondary antibodies, and the mounting medium (Vectashield mounting medium; Vector Laboratories).

Imaging analysis using microscope

The stained images were incorporated into a computer using a fluorescent microscope (Eclipse E400; Nikon). Five non-overlapping fields at a magnification of $\times 200$ within the cross section of each muscle sample were randomly selected and were stored using a CCD camera (DP120; Leica Microsystems), controlled by Viewfinder software (Pixera).

Muscle fiber cross-sectional area was measured in the sections stained by laminin and desmin using ImageJ software (NIH). At least 200 muscle fibers were analyzed in each muscle, and mean muscle fiber cross-sectional area was calculated.

The percentage of the collagen fiber (blue stained) area was determined in the Masson's trichrome staining

sections using ImageJ software (NIH) [38]. Fibers with 130–205 hues of threshold color were classified as collagen fibers.

The number of Pax7-positive (Pax7+) nuclei located within the laminin-positive basal membrane was counted in the laminin-and-Pax7-stained sections using GIMP software (<http://www.gimp.org>) and expressed as Pax7+ satellite cell number per fiber according to the modified method of Oishi et al. [22].

Western-blot analysis

Cytosolic protein extracts of the soleus muscle were solubilized in sample buffer (30 % glycerol, 5 % 2-mercaptoethanol, 2.3 % SDS, 62.5 mM Tris-HCl, pH 6.8, and 0.05 % Bromophenol blue) at 2 mg/ml and incubated at 60°C for 10 min. Equal amounts of protein ($20\ \mu\text{g}$) were separated by 10 % SDS-PAGE. Separated proteins were transferred onto polyvinylidene difluoride membranes (GE Healthcare) using a mini trans-blot cell (Bio-Rad) at 100 V for 60 min at 8°C . After the transfer of protein, the membranes were blocked for 1 h at room temperature in blocking buffer [5 % nonfat dry milk in Tween Tris-buffered saline (T-TBS; 40 mM Tris-HCl, 300 mM NaCl, and 0.1 % Tween 20, pH 7.5)]. After serial washes with T-TBS, the membranes were incubated overnight at 8°C with anti-CD68 (1:1000; AbD Serotec, MCA341GA) or anti-TGF- β (1:1000; Cell Signaling, #3709) antibody diluted in Can Get Signal Solution 1 (TOYOBO, NKB-201). After serial washes with T-TBS, membranes were incubated with horseradish peroxidase-conjugated anti-rabbit IgG (1:20,000; Sigma-Aldrich, A6145) or anti-mouse IgG (1:20,000; Sigma-Aldrich, A4416) secondary antibody diluted in Can Get Signal Solution 2 (TOYOBO, NKB-301) for 1 or 2 h at room temperature. After several washes in T-TBS, protein bands were detected with ECL Prime reagents (GE Healthcare) using ATTO Light Capture system (ATTO), and the band intensities were quantified by using CS Analyzer 2.0 (ATTO).

To determine HSP72 expression levels, membranes were blocked in T-TBS containing 5 % nonfat dry milk for 1 h, and then incubated overnight at 8°C with anti-HSP72 antibody (1:10,000; Stressgen, SPA-812) diluted in T-TBS. After serial washes with T-TBS, the membranes were incubated with alkaline phosphatase (AP)-conjugated anti-rabbit IgG secondary antibody (1:20,000; Sigma-Aldrich, A3687) diluted in T-TBS for 1 h at room temperature. The membranes were reacted with AP Conjugate Substrate Kit (Bio-Rad) at room temperature to visualize the protein bands after several washes in T-TBS. Quantification of the bands was performed using ImageJ software (NIH). In the present study, Ponceau-S staining was also performed to check for equal protein loading.

Statistical analyses

All values were expressed as mean ± SEM. Statistical significance was examined by two-way (treatment × time) analysis of variance (ANOVA) followed by Tukey’s post hoc test. Differences were considered significant at $p < 0.05$.

Results

Muscle weight and protein content

Changes in the soleus weight relative to body weight in all experimental groups are shown in Fig. 1. Two-way ANOVA indicated significant main effects of treatment and time ($p < 0.05$) with a significant interaction ($p < 0.05$). The soleus weight relative to body weight in the Con animals was stable during the experimental period. The relative muscle weight in the BPVC, Ice, and Heat groups was significantly lower than in the Con group at day 7 ($p < 0.05$), and gradually increased thereafter. However, the relative soleus weight in the Heat group was not significantly different from that in the Con group at day 28, whereas the relative weight in the BPVC and Ice groups remained significantly less than the Con group at all time points.

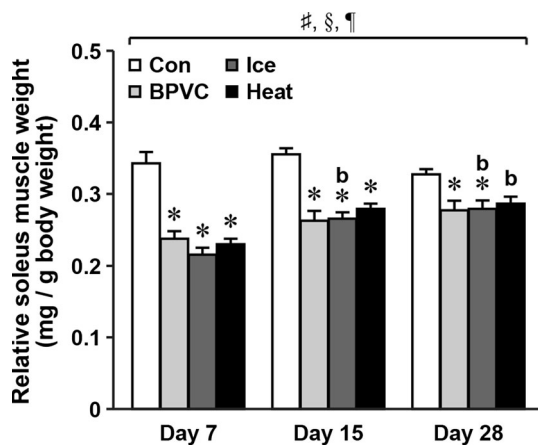


Fig. 1 Changes in the soleus muscle weight relative to body weight of all experimental groups 7, 15, and 28 days after muscle injury. Con, normal control group; BPVC, bupivacaine-injected group; Ice, bupivacaine-injected and with icing applied group; Heat, bupivacaine-injected and with heat stress applied group. Values are means ± SEM. $n = 6-8$ /group at each time point. ^{#,§}Significant main effect of treatment and time revealed by two-way ANOVA, respectively ($p < 0.05$). [¶]Significant interaction between treatment and time revealed by two-way ANOVA ($p < 0.05$). *Significantly different from Con group at the same time point ($p < 0.05$). ^bSignificantly different from day 7 in each group ($p < 0.05$)

There were significant main effects of treatment and time ($p < 0.05$) with a significant interaction ($p < 0.05$) in total and myofibrillar protein contents of soleus muscles. The total protein content in the Con group did not change significantly during the experimental period (Fig. 2a), although the myofibrillar protein content in the Con group at day 28 was significantly greater compared with the value at day 7 (Fig. 2b, $p < 0.05$). Similar to the changes in the relative soleus muscle weight, total and myofibrillar protein concentrations in the BPVC, Ice, and Heat groups were significantly lower than those in the Con group at day 7 ($p < 0.05$). The contents of total and myofibrillar proteins in all bupivacaine-treated groups were then gradually increased, but these values in the BPVC and Ice groups were still significantly less than in the Con group during the remaining recovery period. However, there were no significant differences in the total and myofibrillar protein content between the Con and Heat groups at day 28.

