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Original Article

Considerations for safety of high-frequency repetitive peripheral magnetic stimulation of skeletal muscles in rats: Assessment by histological analysis of muscles and biochemical blood tests

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ABSTRACT

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Objective: The purpose of the present study was to investigate the safety of high-frequency repetitive peripheral magnetic stimulation (rpMS) of skeletal muscles using histological analyses of muscles and biochemical blood tests.

Methods: Twenty male Wistar rats were divided into the rpMS group that received rpMS and the control group (CON group) that did not receive rpMS. Hematoxylin and eosin, modified Gomori trichrome, NADH-tetrazolium reductase, acid phosphatase, and Periodic acid-Schiff (PAS) stains were used for histological analyses of the muscles. Creatine kinase, aldolase, and lactate dehydrogenase (LDH) were measured for the biochemical blood tests. Additionally, the histological analyses of the muscles were performed in two rats at 4 and 12 hours after completion of rpMS application.

Results: PAS stain showed glycogen depletion in the muscle fibers of the rpMS group. However, this finding was not observed in the rats at 4 hours after rpMS application. There was a significantly larger LDH decrease in the rpMS group compared with the CON group. No significant differences were observed for any of the other staining methods or serum enzymes between the two groups. There were no other abnormal findings in the rpMS group.

Conclusion: It was established that rpMS could be utilized safely on skeletal muscles since it did not cause muscle damage in this study.

Key words: magnetic stimulation, skeletal muscle, safety, histological analysis of muscle, biochemical blood test

Introduction

Recently, magnetic stimulation (MS) such as transcranial magnetic stimulation (TMS) has been widely used in both medical treatment [1] and research [2]. However, MS methods that are directly applied to peripheral nerves or skeletal muscles have yet to become as widespread as TMS. The mechanism of contraction of skeletal muscles caused by MS involves the phenomenon that electromagnetic induction produces an eddy current in the body. The eddy current induces depolarization of the cell membrane in the peripheral nerves or skeletal muscles, thereby causing the muscles to contract.

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Until recently, electrical stimulation (ES) was widely used to cause skeletal muscle contraction in the clinical field of rehabilitation medicine. ES has been reported to prevent muscle atrophy caused by immobilization or non-weight bearing condition after orthopedic surgery, in addition to providing muscle-strengthening effects in healthy subjects [3–5]. Furthermore, many researchers have also reported that ES was successfully used to improve or to compensate for affected function in stroke patients with hemiplegia [6–8]. An investigation by Chae et al. [6] into the effectiveness of ES for recovery of motor function in stroke patients with hemiplegia reported that 8 of 46 subjects had to discontinue the experiment due to pain associated with ES. ES-associated pain is produced by stimulation of not only peripheral nerves or muscles but also skin nociceptors, and is a serious problem that cannot be avoided during the application of ES [9, 10]. In contrast, MS can penetrate the skin and adipose tissues, which have a high electrical resistance. Therefore, MS can be used to activate peripheral nerves or muscles without stimulating the skin nociceptors [11, 12]. It has also been reported that application of MS to the quadriceps femoris (QF) produced significantly less pain than ES when the muscle contractions caused by ES and MS were set to the same level of knee joint extension torque [13]. Thus, the lower level of pain associated with the application of MS is a great advantage of using MS.

Another advantage of MS as reported by Ito et al. [14] is that it is useful for stimulating deep muscles such as the iliopsoas. Furthermore, when using MS, patients do not need to take off their clothes, as the procedure does not require the placement of electrodes on the skin. Because of these benefits, other researchers have examined the effectiveness and usefulness of applying high-frequency repetitive peripheral magnetic stimulation (rpMS) to the peripheral nerves or skeletal muscles [14–19] with the hope that MS could be used as an alternative to ES. However, the conventional MS devices have the problem that the stimulation coil is easily heated to a high temperature by repetitive stimulations even during short conduction periods. Therefore, the stimulation protocols of the previous studies were set up in such a way as to avoid heating of the stimulation coil [14–19]. Although long intervals were included between each stimulation period in these protocols, heating of the coil could not be avoided during repeated use of these repetitive stimulations. Thus, it is difficult to use these conventional devices over a long period of time, which may have hindered the widespread use of rpMS.

