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The effect of magnetic stimulation on unloaded soleus muscle of rat: changes in myosin heavy chain mRNA isoforms

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ABSTRACT

This study assessed the potential application and the effectiveness of functional magnetic stimulation (FMS) for preventing skeletal muscle atrophy in adult rats. FMS using magnetic stimulator was performed to rat soleus muscle by placing a round magnetic coil on the back of 3rd-5th lumbar vertebral level at 20 Hz frequency for 60 min/day up to 10 days. A reverse transcriptase-polymerase chain reaction was applied to evaluate relative amounts of mRNAs specific to four myosin heavy chain (MHC) isoforms [MHCIβ, MHCIIa, MHCIIb, and MHCIId(x)] in rat soleus muscle during contractile activity by magnetic stimulation. Ten-day unloading by hindlimb suspension induced a drastic decrease of MHCIβ and MHCIIa mRNA expressions, while MHCIIb and MHCIId(x) mRNA was not decreased. The magnetic stimulation resuscitated the down-regulation of the mRNA levels of MHCIβ and MHCIIa. These results suggest that magnetic stimulation on acute atrophied muscles is useful for preventing the muscle atrophy.

Many patients with upper motoneuron lesion have a difficulty of making useful muscle contraction due to the weak and atrophied muscle (26). Functional electrical stimulation (FES) has been proved to be effective for restoring the gait function of those patients (1, 8, 16, 23). In the first attempt of FES, Liberson *et al.* reported that the hemiplegic patients with foot drop improved their walking (9). This is possible because most of these patients have the intact peripheral nerves below their level of injury that can be stimulated to provide the muscle contractions. In order to maintain the quality and the quantity of their paralysed muscle, therapeutic electrical stimulation (TES) has been performed to increase the muscle force, and to prevent the muscle atrophy (7, 18, 21, 24).

There might be a principal difference among the various approaches such as electrical apparatus or other approaches in terms of restoring the paralysed muscles. Functional magnetic stimulation (FMS) in part, can avoid various complications that are associated with surgery or chronic implants, such as infection and haemorrhage (3, 4). The magnetic fields that are generated from the magnetic coil are able to pass through high-resistance structures such as bone, fat, and skin without harm to the body (4, 5). Lately, FMS has been used effectively to stimulate the spinal nerves below the level of spinal cord injury (SCI), and FMS supported the vital functions such as the ability to cough (11, 14), and to control the bladder (15), and the bowel movement (12, 13, 20). Therefore, we hypothesized that FMS might be an alternative ability for TES as far as the prevention of the muscle atrophy in paralysed muscle is concerned.

It has been said that muscle fibres are able to

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change their phenotype, performing fiber type transitions under the influence of exogenous factors, e.g., altered neuromuscular activity, mechanical activity or hormonal signals. These transitions correspond to alterations in the expression of myosin heavy chain (MHC) isoforms. As recently shown for rat soleus muscle unloaded by hindlimb suspension, the slow-to-fast transition in MHC mRNA and protein isoforms occur in the order (25) : MHCIβ → MHCIIa → MHCIId(x) → MHCIIb. The MHC mRNA was reverse-transcribed with the use of specific oligonucleotide primers, followed by amplification in the polymerase chain reaction (PCR), yielding DNA fragments of defined length (25). The purpose of this study is to evaluate the effects of magnetic stimulation in preventing acute muscle atrophy.

MATERIALS AND METHODS

Nine adult male Wister ST rats with an average body weight of 226 g (range 210–249 g) were used in these experiments. The animals were assigned to 3 groups: the control group (n = 3), the stimulated group (n = 3), and the non-stimulated group (n = 3). The rats of stimulated and non-stimulated groups were suspended by their tails through the silk strings, and their hindlimbs were unweighted to make their muscles atrophied. The height of suspension was adjusted so that the hindlimb just cleared the grid floor. The forelimbs maintained contact with the floor, which allowed the animals access to food and water. The animals adapted rapidly to harnessing and spent long periods of experiment by resting on their chests and forearms. For the hindlimbs suspension and magnetic stimulation, the rats were deeply anaesthetized with an intraperitoneal injection of pentobarbital sodium (40 mg/kg).

A commercially available magnetic stimulator (Daia Medical System, Japan) was used for FMS and the average of diameter in magnetic coil was 90 mm. This stimulator can generate the maximum field strength of 1.0 tesla near the magnetic coil. A computer with an interactive program designed specifically for activating the stimulator was used to control the frequency and length of the stimulation. The continuous stimulation parameters were set up to 750 V (about 93% of maximum intensity of this system) and 20 Hz. An adjustable metal frame supported the magnetic coil, and then it was possible to keep the position of the coil placed on the one side back of 3rd–5th lumbar vertebral level initially for lumbosacral stimulation, which was variable to obtain the maximal movement of their hindlimb as an optimal stimulation (Fig. 1). Magnetic stimulation for the rats of the stimulated group was started 1 day after the operation. This stimulation of rats performed for 60 min/day, and for consecutive 10 days. The animal care protocol for this study was approved by our university's institutional animal care, and followed the guidelines of the US National Institutes of Health (NIH).

