An article for Ultrasound in Medicine and Biology

Effects of therapeutic ultrasound on joint mobility and collagen fibril arrangement in the endomysium of immobilized rat soleus muscle

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Abstract

This study examined effects of therapeutic ultrasound on joint mobility and collagen fibril arrangement in the endomysium of immobilized rat soleus muscle. Twenty-two male Wistar rats were divided randomly into control (n=5) and experimental groups (n=17). In the experimental group, bilateral ankle joints of each rat were fixed in full plantar flexion with a plaster cast over a 4-wk period. Five animals in the experimental group were immobilized throughout the 4-wk (immobilization group) period, whereas the remaining rats in the experimental group were randomly divided into the ultrasound (US, n=6) and sham (n=6) treatment groups. Under anesthesia, continuous ultrasonic energy (frequency, 1 MHz; intensity, 1.0 W/cm^2) was delivered to the triceps surae muscle of the US group for 15 min per d, 6 d per wk over the 4-wk immobilization period. Ultrasonic energy was not delivered to the triceps surae muscle in sham animals; only the transducer head was moved. Ankle joint mobility on dorsiflexion in the immobilization, sham and US groups was significantly smaller than that of the control group, whereas in the US group, this parameter was significantly greater than in the immobilization and sham groups. Collagen fibril arrangement in the endomysium of the control and US groups was longitudinal to the axis of the muscle fibers; in contrast, it was circumferential in the immobilization and sham groups. Our findings revealed that joint immobilization induces decreased joint mobility and collagen fibril movement in the endomysium;

furthermore, ultrasound treatment can prevent these changes. We hypothesized that therapeutic ultrasound during the immobilization process may inhibit deterioration of muscle contracture.

Key Words: Ultrasound, Immobilization, Joint mobility, Collagen fibril arrangement, Endomysium

INTRODUCTION

Clinically, in cases involving prolonged periods of bed rest, immobilization of a joint with a plaster cast or orthosis reduces the range of joint motion (ROM). This condition, which is known as joint contracture, is limiting with respect to the activity of daily living in various aspects; therefore, joint contracture is a problem commonly encountered by rehabilitation professionals.

Several studies documented joint soft tissues believed to be responsible for the limitation in ROM of the joint following immobilization. A recent review proposed that the myogenic limitation predominates during the first 90 d of immobility, whereas the limitation is mainly arthrogenic beyond 90 d of immobility (Harburn and Potter, 1993). Our previous investigation determined that the limitation in joint ROM at 1 mo after immobilization is derived from myogenic change and that the arthrogenic limitation increases at 2-3 mo after immobilization (Okamoto et al., 2004). The limitation in joint ROM due to myogenic change is referred to as muscle contracture; this mechanism may be related to alteration of intramuscular (IM) connective tissue.

IM connective tissue displays a three-tier organization: the epimysium or fascia surrounds whole muscles, the perimysium binds muscle fibers into bundles, and the endomysium outlines individual muscle cells. Collagen is the main fibrous protein in IM connective tissue; additionally, collagen elasticity depends not only on intra- and intermolecular cross-links but also on the arrangement of fibrils (Borg and Caulfield,

1980; Rowe, 1981). Purslow and Trotter (1994) reported that the orientation of collagen fibril distribution in the endomysium exhibits a progressive shift in the circumferential direction at short sarcomere length and in the longitudinal direction at long sarcomere length. Therefore, collagen fibrils in the endomysium run in various directions during muscle relaxation, moving longitudinally to the axis of the muscle fiber on muscle stretching. This re-orientation of the fibrils may reflect the endomysium's role in providing mechanical support to the fiber's surface and its action as an elastic device for contraction-relaxation cycles; moreover, fibril arrangement in the endomysium is thought to be related to muscle elasticity (Borg and Caulfield, 1980).

In terms of changes in the endomysium with respect to the mechanism underlying muscle contracture, earlier investigations consisted predominantly of morphological analysis. J□rvinen et al. (2002) reported that the number of circular and rectangular-oriented collagen fibers increased at the contacts of two adjacent muscle fibers in the endomysium 3 wks after immobilization of rat calf muscles. Our previous study noted that collagen fibril arrangement in the endomysium 1 and 2 wks after immobilization in rat soleus muscles was longitudinal to the axis of the muscle fibers, whereas at 4, 8 and 12 wks after immobilization, fibril arrangement was circumferential. Such changes in collagen fibril arrangement are indicative of decreased collagen fibril movement in the endomysium of immobilized muscle (Okita

et al., 2004).

Ultrasound treatment is commonly used in rehabilitation programs following soft tissue injuries (Locke and Nussbaum, 2001). Ultrasound is thought to exert thermal and mechanical effects on the target tissue resulting in increased local metabolism, circulation, extensibility of connective tissue and tissue regeneration (van der Windt et al., 1999). The current study assessed these effects with respect to increased extensibility of connective tissue. An earlier investigation documented increased elasticity of collagen fibers and facilitated lengthening of contracted tissue at temperature levels greater than 40 °C (Lehman et al., 1970; Zietara and Skorkowski, 1995). The continuous ultrasonic wave is suitable for warming of deep tissues, *e.g.*, skeletal muscle. In fact, Locke and Nussbaum (2001) reported that continuous ultrasonic wave (frequency, 1 MHz; intensity, 1.0 W/cm^2) application at the triceps surae muscle of rat for 15 min led to a peak muscle temperature of 41.9 °C. Upon continuous ultrasound (frequency, 1 MHz; intensity, 1.5 W/cm^2) application at the medial gastrocnemius muscle for 10 min in humans, the mean temperature reached 40.3 °C, which was an increase of 4.9 °C (Draper et al., 1993a). Therapeutic ultrasound focuses primarily on the alteration of extensibility of collagenous tissues in order to improve ROM (Markert et al., 2005); therefore, this treatment is widely utilized to treat joint contracture in a rehabilitative setting. Little is known, however, regarding the effects of therapeutic ultrasound on collagen fibril arrangement in the

endomysium of immobilized muscle. The objective of this study was to clarify the effect of therapeutic ultrasound on joint mobility and collagen fibril arrangement in the endomysium of immobilized rat soleus muscle.

