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# 博　士　論　文

## Effects of stretch on muscle regeneration in the damaged mouse soleus muscle after eccentric exercise.

（伸張性筋活動後の損傷マウスヒラメ筋の再生にストレッチが及ぼす影響）

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# Effects of stretch on muscle regeneration in the damaged mouse soleus muscle after eccentric exercise.

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In the present study, effects of stretch on muscle regeneration were morphologically examined in the soleus muscle injured by downhill running (eccentric exercise). In this study, 133 adult male ddY mice were divided into control (C: n=39), running (R: n=47) and stretch after running (RS: n=47) groups, and at 12 hrs, 1, 2, 3, 5, 7 and 14 days after the running, these animals were sacrificed. To investigate sarcolemmal permeability, Evens blue dye (EBD) was beforehand injected into the peritoneal cavity of some mice. In the R group, area of muscle fibers was larger by 1 day after the running, and recovered to the control level after 5 days. In contrast, in the RS group, the area was smaller by 1 day and recovered to the control level by 2 days. After the running, narrow basophilic sarcoplasms appeared along the margin of the muscle fibers. Electron microscopically, these areas contained several mitochondria and ribosomes, suggesting that these morphological changes are regenerative rather than degenerative events. These areas began to appear earlier and muscle regeneration completed earlier in the RS group than the R group. Our findings suggested that stretch soon after the exercise can reduce delayed onset muscle soreness (DOMS), and promote muscle regeneration after the muscle injury.

**Keywords**: stretch, muscle damage, muscle regeneration, eccentric exercise, DOMS

## Introduction

Muscle soreness and stiffness, so called delayed onset muscle soreness (DOMS), occur usually 24-48 hours after unusual or inappropriate exercise [1, 2]. In many cases, the DOMS naturally vanishes within a few days, but in some cases, muscle soreness and stiffness persist longer than several days, if the care after the exercise was unsuitable. Morphologically, it was reported that disruption of sarcolemma and myofilaments, Z-band streaming, degeneration and necrosis of muscle fibers occurred in the skeletal muscles

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after the extensive eccentric exercise [3-7]. Armstrong et al. [4] reported that these muscle damages were more evident after the downhill running (eccentric exercise) than the level or uphill running (isometric exercise). Since the DOMS occurs after eccentric exercise, rather than after isometric exercise, it is suggested that one of the main causes of the DOMS might be the damage of muscle fibers induced by extensive eccentric exercise. Adequate warm-up is customarily recommended before exercise to prevent muscle, tendon and ligament injuries. After the exercise, though rest and icing are customarily recommended to prevent the secondary injury and inflammation in the sports medicine, adequate exercise including mild stretch is also recommended.

It has been said that stretch, as mechanical signal, promotes the normal development and adaptive growth of muscle fibers in vivo and in vitro, and recovery from disused muscle atrophy after joint fixation and muscle immobilization [8] through additional production of constriction proteins [9, 10] and new sarcomeres [11], changing the muscle phenotype [11-13] and activation of satellite cells [14]. Since activation of satellite cells and production of constriction proteins are essential for muscle recovery from the muscle injury, these findings suggest that stretch might be effective also for muscle recovery after the muscle injury. In the present study, therefore, influence of stretch on muscle regeneration was light and electron microscopically examined in the damaged mouse muscle after the eccentric exercise.

## Materials and Methods

In the present study, 133 male Std-ddY mice (age: 11 weeks, body weight: 40-45g at the start of the experiments) were used. They were housed in standard cages in a temperature-controlled room with a 12:12h light-dark cycle, and food and water were provided ad libitum. All experiments were conducted according to the Guidelines for Animal Experimentation at Kobe University School of Medicine.

These mice were randomly divided into control (C: n=39), running (R: n=47) and stretch (RS: n=47) groups. The mice of the C group were not imposed any exercise or stretch. The animals of the R and RS groups were imposed an intense eccentric exercise on soleus muscle with running on a treadmill (MAT-2100, Fukuda Denshi, Tokyo, Japan) down a 16 degrees at 500 m/h for 90 min [4,5]. In both R and RS group, these animals were anesthetized with an intraperitoneal injection of sodium pentobarbital (Nembutal, 50 mg/kg body weight) at 30 min after the running. The mice of the RS group were received a passive stretch

for 30 min under the anesthesia, while these of the R group were not. In the RS group, the bilateral ankles were fixed in maximally dorsiflexion with non-elastic tape under the anesthesia according to Okita et al. [8]. With this method, the soleus muscles were completely stretched. At 12 hrs, 1, 2, 3, 5, 7 and 14 days after the running, the mice (5 animals at each period) of the R, RS and C groups at the corresponding age were sacrificed under the anesthesia, and bilateral soleus muscles were extracted.

