Influence of icing on muscle regeneration after crush injury to skeletal muscles in rats

Ryo Takagi,1 Naoto Fujita,1 Takamitsu Arakawa,1 Shigeo Kawada,2 Naokata Ishii,2 and Akinori Miki1

1Department of Rehabilitation Sciences, Kobe University Graduate School of Health Sciences, Kobe; and 2Department of Life Science, Graduate School of Arts and Science, The University of Tokyo, Tokyo, Japan

Submitted 8 October 2010; accepted in final form 13 December 2010

Takagi R, Fujita N, Arakawa T, Kawada S, Ishii N, Miki A. Influence of icing on muscle regeneration after crush injury to skeletal muscles in rats. J Appl Physiol 110: 382–388, 2011. First published December 16, 2010; doi:10.1152/japplphysiol.01187.2010.—The influence of icing on muscle regeneration after crush injury was examined in the rat extensor digitorum longus. After the injury, animals were randomly divided into nonicing and icing groups. In the latter, ice packs were applied for 20 min. Due to the icing, degeneration of the necrotic muscle fibers and differentiation of satellite cells at early stages of regeneration were retarded by ~1 day. In the icing group, the ratio of regenerating fibers showing central nucleus at 14 days after the injury was higher, and cross-sectional area of the muscle fibers at 28 days was evidently smaller than in the nonicing group. Besides, the ratio of collagen fibers area at 14 and 28 days after the injury in the icing group was higher than in the nonicing group. These findings suggest that icing applied soon after the injury not only considerably retarded muscle regeneration but also induced impairment of muscle regeneration along with excessive collagen deposition. Macrophages were immunohistochemically demonstrated at the injury site during degeneration and early stages of regeneration. Due to icing, chronological changes in the number of macrophages and immunohistochemical expression of transforming growth factor β1 and IGF-1 were also retarded by 1 to 2 days. Since it has been said that macrophages play important roles not only for degeneration, but also for muscle regeneration, the influence of icing on macrophage activities might be closely related to a delay in muscle regeneration, impairment of muscle regeneration, and redundant collagen synthesis.

When the skeletal muscle is injured, muscle regeneration occurs in four interdependent phases: degeneration, inflammation, regeneration, and fibrosis (19, 21, 35). Skeletal muscle injury often occurs through contusion (4), strain (15), or laceration (19). In sports medicine, icing by using ice packs has been widely used to reduce pain, swelling, degeneration, and inflammation (6, 10, 27, 34), usually soon after the injury (28, 37).

Knight and Merrick examined influences of icing on degeneration and inflammation in injured skeletal muscles (23, 27), and they speculated that since icing can reduce degeneration in injured muscle fibers, it might promote muscle regeneration (20). On the other hand, some researchers claimed that since the cold therapy suppresses inflammation (25, 34), muscle regeneration might be retarded (24). But in these studies, the influence of icing on muscle regeneration was not at all examined. In the present study, therefore, to know the influence of icing on muscle regeneration, morphological and quantitative changes in the necrotic and regenerating muscle fibers after a crush injury were examined in the rat extensor digitorum longus (EDL) muscle.

It was reported that macrophages invading the degenerating muscular tissue produce several kinds of factors that stimulate and promote muscle regeneration in vitro (7, 8, 26). However, there are no definitive findings to show which factors, if any, are produced by macrophages in vivo that affect the process of muscle regeneration. In vitro, it is becoming increasingly clear that some growth factors such as IGF-I and transforming growth factor (TGF)-β1 regulate activation, proliferation, differentiation, and fusion to existing muscle fibers of satellite cells (2, 9, 12, 39). In the present study, therefore, the influence of icing on chronological changes in distribution of macrophages and satellite cells, and expressions of TGF-β1 and IGF-I during the muscle regeneration, were also examined by immunohistochemistry and in situ hybridization.

MATERIALS AND METHODS

Animals

Seventy-eight 8-wk-old male Wistar rats weighing 180–200 g (Japan SLC, Shizuoka, Japan) were used in the present study. The rats were allowed to access food and water freely throughout the experiments. This study was approved by the Institution Animal Care and Use Committee and carried out according to the Kobe University Animal Experimentation Regulations.