MATERIALS AND METHODS

Measurement of core and muscle temperatures

All experiments and procedures were approved by the Ethics Review Committee for Animal Experimentation at Nagasaki University. Animals were housed in cages inside a room with a 12-h dark/light cycle. The temperature and relative humidity of the room were maintained at 25 °C and 50 %, respectively. Food and water were available *ad libitum*.

In a pilot study, time course changes of core and muscle temperatures were measured during therapeutic ultrasound. Ten 8-wk-old male SPF Wistar rats obtained from CLEA (Tokyo, Japan) were randomly divided into the ultrasound (n=5) and sham (n=5) treatment groups. Animals were anesthetized with pentobarbital sodium (40 mg/kg); subsequently, all hair on the lower hind limb was removed and a needle thermo-sensor (PTN-800, Unique Medical Inc, Tokyo, Japan) was carefully inserted along each Achilles tendon into the deep portion of the triceps surae muscle group. The tip of the needle thermo-sensor was positioned at a tissue depth of about 1 cm within the ultrasound treatment area. The diameter of the needle thermo-sensor was

0.6 mm; moreover, the needle surface was coated with epoxy for heat insulation. Simultaneously, a cannular thermo-sensor (PTI-200, Unique Medical Inc, Tokyo, Japan) was inserted 6 to 7 cm past the anal sphincter into the colon. Following attachment of thermo-sensors to a digital thermometer (PTW-301, Unique Medical Inc, Tokyo, Japan), the triceps surae muscle in the right hind limb of each rat was exposed to ultrasound or sham treatment for 15 min. The temperature of the experimental room was maintained at 25 °C. During treatment, core and muscle temperatures were recorded to the nearest 0.1 °C every 2.5 min, or until no temperature increase was observed on three consecutive readings. Additionally, these temperatures were recorded for 10 min following cessation of treatment.

Animals

Twenty-two 8-wk-old male SPF Wistar rats obtained from CLEA (Tokyo, Japan) were randomly divided into the experimental (n=17) and control (n=5) groups. Control rats were untreated. Animals of the experimental group were anesthetized with pentobarbital sodium (40 mg/kg); subsequently, their bilateral ankle joints were fixed in full plantar flexion with plaster casts with the soleus muscles immobilized in a shortened position. The plaster cast, which was positioned from above the knee joint to the distal foot, was changed weekly consequent to loosening due to muscle atrophy. Bilateral ankle joints of five animals in the experimental group were

immobilized throughout the 4-wk experimental period (immobilization group). The remaining rats in the experimental group were randomly divided into the ultrasound (US, n=6) and sham (n=6) treatment groups. For ultrasound or sham treatment, bilateral ankle casts of each rat in the US and sham groups were removed under pentobarbital sodium anesthesia (40 mg/kg) 6 d per wk during the 4-wk immobilization period. Each treatment was performed for 15 min per d, 6 d per wk over the 4-wk immobilization period. Following completion of the daily treatment, bilateral ankle joints were re-immobilized via the identical method.

Ultrasound treatment

To obtain increases in the elastic properties of collagen, Draper et al. (1995a,b) suggested that tissue temperature increases greater than 3 to 4 °C were necessary. Locke and Nussbaum (2001) reported that continuous ultrasonic wave (frequency, 1 MHz; intensity, 1.0 W/cm^2) application at the triceps surae muscle of rat for 15 min increased temperature by 2 to 6 °C. In this study, frequency, intensity and irradiation time were determined for these reports as a reference (Draper et al., 1995a, 1995b; Locke and Nussbaum, 2001).

Therapeutic ultrasound was applied with an Ultrasound US-3 (Itoh Physio-therapy and Rehabilitation Ltd, Tokyo, Japan). The frequency was 1 MHz, beam nonuniformity ration was 5.0, effective radiating area was 0.7 cm^2, area of applicator

radiating surface was 0.785 cm^2 and modulation frequency was 100 Hz±5 %. Aqueous gel for ultrasound treatment served as a coupling medium. The triceps surae muscle in the US group was irradiated for 15 min with a continuous wave (intensity, 1.0 W/cm^2). To deliver ultrasonic irradiation equally to the entire muscle, the ultrasound transducer head was moved in circular fashion over an area approximately twice the size of the effective radiating area. Ultrasonic energy was not delivered to the triceps surae muscle in the sham group; only the transducer head was moved.

Measurement of range of motion on ankle joint dorsiflexion

At 4 wks following immobilization, animals of all groups were anesthetized with pentobarbital sodium (40 mg/kg). Following body weight measurements, the range of motion (ROM) on dorsiflexion of the ankle joint was obtained with a goniometer as described in previous reports (Williams, 1988, Okita et al., 2004).