Two-way ANOVA indicated significant main effects of treatment and time ($p < 0.05$) without a significant interaction in the soluble protein content (Fig. 2c). The soluble protein content was significantly less in the BPVC, Ice, and Heat groups than in the Con group ($p < 0.05$). Moreover, the soluble protein concentration was significantly greater at day 15 and 28 than at day 7 ($p < 0.05$). The protein content at day 28 was also significantly higher than that at day 15 ($p < 0.05$).

Muscle fiber size

Two-way ANOVA revealed significant main effects of treatment and time ($p < 0.05$) with a significant interaction ($p < 0.05$) in the mean muscle fiber cross-sectional area (Fig. 3b). The muscle fiber size of soleus muscle in the Con animals did not change significantly throughout the experimental period. The mean muscle fiber cross-sectional area in all bupivacaine-treated groups was significantly less than that in the Con group at day 7 ($p < 0.05$). The decreased value in all bupivacaine-treated groups was gradually increased toward the control level over time, but the muscle fiber size in the BPVC and Ice groups remained significantly lower compared with the Con value at all time points ($p < 0.05$). At 28 days after bupivacaine injection, however, the muscle fiber cross-sectional area in the Heat group did not significantly differ from the control level, and was somewhat higher than that in the BPVC ($p = 0.09$) and Ice groups ($p > 0.05$).

Collagen area

By using Masson’s trichrome staining of cross sections, collagen-deposition areas were determined in each muscle sample (Fig. 4a). Two-way ANOVA indicated significant

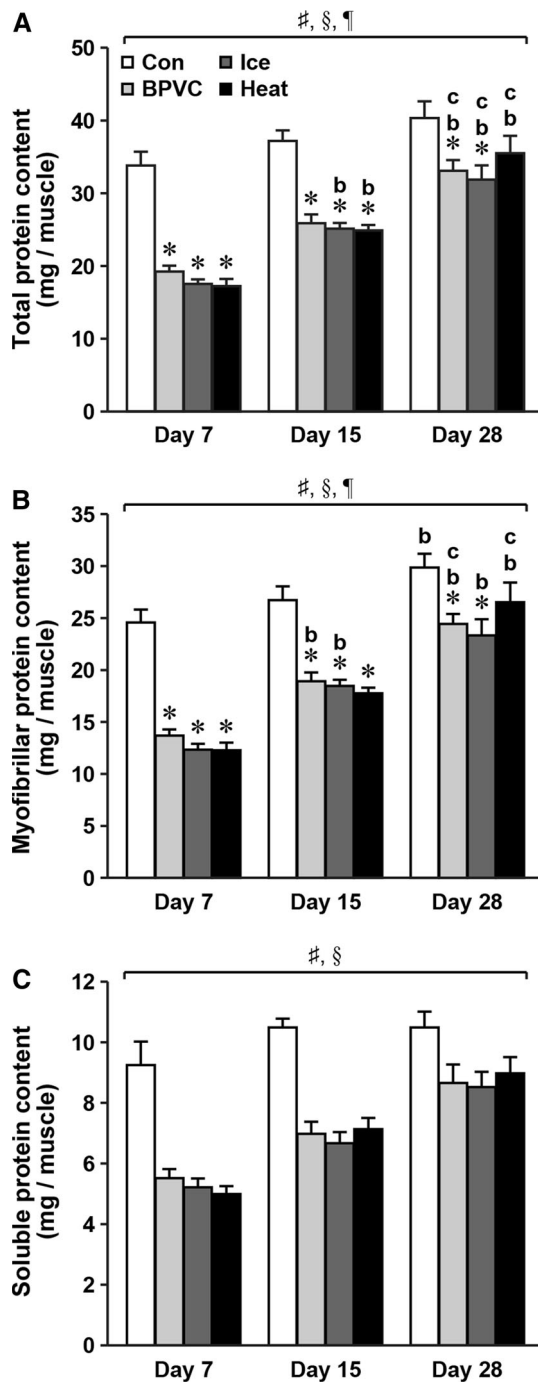


Fig. 2 Changes in total (a), myofibrillar (b), and soluble protein contents (c) of soleus muscle after bupivacaine injection with or without application of icing or heat stress. Values are means \pm SEM. $n = 6$ – 8 /group at each time point. See Fig. 1 for the abbreviations. $\#$, $\$$ Significant main effect of treatment and time revealed by two-way ANOVA, respectively ($p < 0.05$). \dagger Significant interaction between treatment and time revealed by two-way ANOVA ($p < 0.05$). *Significantly different from Con group in the same time point ($p < 0.05$). b,c Significantly different from day 7 and day 15 in each group, respectively ($p < 0.05$)

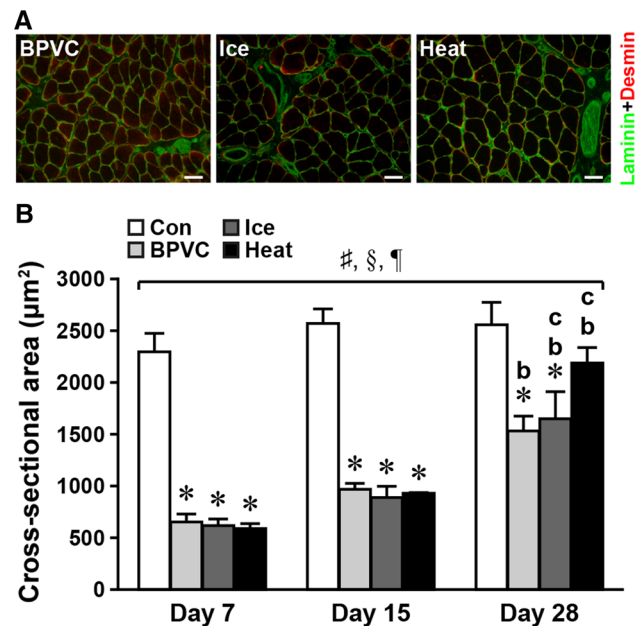


Fig. 3 a Typical immunohistochemical staining images of laminin (green) and desmin (red) in the cross sections of soleus muscles in BPVC, Ice, and Heat groups at day 28. Scale bars 50 μ m. b Changes in the mean cross-sectional area of soleus muscle fibers in each group during the experimental period. Values are means \pm SEM. $n = 3$ /group at each time point. See Fig. 1 for the abbreviations. $\#$, $\$$ Significant main effect of treatment and time revealed by two-way ANOVA, respectively ($p < 0.05$). \dagger Significant interaction between treatment and time revealed by two-way ANOVA ($p < 0.05$). *Significantly different from Con group in the same time point ($p < 0.05$). b,c Significantly different from day 7 and day 15 in each group, respectively ($p < 0.05$)

