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Pulsed Electromagnetic Field Elicits Muscle Recovery via Increase of HSP 70 Expression after Crush Injury of Rat Skeletal Muscle

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Abstract. [Purpose] The present study investigated the effects of electromagnetic field applied at different time points on muscle recovery process through observation of the expression of HSP 70 protein and histological changes in a rat muscle crush injury model. [Subjects and Methods] Fifty-four male Sprague-Dawley rats were subjected to crush injury of the left extensor digitorum longus muscle. The animals were randomly assigned to the control (CON), pulsed electromagnetic fields (PEMF), delayed PEMF (DPEMF) groups and pulsed electromagnetic fields were administered from 1 or 3 days after muscle injury. Western blotting, immunohistochemistry, and hematoxylin-eosin staining was used to evaluate of heat shock protein (HSP) 70 expression to assess muscle recovery. [Results] Muscle injury-induced decrease of HSP 70 protein was increased and recovered by electromagnetic field application in the PEMF and DPEMF groups. Moreover, atrophy and irregular arrangement of muscle fiber caused by crush injury improved in both the PEMF and DPEMF groups. [Conclusion] In the rat model of muscle crush injury, PEMF induced muscle recovery via increased HSJP 70 protein.
Key words: Heat shock protein 70, Muscle crush injury, Pulsed electromagnetic field

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INTRODUCTION

Musculoskeletal impairment is the most common trauma following physical impact and is frequently seen in sports medicine[1)]. Because of the diversity of musculoskeletal diseases and injuries, therapeutic approaches can range from simple analgesics to functional rehabilitation, and can result in chronic pain or loss of mobility and a severe deficit in quality of life[2)]. Despite its clinical importance, few clinical studies have reported on the treatment of musculoskeletal injury and proper therapies are needed for the restoration of muscle volume and function[1, 3)].

From a clinical point of view, muscle healing involves the formation of connective scar tissue, not primarily regeneration. Especially, in the case of serious injuries or broad defects, the healing process in impaired muscle tissue is often limited and characterized by extensive muscle fibrosis creating a tendency of muscle injury to recur. For patients with delayed and imperfect posttraumatic healing process of injured muscle, regeneration enhancing strategies are required[3, 4)].

Pulsed electromagnetic field (PEMF) is the one of the physical rehabilitation modalities. It is generated by external magnetic coils exciting electric fields in the conductive tissues of the body via inductive coupling including eddy current[5)]. PEMF is utilized at extremely low frequencies between 5 and 300 Hz, but can also emphasize typically short was frequencies, such as 27 MHz. PEMF is used in many countries for a wide range of therapeutic applications and for improved well-being[5, 6)]. It has been reported that indications for magnetic field therapy include fracture healing, degenerative diseases of the musculoskeletal system, disorders of the neurological system, muscle spasm, generalized inflammation, and poor circulation[7–11)]. However, it cannot be recommended without more scientific evidence about its therapeutic effect of PEMF. Therefore, the purpose of present study was to confirm the effects of electromagnetic field applied at different time points on the muscle recovery process through observation of the expression of heat shock protein (HSP 70) protein and histological changes in a rat muscle crush injury model.

SUBJECTS AND METHODS

Fifty-four male Sprague-Dawley rats, weighing between 250 g and 300 g, were used in the experiment. They were kept on a light/dark cycle with *ad libitum* access to food and water. All experiments were performed in accordance with protocols approved by the Animal Experiment Committee of University of Daegu, which are based on the NIH Guidelines for the Care and Use of Laboratory Animals (NIH publication, 1996). The animals were randomly divided