Measurement of ROM was defined as the angle (0 to 180 degrees) between a straight line connecting the fifth metatarsal and malleolus lateralis of the fibula to a line connecting the malleolus lateralis of the fibula and the center of the knee joint when the ankle was passively dorsiflexed maximally and the knee joint flexed 90 degrees. One-way analysis of variance (ANOVA) was used for statistical evaluation. When ANOVA yielded a significant F value ($p<0.05$), pair-wise comparisons were

conducted with Fisher's PLSD method.

Morphological analysis of the endomysium

Following measurement of ROM, the right soleus muscle was excised, stretched with a 4-g sinker as described in our previous study (Okita et al., 2004), and fixed in 3 % glutaraldehyde in 0.1 mol/L cacodylate buffer, pH 7.4. The cell-maceration method of Ohtani et al. (1988) was utilized. After initial fixation in 3 % glutaraldehyde, right soleus muscles were cut into 3×3×3 mm blocks and immersed in 10 % aqueous sodium hydroxide solution for 14 d at room temperature; subsequently, tissues were rinsed in distilled water for 3 d. These samples were placed in a 1 % aqueous tannic acid solution for 2 h and rinsed in distilled water for 1 h, followed by postfixing in a 1 % aqueous solution of osmium tetroxide for 2 h, after which they were dehydrated in a series of graded concentrations of ethanol and dried by the t-butyl alcohol freeze-drying method (Inoue and Osatake, 1988). The dried specimens were mounted on metal stubs and coated with gold, after which the collagen fibril arrangement in the endomysium was examined and micrographs produced with a scanning electron microscope (SEM; JSM-6700F/IV, JEOL. Tokyo, Japan) at final magnification of x20,000. SEM micrographs of the longitudinal surfaces of the cut strips were generated. Care was taken to ensure that micrographs used to analyze collagen fibril orientation in the endomysium were obtained from specimens oriented

completely perpendicular to the line of view. The upper left angles formed by the two lines of the collagen fibril segment and the direction parallel to the muscle fiber axis on the photomicrographs (0 to 180 degrees) were determined. The photomicrograph area was 27 μm^2 (4.5×6 μm). All angles formed by the two lines on a photomicrograph were measured; 20 photomicrographs were acquired from 20 random muscle fibers per soleus muscle. More than 3,000 angles were obtained from a group. Morphometric analyses of the photomicrographs were conducted with the double blind method employing an Apple Macintosh personal computer and the public domain NIH Image program.

Histological observation

Extracted left muscle samples were frozen in isopentane cooled to the freezing point with liquid nitrogen. Serial cross sections (7 μm) were prepared on a cryostat, followed by staining with hematoxylin and eosin (H&E).

RESULTS

Core and muscle temperatures

Figure 1 presents data for the time course changes in core and muscle temperatures in the US and sham groups. Before ultrasound or sham treatment, means ± SD for core temperature were 36.0±0.9 °C in the US and 36.5±0.6 °C in the sham groups.

The core temperature was unaltered during ultrasound or sham treatment (Fig. 1A). Before ultrasound or sham treatment, means ± SD for muscle temperature were 34.3±0.8 °C in the US and 34.3±0.9 °C in the sham groups. The muscle temperature in the sham group was unchanged during treatment. During irradiation with ultrasound, however, muscle temperature in the US group gradually increased for seven minutes, reaching a level of 40.6±0.4 °C. Subsequently, muscle temperature in the US group scarcely changed until the cessation of ultrasound treatment, after which it gradually decreased (Fig. 1B).

Range of motion (ROM)

Figure 2 presents data for ROM on dorsiflexion for all groups. Means ± SD for ROM on dorsiflexion in experimental animals were 68.5±11.1 degrees in the immobilization, 63.8±5.3 degrees in the sham and 81.3±6.1 degrees in the US groups. ROM on dorsiflexion in the immobilization, sham and US groups was significantly smaller than that of the control group; on the other hand, ROM in the US group was significantly larger than ROM in the immobilization and sham groups.

Collagen fibril arrangement in the endomysium

A representative scanning electron micrograph of collagen fibril arrangement in the endomysium is shown in Fig. 3. The muscle fiber axis runs horizontally. Many

longitudinal collagen fibril components are present in the control group; however, in the experimental group, this arrangement is apparent only in the US group (Fig. 3A and D). In the immobilization and sham groups, however, circumferential collagen fibril components are increased (Fig. 3B and C). The percent distributions of the form angle of the direction parallel to the muscle fiber axis and directional angle of the collagen fibrils appear in Fig. 4. Two angle peaks of 0 to 50 and 130 to 180 degrees are evident in the control group (Fig. 4A). The US group also exhibits two peaks for the same angles (Fig. 4D); in contrast, only one peak is observed for the angle between 50 to 130 degrees in the immobilization and sham groups (Fig. 4B and C).

Histological changes

Routine histological analysis of the immobilization group revealed atrophic changes, including decreased fiber diameter, increased density of nuclei and expansion of the perimysium (Fig. 5B), relative to the control group (Fig. 5A). The abnormal findings, with the exception of the atrophic changes, were not apparent in the sham and US groups (Fig. 5C and D). Furthermore, the remarkable change related to connective tissue of the US group was not evident (Fig. 5D).

DISCUSSION

The findings of our pilot study revealed that core temperature did not change;

rather, peak muscle temperature increased by approximately 6 °C during ultrasound irradiation. Upon application of ultrasound (frequency, 1 MHz; intensity, 1.0 W/cm^2) for 15 min at the triceps surae muscle of rat in a previous investigation, muscle temperature increased by 2 to 6 °C (Locke and Nussbaum, 2001), which was consistent with the current results.