To solve this problem, in the present study we developed a new stimulation device that was designed to minimize heating of the stimulation coil during repeated use. This new stimulation device employs two countermeasures against coil heating. The first involves the use of an iron-core coil. Charles et al. [20] reported that the electrical resistance of a coil could be decreased by using an iron-core coil and that this would inhibit the rise in temperature during stimulations. The other is the installation of an air-cooling device for the stimulation coil. A constant flow of air is directed at the coil so that it is continuously cooled throughout the application of the rpMS. These countermeasures enabled us to continuously use the new stimulation device for much longer periods of time compared to the conventional devices. rpMS is expected to be widely used in clinical practice in the future. Nevertheless, no clear criteria have been established and there are many unclear points regarding the riskless stimulation intensity and the length of time of continuous use. Therefore, the present study investigated the safety of applying rpMS to skeletal muscles in rats by using histological analyses of the muscles and biochemical blood tests.

Methods

1. Subjects

The present study used 24 male Wistar rats aged 8 weeks (weighing 185–215 g). They were randomly assigned to either the group given rpMS (rpMS group: n=10) or the control group, which received no stimulation (CON group: n=10). In addition, we set up a further experiment designed to clarify the histological changes of the muscles that occur over time after the cessation of rpMS application. Sections of muscles were taken from two rats at 4 hours after the application of rpMS (rpMS4h group) and two rats at 12 hours after the application of rpMS (rpMS12h group). All rats were housed in an environment where the room temperature was kept at 22 ± 2°C and the humidity at 20–60%. The room lights were turned on from 7:00 to 21:00, with food and water available ad libitum. The present study was approved by the Animal Research Committee of Kawasaki Medical School (No. 14-018).

2. Experimental procedure

At the beginning of the experiment, the rats were anesthetized by inhalation of sevoflurane. Approximately 2 ml of blood sample was collected from the right femoral vein in both the CON and rpMS groups before the intervention. In the rpMS group, MS was applied to the anterior surface of the left femoral region of the rats for approximately one hour according to the MS protocol described below. Rats in the CON group were kept at absolute rest without MS for the same length of time as the MS protocol. After the intervention, approximately 2 ml of blood sample was also taken from the right femoral vein in both groups. Immediately after the blood collection, the left QF muscles were removed for muscle preparation for the histological analyses. In the rpMS4h and rpMS12h groups, the rats were put back into their cages for recovery from anesthesia after the application of the MS. After 4 or

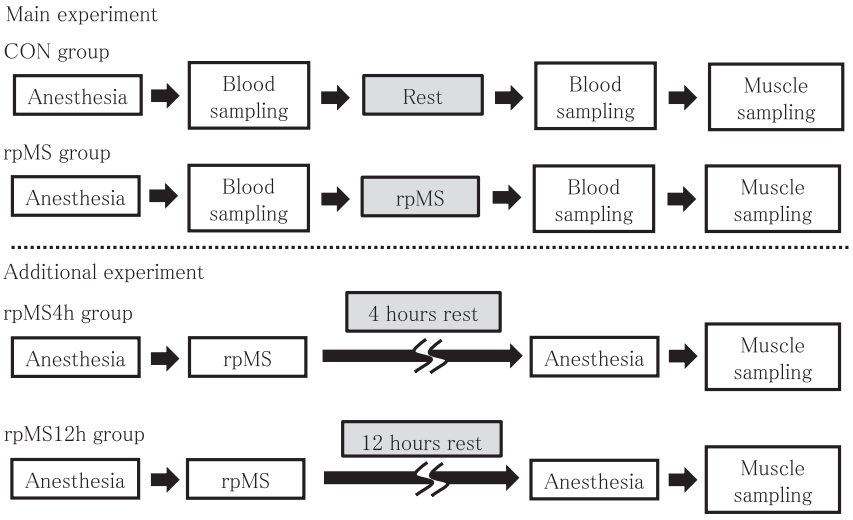

Figure 1. Experimental procedure.