The day after the stimulation period ended, the soleus muscle was surgically removed from both legs, immersed in RNA later ™ (TAKARA BIO, Japan) and incubated for twelve hours at 4°C. The muscle samples were pulverized under liquid nitrogen and homogenized in cold ISOGEN (NIPPON GENE, Japan). After homogenisation, total RNA was extracted according to the manufacturer's instruction. RNA concentration was assessed spectrophotometrically. Total RNA stock solution (2 μg) was reverse-transcribed in a 20 μl volume using the following assay mixture: RT buffer (10 mM Tris-HCl, pH 8.3, 5 mM $MgCl_2$, and 50 mM KCl), 0.25 units avian myoblastosis virus reverse transcriptase XL, 20 units RNase inhibitor (TAKARA BIO), 2.5 μM random 9 mers and 2.50 mM deoxynucleoside triphosphate (dNTP; TAKARA BIO). Incubation was performed for 30 min at 42°C. PCR were performed using Takara *Taq*™ polymerase (TAKARA BIO) and the primers specific to MHCIβ, MHCIIa, MHCIIb, and MHCIId(x) which were derived from published cDNA sequences (Table 1). Depending on the initial amount of template, the number of cycles was determined to 28 cycles to allow product detection in the exponential range of amplification. PCR products were analyzed by agarose gel electrophoresis, and aliquots (10.0 μl) were loaded in a 1.5%

Fig. 1 Experimental rat with a round magnetic coil placed over the lumbosacral portion

Table 1 *Primers for RT-PCR of MHC isoforms*

Isoform	Product Length (bp)	Primer Sequence	Reference No.
MHCIβ	288	Antisense GGGCTTCACAGGCATCCTTAG Sense ACAGAGGAAGACAGGAAGAACCTAC	17
MHCIIa	310	Antisense TAAATAGAATCACATGGGGACA Sense TATCCTCAGGCTTCAAGATTTG	6, 10
MHCIIb	197	Antisense TTGTGTGATTTCTTCTGTCACCT Sense CTGAGGAACAATCCAACGTC	6, 10
MHCIId(x)	120	Antisense TCCCAAAGTCGTAAGTACAAAATGG Sense CGCGAGGTTCACACCAAA	6

MHC, myosin heavy chain; bp, base pair; RT-PCR, reverse transcriptase-polymerase chain reaction

agarose gel containing 0.5 μg/ml ethisium bromide and separated in a 1 × Tris-borate, EDTA (TBE) buffer. The intensity of each bands were calculated using NIH image. The relative ratio of each MHC isoform band density to the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was calculated.

RESULTS

To investigate the amounts of the different MHC mRNA isoforms in total RNA extracts, semi-quantitative RT-PCR was performed. Oligonucleotide primers for the different isoforms were selected to yield amplification products of different length, i.e., 288 bp for MHCIβ, 310 bp for MHCIIa, 197 bp for MHCIIb, and 120 bp for MHCIId(x) (Fig. 2). Unloading of hindlimb for 10 days changed the expression pattern of the MHC mRNA isoforms (Fig. 3). The mean relative expression ratio of each mRNA of MHCIβ, MHCIIa, MHCIIb, and MHCIId(x) to GAPDH were, respectively, 1.16, 1.10, 1.15 and 0.60 in control group, 0.54, 0.38, 0.65 and 0.32 in non-stimulated group, and 1.18, 1.31, 1.09 and 0.42 in stimulated group. Unloading of hindlimb drastically decreased MHCIβ mRNA expression from 1.16 to 0.54 in relative ratio. Whereas, magnetic stimulation of soleus muscle did not decrease its relative ratio (1.16 to 1.18). MHCIIa mRNA of soleus muscle was also down-regulated by unloading (1.10 to 0.38), meanwhile, up-regulated by magnetic stimulation (1.10 to 1.31). MHCIIb and MHCIId(x) mRNA expression of each group showed a similar trend, however, did not changed much less significantly.

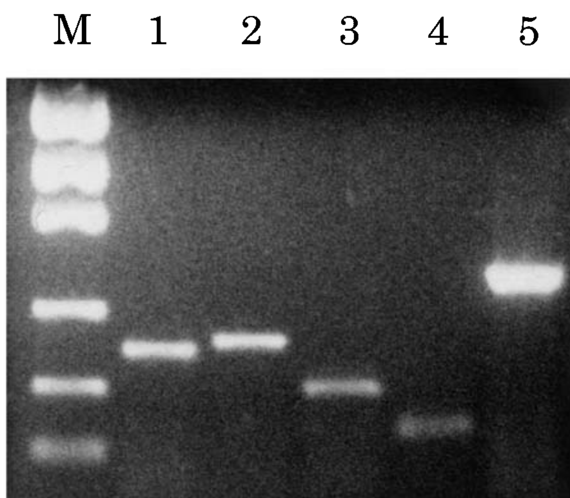

Fig. 2 Electrophoretically separated reaction products from reverse transcriptase-polymerase chain reactions (RT-PCR). To investigate the amounts of the different MHC mRNA isoforms in total RNA extracts, RT-PCR was performed. Oligonucleotide primers for the different isoforms were selected to yield amplification products of different length, i.e., 288 bp for MHCI*β*, 310 bp for MHCIIa, 197 bp for MHCIIb, and 120 bp for MHCIId(x). Lane 1, MHCI*β* isoform; Lane 2, MHCIIa isoform; Lane 3, MHCIIb isoform; Lane 4, MHCIId(x); Lane 5, GAPDH; M, DNA molecular weight marker