**Morphological examination**

The right soleus muscles were immediately frozen in acetone cooled by dry ice. For light microscopy, cross serial sections 5 μm in thickness were cut with a cryostat (5030 Microtome, Bright Instruments, Japan) at –20℃, mounted on glass slides, stained with Carazzi's hematoxylin and eosin (H-E stain), and examined under an ordinary light microscope (BX50F4, Olympus, Tokyo, Japan).

The left soleus muscles were immediately cut into small pieces with razor blade, fixed with a mixture of 4% paraformaldehyde and 2.5% glutaraldehyde dissolved in Millonig's phosphate buffer (pH 7.4) for 24 hrs at 4℃, washed for 2 hrs in the same buffer, and postfixed in 1% osmium tetroxide dissolved in the same buffer for 2 hrs at 4 ℃ . These materials were then dehydrated in grades alcohols, cleared with propylenoxide and embedded in an epoxy resin mixture (Quetol 812, Nissin EM, Tokyo, Japan) as usual. Longitudinal sections 1 μm in thickness were serially cut with a diamond knife, stained with 1% toluidine blue solution and examined under the light microscope. For electron microscopy, longitudinal ulrathin sections were cut serially with a diamond knife, contrasted with uranyl acetate and lead citrate and examined in a transmission electron microscope (JEM-1200, JEOL, Tokyo, Japan) at 80 kv.

To examine the sarcoplasmic permeability in the damaged skeletal muscle fibers, 1% Evans blue dye (EBD, Sigma, St Louis, USA) dissolved in phosphate buffered saline (PBS, pH 7.5) was injected into the peritoneal cavity of some mice of each group (C: n=1, R: n=3 and RS: n=3) at 24 hrs before the sacrifice [15, 16], and the animals were killed at 12 hrs, 1, 2 and 3 days after the running. Bilateral soleus muscles from the EBD-injected animals were removed and frozen in acetone cooled by dry ice. Cross sections 5 μm thick were cut serially with the cryostat, and examined under a fluorescent light microscope (Fluoview, FV300, Olympus, Tokyo, Japan). Some next sections were stained with H-E for comparison with the EBD stain.

**Quantitative analysis**

Muscle fiber area

The analysis for the muscle fiber area was performed with a computerized image processing system program (Scion Image Beta 4.02, Scion) and a statistical software program (Excel, Microsoft). At least 400 fibers from each sample were used for statistical analysis. A one-way analysis of variance (ANOVA) was used to determine relative differences of muscle fiber area among the three groups (C, R and RS) at each period. If the difference was significant ($p < 0.05$) by the ANOVA, pairwise comparisons were further examined with Fisher's PLSD method.

Necrotic fiber

The necrotic fiber in the entire cross section of the muscle was quantified, and was assessed for presence of intracellular infiltration of phagocyte and centrally located nuclei, according to Rosenberg *et al.* [17]. Kruskal-Wallis was used to test relative differences of necrotic fibers among the three groups at each period, and pairwise comparisons were further examined with Mann-Whitney when the difference was significant ($p < 0.05$).