Experimental Protocol

The animals were anesthetized by an intraperitoneal injection of pentobarbital sodium (50 mg/kg), the anterolateral aspect of the left hindlimb was shaved, and a longitudinal incision ~20 mm long was made to expose the EDL muscle. The middle part of the muscle belly was crushed for 30 s by using forceps, to which a weight (500 g) was attached, according to the crush injury method described by Furuta (14). Immediately after the injury, the skin was closed with a 4–0 suture. These animals were then randomly divided into two groups: icing group and nonicing group. The ice pack was prepared by enclosing crushed ice in a polyethylene bag. The temperature at the surface of the ice pack was 0.3–1.3°C. Five minutes after the injury, animals in the icing group received icing, but those in the nonicing group did not. The ice pack was transcutaneously applied to the injured EDL muscle in vivo for 20 min under minimum compression controlled to touch the ice pack to the skin. At 6 and 12 h and 1, 2, 3, 4, 5, 6, 7 (n = 3), 14 and 28 (n = 6) days after the injury, the animals were killed by an overdose intraperitoneal injection of pentobarbital sodium. Segments of the EDL muscle were harvested, and these unfixed samples were embedded in OCT compound. They were immediately frozen in dry ice-cooled acetone, and stored at ~80°C until used. In four of the animals used in the present study, the right EDL muscle was also crushed in the same manner as described above,
and temperature at the surface of the right EDL muscle and rectal temperature were monitored during and after the icing by using a thermometer (Tsuruga Electric Works, Osaka, Japan). During the temperature measurements, room temperature was kept at 25°C.

Morphological Analyses

Cross sections were cut with a cryostat (Shiraimatsu, Osaka, Japan) and mounted on glass slides. Some sections (9 µm) were used for hematoxylin and eosin staining, and van Gieson’s staining for histological investigation, and the others were used for immunohistochemistry and in situ hybridization.

Immunohistochemistry

Immunohistochemistry was performed according to the conventional single-labeling protocol described by Watanabe and Nakane (36). Sections (9 µm) were air-dried for 15 min at room temperature and rinsed in TBS for 5 min, and endogenous peroxidase activity was quenched with 0.3% hydrogen peroxide diluted with methanol for 35 min at room temperature. After washing in TBS, these sections were reacted for 1 h at room temperature with the primary antibodies, TGF-β1, IGF-I, PAX7 (Santa Cruz Biotechnology), and ED1 (Sero-tec). ED1 binds the antigen specific for subpopulations of monocyte and macrophage (11). PAX-7 binds the antigen specific for quiescent tec). ED1 binds the antigen specific for subpopulations of monocyte and macrophage (11). PAX-7 binds the antigen specific for quiescent and activated satellite cells (33). After washing three times in TBS, the secondary antibodies, MAX-PO (R) and (M) (Nichirei Biosciences, Tokyo, Japan) were applied for 30 min at room temperature.

In Situ Hybridization

To produce the in situ hybridization probes, total RNA was isolated from the rat plantaris muscle using Isogen (Nippon gene, Tokyo, Japan), digested with DNase 1 (RQ 1; Promega). Then the RNA was reverse-transcribed using the PrimerScript II first-strand cDNA synthesis kit (Takara, Tokyo, Japan). For the TGF-β1 and IGF-I probes, oligonucleotide primers containing T7 promoter sequence were used to amplify each cDNA as follows: TGF-β1 forward, 5’-taatcactactggtgagcagaa-3’; TGF-β1 reverse, 5’-taatcactactataaggcagacaagttagctgtcgc-3’; IGF-I forward, 5’-gagggccttactaacaagctgagagatc-3’; and IGF-I reverse, 5’-taatcactactataggcagacaagttagctgactgactgagcagaa-3’. The cRNA probes labeled with DIG were obtained using DIG RNA Labeling kit (Roche Diagnostics, Basel, Switzerland) according to the manufacturer’s instructions.