12 hours, these rats were anesthetized again, and the left QF muscles were removed in the same way. All rats were euthanized by excessive administration of sevoflurane promptly after the completion of the experimental procedures, which are shown in Figure 1.

3. Magnetic stimulation conditions

A newly-developed magnetic stimulator (IFG Corp., Miyagi, Japan) was used for application of rpMS in this study (Fig. 2). The stimulation intensity was set at 600 V, which was the maximum output voltage of this device. The magnetic flux density was 0.9 Tesla, and the stimulation frequency was 40 Hz. The stimulation protocols were as follows. Rats received repetitive stimulation 10 times, each stimulation lasting 10 seconds, followed by a 30-second interval. The 10 times of stimulation were defined as one set. Between each set, the rats were given a 4-minute resting period. A total of six sets were imposed on each rat (Fig. 3). The total time required to complete the MS intervention

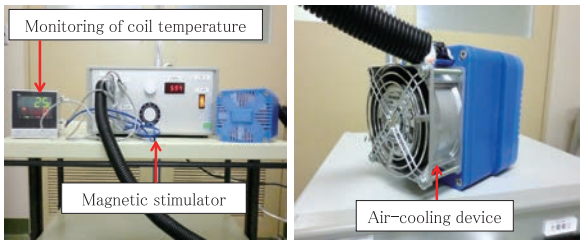

Figure 2. Magnetic stimulator.

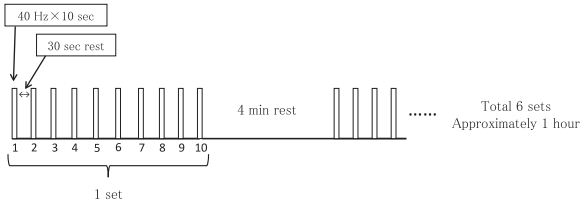

Figure 3. Magnetic stimulation protocol.

was approximately one hour. An investigator held the stimulation coil by hand so that the center of the coil could be attached firmly to the anterior surface of the left femoral region during rpMS application. Extension of the left knee joint caused by the rpMS was confirmed visually. In addition, the internal temperature of the coil during rpMS application was continuously monitored by an external device that was connected to a thermometer placed in the coil. The investigator confirmed that the internal temperature did not exceed 37°C.

4. Histological analysis of the muscles

The test muscle examined in the study was the left QF. After first exposing the left QF and marking the spot at the midpoint of the longitudinal axis of the muscle belly, it was then removed. After the removed muscles were trimmed to around 1 cm in length and 0.5 cm in diameter, they were rapidly frozen in normal-hexane solution cooled to below −80°C by dry ice. The frozen muscle samples were stored in a deep freezer maintained at −70°C until slicing and staining of the tissue could be performed. The middle parts of the frozen muscle samples were sliced into 12-μm thick cross-sectional slices using a cryostat Leica CM3050S (Leica Microsystems Co., Ltd., Wetzlar, Germany) and serially mounted on separate glass slides. After being dried for more than 2 hours, all serial sections were stained with hematoxylin and eosin (HE), modified Gomori trichrome (Gomori), NADH-tetrazolium reductase (NADH), acid phosphatase (Acp), and Periodic acid-Schiff (PAS) stains. A specialized pathologist observed all stained samples using an optical microscope, and confirmed whether there were any histological characteristics of the muscles.

5. Biochemical blood tests

The three serum enzymes measured as markers

of muscle damage included creatine kinase (CK), aldolase, and lactate dehydrogenase (LDH). In the first step, the skin over the right femoral region was incised, and the right femoral vein was exposed for blood collection. After inserting a disposable plastic indwelling cannula with a needle (Terumo Corp., Tokyo, Japan) into the right femoral vein, a blood sample was obtained. Approximately 2 ml of blood was taken from the right femoral vein of all rats in the CON and rpMS groups before and after the intervention. To extract the serum, all blood samples were centrifuged at 3000 rpm for 5 min at 4°C. Measurements of enzyme levels in the serum were performed by an outside laboratory (SRL, Inc., Tokyo, Japan).