main effects of treatment and time ($p < 0.05$) with a significant interaction ($p < 0.05$). The collagen area in the Con group was stable during the experimental period (Fig. 4b). An increase in collagen deposition was observed during the recovery from bupivacaine-induced muscle injury. The collagen-stained area in the BPVC group was significantly greater than in the Con group at day 15 ($p < 0.05$). This area in the BPVC group was still tended to be greater than the Con group at day 28 ($p > 0.05$). In addition, the injury-related collagen deposition was accelerated by the application of icing after muscle injury. The collagen area in the Ice group was significantly greater than in the Con and BPVC groups at day 7 ($p < 0.05$). The collagen-stained area in the Ice group was also significantly greater than that in all other groups at day 15 ($p < 0.05$), and then remained significantly greater than in the Con and Heat groups at day 28 ($p < 0.05$). However, the application of heat stress after muscle injury inhibited the injury-related collagen deposition. There were no significant differences in the collagen area between the Con and Heat

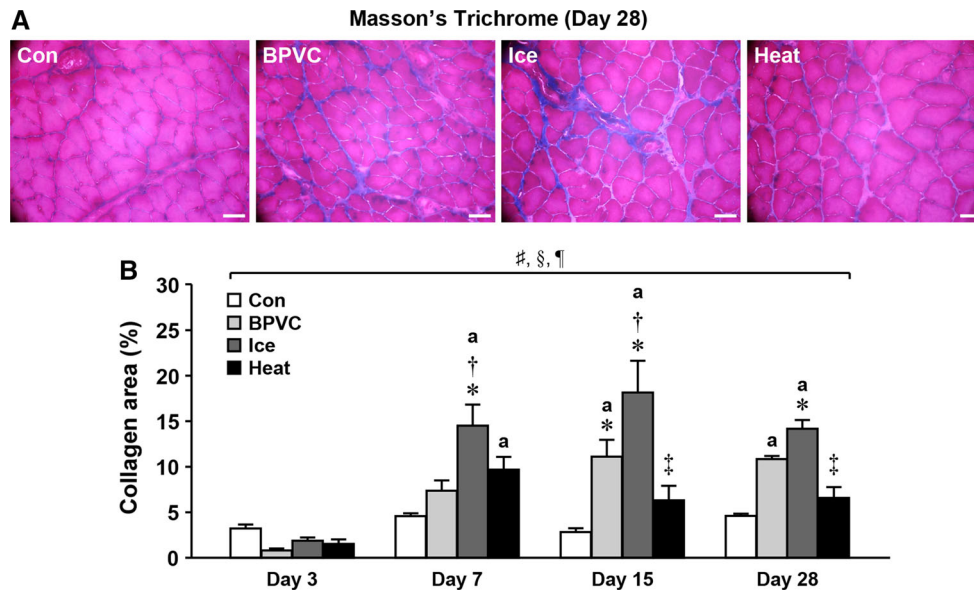


Fig. 4 **a** Typical images of cross sections stained with Masson's trichrome (collagen: blue, muscle fibers: red) in all experimental groups at day 28. Scale bars 50 μ m. **b** The percentage of collagen area in all experimental groups at 3, 7, 15, and 28 days after muscle injury. Values are means \pm SEM. $n = 3\text{--}5/\text{group}$ at each time point. See Fig. 1 for the abbreviations. #, § Significant main effect of

treatment and time revealed by two-way ANOVA, respectively ($p < 0.05$). ¶ Significant interaction between treatment and time revealed by two-way ANOVA ($p < 0.05$). *, †, ‡ Significantly different from Con, BPVC, and Ice groups in the same time point, respectively ($p < 0.05$). ^aSignificantly different from day 3 in each group ($p < 0.05$)

groups at all time points, although the mean area with collagen deposition in the Heat group was significantly greater at day 7 compared with the value at day 3 ($p < 0.05$).

Number of Pax7+ satellite cells

Pax7+ satellite cells were identified by using immunohistochemical staining for Pax7, laminin, and DAPI (Fig. 5a). Changes in the number of Pax7+ satellite cells after muscle injury in all experimental groups are shown in Fig. 5b. There were significant main effects of treatment and time ($p < 0.05$) with a significant interaction ($p < 0.05$) in the Pax7+ satellite cell number. The number of satellite cells expressing Pax7 in the Con group was stable during the experimental period. The number of Pax7+ satellite cells in the BPVC, Ice, and Heat groups was significantly higher than that in the Con group at day 3 ($p < 0.05$), and gradually decreased toward the control level thereafter. The levels of these cells, except in BPVC and Heat group at day 7, were significantly less than those at day 3. The number in Heat group was still greater than Con. Further, the mean levels in Heat group tended to be greater than other groups during recovery period ($p > 0.05$).

Protein levels of CD68, TGF- β , and HSP72

Figure 6 illustrates changes in the protein expression of CD68, TGF- β , and HSP72 after muscle injury in all

groups. Two-way ANOVA indicated significant main effects of treatment and time ($p < 0.05$) with a significant interaction ($p < 0.05$) in the protein levels of CD68, TGF- β , and HSP72. The levels of CD68 protein in the Con group were stable during the experimental period (Fig. 6b). The protein levels of CD68 in the BPVC, Ice, and Heat groups were significantly greater than in the Con group at day 3 ($p < 0.05$). The CD68 protein levels in the Heat group also tended to be greater than that in the BPVC ($p > 0.05$) and Ice groups ($p = 0.07$) at day 3. During the remaining recovery period, the CD68 levels in the BPVC, Ice, and Heat groups gradually decreased toward the control level.

The expression levels of TGF- β in the Con group did not change significantly during the experimental period (Fig. 6c). TGF- β protein levels in the BPVC, Ice, and Heat groups were significantly greater than in the Con group at day 3 ($p < 0.05$). However, the TGF- β levels then gradually decreased during recovery. Significant decrease at day 7 and 15 in the BPVC and Heat, not Ice, groups was noted, although the decrease in the Ice group also became significant at day 28 ($p < 0.05$). However, there were no significant differences in the TGF- β levels among the four groups at day 7, 15, and 28.