The sections were fixed with 4% paraformaldehyde in 0.1 M PBS for 20 min and were washed twice with PBS for 15 min. After incubation in 5 × SSC for 15 min, the sections were prehybridized in hybridization buffer (5 × SSC, Denhardt’s, 10% dextran sulfate, 0.5 mg/ml yeast tRNA, and 40 µg/ml salmon sperm DNA) at 58°C for 2 h. Then the hybridization buffer containing probe was dispersed on the sections. The sections were incubated at 58°C overnight and were washed in 2 × SSC at room temperature for 30 min, 2 × SSC containing 50% formamide at 60°C for 1 h, and 0.1 × SSC at 60°C for 1 h. The sections were incubated with anti-DIG-antibody [1:5,000 with 0.5% blocking reagent (Roche) in 100 mM Tris-HCl, pH 7.5, containing 150 mM NaCl] for 1 h and were washed in 100 mM Tris-HCl (pH 7.5) containing 150 mM NaCl for 15 min, and 100 mM Tris-HCl (pH 9.5) containing 150 mM NaCl and 50 mM MgCl2 for 5 min. For color development, the sections were incubated in NBT/BCIP Stock Solution (20 µl/ml) in 100 mM Tris-HCl (pH 9.5) containing 150 mM NaCl and 50 mM MgCl2. The sections were washed with 10 nM Tris-HCl (pH 7.5) containing 1 mM EDTA to stop the reaction and then were washed with water for 15 min.

Quantitative Analyses

Regenerating muscle fibers. Previously, it was reported that regenerating muscle fiber exhibited several characteristic features, such as a large central nucleus, a small cross-sectional area, and basophilic sarcoplasm (30). In the sections used for hematoxylin and eosin staining, we examined proportions of the muscle fibers containing central nucleus to total muscle fibers, and cross-sectional areas of 100 muscle fibers that have central nucleus at 14 and 28 days after the injury as indicators of the regenerating muscle fibers maturation.

Distribution of macrophages. To know the chronological changes in number of macrophages, some immunostained sections were randomly selected from each group, and the number of the ED1-positive macrophages was counted in 10 areas (1 area = 0.0625 mm²) at each period after the injury.

Collagen fibers. In the sections used for van Gieson’s staining, proportions of the collagen fibers area that were stained red were calculated in 10 areas (1 area = 0.0625 mm²) at 14 and 28 days after the injury by using Scion Image (Scion).

Statistical Analyses

The values were expressed as means ± SE. The difference between means in the icing and nonicing groups was determined by parametric or Welch t-test with a significance level set at P < 0.05.

RESULTS

Temperature at the Surface of the EDL Muscle and in the Rectum

During the icing applied to the anterior surface of the lower leg, temperature at the surface of the EDL muscle decreased linearly with time, and at the end of 20 min icing, the temperature decreased by 13.3 ± 1.2°C (Fig. 1). After removal of the ice pack, the temperature began to rise gradually. But the rectal temperature decreased only slightly, and at the end of the icing, it decreased by 3.2 ± 0.8°C (Fig. 1). After removal of the ice pack, the rectal temperature recovered to the control level within 1 h.

Degeneration and Early Stages of Regeneration

At 6 h after the injury, both in the nonicing and icing groups, several degenerating muscle fibers were observed near the crushed site. In the nonicing group, the degenerating muscle fibers were swollen and round in shape, and the sarcoplasm stained very faintly (Fig. 2a). These features are characteristics of necrosis. In some necrotic muscle fibers, the contour became unclear (Fig. 2a). On the other hand, in the icing group, the

Fig. 1. Alterations of the temperatures at the surface of the extensor digitorum longus (EDL) muscle (solid line) and in the rectum (dotted line) during and after icing (n = 4). Vertical axis shows the temperature. Horizontal axis shows the time. Values are presented as means ± SE. For clarity the error bars have been plotted for every 5 min only.

J Appl Physiol • VOL. 110 • FEBRUARY 2011 • www.jap.org
contour was clearly defined, and sarcoplasm stained still deeply (Fig. 2b). At 12 h after the injury, the sarcoplasm of the necrotic muscle fibers in the nonicing group (Fig. 2c) stained fainter than that in the icing group (Fig. 2d). In the nonicing group, almost all cell membranes could not be observable (Fig. 2c), but in the icing group, some cell membranes could still be discerned clearly (Fig. 2d). Besides cellular elements were noted in the necrotic muscle fibers in the nonicing group (Fig. 2c), but in the icing group, they were not yet found in the necrotic muscle fibers (Fig. 2d). Those were likely macrophages, regarding their shape and size.