6. Statistical analysis

IBM SPSS Statistics 20 (IBM SPSS Statistics Inc., Tokyo, Japan) was used for statistical analysis. Student's *t*-test was used to compare enzyme levels in the serum obtained before the intervention between the CON and rpMS groups. A paired *t*-test was used to assess the changes in the serum enzyme levels before and after intervention. After calculation of the rate of change in the serum enzyme levels before and after the intervention, the differences between the CON and rpMS groups were compared using Student's *t*-test. The level of significance was set at less than 5%.

Results

Histological analysis of the muscles

Each figure shows representative histological images of the muscles for each group. Histological observations for the PAS stain indicated that paler-staining muscle fibers were extensively observed in 8 of the 10 rats in the rpMS group. In contrast, paler-staining fibers were not observed in the CON, rpMS4h, and rpMS12h groups (Fig. 4). Moreover, the paler-staining fibers shown by the PAS stain in the rpMS group were found to roughly correspond to the type II fibers that were stained light blue by the NADH stain (Fig. 5). No abnormal histological findings were observed in any of the groups for the HE and Gomori stains (Figs. 6, 7). Furthermore, the Acp stain indicated that no macrophage migrations were observed in any of the stained samples (Fig. 8).

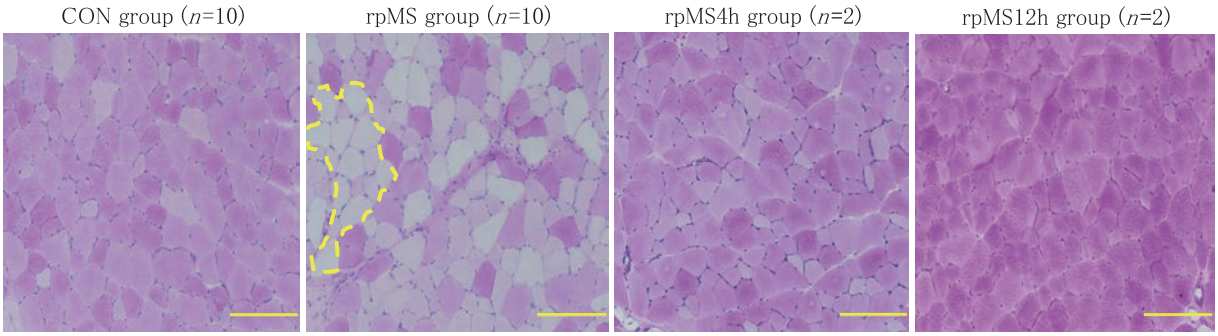

Figure 4. PAS stain.
Bars: 200 μm; ×200.
Paler-staining fibers were observed extensively in the rpMS group. In contrast, the paler-staining fibers found in the rpMS group were not observed in the rpMS4h group.

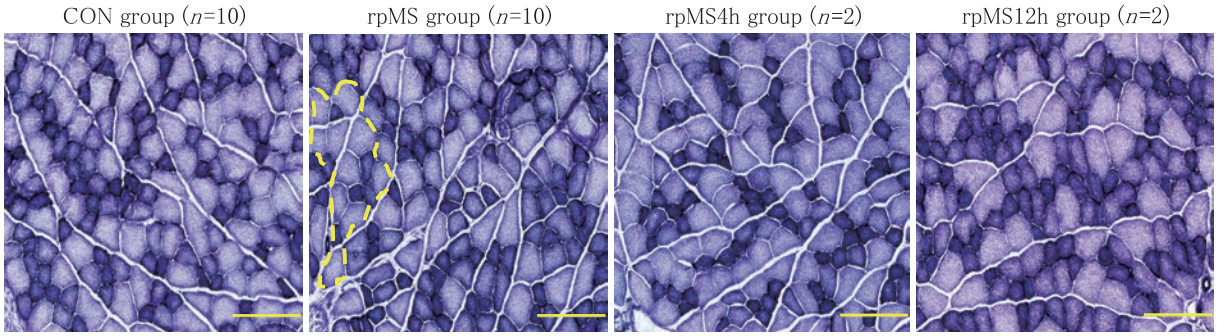

Figure 5. NADH stain.
Bars: 200 μm; ×200.
The paler-staining fibers of the rpMS group seen with the PAS stain (yellow frame line in Fig. 4) corresponded to type II fibers (yellow frame line) that were stained light blue by the NADH stain.

