Icing at Early Stage Depresses Skeletal Muscle Regeneration

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Abstract

In the present study, we investigated the effects of icing on skeletal muscle regeneration by using histochemical techniques. Eight-week-old male mice (CL57BL/6J) were divided into 3 groups: control (C) group; IE group, in which icing was performed at early stage (immediately) after muscle injury; and ID group, in which icing was performed at delayed stage (8 days) after muscle injury. Muscle injury was induced by using intramuscular injection of 0.1 mL cardiotoxin (CTX: 10 μM) into the left tibialis anterior (TA) muscle of all mice. Icing in IE and ID groups was carried out 1 hour and 8 days after the CTX-injection, respectively. TA muscles were removed 2 and 4 weeks after CTX-injection.

Icing immediately after the injury (early stage of muscle injury, IE group) partially depressed the increases in muscle wet weight, in the mean CSA of fibers with central nuclei and in the number of Pax7-positive nuclei, and the decrease in the percentage of fibers with central nuclei, compared with icing 8 days after the injury (delayed stage of muscle injury, ID group) and non-icing (control: C group). On the contrary, icing 8 days after the injury enhanced the increase in the number of Pax7-positive nuclei and the decrease in the number of fibers with central nuclei, compared with C and IE groups.

These results suggested that icing at delayed stage of muscle injury might facilitate the recovery of CTX-injured TA muscle fibers. However, the effects of icing on skeletal muscle regeneration might be dependent on the timing of icing (elapsed time after muscle injury). Icing immediately after injury might delay the recovery of muscle injury although it is generally accepted that icing immediately after muscle injury is useful for the first-aid treatment of muscle strain and contusion.

Key words

inging, skeletal muscle, satellite cell, skeletal muscle regeneration

Introduction

Skeletal muscle injury is a trauma that frequently occurs during sports¹-³, and cryotherapy (icing) is widely used as an emergency treatment given immediately after the injury at the scene of the sports activity⁴-⁵. As peripheral vasoconstriction, inhibition of enzyme activity, inhibition of spasms, increased pain thresholds, and hypertonia are well known as physiological effects of icing⁶-¹⁰, it has been believed that icing on muscle injury can be effective for the reduction of pain, swelling, degeneration, inflammation, bleeding, and other symptoms¹¹-¹⁵.

Regarding the effects of icing on skeletal muscle regeneration, there were contrary reports. Some re
ports showed that icing attenuates degeneration and enhances muscle regeneration\cite{22,30}. Conversely, icing controls inflammation and delays muscle regeneration\cite{19,17}. The effects of icing on skeletal muscle injury are still not elucidated. In addition, icing is generally carried out immediately after injury, so-called “acute phase.” However, the optimal timing (time) of icing in response to tissue damage is still unclear.

It is well-known that satellite cells are responsible for the repair and the regeneration of adult skeletal muscle. Following skeletal muscle injury, the number of muscle satellite cells increases and those cells differentiate into myotubes and myofibers\cite{26}. It is reported that most of the satellite cells are quiescent in non-injured conditions, and that the cells proliferate and fuse to form multinucleated myotubes, when they are activated in response to injury\cite{20,28}. However, it is still unclear whether the satellite cells would be activated by injury or not. It has been reported that muscle satellite cells are activated by mechanical stretch as well as heat stress, which are hypertrophic stimuli, and these stresses facilitates skeletal muscle regeneration\cite{20-23}. On the other hand, there has been no report regarding the effects of icing on muscle satellite cells following skeletal muscle injury.

In this study, we investigated the effects of icing on skeletal muscle regeneration by using histochemical techniques. The optimal timing of icing on the repair of skeletal muscle injuries was also examined.

Materials and Methods

Animals and grouping

The experimental procedures were carried out in accordance with St. Marianna University School of Medicine’s Animal Experiment Guidelines and were approved by the Animal Experiment Committee of the Experimental Animal Research Facilities, St. Marianna University School of Medicine (Approval No. 1106009). Three mice were housed in a cage (20 × 31 cm and 13.5 cm height) in a vivarium room with 12:12-h light: dark cycle and with maintained temperature and humidity at 24 ± 1°C and 55 ± 10% (Mean ± SEM). Solid food and water were provided ad libitum.