The levels of HSP72 protein in the Con group were stable during the experimental period (Fig. 6d). The HSP72 levels in the BPVC, Ice, and Heat groups were significantly lower than those in the Con group at day 3 ($p < 0.05$), and gradually increased toward the control level thereafter.

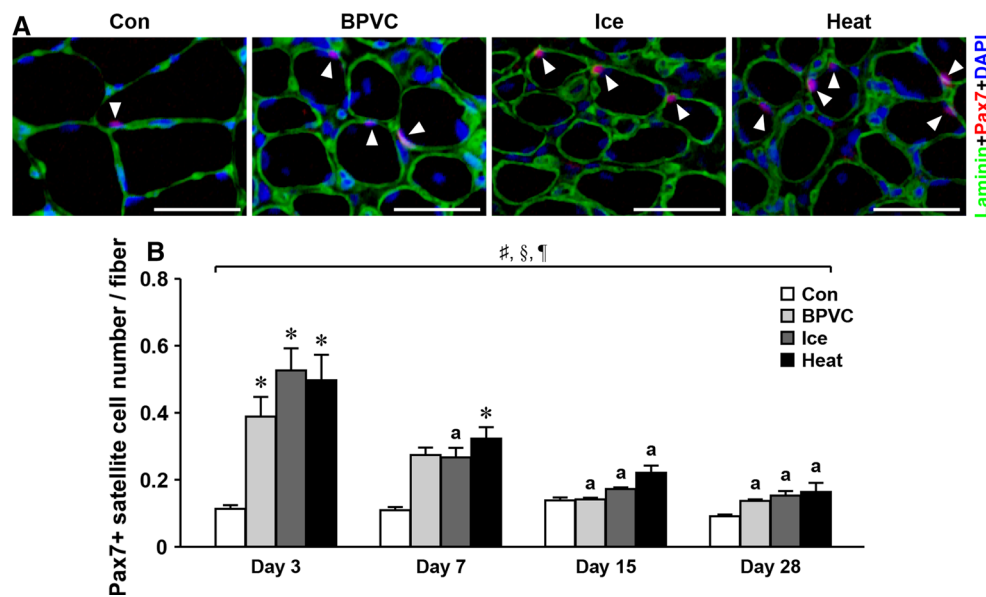


Fig. 5 **a** Typical immunohistochemical staining images of Pax7 (red) and laminin (green) with nuclear staining by 4',6-diamidino-2-phenylindole (DAPI, blue) in the cross sections of soleus muscles in all experimental groups at day 7. Arrowheads indicate the Pax7-positive (Pax7+) satellite cells. Scale bars 50 μ m. **b** Changes in the number of Pax7+ satellite cells per fiber in Con, BPVC, Ice, and Heat groups during the experimental periods. Values are means \pm SEM.

$n = 3\text{--}5$ /group at each time point. See Fig. 1 for the abbreviations. [#],^{\$}Significant main effect of treatment and time revealed by two-way ANOVA, respectively ($p < 0.05$). [¶]Significant interaction between treatment and time revealed by two-way ANOVA ($p < 0.05$). *Significantly different from Con in the same time point ($p < 0.05$). ^aSignificantly different from day 3 in each group ($p < 0.05$)

Application of heat stress stimulated the recovery. The protein levels of HSP72 in the Heat group was not significantly different from those in the Con group at day 7, whereas the HSP levels in the BPVC and Ice groups were still significantly less than in the Con group at this time point ($p < 0.05$).

Discussion

Overview of principal findings

The present study was performed to examine the effects of icing or heat stress on the regeneration after bupivacaine-induced soleus muscle injury in rats. Icing immediately after the injury accelerated and enhanced the injury-related increase in the collagen area. On the other hand, intermittent heat stress during regenerative process after bupivacaine injection not only inhibited the injury-related increase in the collagen area but also partially facilitated the recovery of muscle mass, protein content, and muscle fiber size toward the control level. To our knowledge, this is the first study showing that heat stress caused a lower collagen deposition in injured soleus muscle versus icing.

Effects of icing or heat stress on fibrosis

Fibrosis is characterized by an excessive accumulation of extracellular matrix proteins, particularly collagen, and occurs in response to skeletal muscle injury [1, 39]. It has been reported that fibrosis can hinder complete muscle regeneration and lead to incomplete functional recovery in the injured skeletal muscle [1, 40–42]. It was also suggested that decreased collagen deposition during regenerative process after skeletal muscle injury might partially contribute to the enhancement of muscle regeneration [17, 23, 43]. Thus, these findings suggest that a reduction of the development of fibrosis may result in a successful regeneration after skeletal muscle injury.

Consistent with the results of Takagi et al. [17] who investigated the effects of icing on rat fast EDL muscle regeneration, icing applied immediately after soleus muscle injury caused an early and a further accumulation of collagen deposition during the regeneration process in the present study (Fig. 4b). Although no significant influence of the application of icing on the recovery of muscle mass (Fig. 1), protein content (Fig. 2), and muscle fiber size (Fig. 3) was observed in the present study, these previous and present results indicate that icing immediately after muscle injury accelerated and enhanced the development