Ultrasound with continuous mode is a method for the application of deep heat to connective tissue. The extensibility of animal tendons has been shown to increase with the application of ultrasound (Lehman et al., 1970; Gersten, 1955). Draper et al. (1993b; 1995a) found that continuous ultrasound (frequency, 1 MHz; intensity, 1.5 W/cm^2) for 7 to 8 min was sufficient to increase the tissue temperature of the triceps surae muscle in humans, which resulted in elastic changes in collagen. To obtain increases in the elastic properties of collagen, an elevation in tissue temperature of greater than 3 to 4 °C is indicated (Draper et al., 1995a, 1995b). Accordingly, in this study, we surmised that muscle contracture in an animal model may be enhanced in the presence of ultrasonic irradiation methodology.

The current findings demonstrated declines in ROM of dorsiflexion to 42.8% and 39.9% of the control value in the immobilization and sham groups, respectively; no significant difference was observed between the immobilization and sham groups in terms of ROM on dorsiflexion. ROM on dorsiflexion in the US group decreased to 50.8% of the control value; on the other hand, in the US group, ROM was significantly

larger than that in the immobilization and sham groups. These data suggested that therapeutic ultrasound with continuous wave at an intensity of 1.0 W/cm^2 inhibits the progress of the limitation in ROM.

Many collagen fibrils in the endomysia of the control group were longitudinal. However, the arrangement of approximately 70% of the collagen fibrils had shifted in the circumferential direction in the immobilization and sham groups. In general, collagen fibrils in the endomysium are oriented in various directions during muscle relaxation and move longitudinally toward the axis of the muscle fiber during muscle stretching (Borg and Caulfield, 1980; Purslow and Trotter, 1994). When normal muscle is stretched, collagen fibers are extended in advance of muscle fibers (Stolov et al., 1973; Spector et al., 1982). Collagen fibril orientation displays a progressive shift in the circumferential direction at short sarcomere length and in the longitudinal direction at longer sarcomere length (Purslow and Trotter, 1994). In this study, muscle samples were stretched with a 4-g sinker, after which they were fixed for comparison of the collagen fibril arrangement in the endomysium in all groups during stretching under identical force. In the immobilization and sham groups, collagen fibril distribution increased in the circumferential direction; this result was consistent with that of J□rvinen et al. (2002) as well as that of our previous investigation (Okita et al., 2004). This change was suggestive of the decrease in collagen fibril movement and reduction of extensibility in the endomysium. After 9 wk of

immobilization, collagen fiber arrangement in the cruciate ligament of rabbit exhibited obvious distortion, which is indicative of a more random matrix organization (Akeson et al., 1980). This report also documented reduced water and glucosaminoglycan contents in rabbit cruciate ligament 9 wk after immobilization. Such changes facilitate the formation of cross-links between adjacent collagen fibrils and/or intra- and intermolecular cross-links, which become more stable and mature with time. These cross-links provide greater resistance to deformation and decrease collagen fiber movement, which is indicative of a circumferential component (Akeson et al., 1980).

In the US group, the collagen fibril arrangement in the endomysium was approximated to that of the control group. Additionally, two angle peaks of 0 to 50 and 130 to 180 degrees in the percent distributions of the form angle of the direction parallel to the muscle fiber axis were present in the US group, which was similar to the control group. Therefore, our data suggested that ultrasound irradiation with continuous mode during the immobilization process may prevent decreased collagen fibril movement in the endomysium.

Continuous ultrasound is believed to exert thermal and mechanical effects (van der Windt et al., 1999). However, we hypothesized that inhibition of decrease in collagen fibril movement in the US group may reflect an elevation in muscle temperature. Very few studies have been conducted in animals and humans involving heat with respect to extensibility of soft tissues and ROM. Research

performed on rat tail tendons indicates that stretching while the tissue is heated to temperatures between 39 °C and 45 °C results in permanent elongation of the tissue (Lehmann et al., 1970; Warren et al., 1971, 1976). Usuba et al. (2006) reported that stretching with infrared or ultrasound was more effective than stretching in the absence of heat in terms of increasing the ROM and decreasing the phase lag of moderately severe joint contracture. In a human study, Taylor et al. (1995) found that hamstring flexibility can be improved by the application of heat packs before stretching. Wessling et al. (1987) noted that ultrasound applied to the muscle belly before stretching promotes significantly greater immediate gains in ankle dorsiflexion in comparison with stretching alone. Knight et al. (2001) reported that the use of ultrasound for 7 min before stretching may be the most effective approach for increasing ankle dorsiflexion ROM. These investigations suggested the effectiveness of the combination of stretching and warming of the tissue by therapeutic ultrasound in terms of improvement of extensibility in tissue and joint ROM. In previous reports, however, the effect of therapeutic ultrasound alone is unclear. The current study is the first to document that ultrasound intervention alone during immobilization can prevent decreased joint mobility and collagen fibril movement in the endomysium. Immobilization of a joint induces the formation of cross-links in IM collagen (Hibino et al., 2008); additionally, ultrasound treatment with continuous mode may inhibit the formation of cross-links. Moreover, this mechanism may be

related to our findings.