Eight-week-old male mice (C57BL/6J, n=36) were randomly divided into 3 groups: control (C), icing at early stage of muscle injury (IE), icing at delayed stage of muscle injury (ID) groups (n=12 in each group). We investigated the time-dependent changes in the protein expression of Pax7, which is well known as a specific marker for muscle satellite cell, following muscle injury induced by cardiotoxin (CTX: see the Initiation of necrosis-regeneration cycle for the details). By using Western blotting analyses\cite{34}, the peak expression of Pax7 was observed at Day 7 following muscle injury (data not shown). Therefore, icing was performed 1 hour after muscle injury in IE group and 8 days after muscle injury in ID group, respectively.

Initiation of necrosis-regeneration cycle

Necrosis-regeneration cycle was induced by using intramuscular injection of 0.1 mL CTX (10 μM in physiological saline (PS), Sigma, St. Louis, MO, USA) of Naja naja atra venom. Injection of CTX was performed into the proximal, middle, and distal part of the left tibialis anterior muscle (TA) of all mice in the IE and ID groups using a 27-gauge needle under anesthesia with Forane (Abbott Japan, Tokyo, Japan). This procedure for the initiation of necrosis-regeneration was performed carefully to avoid the damage in the nerves and blood vessels, as was suggested elsewhere\cite{36}. The mice were housed in the same cages for 2-4 weeks.

Experimental protocol

Under anesthesia with Forane, both hindlimbs of mice were completely soaked in ice-cold water for 20 minutes. During icing, the abdomen was wrapped with an expanded polystyrene board and the tail was clipped to the back to avoid direct contact with cold water. The muscle temperature of the TA and the esophageal temperature were monitored with a thermocouple thermometer PTW-301 (Unique Medical Co., Ltd., Tokyo, Japan), and the muscle and esophageal temperature were ~4°C immediately after icing and ~30°C for 15 minutes of icing, respectively. The right TA was dissected 2 (3 groups; n=6 per each group) and 4 weeks (3 groups; n=6 per each group) after CTX-injection. After the muscle weight was weighed, the TA was frozen in isopentane cooled by liquid nitrogen, and stored at −80°C.

Histochemical and immunohistochemical analyses

Serial transverse cryosections (6 μm thick) of the midbelly region of frozen left TA muscles were cut at −20°C by using a Cryostat (CM1900, Leica, Wetzlar, Germany) and mounted on the slide glasses. The sections were air-dried and stained to
analyze the histological stages by using hematoxylin and eosin (HE) and the profiles of Pax7-positive nuclei by the standard immunohistochemical technique, respectively\(^2\). General pathological observations including centrally located myonuclei were based on HE.

Briefly, monoclonal anti-Pax7 antibody (Developmental Studies Hybridoma Bank, Iowa, IA, USA) was used for the detection of muscle satellite cells\(^2\). Cross-sections were fixed with paraformaldehyde (4%) at 4°C for 15 minutes. After blocking for 7 minutes by using a reagent (Large Volume Ultra V Block, Thermo Scientific, Cheshire, UK), samples were incubated with the primary antibodies for Pax7 and rabbit polyclonal anti-laminin (Z0097, Dako Cytomation, Glostrup, Denmark; diluted 1:1000) at room temperature for 3 hours. Sections were also incubated with the second primary antibodies for Alexa Fluor 568-labeled goat anti-mouse IgG1 (1:500; Invitrogen, Molecular Probes, Eugene, OR, USA) and Alexa Fluor 488-labeled goat anti-rabbit IgG (1:1000; Invitrogen, Molecular Probes, USA). Then, nuclei were stained by 5-minute incubation in a solution of 4',6-diamidino-2-phenylindole (DAPI, 01:10000; Sigma-Aldrich, St Louis, MO, USA).