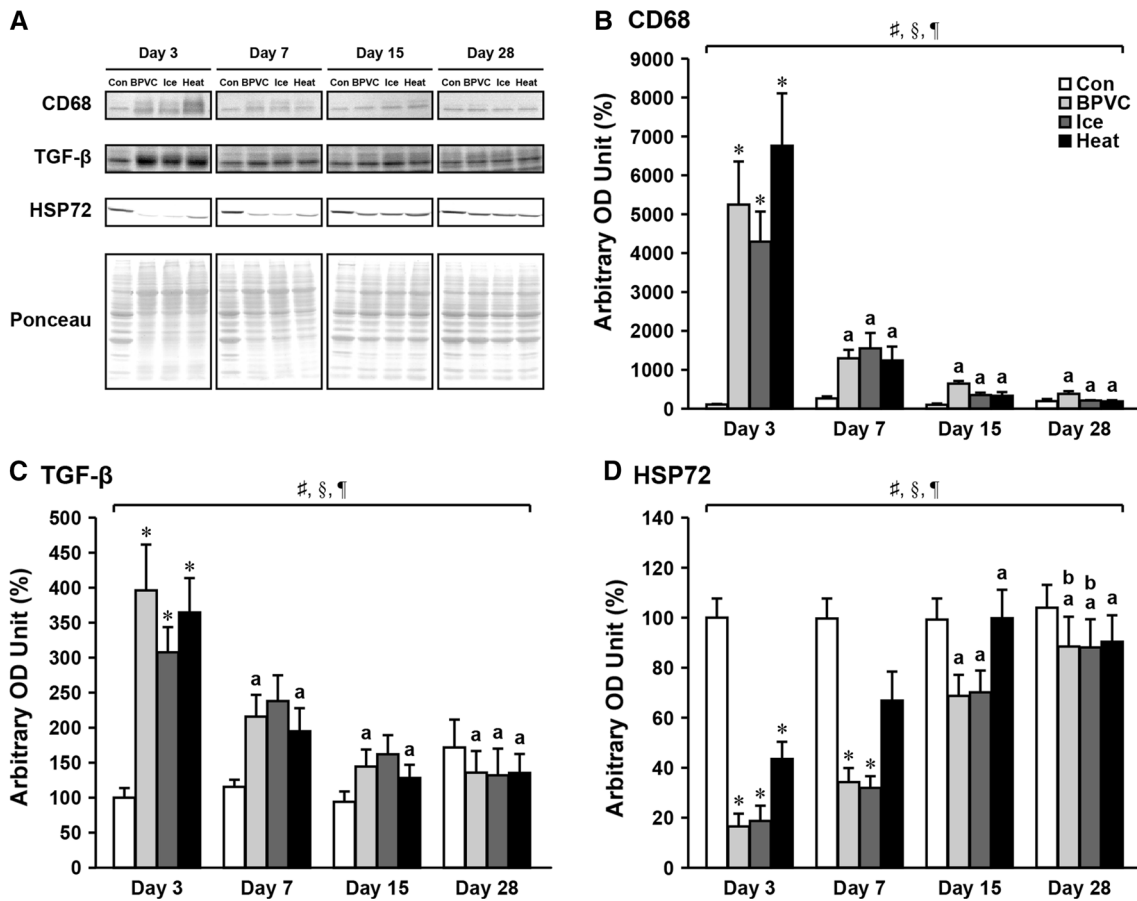


Fig. 6 **a** Representative Western-blot patterns of CD68, transforming growth factor-β (TGF-β), heat shock protein 72 (HSP72), and Ponceau-S staining in soleus muscles of Con, BPVC, Ice, and Heat groups 3, 7, 15, and 28 days after bupivacaine injection. **b–d** The protein expression levels of CD68 (**b**), TGF-β (**c**), and HSP72 (**d**) in each group during the experimental periods. The optical density (OD) values of CD68, TGF-β, and HSP72 are expressed as percentages relative to the average level in the Con group at day 3 (100 %).

Values are means ± SEM. *n* = 6–12/group at each time point. See Fig. 1 for the abbreviations. #, \$, ¶ Significant main effect of treatment and time revealed by two-way ANOVA, respectively (*p* < 0.05). ¶ Significant interaction between treatment and time revealed by two-way ANOVA (*p* < 0.05). *Significantly different from Con group in the same time point (*p* < 0.05). ^{a, b}Significantly different from day 3 and day 7 in each group, respectively (*p* < 0.05)

of fibrosis, which may result in incomplete muscle regeneration.

On the other hand, no significant differences in the collagen area of soleus muscle between the Con and Heat groups were observed at all time point in the present study (Fig. 4b). In addition, the recovery of muscle fiber size of injured soleus toward the control level was partially promoted by heat stress (Fig. 3). We also observed the similar effects of heat stress on the recovery of muscle mass and protein content, especially myofibrillar (contractile) proteins, of injured soleus muscles (Figs. 1, 2). These results are consistent with the previous studies that application of heat stress to injured rat slow and/or fast skeletal muscles promoted the recovery of these parameters [21–23]. Furthermore, this is the first study showing that the collagen area was significantly lower in the Heat than in the Ice group at 15th and 28th days after bupivacaine injection

(Fig. 4b). Therefore, all the aforementioned findings suggest that heat stress may be an effective treatment for reducing fibrosis and enhancing muscle regeneration after injury compared with icing.

Effects of icing or heat stress on TGF-β

TGF-β is one of the most powerful fibrotic cytokines and plays a central role in the development of fibrosis in various tissues including skeletal muscle [1, 39]. Numerous studies have shown that TGF-β stimulates collagen synthesis, fibroblast proliferation, and differentiation of myogenic cells into fibrotic cells [19, 39, 44], which leads to excessive accumulation of fibrotic tissue. It has been also reported that elevated levels of TGF-β are related to the development of fibrosis in skeletal muscle [1, 19, 39, 45, 46].

We found that the recovery from injury-related increase in the expression level of TGF- β was delayed by the application of icing (Fig. 6c). Takagi et al. [17] showed that icing immediately after crush injury of rat fast EDL muscle caused excessive collagen deposition and suggested that the timing of appearance and disappearance of TGF- β during the regeneration process of skeletal muscle might be important in the control of appropriate collagen synthesis in the regenerating muscle. Therefore, these findings suggest that the icing-related acceleration and enhancement of the development of fibrosis observed in the present study might result from the stimulation of collagen synthesis through a delay in the timing of disappearance of TGF- β .

In the present study, however, intermittent heat stress had no significant effect on the injury-related changes in the expression levels of TGF- β during regenerative process of injured soleus muscle (Fig. 6c), suggesting that heat stress-related inhibition of the development of fibrosis observed in this study was induced by TGF- β -independent mechanisms. These findings are inconsistent with the results of Takeuchi et al. [23] who reported that heat stress applied soon after crush injury of rat fast EDL muscle accelerated a decrease in the expression of TGF- β and lead to a lower collagen deposition. We have no clear explanation for these different results at present. Further studies are needed to clarify these phenomena.

Effects of icing or heat stress on CD68, Pax7+ satellite cells, and HSP72

It has been generally accepted that macrophages, such as CD68-expressed pro-inflammatory M1 macrophages, and muscle satellite cells are essential for regeneration after skeletal muscle injury [2, 6, 8]. For example, suppression of macrophage infiltration by administration of anti-M-CSF receptor antibody or genetic knockout of CXC chemokine ligand 16 impaired mouse fast TA muscle regeneration, as well as satellite cell proliferation and differentiation, and induced severe fibrosis [9, 10]. It was also reported that genetic ablation of Pax7, which is specifically expressed in quiescent, mitotic active, and proliferating satellite cells [6, 8, 47] and is required for specification, survival, and maintenance of satellite cells [47–50], to specifically deplete satellite cells in adult mouse muscle resulted in poor fast TA muscle regeneration along with massive fibrosis [11, 12]. These findings suggest that macrophages and muscle satellite cells may regulate not only muscle regeneration but also the development of fibrosis after skeletal muscle injury.