into 3 groups: the control (CON), pulsed electromagnetic fields (PEMF), and delayed pulsed electromagnetic fields (DPEMF) groups. Rats were anesthetized by intraperitoneal injection with a mixture of 2 mL/kg 50% zoletil and 50% xylazine hydrochloride. The left lower limb was shaved and disinfected with 70% ethanol. Through a 2 cm anterolateral longitudinal incision of the skins the underlying fascia from the extensor digitorum longus was mobilized. A blunt injury was induced on the left extensor digitorum muscle via an instrumented clamp (area of contact between muscle tissue and clamp: 5 mm^2), which allowed a standardized force application of 25 N for 10 s (DMC PLUS, HBM Hottinger Baldwin Messtechnik GmbH Germany). PEMF was conducted for the PEMF and DFEMF groups. The frequency of the electromagnetic field used in the present study was 27.12 MHz, and the application time was 20 minutes; the instrument used was a Diapulse (Diapulse Corp., America). The PEMF irradiation was performed once a day for 5 days from 1 day after injury for the PEMF group, and once a day for 3 days from 3 days after injury for the DPEMF. For sacrifice, the animals were anesthetized with a mixture of 2 mL/kg 50% zoletil and 50% xylazine hydrochloride and perfused through the heart with 200 mL of 0.9% NaCl solution, followed by 4% paraformaldehyde solution. The obtained samples were embedded in paraffin and sectioned at a thickness of 10 μm for immunohistochemistry and H-E staining. In brief, the sections were washed (3×10 min) in 0.01 M phosphate-buffered saline solution (PBS; pH 7.2) and incubated with appropriate primary antibodies for 12 h at room temperature. The antibody was diluted to 1:200 with a solution of Triton X-100 and normal donkey serum. After incubation in primary antibody, the sections were rinsed (3×10 min) in PBS, incubated for 90 min at room temperature with anti-mouse immunoglobulin (Ig) G (Vector Laboratories Inc, USA), diluted 1:25 in a solution of Triton X-100 and normal donkey serum. After incubation in secondary antibody, the sections were rinsed (3×10 min) in PBS, and incubated for 1 hour at room temperature with Vectastain Elite ABC-kit (Vector Laboratories Inc, USA). Then, the sections were rinsed again with PBS and incubated for 10 minutes in 0.04 mg of 3,3′-diaminobenzidine (DAB) in 200 mL distilled water. The sections were then incubated for 1 minute in DAB solution with 35% H_2O_2. The DAB sections were rinsed again with PBS (3×10 min) to halt the chromagen reaction, wet-mounted on gelatin/chromium-coated slides, and allowed to air-dry overnight. The sections were then dehydrated through a series of alcohols, soaked in xylene, and cover-slipped with Clarion (Biomedia, USA). To confirm the histological changes in brain tissue, the sections were washed (3×10 min) in 0.01 M phosphate-buffered saline (PBS, pH 7.2). Then, they were dipped in hematoxylin solution for 5 minutes and washed under running tap water. The sections were dipped in 1% HCl-alcohol solution 4 times, then, washed in running tap water for 5 minutes. After washing, they were dipped in eosin solution for 2 minutes, dehydrated via a graded series of alcohols, soaked in xylene and cover-slipped with Clarion (Biomedia, USA). Western blotting analysis was performed for quantitative investigation of the protein expression in the muscle tissue. Muscle samples from each group of rats were collected, washed twice in PBS, and then homogenized and lysated with buffer (137 mM NaCl, 8.1 mM Na_2HPO_4, 2.7 mM KCl, 1.5 mM KH_2PO_4, 2.5 mM EDTA, 1 mM dithiothreitol, 0.1 mM PMSF, and 10 μg/ml leupeptin [pH 7.5]) for 30 minutes on ice. Then, the lysates were centrifuged for 10 minutes at 15,000 rpm at 4 °C, and the protein concentration was determined, as described previously[12]. Equal amounts of protein (i.e. 40 μg) were resolved via 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. The blots were then washed with TBST (10 mM Tris · HCl [pH 7.6], 150 mM NaCl, 0.05% Tween 20), blocked with 5% skim milk for 1 hour, then incubated with the appropriate primary antibodies at the dilutions recommended by the manufacturers. The membranes were washed, and the primary antibodies were detected using horseradish peroxidase-conjugated goat anti-rabbit IgG or goat-anti mouse IgG. The bands were then visualized via enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The results are expressed as mean ± standard error (S.E.). All results were analyzed with analysis of variance (ANOVA), and comparisons of the mean values between the treatment and control groups were made using the Bonferroni-Dunn test. Differences were considered statistically significant at values of $p < 0.05$. All analyses were performed using SPSS for Windows (v. 12.0 K, SPSS Inc., USA).

RESULTS

Immunoblotting of HSP 70 expression was performed for each group. The optical density values relative to the CON group values on day 1 after injury were compared between groups and time points. The expression of HSP 70 tended to increase significantly with lapse of time in the PEMF and DPEMF groups, however, it decreased in CON ($p<0.05$). At 1 day after injury, there were not significant differences among the three groups ($p>0.05$), though there was a small increase in the PEMF group. At 3 days after injury, a more significant increase in HSP 70 expression presented in the PEMF group than in the DPEMF group ($p<0.05$). There were significant increases in the PEMF and DPEMF groups 5 days after injury with a greater increase in the PEMF group ($p<0.05$) (Table 1A). The immunohistochemistry results of HSP 70 expression were similar to those of immunoblotting at 5 days after injury (Table 1B).

To observe the histological changes induced by crush injury and therapeutic application, hematoxylin-eosin (H-E) staining was carried out 5 days after injury. General severe atrophy of muscle fiber and irregular arrangement was seen in the CON group. However, muscle atrophy and arrangement were improved in the PEMF and DPEMF groups with greater recovery in the PEMF group (Fig. 1).

DISCUSSION

The principal clinical indications for PEMF are fracture healing, degenerative diseases of the musculoskeletal system, disorders of the neurological system, muscle spasm,

Table 1A. The effect of pulsed electromagnetic field on the expression of HSP 70 after muscle crush injury

Group	Relative optical density (% of CON of on day 1 after injury)		
	1 day	3 days	5 days
CON	99.6 ± 1.4	84.6 ± 1.6*	65.2 ± 1.4*
PEMF	103.9 ± 1.5	134.1 ± 3.4*§	141.4 ± 4.4*§
DPEMF	96.2 ± 1.7	104.3 ± 2.6§	115.6 ± 2.4*§

Table 1B.