## Results

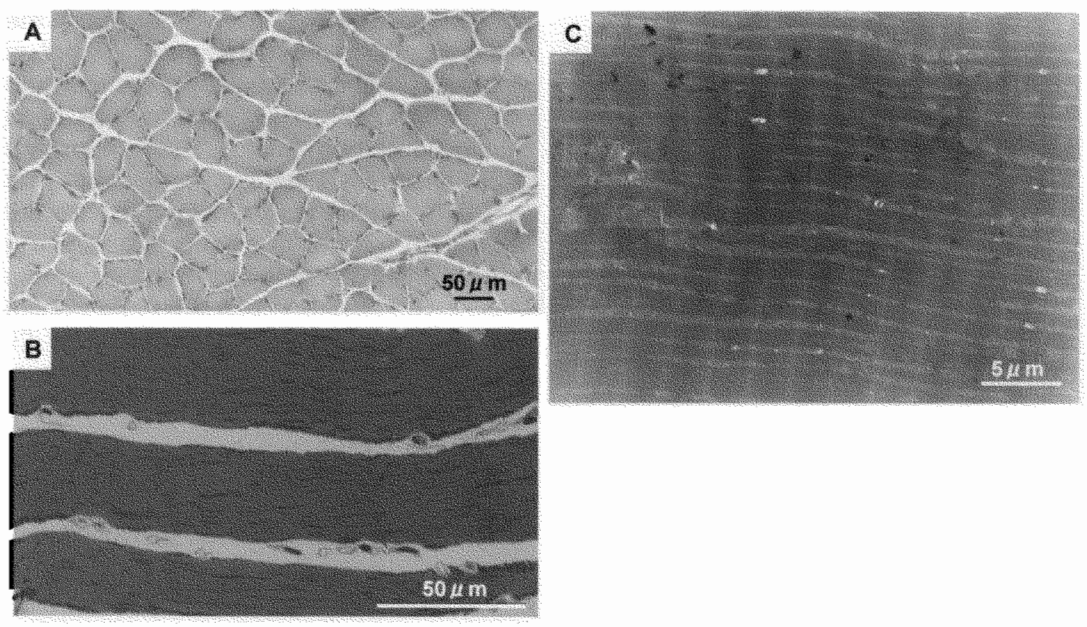


Fig. 1 Normal appearance of the soleus muscle. Muscle fibers are polygonal in shape, the spaces among the muscle fibers are narrow, and 10-20 muscle fibers are grouped by thin connective tissue (A). The muscle fibers run almost parallel with each other, and the contour of the muscle fibers is smooth (B). In the sarcoplasm, regular cross striations are observed (C).