DISCUSSION

As for electric stimulation, the electrophysiological detection of pathology affecting lumbosacral nerve roots has been difficult because of their deep, relatively inaccessible location. Electrical stimulation of these roots can only be performed using high voltage techniques or by using needle electrodes inserted to the depth of the vertebral lamina. Both methods are painful, besides the latter is invasive. The disadvantages of the surface electrodes were potential loosening, skin irritation, and poor cosmetic appearance (23). In the last decade, magnetic stimulation has emerged as a useful method for stimulating nerves (2–4). The recent development of the surface magnetic coil system has allowed deeply situated nerve fibers to be stimulated less painfully

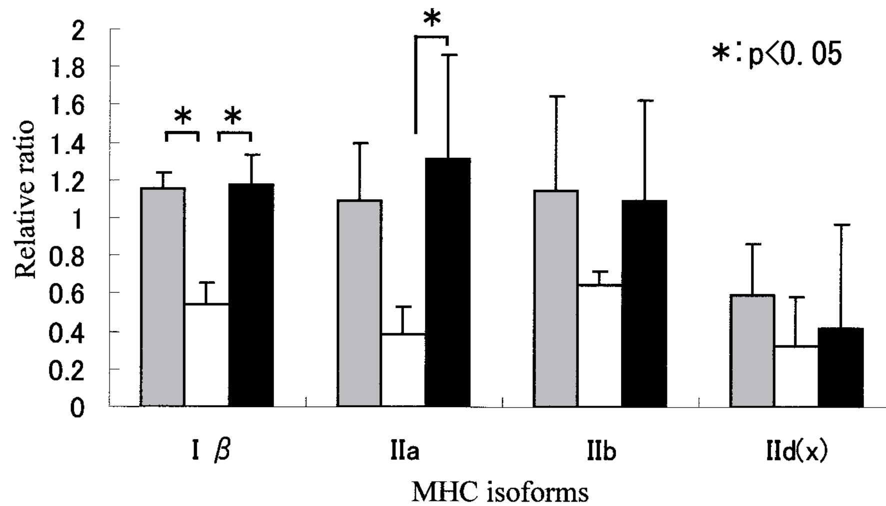

Fig. 3 The mean relative expression ratio of each mRNA of MHCI*β*, MHCIIa, MHCIIb, and MHCIId(x) to GAPDH in each group were calculated. Gray column, control group; white column, non-stimulated group; black column, stimulated group.

(22). The magnetic coil stimulator offers a theoretic advantage over a conventional electrical stimulation in depolarizing deep nerves, since its time-varying magnetic field is not attenuated by intervening high-impedance structures such as skin and bone.

The present study investigates the time courses of adaptive changes in MHC isoforms expression of rat soleus muscle in response to magnetic stimulation. Numerous studies using rats have shown that mechanical unloading of soleus muscle induces slow-to-fast transitions (19, 27). Stevens *et al.* (25) reported that MHCIβ, the predominant isoform in normal soleus muscle (~95%), decreased within the first week of unloading, reaching a relative concentration of ~75% at day 7. MHCIIa, which represented 5% of total MHC isoforms in control soleus muscle, was elevated by the muscle unloading and reached a maximum of ~25% after 1 week. In second week, the protein expression of MHCIIa in unloaded soleus muscle was gradually decreased. The decreases of protein expression in MHCIβ and MHCIIa indicate the evoking of slow-to-fast transition (25).

In our study, MHCIβ and MHCIIa mRNA expressions were drastically decreased by 10-day unloading, however, MHCIIb and MHCIId(x) mRNA were not decreased. These changes indicate that the soleus muscle unloaded for 10 days was in midcourse of slow-to-fast transition. The magnetic stimulation could resuscitate the down-regulation of the mRNA levels of MHCIβ and MHCIIa, supporting the availability of the magnetic stimulation for preventing the slow muscle from slow-to-fast transition.

This preliminary finding in rats showed that FMS might improve the muscle fiber property in acute stage. Over the last decade, our laboratory has explored the potential therapeutic benefits of FES as a rehabilitation technology. Perhaps the most interesting applications of magnetic stimulation to peripheral nerve, would be in the rehabilitation area, where adequate stimulation would be required to produce useful movement of the innervated muscle.

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