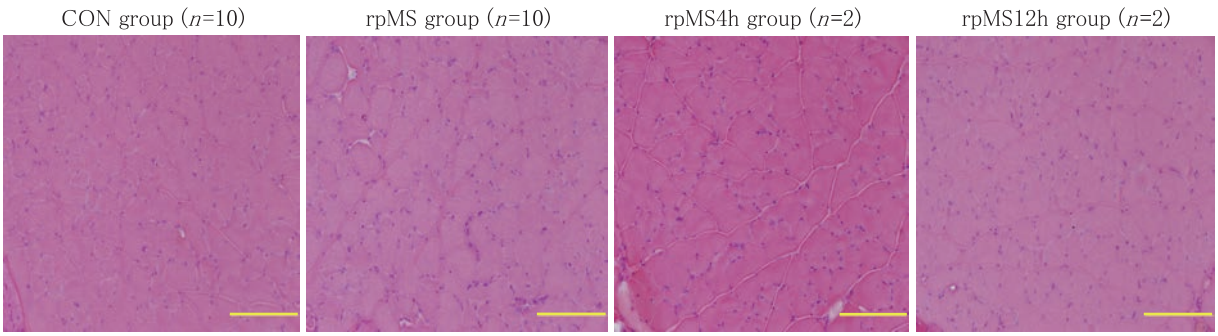

Figure 6. HE stain.
Bars: 200 μm; ×200.
No disturbances in the basic structure or any abnormal findings such as opaque fibers were observed in any of the samples.

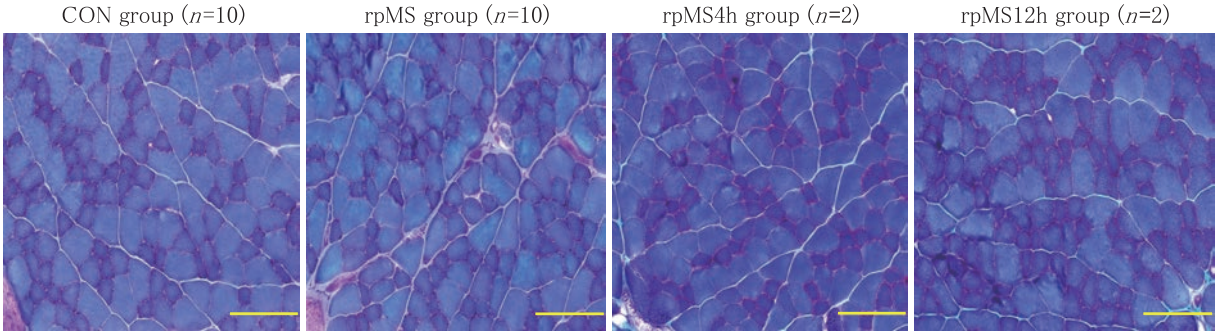

Figure 7. Gomori stain.
Bars: 200 μm; ×200.
No abnormal findings were observed in any of the samples.