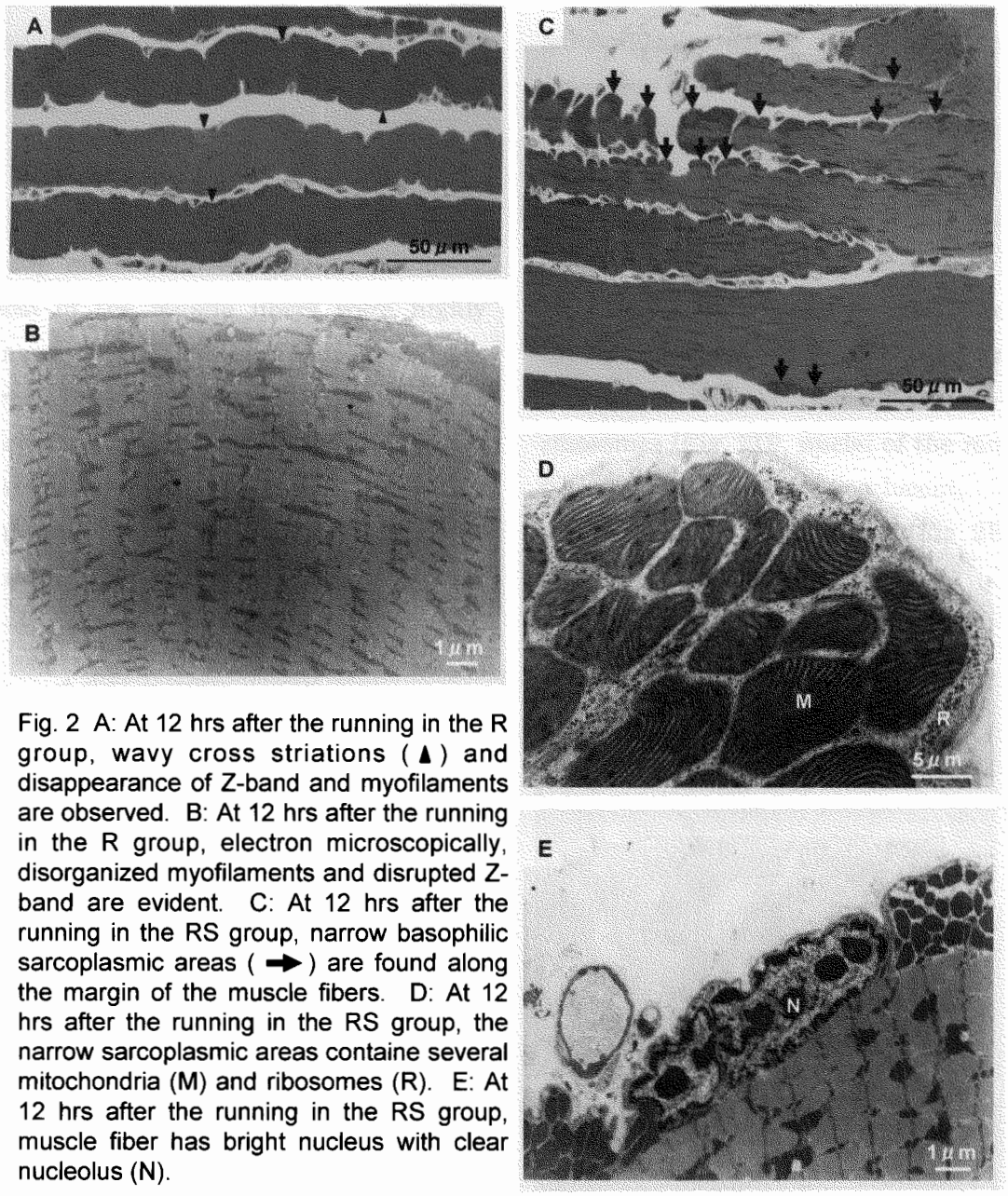


Fig. 2 A: At 12 hrs after the running in the R group, wavy cross striations (▲) and disappearance of Z-band and myofilaments are observed. B: At 12 hrs after the running in the R group, electron microscopically, disorganized myofilaments and disrupted Z-band are evident. C: At 12 hrs after the running in the RS group, narrow basophilic sarcoplasmic areas (➡) are found along the margin of the muscle fibers. D: At 12 hrs after the running in the RS group, the narrow sarcoplasmic areas containe several mitochondria (M) and ribosomes (R). E: At 12 hrs after the running in the RS group, muscle fiber has bright nucleus with clear nucleolus (N).

**Morphological observations**

1) Normal appearance of the soleus muscle

In the transverse sections of the normal mouse soleus muscle (control group), muscle fibers were polygonal in shape, and the sarcoplasm was uniformly stained throughout the muscle fibers. Spaces among the muscle fibers were narrow, and 10-20 muscle fibers were grouped by thin connective tissue (Fig. 1A). In the longitudinal sections, the muscle fibers run almost parallel with each other, and the contour of the muscle

fibers was smooth (Fig. 1B). In the sarcoplasm, regular cross striations were observed (Fig. 1C)

2) Twelve hours after the running

Light microscopically, both in the R group and in the RS group, contour of most of the muscle fibers were highly waved. In these muscle fibers, arrangement of cross striations was disturbed (Fig. 2A). In the RS group, narrow sarcoplasmic areas exhibiting basophilic stain were found along the margin of the muscle fibers (Fig. 2C). But in the R group, these basophilic sarcoplasmic areas were not noted anywhere in the muscle fibers. Electron microscopically, disorganized myofilaments and disrupted Z-band were evident in these injured muscle fibers in both R and in the RS groups (Fig. 2B). Electron microscopically, the narrow sarcoplasmic areas seen in the RS group contained several mitochondria and ribosomes (Fig. 2D), nuclei of the muscle fibers became electron-lucent, and nucleoli became more evident (Fig. 2E).