**Imaging of muscular sections and analyses**

Cross-sectional area of fibers having central nuclei of 250-500 muscle fibers was calculated by using the image analysis software Image J (Ver. 1.45i, Wayne Rasband, National Institutes of Health, USA). The percentage of fibers having central nuclei relative to total muscle fibers in the whole transverse section of 250–500 muscle fibers was also calculated. Both Pax7- and dapi-positive nuclei, located within laminin-positive basal membrane were counted in the whole transverse section of 250–500 fibers and were expressed as the total number of both Pax7-positive nuclei and myonuclei per cross-section of TA muscle. Further, the percentage of Pax7-positive nuclei relative to the total number of dapi-positive nuclei were calculated\(^2\).

**Statistical analyses**

All values were expressed as mean ± SD. Statistical significance was analyzed by using two-way (treatment x time) ANOVA. When a significant interaction between two effects was observed, Tukey post hoc test was carried out by using SPSS Statistics 20.0J (IBM Japan, Tokyo, Japan). Statistical significance was established at p<0.05.

**Results**

**Muscle wet weight**

Two-way (treatment x time) ANOVA revealed that significant main effects of treatment and time (p <0.05) were observed in absolute muscle wet weight without a significant interaction. There were significant differences in muscle wet weight between C and IE groups and between IE and ID groups (p<0.05) (Fig. 1). Following 2 and 4 weeks after CTX-injection, there were significant differences in muscle wet weight between C and IE groups and

![Fig. 1. Changes in absolute muscle wet weight.](image)

**Fig. 1. Changes in absolute muscle wet weight.**

C, control group; IE, icing at early stage of muscle injury (immediately after muscle injury); ID, icing at delayed stage of muscle injury (8 days after muscle injury); 2W, 2 weeks after muscle injury; 4W, 4 weeks after muscle injury. Values are means ± SD. n=6/group at each time point.

\(^*\), significant difference at p<0.05.
between IE and ID groups (p<0.05).

**Cross-sectional area (CSA) for central nuclei fibers**

Changes in fiber CSA having central nuclei are shown in Figure 2. Two-way ANOVA revealed a significant main effect of treatment (p<0.05) without a significant interaction. There was a significant difference in fiber CSA between C and IE groups (p<0.05) (Fig. 2). Following 2 weeks after CTX-injection, there were significant differences in fiber CSA having central nuclei between C and IE groups and between IE and ID groups (p<0.05). However, no significant differences were observed following 4 weeks after the injection.

**Muscle fiber with central nuclei**

The percentages of fibers with central nuclei after CTX-injection are shown in Figure 3. Two-way ANOVA revealed significant main effects of treatment and time (p<0.05) without a significant interaction. There were significant differences in the percentage of fibers with central nuclei between C and ID groups and between IE and ID groups (p<0.05). Following 2 weeks after the injection, there was no significant difference in the mean percentage of these fibers among three experimental groups. Following 4 weeks after CTX-injection, the number of these fibers decreased. Larger decrease in the number of these fibers in IE group was observed, compared with C and ID groups. There was a significant difference in the mean percentage of these fibers between IE and ID groups (p<0.05).

**Pax7-positive nuclei**

Pax7-positive nuclei were located inside the laminin-positive basal lamina (Fig. 4). Changes in

![Fig. 2. Responses of the mean fiber cross-sectional area (CSA) having central nuclei. See Fig. 1 for the abbreviations. Values are means ± SD. n=6/group at each time point. *p<0.05.](image)

![Fig. 3. Changes in the percentage of centrally-nucleated fibers relative to the total muscle fibers. See Fig. 1 for the abbreviations. Values are means ± SD. n=6/group at each time point. *p<0.05.](image)
the relative numbers of Pax7-positive nuclei were shown in Figure 5. Two-way ANOVA revealed significant main effects of treatment and time (p<0.05) with a significant interaction (p<0.05). There were significant differences in the relative numbers of Pax7-positive nuclei between C and ID groups and between IE and ID groups (p<0.05). Following 2 weeks after CTX-injection, larger number of Pax7-positive nuclei was observed in ID group, compared with that in C and IE groups. There were significant differences in the relative number of Pax7-positive nuclei between C and IE groups and between IE and ID groups (p<0.05). No significant differences in the Pax7-positive nuclei were observed following 4 weeks after the injection.