At 3 days after the injury, in the nonicing group, thin regenerating muscle cells showing a basophilic sarcoplasm and large centrally located nucleus (central nucleus) were distributed (Fig. 2e), but in the icing group, these cells were not found anywhere (Fig. 2f).

At 4 days after the injury, in the nonicing group, regenerating muscle cells were evidently larger than those in the icing group (Fig. 2, g and h). In the nonicing group, some regenerating muscle cells (myotubes) which have two nuclei in the sarcoplasm were also noted (Fig. 2g).

Late Stages of Regeneration

To examine the influence of icing on maturation of the regenerating muscle fibers, morphological analyses were performed. At the late stages of regeneration, as the regenerating muscle fiber matures, the nucleus migrates from the center to the periphery of the muscle fiber, and the size of the muscle fiber increases gradually (30).

At 14 days after the crush injury, the proportion of the regenerating fibers showing central nucleus to the total muscle fibers in the nonicing group was 19.1 ± 2.7% (Fig. 3A), and in the icing group 26.0 ± 2.1% (Fig. 3A). This proportion in the icing group was significantly higher than that in the nonicing group (P < 0.05). At 28 days after the injury, the proportion in the nonicing group was 11.9 ± 2.5%, and in the icing group 15.1 ± 1.8% (Fig. 3A), respectively.

At 14 days after the injury, the cross-sectional area of the regenerating muscle fibers in the nonicing group was 9.6% above that in the icing group (Fig. 3B). At 28 days after the injury, the cross-sectional area in the nonicing group was 64.5% above that in the icing group (Fig. 3B). At 28 days after the injury, the area in the nonicing group was significantly larger than that in the icing group (P < 0.01).

Histochemical Observations

Distribution of macrophages. At 12 h after the crush injury, in the nonicing group, several ED1⁺ macrophages were demonstrated in the degenerating areas near the crushed site, and some macrophages were found within the necrotic muscle fibers (Fig. 4a). In contrast, in the icing group, although some macrophages were found in the degenerating area, macrophages invading the necrotic muscle fibers were not yet noted (Fig. 4b).
At 2 days after the injury, in the non-icing group, several macrophages were distributed within and among the necrotic muscle fibers (Fig. 4c). On the other hand, in the icing group, although macrophages were found in and among the necrotic muscle fibers (Fig. 4d), the number of the macrophages was evidently less than in the non-icing group.

At 4 days after the injury, in the non-icing group, macrophages were observed mainly among the muscle fibers (Fig. 4e). On the other hand, in the icing group, although several macrophages were observed among the muscle fibers, there were some muscle fibers containing macrophages observed (Fig. 4f).

Chronological changes in the number of macrophages in the non-icing (dashed line) and icing (solid line) groups are presented in Fig. 5. In the icing group, the time when the number of macrophages had peaked, and chronological changes in number of macrophages were delayed by 1 day compared with those in the non-icing group.

**TGF-β1 mRNA and IGF-I mRNA expressions.** Immunohistochemistry (ED1) and in situ hybridization were performed in serial sections (6 μm) to elucidate that the same cell shows positive signals in both sections.

TGF-β1 mRNA positive cells (Fig. 6a) corresponded to macrophages (Fig. 6b) invading the necrotic muscle fibers at 2 days after the injury in the non-icing group. In the icing group at 5 days after the injury, TGF-β1 mRNA expressions (Fig. 6c) corresponded to macrophages (Fig. 6d) among the regenerating muscle fibers and demonstrated in the regenerating muscle fibers (Fig. 6c).

IGF-I mRNA expressions (Fig. 6e) also corresponded to macrophages (Fig. 6f) among the regenerating muscle fibers and demonstrated in the regenerating muscle fibers (Fig. 6e) at 4 days after the injury in the non-icing group.

**TGF-β1, IGF-I, and satellite cell expressions.** TGF-β1 expression started to be demonstrated faintly in the necrotic muscle fibers at 12 h after the injury in the non-icing group (Fig. 7a), and at 1 day after the injury in the icing group (Fig. 7b). At 2 days after the injury, the expression became the most pronounced in the non-icing group (Fig. 7c), but in the icing group, it did not yet peak (Fig. 7d). It could be seen until 3 days after the injury in the non-icing group (Fig. 7e), but in the icing group, it was still demonstrable in the regenerating muscle fibers (Fig. 7f) at 5 days after the injury.