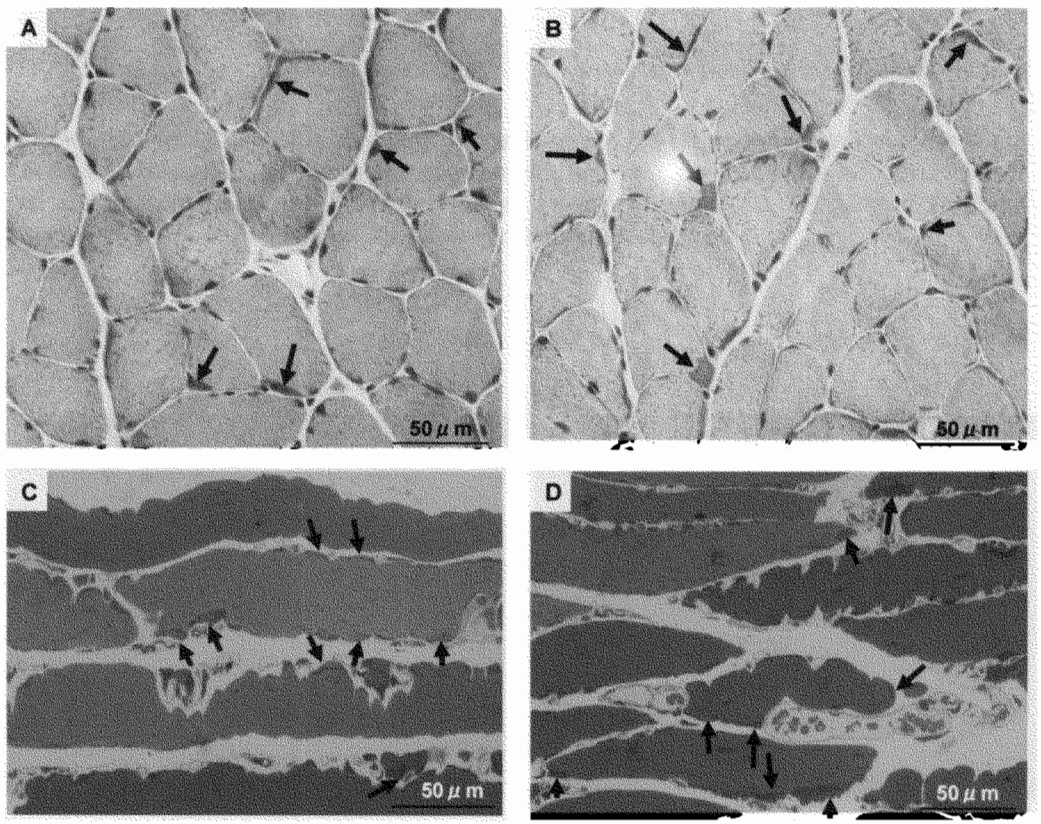


Fig. 3 At 1 day after the running. In the R group (A, C), basophilic sarcoplasms (➡) first appear at this time. In the RS group (B, D), basophilic sarcoplasms are often observed than the R group.