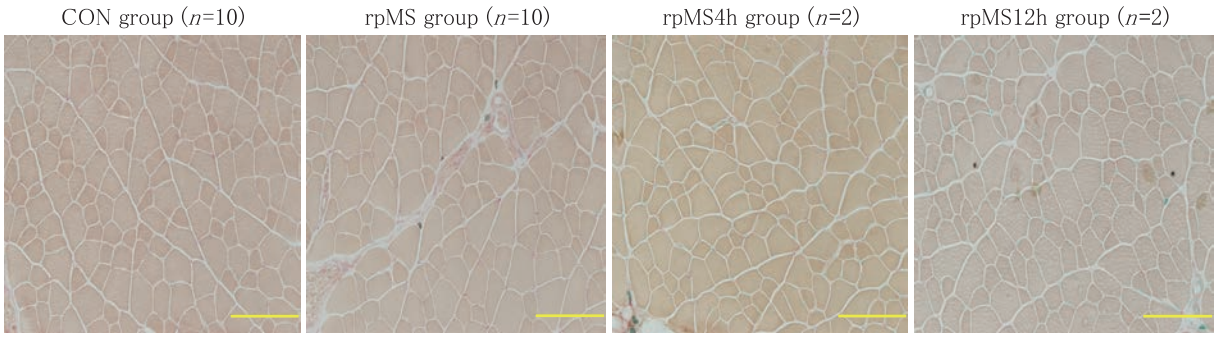

Figure 8. Acp stain.
Bars: 200 μm; ×200.
No fibers stained red indicating macrophage migration were observed in any of the samples.

Biochemical blood tests

Mean and standard deviation of the serum enzyme levels for CK, aldolase, and LDH in the CON and rpMS groups are shown in Table 1. There were no significant differences in any of the serum enzyme levels between the two groups before the intervention. In addition, there were no significant changes in any of the serum enzyme levels before and after the intervention. The rates of change in each serum enzyme level of the two groups are shown in Table 2. The rate of change in LDH of the rpMS group was significantly smaller than that of the CON group. In the two cases of the rpMS group where the PAS stain did not show any paler-staining muscle fibers, the rates of change in LDH were 32% and −7%, respectively, and were not as low as the mean value observed for the rpMS group.

Table 1. Changes in biochemical markers before and after the intervention.

Serum enzyme	Group	Before	After	*t*-Test
CK (IU/L)	CON	180.8 ± 103.6	176.3 ± 79.8	N.S.
	rpMS	198.7 ± 94.2	186.3 ± 61.5	N.S.
Aldolase (IU/L)	CON	18.6 ± 4.6	18.2 ± 3.4	N.S.
	rpMS	20.1 ± 5.3	19.3 ± 2.9	N.S.
LDH (IU/L)	CON	200.3 ± 184.3	213.1 ± 127.4	N.S.
	rpMS	276.4 ± 141.3	215.0 ± 120.1	N.S.

The data shown are the mean ± SD.
CK, creatine kinase; LDH, lactate dehydrogenase; CON, control group; rpMS, magnetic stimulation group; N.S., not significant.

Table 2. Rates of change in serum enzyme activity.

Serum enzyme	CON	rpMS	*t*-Test
CK (%)	13 ± 53	2 ± 32	N.S.
Aldolase (%)	3 ± 27	− 1 ± 16	N.S.
LDH (%)	55 ± 95	− 21 ± 33	$p < 0.05$

The data shown are the mean ± SD.
CK, creatine kinase; LDH, lactate dehydrogenase; CON, control group; rpMS, magnetic stimulation group; N.S., not significant.

Discussion

Histological analysis of the muscles

Histological analysis of the muscles when using the PAS stain showed extensive paler-staining muscle fibers in 8 of the 10 rats in the rpMS group. Since it has been reported that the PAS stain is able to estimate the relative glycogen content of muscle fibers [21, 22], this staining method can be used to confirm whether muscle contractions occurred immediately before the histological assessment [21, 23]. When muscle fibers are repeatedly stimulated in order to induce muscular contraction under anaerobic conditions, the muscle fibers selectively consume intracellular glycogen as an energy source. In other words, glycogen must be depleted in the contracting muscle fibers when anaerobic metabolism is highly activated in the muscle fibers. In contrast, glycogen remains unchanged in muscle fibers that are not contracting [24]. Thus, the existence of paler-staining muscle fibers on PAS stain observed in the present study was attributed to the fact that the adenosine triphosphate (ATP) necessary for muscle contraction was supplied by anaerobic glycolysis as a result of the muscle contraction induced by the rpMS under anaerobic conditions. Nakatani et al. [25] reported that accumulation of glycogen increased many times over the baseline condition at four hours after the cessation of excessive exercise designed to deplete glycogen in the muscle. Although our additional experiment investigated alterations in the glycogen of the muscles in two rats at four hours after the cessation of rpMS application, the PAS stain did not show any paler-staining muscle fibers in the rpMS4h group, unlike that found for the rpMS group. Therefore, the glycogen depletion of the muscle fibers in the rpMS group was a transitory change.