IGF-I expression started to be seen faintly in the necrotic muscle fibers at 1 day after the injury in the non-icing group (Fig. 8a), and at 2 days after the injury in the icing group.

---

**Fig. 4.** Immunohistochemistry of ED1 in transverse sections of the EDL muscle in the non-icing group (a, c, and e) and icing group (b, d, and f) at 12 h, 2 days, and 4 days after the injury. Arrow and arrowhead show the macrophage. Asterisk shows the necrotic muscle fiber. Bars = 50 μm.

**Fig. 5.** Chronological changes in number of the ED1+ macrophages after the injury. Vertical axis shows number of the ED1+ macrophages. Horizontal axis shows the time after the injury. These values are presented as means ± SE. Dashed line, non-icing group; solid line, icing group.

**Fig. 6.** Serial sections used for immunohistochemistry (ED1), eosin staining, and in situ hybridization. a and b: transforming growth factor (TGF)-β1 mRNA in the non-icing group at 2 days after the injury. Bilateral arrows show same cells, respectively. Asterisk shows the necrotic muscle fiber. c and d: TGF-β1 mRNA in the icing group at 5 days after the injury. Bilateral arrows show same cells, respectively. Arrowhead shows the regenerating muscle fiber. e and f: IGF-I mRNA in the non-icing group at 4 days after the injury. Bilateral arrows show same cells, respectively. Arrowhead shows the regenerating muscle fiber. Bars = 50 μm.
At 5 days after the injury, the expressions reached almost peak in the regenerating muscle fibers in both groups (Fig. 8, c and d). At 7 days thereafter, the expression became very weak in both groups (Fig. 8, e and f).

Numerous satellite cells were noted at 3 days after the injury in the non icing group (Fig. 9a), but in the icing group at the same period, they could be hardly seen (Fig. 9b). At 4 days after the injury, a satellite cell which fused into the muscle fiber (Fig. 9c) could be seen in the non icing group, but in the icing group, satellite cells (Fig. 9d) still remained near the muscle fibers.

Morphological and Quantitative Analyses of Collagen Fibers

Both at 14 and 28 days after the injury, collagen deposition occurred more excessively in the icing group than in the non icing group (Fig. 10A). In the non icing group, collagen fibers were seen only among the bundles of muscle fibers (Fig. 10Aa and Ac). In contrast, in the icing group, each muscle fiber was surrounded by collagen fibers (Fig. 10Ab and Ad). Thus in the non icing group, muscle fibers were polygonal in shape, but in the icing group, they were round in shape.

The proportion of collagen fibers area was 8.5 ± 0.9% in the non icing group (Fig. 10B) and 12.5 ± 0.6% in the icing group (Fig. 10B) at 14 days after the injury. At 28 days after the injury, the proportion was 8.9 ± 1.3% in the non icing group and 18.7 ± 1.3% in the icing group (Fig. 10B). At both 14 and 28 days after the injury, the proportion in the icing group was significantly higher than that in the non icing group, respectively (P < 0.01).

**DISCUSSION**

In the present study, the influence of icing on muscle regeneration was examined in the crushed rat EDL muscle. Due to icing applied soon after the injury for 20 min, degeneration and inflammatory reaction in the injured muscle were delayed ~1 day, and not only a delay in muscle regeneration but also impairment, at least in part, of muscle regeneration, and excessive collagen deposition were caused.

In the present study, due to icing for 20 min, the temperature at the surface of the EDL muscle decreased more than 10°, and
morphological changes accompanied with degeneration in the necrotic muscle fibers were evidently retarded at 6 and 12 h after the injury. When skeletal muscles are crushed, the sarcotlemma is disrupted (14), and extracellular Ca²⁺ immediately enters the injured muscle fibers through the disruption; thereby degeneration of the injured muscle fibers begins by activated calpain, which is a calcium-dependent neutral protease (5, 16, 18, 32, 38). A decrement of 10° slows the enzyme activity by about half (29). These data indicate that icing could have retarded muscle degeneration after the injury through the decrement of the muscle temperature, which might slow calpain activity necessary for the primary reaction of degeneration.