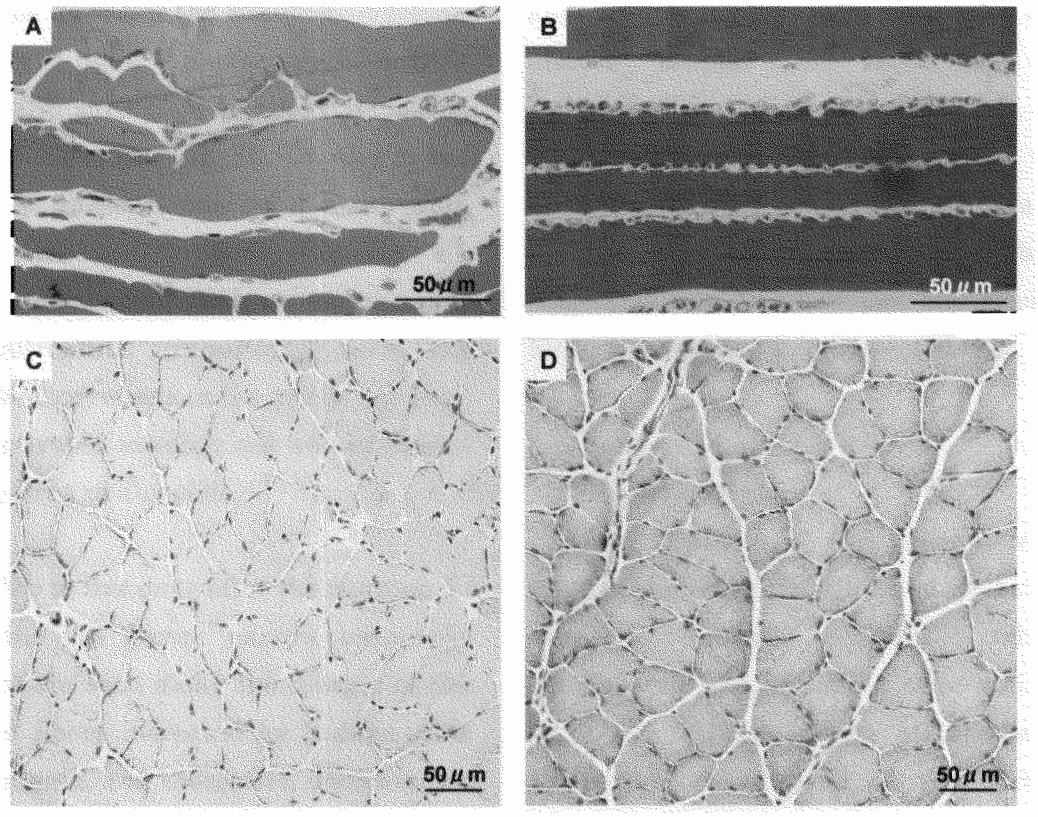


Fig. 4 At 14days after the running. In the R group (A, C), regenerating muscle fibers exhibiting basophilic sarcoplasms still remain, and fascicle formation of muscle fibers is not yet completed. On the other hand, in the RS group (B, D), almost all fibers appear to be normal and fascicle formation becomes clear.

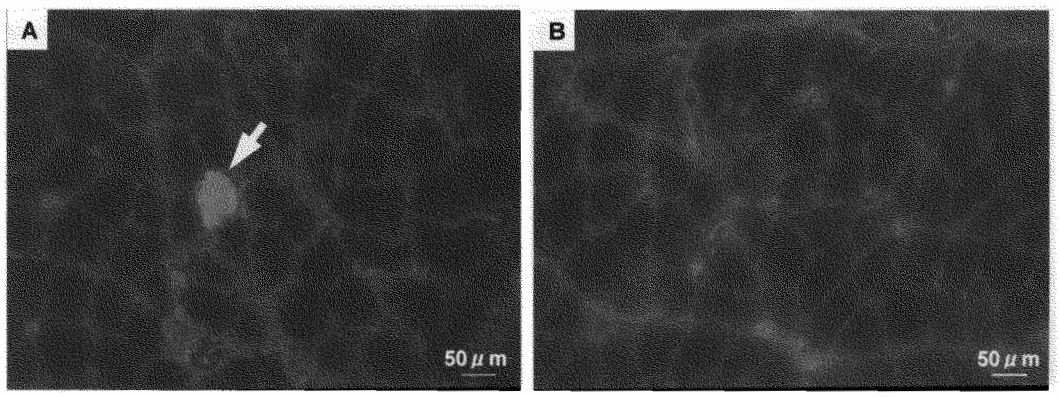


Fig. 5 EBD staining in the normal (A) and R (B) group at 1day after the running. An EBD-positive fiber (➡) is seen in the normal.

3) One day after the running

By light microscopy, narrow basophilic sarcoplasmic areas increased in the RS groups (Fig. 3B, D), and in the R group, these sarcoplasmic areas were first found at this time (Fig. 3A, C). Inflammatory cells such as macrophages or neutrophilic granulocytes were not observed anywhere in the soleus muscles. The activated satellite cell was not found in any area in any slide.

4) Two to five days after the running

In the RS group, narrow basophilic sacroplasm seen along the margin of the muscle fibers increased by 3 days after the running, and thereafter, decreased in number. While in the R group, these areas gradually increased in number by 5 days after the running.

5) Seven days after the running

In the RS group, the narrow basophilic sarcoplasmic areas were almost no longer observed. Wavy contour was still observed in some muscle fibers, cross striations became clear again, and muscle fibers reformed small bundles, as seen in the normal soleus muscle. In the R group, however, several muscle fibers exhibited highly waved contour, and arrangement of cross striations was still disturbed.

6) Fourteen days after the running.

In the RS group, morphological appearance in the soleus muscle was almost normal as seen in the corresponding control group (Fig. 4B, D). In the R group, however, though the arrangement of cross striations became normal, there were some muscle fibers still exhibiting wavy contour, and fascicle formation was not yet completed (Fig. 4A, C).

7) Evans blue stain

When the mouse was treated with Evens blue dye (EBD) 1 day before sacrifice, muscle fibers whose sarcolemma was disrupted were stained by EBD. Muscle fibers stained with EBD were found even in the normal mouse, but only occasionally (Fig. 5A). In the present study, muscle fibers stained with EBD were found only occasionally in the R (Fig. 5B) and RS groups.