Application of icing immediately after slow soleus muscle injury did not influence the injury-related changes in the expression level of CD68 (Fig. 6b) and the number of Pax7+ satellite cells (Fig. 5b), as well as muscle mass

(Fig. 1), protein content (Fig. 2), and muscle fiber size (Fig. 3), in our study. These results suggest that M1 macrophage infiltration and proliferative and differentiative potential of muscle satellite cells during regeneration after muscle injury might not be affected by the application of icing in the present study. However, Takagi et al. [17] reported that application of icing applied soon after crush injury of rat fast EDL muscle, which impaired muscle regeneration and induced excessive collagen deposition, retarded the migration of macrophages into the injury site and the proliferation and differentiation of muscle satellite cells. We have no clear explanation for these divergent results at present. Further studies are required to elucidate this issue.

On the other hand, the present study showed that heat stress tended to stimulate the injury-related increase in the CD68 expressions at 3 days after bupivacaine injection (Fig. 6b). We also found that a large number of Pax7+ satellite cells at 7 days after bupivacaine injection was still kept only in the Heat group compared with the Con group (Fig. 5b). These findings are consistent with the previous studies reported that application of heat stress to injured rat slow and/or fast skeletal muscles facilitated the migration of macrophages [23] and the proliferation and differentiation of muscle satellite cells [21–23]. Recently, Novak et al. [43] showed that injection of M1 macrophages into lacerated mouse fast gastrocnemius muscle improved muscle regeneration and reduced fibrosis, and suggested the possible mechanisms that M1 macrophages might accelerate debris clearance, enhance myoblast proliferation, and inhibit fibroblast collagen production [3, 51]. In addition, it was reported that higher level of Pax7+ satellite cells might promote the regeneration of injured both rodent slow and fast skeletal muscles [21–23, 52–54]. It was also suggested that Pax7+ satellite cells could limit the development of fibrosis through negative regulation of fibroblasts during fast plantaris muscle hypertrophy [55] or fast TA muscle regeneration [11] in mice. Therefore, all aforementioned findings suggest that heat stress-related inhibition of fibrosis and enhancement of muscle mass, protein content, and muscle fiber size recoveries, observed in the present study, might be attribute to the M1 macrophage- and/or muscle satellite cell-dependent mechanisms.

Numerous studies reported that increased levels of HSP72 could facilitate the regrowth of atrophied skeletal muscle [26, 27] and the regeneration of injured skeletal muscle [21, 22], although the precise mechanisms are still unclear. Recently, Senf et al. [56] reported that a deficit in regeneration of injured fast TA muscle fibers with fibrosis was observed in HSP72-null mice, but these negative effects were prevented when HSP72 plasmid was introduced into the muscle before the injury. Gehrig et al. [57] also indicated that induction of HSP72 in dystrophin-null

mdx dystrophic mouse skeletal muscle via either transgenic overexpression or treatment with a pharmacological inducer of HSP72 reduced fibrosis. Therefore, these findings suggest that HSP72 may be also important for successful regeneration after skeletal muscle injury.

The present data showed that application of icing immediately after bupivacaine injection had no effect on the injury-related changes in HSP72 levels (Fig. 6d). Locke and Celotti [58] reported that neither 8 nor 20 °C cold stress for 20 min increased HSP72 content in slow and fast rat skeletal muscles. Thus, these findings suggest that the application of icing did not affect the expression level of HSP72 during the regeneration after skeletal muscle injury.

However, a faster recovery of HSP72 levels to near control levels was noted in regenerating soleus muscles when intermittent heat stress was applied (Fig. 6d). This result agrees with the previous reports that showed that application of heat stress to injured both rat slow and fast skeletal muscles enhanced the recovery from the injury-related decrease in the expression of HSP72 during the regenerative process [21, 22]. Considering the roles of HSP72 during regenerative process after skeletal muscle injury described above, these findings suggest that heat stress-related enhancement of HSP72 noted in the present study might result in reduced fibrosis and enhanced recovery of muscle mass, protein content, and muscle fiber size.

Conclusions

The present study demonstrated that icing immediately after skeletal muscle injury accelerated and enhanced the development of fibrosis in muscle due, in part, to a delay in the timing of disappearance of TGF- β during the regeneration. In contrast, intermittent application of heat stress inhibited the development of fibrosis and partially promoted the recovery of muscle mass, protein content, and muscle fiber size of injured skeletal muscle toward control levels through enhancement of the macrophage infiltration, the proliferation and differentiation of satellite cells, and the expression of HSP72. It was suggested that icing may not be the optimal therapeutic treatment for skeletal muscle injuries resulting from strain, contusion, or laceration that occurs in sports and after accidents, although it has been generally accepted that icing is recommended as the first-aid treatment immediately after muscle injury. However, application of heat stress may have a beneficial therapeutic potential for successful skeletal muscle regeneration at least by reducing fibrosis.

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Compliance with ethical standards

Conflict of interest The authors declare that there are no conflicts of interest.