Nonaka (30) reported that after removal of degenerated muscle fibers, regenerating muscle cells appear. In the present study, at the early stages of muscle regeneration in the nonicing group, the regenerating muscle cells first appeared at 3 days after the injury, and at 4 days after the injury, muscle cells having two or more nuclei (myotubes) were noted, while in the icing group, these regenerating muscle cells were first found at 4 days after the injury, and they were evidently smaller than those in the nonicing group. At the late stages of regeneration, centrally nucleated (immature) muscle fibers remained more prevalent in the icing group than those in the nonicing group at 14 days after the injury. Additionally, the size of the regenerating muscle fibers in the icing group was significantly smaller than that in the nonicing group at 28 days after the injury. These results indicate that icing could have retarded differentiation at the early stage of regeneration and maturation of the regenerating muscle fibers at the late stage of regeneration considerably.

Akiyama et al. (1) reported that muscle degeneration induces macrophage migration to injured site to phagocytose the necrotic muscle fibers. In the present study, in the icing group, macrophages found in the degenerating area at 6 and 12 h after the injury were less than in the nonicing group, and chronological changes in the number of macrophages in the degenerating and regenerating area was retarded by 1 day. These findings suggest that icing could have retarded macrophage migration to the injured site probably through the retardation of muscle degeneration after the injury.

As shown in the present study, when degeneration was occurring, macrophages were seen mainly in the necrotic muscle fibers, but when degeneration had been completed and regeneration started, they were observed mainly among the regenerating muscle fibers. These findings suggest that macrophages might play several important roles besides the phagocytosis in the necrotic muscle fibers.

As shown by in situ hybridization, macrophages produce TGF-β1 and IGF-I. In the icing group, immunohistochemical reactions for TGF-β1 and IGF-I began to be demonstrated later than those in the nonicing group. These data indicate that icing might retard the chronological changes in expressions of TGF-β1 and IGF-I through the retardation of macrophage migration. It has been demonstrated that TGF-β1 regulates proliferation and differentiation of satellite cells (9, 39). On the other hand, IGF-I was reported to stimulate the differentiation (2, 12) and growth of regenerating muscle cells by increasing the size of myotubes and protein synthesis (3, 31). In the nonicing group, both numerous satellite cells and the regenerating muscle cells (myotubes) were observed earlier than in the icing group. In the icing group, the regenerating muscle fibers at 14 days after the injury were more immature, and the size at 28 days after the injury was smaller than in the nonicing group. These findings suggest that icing might retard the proliferation and differentiation of satellite cells and maturation of the regenerating muscle fibers through the retardation of chronological changes in TGF-β1 and IGF-I expressions essential for muscle regeneration.

Collagen deposition in the icing group at 14 and 28 days after the injury was significantly more excessive than that in the nonicing group. In the nonicing group, the immunohistochemical reaction for TGF-β1 was demonstrated only until 3 days after the injury, but in the icing group, it could be still seen at 5 days after the injury. Fu et al. and Klein et al. showed that TGF-β1 stimulates collagen synthesis, which leads to the development of fibrosis (13, 22). Our findings suggest that the timing of appearance and disappearance of TGF-β1 during the course of the muscle regeneration might be important to regulation of appropriate collagen synthesis in the regenerating muscle.

In summary, icing applied soon after a muscle crush injury could have retarded proliferation and differentiation of satellite cells at the early stages of regeneration through retardation of degeneration and macrophage migration, which play a crucial role in muscle regeneration, and could have induced not only a
delay in late stages of muscle regeneration but also impairment of muscle regeneration along with a thicker collagen deposition around the regenerating muscle fibers. Judging from these findings, it might be better to avoid icing, although it has been widely used in sports medicine.

ACKNOWLEDGMENTS
We thank Dr. Michiko Shintani (Dept. of Pathological Analytics, Kobe Univ. Graduate School of Health Sciences) and our laboratory staff for help with the experiments. We also thank Mr. Bondje Peter (Kobe Univ. Graduate School of Health Sciences) for giving helpful comments during the creation of the manuscript.

GRANTS
This work was supported by The Ministry of Education, Culture, Sports, Science and Technology (MEXT) Grant-in-Aid for Scientific Research (21700530, 22500460).

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

REFERENCES
No conflicts of interest, financial or otherwise, are declared by the author(s).