**Quantitative analysis**

Comparison of muscle fiber area (Fig. 6) At 1 day after the running, the mean ± SD of muscle fibers in the R (1804.7 ± 562.3 $\mu m^2$) was significantly larger than the C (1749.8 ± 556.1 $\mu m^2$) and RS (1702.3 ± 579.6 $\mu m^2$) groups, but at 3 days after the running, significantly smaller (R: 1545.2 ± 529.0$\mu m^2$) than the C (1716.3 ± 540.6 $\mu m^2$) and RS (1733.6 ± 503.6 $\mu m^2$), and recovered to the control level by 5 days after the running.

In contrast, the muscle fiber area in the RS group was slightly smaller than the C

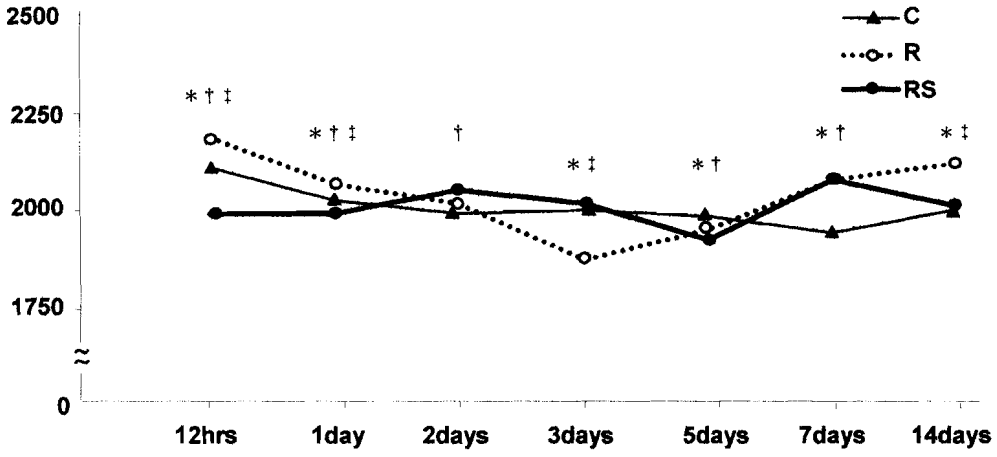


Fig. 6 Comparison of muscle fiber area
In the R group, muscle fiber area was larger by 1day after the exercise, but smaller at 3days than the other groups and recovered to the level of the C group at 5days. In the RS group, it was smaller than the other groups by 1day and recovered to the C group at 2days.
*: $p < 0.05$ C vs. R group
†: $p < 0.05$ C vs. RS group
‡: $p < 0.05$ R vs. RS group

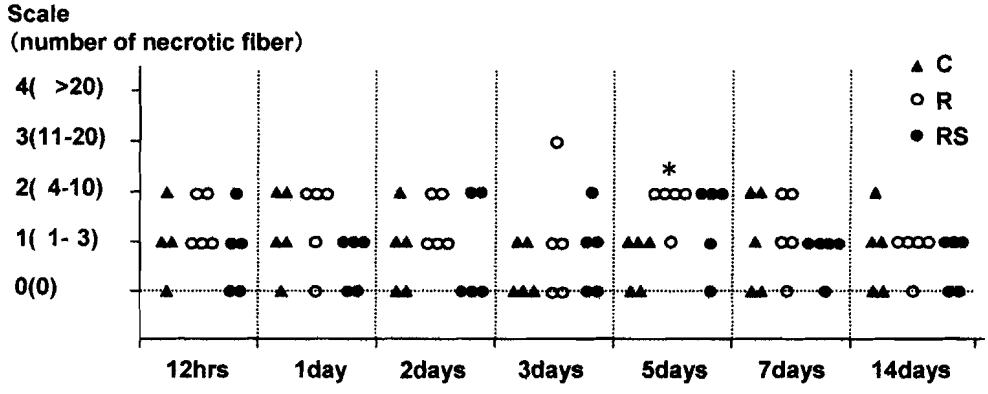


Fig. 7 Comparison of necrotic fibers
Necrotic fibers were very few in each group. There were no relative differences in number of necrotic fibers among the three groups at each period but at 5days after the exercise.
*: $p < 0.05$ C vs. R group

group at 1 day after the running, and recovered to the control level by 2 days after the running.

Incidence of necrotic fibers (Fig. 7)

Degenerating or necrotic muscle fibers were found very rarely in C, R and RS groups. Only at 5 days after the running, it appeared that necrotic fibers were slightly more often found in the R

group than the C group, there were no significant differences in the incidence of necrotic fibers among these 3 groups at any period examined in the present study.