Group	Relative optical density (% of CON of on day 5 after injury)		
	CON	PEMF	DPEMF
5 days	99.4 ± 1.0	141.8 ± 3.4*	113.3 ± 2.1*

To confirm the expression of HSP 70, western blotting (A) and immunohistochemistry (B) were conducted on samples from the CON, PEMF, and DPEMF groups. Each example shown is representative of three experiments. The optical density values are the mean ± S.E. of the values of relative to the CON group on days 1 and 5 after injury. They were determined by densitometry relative to β-actin. Statistical analysis was performed by using two-way ANOVA. * $p < 0.05$ versus day 1 after injury between the period; §$p < 0.05$ versus CON between the group

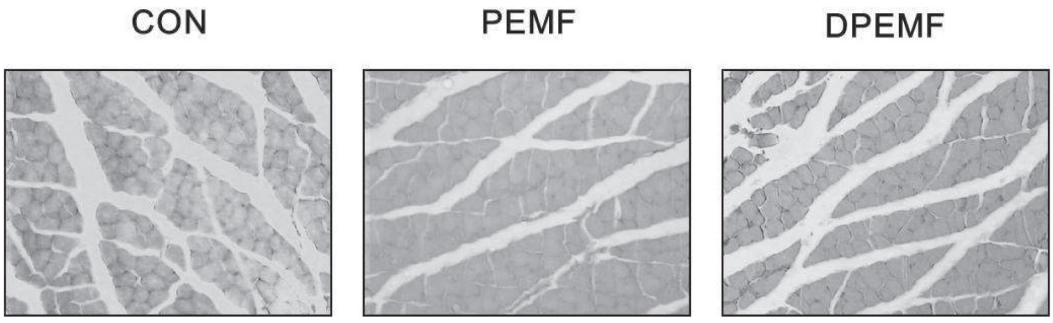

Fig. 1. The effect of pulsed electromagnetic field after muscle crush injury from H-E staining . To confirm the histological change, H-E staining was conducted on samples the CON, IST, and CST groups (×100).

generalized inflammation, and poor circulation[7]. These therapeutic properties of PEMF are well known, however, studies of interactions between organic tissues and PEMFs are few[13]. The purpose of present study was to confirm the effects of electromagnetic fields applied at different time points on muscle recovery through the observation of the expression of HSP 70 protein and histological changes in a rat muscle crush injury model.

It has been reported that the synthesis of HSP 70, which can be defined as HSP 70 expression, tends to be elevated in the acute phase of tissue injury. HSP, a stress protein, is synthesized shortly after cells are exposed to stress or a rapid rise in temperature[14]. It functions to minimize cell injury by maintaining proper protein folding with degenerative enzyme proteins and by supporting intracellular homeostasis, including protein transport and synthesis[4, 15]. Although HSP 70 is spontaneously expressed under rapid-onset pathological conditions, it does not sufficiently protect against cell injury.

In the present study, the application of PEMF increased the expression of HSP 70 protein in injured muscle which also showed histological improvement. A previous study reported that PEMF induced the cellular heat shock factor (HSF) 1 response, and released HSP 90 from the heat shock transcription factor 1 in primary human T lymphocytes and fibroblast cell lines[16]. PEMF increased HSF1 phosphorylation and HSF1–DNA binding together; these factors improve HSP 70 expression. Goodman et al.[17] demonstrated that magnetic field-exposed cells exhibited HSF1 DNA-binding activity and heat shock element-binding in a sequence of events that mediated the transcription of the HSP 70 gene and synthesis of the HSP 70 protein. Moreover, George et al.[18] reported that electromagnetic field induction of HSP 70 improved myocardial function. These results indicate that PEMF application is beneficial for the prevention of the pathological progression of muscle injury and the promotion of the repair mechanism by maintaining HSP 70 expression.

The healing process of the muscle generally includes three continuous processes of destruction, repair, and remodeling[1]. In the primary phase, the rupture and necrosis of myofibers and hematoma formation occurs in the injured portion, and chemokines, growth factors, and cytokines are required by inflammatory cells for recovery[19, 20]. This phase induces and overlaps with the repair phase. In addition, muscle healing involves the formation of connective scar tissue, not primarily regeneration. The healing process of injured muscle can frequently be hampered by the formation of scar tissue which is characterized by extensive muscle fibrosis and can lead to recurrence of muscle injury[4]. Thus, a therapeutic approach in this phase is important to decrease scar tissue and promote the alignment of muscle fiber for normal contraction. Our results show there was a the more significant increase in HSP 70 expression and greater histological improvement in the PEMF group than in the DPEMF group. This suggests that application of PEMF is helpful in the treatment of muscle repair.

Our results suggest that PEMF enhances the recovery of injured muscle via HSP 70 expression. More study will be needed to establish the clinical aspects for humans. The present study provides useful evidence of the biological effects of PEMF, which might exert a positive effect on intracellular metabolism stimulate metabolic activation in the extracellular matrix. It will be necessary to perform experiments in various injury models and environments to provide data for evidence-based practice.

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