Fig. 4. Identification of satellite cell and myonuclei.
Transverse sections of TA muscle were immunostained with anti-Pax7 (A: red), anti-laminin (B: green) and Dapi (C: blue). D is merged image of A, B and C. The Pax7-positive nuclei located inside the basal membrane are indicated by arrow heads. Scale bars=100 μm.

Fig. 5. Changes in the percentage of Pax7-positive nuclei relative to the total myonuclei.
See Fig. 1 for the abbreviations. Values are means ± SD. n=6/group at each time point.
*, significant difference at p<0.05.
Discussion

This study showed that the effects of icing on skeletal muscle regeneration were dependent on the time of icing. Icing immediately after the injury (at early stage of muscle injury, IE group) partially depressed the increases in muscle wet weight, in the mean CSA of fibers with central nuclei and in the number of Pax7-positive nuclei, compared with non-icing 8 days after the injury (at delayed stage of muscle injury, ID group) and non-icing (C group). On the contrary, icing 8 days after the injury enhanced the increase in the number of Pax7-positive nuclei and the decrease in the number of fibers with central nuclei, compared with C and IE groups.

It has been suggested that high level of Pax7-positive satellite cells in injured skeletal muscle might show high regenerative potential of injured skeletal muscle [12-21]. In addition, it is generally accepted that the population of Pax7-positive satellite cells increases in muscle following CTX-injection-associated injury. In this study, icing at delayed stage of muscle injury enhanced the increase in Pax7-positive nuclei following skeletal muscle injury.

It is well known that central nucleus is an indicator of myofiber regeneration [12-21]. In our present study, the number of the fibers with central nuclei was measured as an indicator for the recovery from muscle injury. The mean percentage of these fibers was ~96% 2 weeks after CTX-injection without icing, and then gradually decreased to ~76% after 4 weeks of ambulation recovery. Mean level of fibers with central nuclei was not changed by icing at early stage of muscle injury (~100 and 77% at 2 and 4 weeks after CTX-injection). On the contrary, icing at delayed stage of muscle injury caused a further decrease in the number of these fibers (~91 and 55% at 2 and 4 weeks after CTX-injection). Therefore, the morphological data strongly suggested that the icing at delayed stage of muscle injury might facilitate the recovery of CTX-injured TA muscle fibers.

Recently, Takagi et al. [20] reported the effects of 20-minutes of icing on injured extensor digitorum longus (EDL) muscle 5-minutes after crush injury. Smaller fiber CSA and larger number of fiber with central nuclei were observed ice-cooled injured EDL muscle, compared with non-icing group. In our present study, icing at early stage of muscle injury did not affect on the changes in the numbers of Pax7-positive satellite cells and of fibers with central nuclei. Muscle wet weight (2 and 4 weeks after the injection) and mean fiber CSA (2 weeks after the injection) in IE group were significantly lower than those in C and ID groups (p<0.05). Therefore, the effects of icing on skeletal muscle regeneration might be dependent on the timing of icing (elapsed time after muscle injury).

There was no report regarding the effect of differences in elapsed time of icing after muscle injury. The time of icing was determined by the changes in the protein expression levels of Pax7 in this study. As Pax7 is exclusively expressed in muscle satellite cells in skeletal muscle, the expression level of Pax7 might implicate the number of satellite cells. Seven days after muscle injury, the peak expression of Pax7 protein was observed in regenerating TA muscle in this investigation (data not shown). Although the effects of icing on proliferation and differentiation of muscle satellite cells are unclear, it is generally considered that low temperature-environment would depress these activities of muscle satellite cells.

Perspective

It is generally accepted that icing immediately after muscle injury is useful for the first-aid treatment of muscle strain and contusion. Judging from the results of this study, icing immediately after injury might delay the recovery of muscle injury. Therefore, we should reconsider the treatment for muscle injuries.

Conclusion

1. The effects of icing on skeletal muscle regeneration were dependent on the time of icing.
2. Icing at early stage of muscle injury (immediately after the injury) partially depressed skeletal muscle regeneration.
3. Icing at delayed stage of muscle injury (8 days after the injury) enhanced the increase in the number of Pax7-positive nuclei and the decrease in the number of fibers with central nuclei.

References