## DISCUSSION

Muscle soreness often occurs 1-2 days after unusual or inappropriate exercise. This delayed onset muscle soreness (DOMS) occurs after the eccentric rather than isometric exercise, downhill running used in the present study has been widely applied as animal experimental model to study the muscle injury and DOMS [4, 5]. In the present study, disorganization of cross striations due to disruption of myofibrils and Z-band were observed after the eccentric exercise, as reported by Armstrong et al. [2, 4, 5, 18] and Friden et al. [6, 19, 20] in human and rat skeletal muscles. Besides these morphological alterations, it was reported that disruption of sarcolemma and degeneration of muscle fibers occurred after the extensive eccentric exercise [19, 21-24]. If the sarcolemmal disruption occurred, $Ca^{2+}$ entered the muscle fibers at the disrupted portions from outside, and muscle fibers began degeneration soon after the injury [18, 25-27]. These degenerating muscle fibers were stained by Evans blue dye (EBD) [15, 16], when the EBD was injected into peritoneal cavity one day before sacrifice. In the present study, however, muscle fibers stained with EBD or degenerating muscle fibers in the H-E stain sections were very rare in the mouse soleus muscle after the downhill running. It was also reported that neither necrosis of muscle fibers nor sarcolemmal disruption occurred in the muscle fibers after eccentric contraction [28-30]. These findings including ours suggest that muscle injury occurred after the eccentric exercise might be microinjury limited only in the muscle fibers.

Characteristic alteration seen in the injured muscle fibers after the downhill running is appearance of narrow sarcoplasmic areas along the margin of muscle fibers. These areas were stained basophilic in H-E stain sections. Electron microscopically, these sarcoplasmic areas contained several mitochondria and ribosomes. Since these basophilic areas were not found anywhere in the normal or necrotic muscle fibers, these morphological features can be regarded as regenerative rather than degenerative reactions. Inflammatory cells such as macrophages or neutrophilic granulocytes were not observed anywhere in the soleus muscle after the downhill running. Satellite cells [31] locating along the basal lamina of muscle fibers were long spindle in shape and had a dark nucleus and scanty cytoplasm in normal, injured and

regenerating muscle. These findings suggest that muscle regeneration in the injured muscle after the eccentric exercise might be intracellular event, and inflammatory cells or satellite cells [32-34] might not take part in the repair and regeneration occurring only within the muscle fibers.

Stretch after the exercise is commonly recommended and practiced as cool-down for inhibition of muscle tension, increasing flexibility, and repair of fatigue [35]. On the other hand, rest is also recommended to minimize the reinjury and cicatrisation. Jarvinen et al. [36] suggested that early mobilizaton should be begun after sufficient periods, e.g. 3-5 days after the injury. Lund et al. [37] and High et al. [38] reported that stretch before or after exercise was not effective for prevention of muscle soreness or damage. Clinically, muscle soreness is chronologically related with muscle swelling and stiffness. Thus, it has been said that one of the main cause of DOMS might be muscle swelling due to muscle injury induced by unusual exercise [3, 19, 39-41]. In the present study, by 1 day after the downhill running (R group), area of muscle fibers was significantly larger than the normal muscle fibers, smaller at 3 days after the running and recovered to the normal level at 5 days after the running. In contrast, by stretch after the running (RS group), area of muscle fibers was smaller by 1 day, and recovered to the control level at 2 days after the running. These findings suggest that stretch soon after the exercise can prevent muscle swelling. Thus, it is possible that stretch might reduce the DOMS.

In the RS group, narrow basophilic sarcoplasm began to appear earlier than in the R group, and morphological appearance of muscle fibers became almost normal by 7 days after the running. On the other hand, in the R group, regenerating features were still observed at 14 days after the running. It was reported that mechanical stimulation, such as passive stretch and contraction induce secretion of insulin-like growth factor I (IGF-I) [10-13, 42, 43], which is consider to stimulate not only growth but also repair, maintenance and remodeling of tissues. Thus, Bamman et al. [44] suggested that IGF-I system in the muscle might modulate muscle regeneration after injury. These findings taken together suggest that stretch soon after the exercise might not only reduce muscle soreness but also promote muscle repair and regeneration in the injured muscle fibers.

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