The initial process of muscle fiber necrosis is generally caused by an influx of extracellular fluid into the cell due to damage to the plasma membrane. Since extracellular fluid contains high concentrations of calcium ions, an influx of extracellular fluid into the cell will cause an excessive contraction of the myofibrils. Excessively contracted muscle fibers are referred to as “opaque fibers” and are darkly stained by HE and Gomori stains, among others [26]. In the present study, however, muscle fibers considered to be opaque fibers were not identified by either HE or Gomori stains in any of the samples that received rpMS. Based on these results, we speculate that application of rpMS is unlikely to cause necrosis of muscle fibers.

During the process of muscle fiber necrosis, several myofibrils around the excessively contracted myofibrils ruptured, and the ruptured myofibrils were then degraded by calcium-dependent neutral proteases. Subsequently, macrophages infiltrated these necrotic fibers to clean up the necrotic area, with the spaces created considered to become areas for proliferation of myoblasts after the differentiation of satellite cells into myoblasts [26]. Since Acp stain turns macrophages red, we used this stain to determine the absence or presence of necrotic fibers in the present study. Our findings confirmed the absence of macrophage migration in all samples from the CON and rpMS groups. This indicates that necrotic fibers are not present immediately after the cessation of the MS protocols. With regard to the appearance of macrophages, Nonaka et al. [26] administered bupivacaine hydrochloride intramuscularly in their study to cause necrosis of rat skeletal muscles. Macrophage migrations were identified around the necrotic fibers at 12 hours after

the injection. Since our histological findings showed that none of the cells were turned red by the Acp stain in the rpMS12h group, the MS protocol used in the current study did not cause any muscle fiber necrosis.

Biochemical blood tests

In this study, there were no significant differences in the CK and aldolase levels before and after the intervention in either the CON or rpMS group. Furthermore, there were no significant differences in the rates of change in the CK and aldolase levels between the two groups. Damage to cell membranes in the muscles causes a CK efflux from the cells, leading to an increase of the CK concentration in the blood. Therefore, CK has been used as a marker of muscle damage [27–29]. In addition, aldolase is present in tissues that require high levels of energy metabolism, such as the skeletal muscles. Thus, damage to these tissues can be estimated by measuring the level of aldolase in the blood [30]. The present result suggests that rpMS application did not cause damage in any tissues containing CK and aldolase, such as the skeletal muscles.

The rate of change in LDH of the rpMS group was a negative value, and was significantly smaller than that observed for the CON group. LDH is present in almost all cells and thus cellular damage causes elevation of LDH in the blood. Therefore, LDH is a signal that indicates an abnormality within the body [31], and has been widely used as a marker of muscle damage [27, 32, 33]. Moreover, LDH is an enzyme that catalyzes the interconversion between pyruvate and lactate during the last stage of glycolysis [31]. As described above, since glycogen depletion was confirmed by the histological findings on the PAS stain in the rpMS group, it is suggested that ATP was supplied by glycolysis that occurred within the muscle fibers. The glycolytic system is an anaerobic pathway for the degradation of glucose in the absence of oxygen supply, with lactate produced as an end product. Therefore, we believe that the activated chemical reaction that converts pyruvate to lactate affected the rate of change in LDH within the muscle fibers that had muscle contractions induced by rpMS. This means that the observed change in LDH in the rpMS group was not indicative of any damage in the muscular tissue. In the two cases of the rpMS group that did not exhibit paler-staining muscle fibers with the PAS stain, the rates of change in LDH were not as low as the mean value observed for the rpMS group. These results suggest that there were no activated glycolyses in these two cases of the rpMS group during muscle contraction. Therefore, since the glycogen persisted and remained unchanged, no histological alterations were observed on the PAS stain.

In conclusion, histological analysis of the muscles and biochemical blood tests performed to investigate the safety of rpMS on skeletal muscle in the current study demonstrated that there was no damage to the muscular tissue after the application of rpMS. Thus, when applied in accordance with the stimulus conditions used in the present study, rpMS can safely be administered without any muscle damage.

Acknowledgments

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