On the other hand, Sugama et al. (1998) suggested continuous ultrasound irradiation (frequency, 3 MHz; intensity, 0.5 W/cm^2) can accelerate amelioration of intra- and intermolecular cross-links in IM collagen following immobilization of rat soleus muscle; however, in comparison with our experiment, little irradiated ultrasonic energy was applied. We initially presumed that the result associated with the US group in the current study was attributable exclusively to the thermal effect. However, it may be appropriate to consider the influence of a mechanical effect based on the report of Sugama et al. (1998).

Histological observation revealed muscle fiber atrophy, increased density of nuclei and expansion of the perimysium in the immobilization, sham and US groups. These changes were caused by immobilization during the 4-wk period. On the other hand, despite continuous ultrasonic irradiation over the 4-wk immobilization period, necrosis of muscle fibers was not induced. Therefore, it appears that therapeutic ultrasound with continuous mode does not negatively influence atrophied muscle fibers. The mechanical stimulus of ultrasound is known to increase collagen fibers and proteoglycan synthesis (Da Chuha et al., 2001, Parvizi et al., 1999). In contrast, in this investigation, a specific change in connective tissue was not observed in the US group. We hypothesize that biochemical evaluation is required in future experiments.

In conclusion, the current results suggested that 4-wk immobilization of rat soleus muscle induced a decrease in ROM on dorsiflexion of the ankle joint and an increase in the circumferential direction in collagen fibrils in the endomysium. This alteration in arrangement was indicative of the decrease in collagen fibril movement and reduction of extensibility in the endomysium. On the other hand, although intervention with ultrasound (frequency, 1 MHz; intensity, 1.0 W/cm^2) for 15 min per d during the 4-wk immobilization process is not perfect, it can inhibit these changes. Consequently, ultrasound treatment with continuous mode may be effective with respect to prevention of the deterioration of muscle contracture.

Acknowledgments- The authors thank Mr. T. Suematsu, of the Microscopic Center, Nagasaki University School of Medicine, for his assistance with scanning electron microscopy. This research was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan (Grant no. 18500428) and a Nagasaki University President's Fund Grant.

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Figure legends

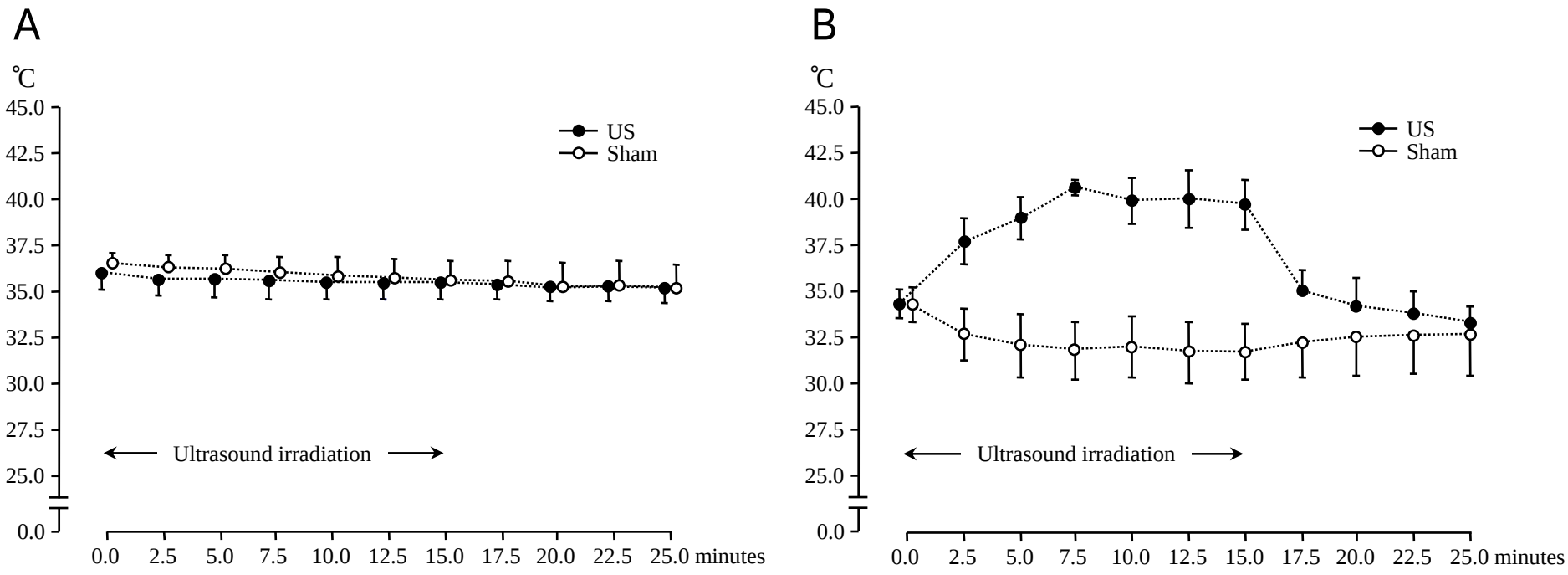
Fig. 1. Time course changes in core and muscle temperatures in the US and sham groups. Values are means ± SD. (A) Core temperature. (B) muscle temperature.

Fig. 2. Range of motion on ankle dorsiflexion in all groups. Values are means ± SD. *compared with control group; † compared with immobilization group; ‡ compared with sham group.

Fig. 3. Scanning electron micrographs of soleus muscle endomysia. (A) Control. (B) Immobilization. (C)Sham. (D) US. Bar = 1 μm.