References

- Huard J, Li Y, Fu FH (2002) Muscle injuries and repair: current trends in research. *J Bone Joint Surg Am* 84-A:822–832
- Tidball JG, Villalta SA (2010) Regulatory interactions between muscle and the immune system during muscle regeneration. *Am J Physiol Regul Integr Comp Physiol* 298:R1173–R1187
- Arnold L, Henry A, Poron F, Baba-Amer Y, van Rooijen N, Plonquet A, Gherardi RK, Chazaud B (2007) Inflammatory monocytes recruited after skeletal muscle injury switch into antiinflammatory macrophages to support myogenesis. *J Exp Med* 204:1057–1069
- Bencze M, Negroni E, Vallese D, Yacoub-Youssef H, Chaouch S, Wolff A, Aamiri A, Di Santo JP, Chazaud B, Butler-Browne G, Savino W, Mouly V, Riederer I (2012) Proinflammatory macrophages enhance the regenerative capacity of human myoblasts by modifying their kinetics of proliferation and differentiation. *Mol Ther* 20:2168–2179
- Mauro A (1961) Satellite cell of skeletal muscle fibers. *J Biophys Biochem Cytol* 9:493–495
- Chargé SB, Rudnicki MA (2004) Cellular and molecular regulation of muscle regeneration. *Physiol Rev* 84:209–238
- Ciciliot S, Schiaffino S (2010) Regeneration of mammalian skeletal muscle. Basic mechanisms and clinical implications. *Curr Pharm Des* 16:906–914
- Hawke TJ, Garry DJ (2001) Myogenic satellite cells: physiology to molecular biology. *J Appl Physiol* 91:534–551
- Segawa M, Fukada S, Yamamoto Y, Yahagi H, Kanematsu M, Sato M, Ito T, Uezumi A, Hayashi S, Miyagoe-Suzuki Y, Takeda S, Tsujikawa K, Yamamoto H (2008) Suppression of macrophage functions impairs skeletal muscle regeneration with severe fibrosis. *Exp Cell Res* 314:3232–3244
- Zhang L, Ran L, Garcia GE, Wang XH, Han S, Du J, Mitch WE (2009) Chemokine CXCL16 regulates neutrophil and macrophage infiltration into injured muscle, promoting muscle regeneration. *Am J Pathol* 175:2518–2527
- Murphy MM, Lawson JA, Mathew SJ, Hutcheson DA, Kardon G (2011) Satellite cells, connective tissue fibroblasts and their interactions are crucial for muscle regeneration. *Development* 138:3625–3637
- von Maltzahn J, Jones AE, Parks RJ, Rudnicki MA (2013) Pax7 is critical for the normal function of satellite cells in adult skeletal muscle. *Proc Natl Acad Sci USA* 110:16474–16479
- Ohira T, Wang XD, Ito T, Kawano F, Goto K, Izawa T, Ohno H, Kizaki T, Ohira Y (2015) Macrophage deficiency in osteopetrotic (*op/op*) mice inhibits activation of satellite cells and prevents

- hypertrophy in single soleus fibers. *Am J Physiol Cell Physiol* 308:C848–C855
14. Knight KL (1995) Cryotherapy in sport injury management. Human Kinetics, Portland
 15. Merrick MA (2002) Secondary injury after musculoskeletal trauma: a review and update. *J Athl Train* 37:209–217
 16. Hubbard TJ, Aronson SL, Denegar CR (2004) Does cryotherapy hasten return to participation? A systematic review. *J Athl Train* 39:88–94
 17. Takagi R, Fujita N, Arakawa T, Kawada S, Ishii N, Miki A (2011) Influence of icing on muscle regeneration after crush injury to skeletal muscles in rats. *J Appl Physiol* 110:382–388
 18. Allen RE, Boxhorn LK (1987) Inhibition of skeletal muscle satellite cell differentiation by transforming growth factor-beta. *J Cell Physiol* 133:567–572
 19. Li Y, Foster W, Deasy BM, Chan Y, Prisk V, Tang Y, Cummins J, Huard J (2004) Transforming growth factor-beta1 induces the differentiation of myogenic cells into fibrotic cells in injured skeletal muscle: a key event in muscle fibrogenesis. *Am J Pathol* 164:1007–1019
 20. Ito T, Fujiya H, Goto K, Ogura Y, Kurosaka M, Yatabe K, Kishiro S, Yoshida A, Yoshioka H, Terauchi K, Beppu M, Funabashi T, Akema T, Musha H (2013) Icing at early stage depresses skeletal muscle regeneration. *J St Marianna Univ* 4:61–67
 21. Kojima A, Goto K, Morioka S, Naito T, Akema T, Fujiya H, Sugiura T, Ohira Y, Beppu M, Aoki H, Yoshioka T (2007) Heat stress facilitates the regeneration of injured skeletal muscle in rats. *J Orthop Sci* 12:74–82
 22. Oishi Y, Hayashida M, Tsukiashi S, Taniguchi K, Kami K, Roy RR, Ohira Y (2009) Heat stress increases myonuclear number and fiber size via satellite cell activation in rat regenerating soleus fibers. *J Appl Physiol* 107:1612–1621
 23. Takeuchi K, Hatade T, Wakamiya S, Fujita N, Arakawa T, Miki A (2014) Heat stress promotes skeletal muscle regeneration after crush injury in rats. *Acta Histochem* 116:327–334
 24. Naito H, Powers SK, Demirel HA, Sugiura T, Dodd SL, Aoki J (2000) Heat stress attenuates skeletal muscle atrophy in hindlimb-unweighted rats. *J Appl Physiol* 88:359–363
 25. Selsby JT, Dodd SL (2005) Heat treatment reduces oxidative stress and protects muscle mass during immobilization. *Am J Physiol Regul Integr Comp Physiol* 289:R134–R139
 26. Goto K, Honda M, Kobayashi T, Uehara K, Kojima A, Akema T, Sugiura T, Yamada S, Ohira Y, Yoshioka T (2004) Heat stress facilitates the recovery of atrophied soleus muscle in rat. *Jpn J Physiol* 54:285–293
 27. Selsby JT, Rother S, Tsuda S, Prakash O, Quindry J, Dodd SL (2007) Intermittent hyperthermia enhances skeletal muscle regrowth and attenuates oxidative damage following reloading. *J Appl Physiol* 102:1702–1707
 28. Uehara K, Goto K, Kobayashi T, Kojima A, Akema T, Sugiura T, Yamada S, Ohira Y, Yoshioka T, Aoki H (2004) Heat-stress enhances proliferative potential in rat soleus muscle. *Jpn J Physiol* 54:263–271
 29. Kobayashi T, Goto K, Kojima A, Akema T, Uehara K, Aoki H, Sugiura T, Ohira Y, Yoshioka T (2005) Possible role of calcineurin in heating-related increase of rat muscle mass. *Biochem Biophys Res Commun* 331:1301–1309
 30. Ohno Y, Yamada S, Sugiura T, Ohira Y, Yoshioka T, Goto K (2010) A possible role of NF-kappaB and HSP72 in skeletal muscle hypertrophy induced by heat stress in rats. *Gen Physiol Biophys* 29:234–242
 31. Locke M (1997) The cellular stress response to exercise: role of stress proteins. *Exerc Sport Sci Rev* 25:105–136
 32. Kilgore JL, Musch TI, Ross CR (1998) Physical activity, muscle, and the HSP70 response. *Can J Appl Physiol* 23:245–260
 33. Yoshihara T, Naito H, Kakigi R, Ichinoseki-Sekine N, Ogura Y, Sugiura T, Katamoto S (2013) Heat stress activates the Akt/mTOR signalling pathway in rat skeletal muscle. *Acta Physiol (Oxf)* 207:416–426
 34. Ogata T, Oishi Y, Ohmori H (2002) Changes in heat shock protein 72 of regenerating rat skeletal muscle. *Jpn J Phys Fit Sports Med* 51:75–84 (in Japanese)
 35. Orimo S, Hiyamuta E, Arahata K, Sugita H (1991) Analysis of inflammatory cells and complement C3 in bupivacaine-induced myonecrosis. *Muscle Nerve* 14:515–520
 36. Solaro RJ, Pang DC, Briggs FN (1971) The purification of cardiac myofibrils with Triton X-100. *Biochim Biophys Acta* 245:259–262
 37. Kim JH, Kwak HB, Leeuwenburgh C, Lawler JM (2008) Life-long exercise and mild (8%) caloric restriction attenuate age-induced alterations in plantaris muscle morphology, oxidative stress and IGF-1 in the Fischer-344 rat. *Exp Gerontol* 43:317–329
 38. Papadopoulos F, Spinelli M, Valente S, Foroni L, Orrico C, Alviano F, Pasquinelli G (2007) Common tasks in microscopic and ultrastructural image analysis using ImageJ. *Ultrastruct Pathol* 31:401–407
 39. Mann CJ, Perdiguero E, Kharraz Y, Aguilar S, Pessina P, Serrano AL, Muñoz-Cánoves P (2011) Aberrant repair and fibrosis development in skeletal muscle. *Skelet Muscle* 1:21
 40. Fukushima K, Badlani N, Usas A, Riano F, Fu F, Huard J (2001) The use of an antifibrosis agent to improve muscle recovery after laceration. *Am J Sports Med* 29:394–402
 41. Sato K, Li Y, Foster W, Fukushima K, Badlani N, Adachi N, Usas A, Fu FH, Huard J (2003) Improvement of muscle healing through enhancement of muscle regeneration and prevention of fibrosis. *Muscle Nerve* 28:365–372
 42. Hwang JH, Ra YJ, Lee KM, Lee JY, Ghil SH (2006) Therapeutic effect of passive mobilization exercise on improvement of muscle regeneration and prevention of fibrosis after laceration injury of rat. *Arch Phys Med Rehabil* 87:20–26
 43. Novak ML, Weinheimer-Haus EM, Koh TJ (2014) Macrophage activation and skeletal muscle healing following traumatic injury. *J Pathol* 232:344–355
 44. Cannon JG, St Pierre BA (1998) Cytokines in exertion-induced skeletal muscle injury. *Mol Cell Biochem* 179:159–167
 45. Bernasconi P, Torchiana E, Confalonieri P, Brugnoli R, Barresi R, Mora M, Cornelio F, Morandi L, Mantegazza R (1995) Expression of transforming growth factor-beta 1 in dystrophic patient muscles correlates with fibrosis. Pathogenetic role of a fibrogenic cytokine. *J Clin Invest* 96:1137–1144
 46. Pessina P, Cabrera D, Morales MG, Riquelme CA, Gutiérrez J, Serrano AL, Brandan E, Muñoz-Cánoves P (2014) Novel and optimized strategies for inducing fibrosis in vivo: focus on Duchenne Muscular Dystrophy. *Skelet Muscle* 4:7
 47. Seale P, Sabourin LA, Girgis-Gabardo A, Mansouri A, Gruss P, Rudnicki MA (2000) Pax7 is required for the specification of myogenic satellite cells. *Cell* 102:777–786
 48. Kuang S, Chargé SB, Seale P, Huh M, Rudnicki MA (2006) Distinct roles for Pax7 and Pax3 in adult regenerative myogenesis. *J Cell Biol* 172:103–113
 49. Oustanina S, Hause G, Braun T (2004) Pax7 directs postnatal renewal and propagation of myogenic satellite cells but not their specification. *EMBO J* 23:3430–3439
 50. Relaix F, Montarras D, Zaffran S, Gayraud-Morel B, Rocancourt D, Tajbakhsh S, Mansouri A, Cumano A, Buckingham M (2006) Pax3 and Pax7 have distinct and overlapping functions in adult muscle progenitor cells. *J Cell Biol* 172:91–102
 51. Song E, Ouyang N, Hörbelt M, Antus B, Wang M, Exton MS (2000) Influence of alternatively and classically activated macrophages on fibrogenic activities of human fibroblasts. *Cell Immunol* 204:19–28