Fig. 4. Percent distributions of the angle of direction formed parallel to the muscle fiber axis and the directional angles of collagen fibrils. Two peaks displaying angles of 0 to 50 and 130 to 180 degrees are evident in the control group. In the experimental group, US rats also exhibit two peaks for the same angles; in contrast, only one peak corresponding to the 50 to 130 degree angles is observed in the immobilization and sham groups. (A) Control. (B) Immobilization. (C) Sham. (D) US.

Fig. 5. Light micrographs of soleus muscle (H&E staining). (A)Control. (B)Immobilization. (C)Sham. (D)US. Bar = 100 μm.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32.5
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27.5
25.0
0.0
US
Sham
Ultrasound irradiation
0.0 2.5 5.0 7.5 10.0 12.5 15.0 17.5 20.0 22.5 25.0 minutes

Fig. 1.

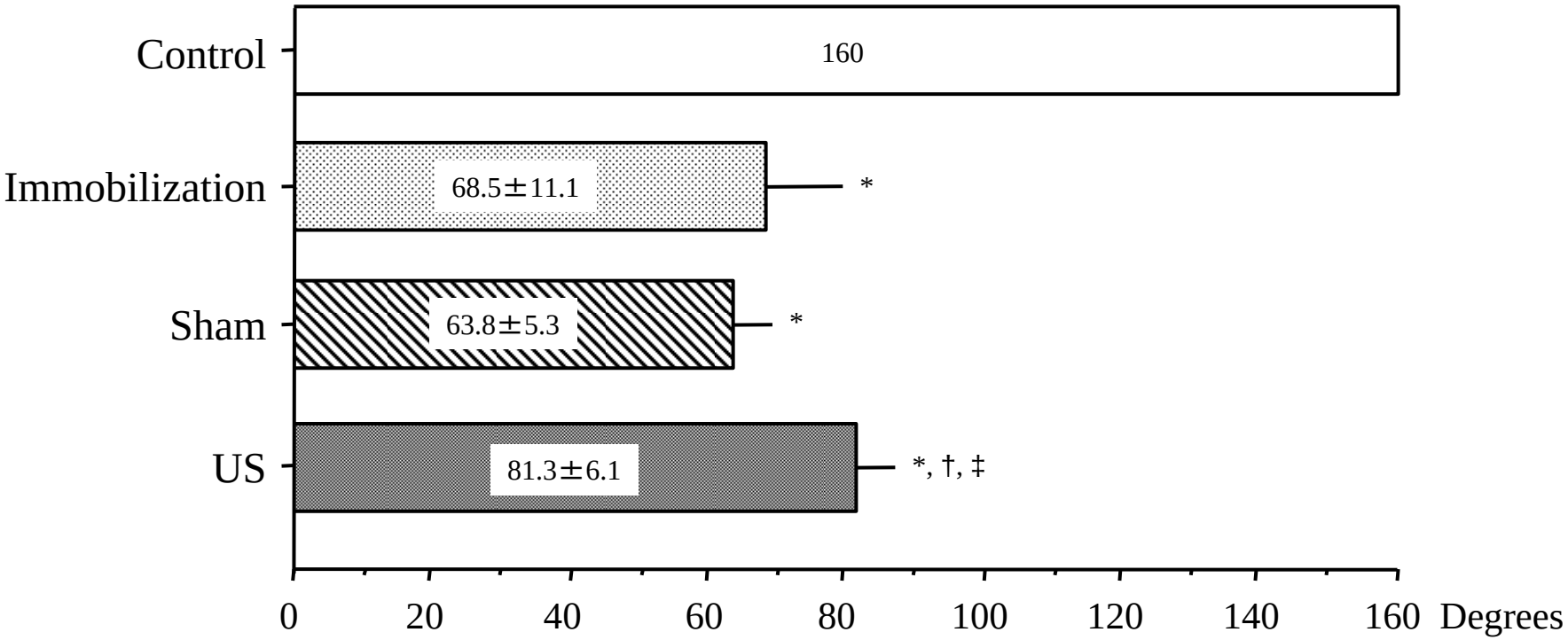
Control
Immobilization
Sham
US
160
68.5±11.1
63.8±5.3
81.3±6.1
*
*
*, †, ‡
0
20
40
60
80
100
120
140
160
Degrees

Fig. 2.