52. Matsuba Y, Goto K, Morioka S, Naito T, Akema T, Hashimoto N, Sugiura T, Ohira Y, Beppu M, Yoshioka T (2009) Gravitational unloading inhibits the regenerative potential of atrophied soleus muscle in mice. *Acta Physiol (Oxf)* 196:329–339
53. Morioka S, Goto K, Kojima A, Naito T, Matsuba Y, Akema T, Fujiya H, Sugiura T, Ohira Y, Beppu M, Aoki H, Yoshioka T (2008) Functional overloading facilitates the regeneration of injured soleus muscles in mice. *J Physiol Sci* 58:397–404
54. Naito T, Goto K, Morioka S, Matsuba Y, Akema T, Sugiura T, Ohira Y, Beppu M, Yoshioka T (2009) Administration of granulocyte colony-stimulating factor facilitates the regenerative process of injured mice skeletal muscle via the activation of Akt/GSK3 α signals. *Eur J Appl Physiol* 105:643–651
55. Fry CS, Lee JD, Jackson JR, Kirby TJ, Stasko SA, Liu H, Dupont-Versteegden EE, McCarthy JJ, Peterson CA (2014) Regulation of the muscle fiber microenvironment by activated satellite cells during hypertrophy. *FASEB J* 28:1654–1665
56. Senf SM, Howard TM, Ahn B, Ferreira LF, Judge AR (2013) Loss of the inducible Hsp70 delays the inflammatory response to skeletal muscle injury and severely impairs muscle regeneration. *PLoS ONE* 8:e62687
57. Gehrig SM, van der Poel C, Sayer TA, Schertzer JD, Henstridge DC, Church JE, Lamon S, Russell AP, Davies KE, Febbraio MA, Lynch GS (2012) Hsp72 preserves muscle function and slows progression of severe muscular dystrophy. *Nature* 484:394–398
58. Locke M, Celotti C (2001) Cold stress does not induce stress proteins SP 25 and SP 72 in rat skeletal muscle. *Cryobiology* 43:54–62