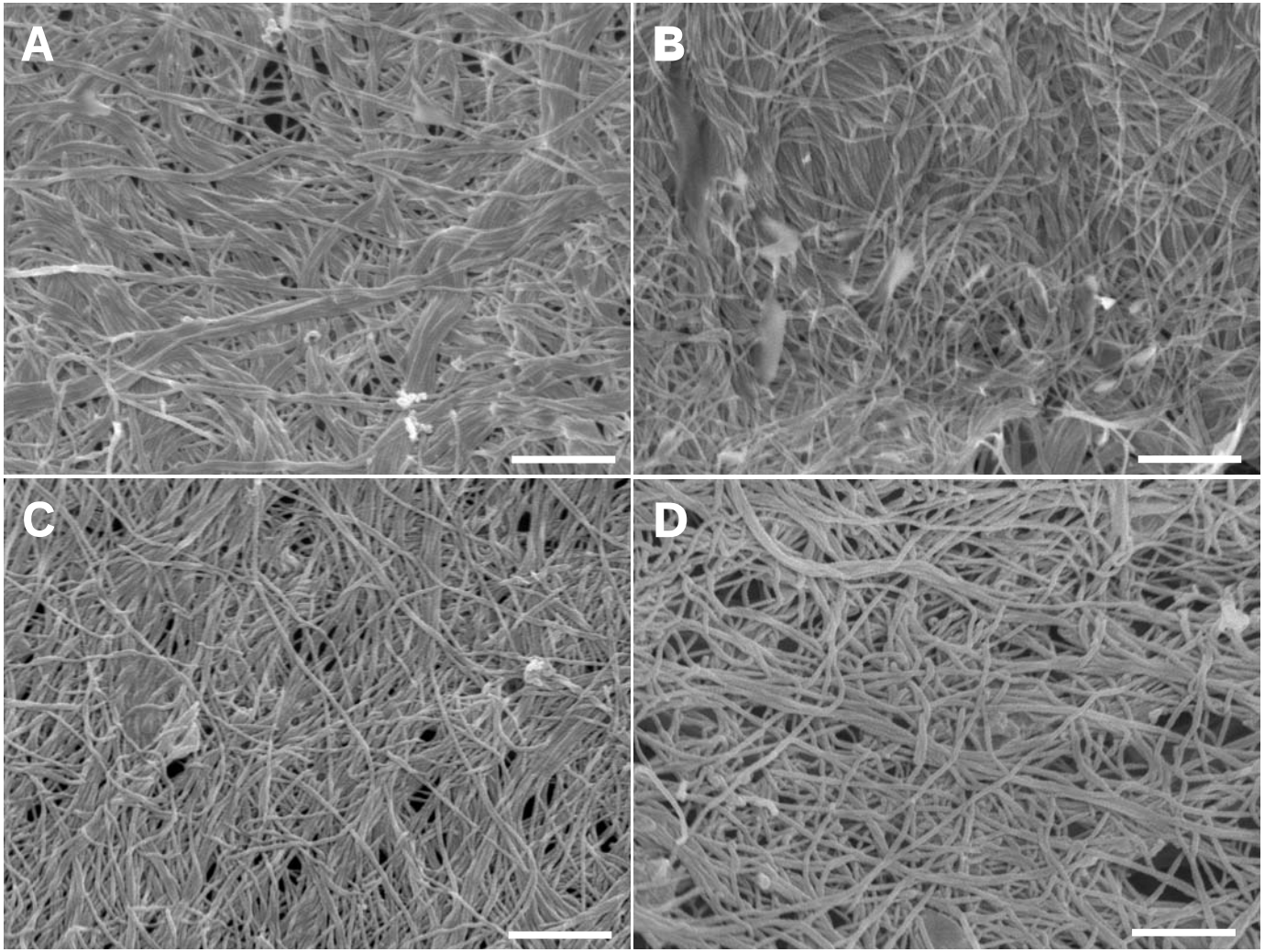

Fig. 3.

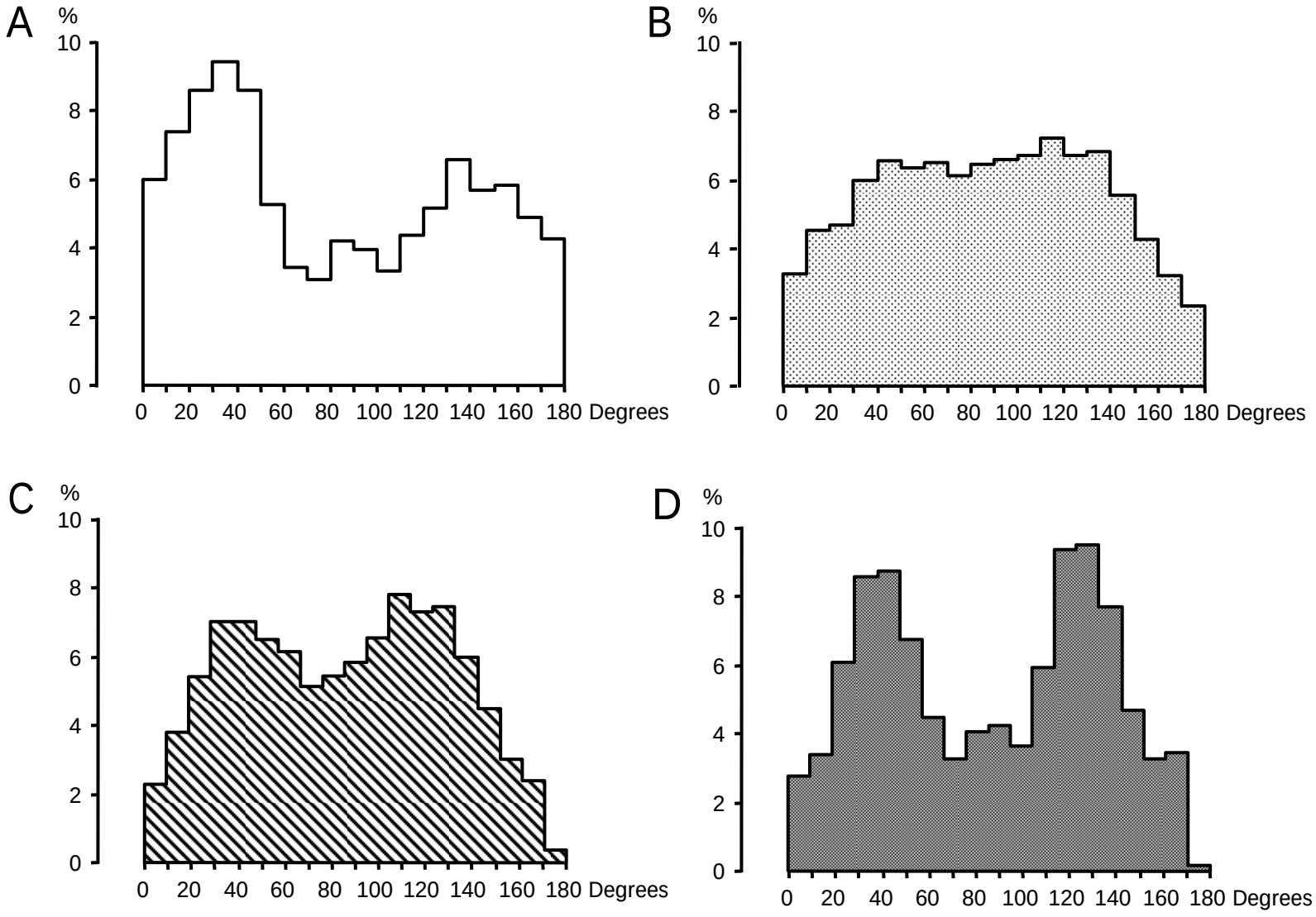
A
%
10
8
6
4
2
0
0 20 40 60 80 100 120 140 160 180 Degrees
B
%
10
8
6
4
2
0
0 20 40 60 80 100 120 140 160 180 Degrees
C
%
10
8
6
4
2
0
0 20 40 60 80 100 120 140 160 180 Degrees
D
%
10
8
6
4
2
0
0 20 40 60 80 100 120 140 160 180 Degrees

Fig. 4.

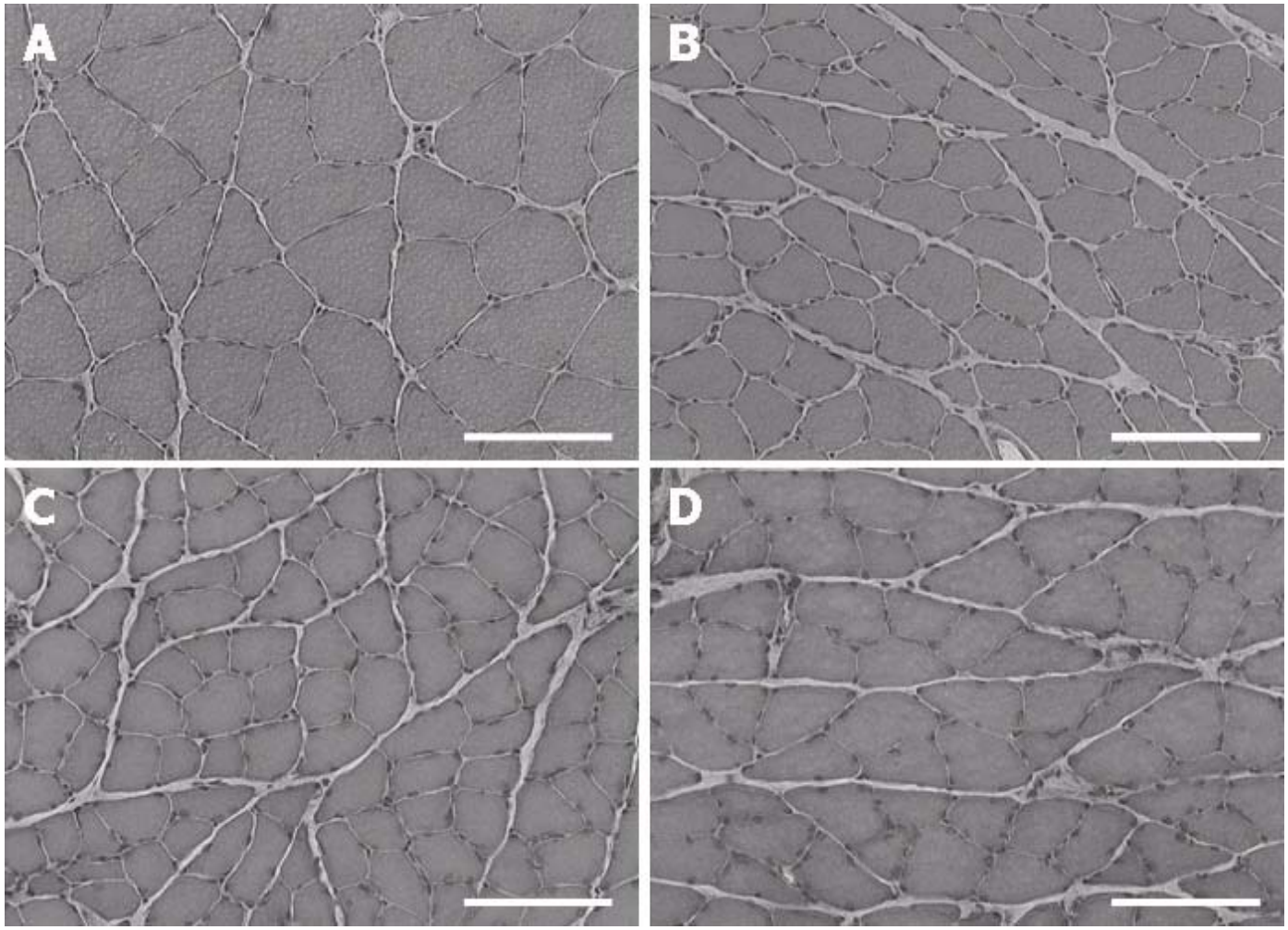

Fig. 5.