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Original Article

Pulsed and Continuous Ultrasound Increase Chondrogenesis through the Increase of Heat Shock Protein 70 Expression in Rat Articular Cartilage

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Abstract. [Purpose] The present study was aimed to investigate the effects of pulsed and continuous ultrasound (US) irradiation on heat shock protein (HSP) 70 and mRNA levels of chondrogenesis-related gene expression in rat tibial articular cartilage. [Subjects and Methods] Forty-eight rats with body weights of 200−250 g were randomly divided into three groups. In the control (CON) group, three rats were treated with sham sonication. The pulsed US irradiation group was irradiated with a pulse rate of 20%, a frequency of 1 MHz, and an intensity of 1.5 W/cm^2 for 10 minutes. The continuous US irradiation group was continuously with a frequency of 1 MHz and an intensity of 1.5 W/cm^2 for 10 minutes. Immunohistochemistry for evaluation of HSP 70 and RT-PCR for expression of the chondrogenesis-related mRNA were used. [Results] The expression of HSP70 protein was increased in the pulsed and continuous US groups. The increase in the continuous US group was more prominent than in the pulsed US group. In addition, pulsed and continuous US irradiation increased the expression of Mustn1 and Sox9. [Conclusion] The results of this study show that US increases chondrogenesis via the increase of HSP 70 and chondrogenesis-related mRNA expressions in rat articular cartilage.
Key words: Articular cartilage, Heat shock protein 70, Ultrasound

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INTRODUCTION

Since therapeutic ultrasound (US) was adopted by the American Council of Physical Medicine and Rehabilitation as one of the general modalities for physical therapy in 1952, it has been applied and frequently studied in patients with musculoskeletal impairment[1)]. The thermal and mechanical effects of US are important when attempting to induce the physiological responses in injured regions[2)]. It influences the tissue-healing process of angiogenesis, chondrogenesis, intramembranous ossification, and bone remodeling by increasing the local circulation, the tensile strength of connective tissues, and tissue regeneration, and involving the movement of biochemical molecules such as Ca^{2+} and K^+ through the cellular membrane[3, 4)]. In particular, the recent study reported treatment with US one or a few times a day for up to a month for various kinds of degeneration in articular cartilage[5)].

A lot of diseases in articular cartilage including osteoarthritis are classified as progressive degeneration of articular cartilage and leading cause of musculoskeletal stiffness and weakness, joint instability, inflammation, deformity and a decreased range of joint motion in older adults worldwide[6)]. It is a multifactorial disease resulting from loss of equilibrium between matrix deposition and degradation as well as failure of joint cartilage repair after an initial insult[7, 8)]. Because of limitation of the limited capacity for repair due to avascularity and low cellular mitotic activity in articular cartilage, the treatment options for articular cartilage are few and unsatisfactory[6)]. Various new surgical strategies have been developed newly. However, they might disturb the structure of the cartilage and may even increase the risk of further damage[9)]. Due to these reasons, although some reports emphasize the importance of US therapy, one of the nonoperative treatments, in patients with degeneration in articular cartilage, which could influence chondrogenesis, studies concerning the effects of US are insufficient[7)].

Therefore, this study was designed to investigate the effects of pulsed and continuous US on the confirmation of HSP 70 expression, which protects cells and enhances cell viability in arthritis condition[10, 11)], and mRNA levels of chondrogenesis-related genes including Sox9 and Mustn1[8, 12)] in rat articular cartilage. This could contribute to establishment of the evidence of US treatment effects in articular cartilage and the possibility of finding factors

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having potential to be developed into therapeutic agents for arthritis.

SUBJECTS AND METHODS

We used 48 Sprague-Dawley rats (body weight 200–250 g) as experimental animals and housed them in standard cages (20 × 15 × 45 cm). The experimental animals were provided with sufficient feed and water during the experimental period. The housing room conditions were maintained as follows: 23 ± 2 °C temperature, 40–60% humidity, and 12 hours on/12 hours off light/dark cycle, in order to standardize environmental influences. All the experiments were performed in accordance with protocols approved by the University of Daegu Animal Experiment Committee, based on the NIH Guidelines for the Care and Use of Laboratory Animals (NIH publication, 1996). All 48 experimental animals were randomly divided into the control (CON), continuous US, and pulsed US groups and employed for each experiment.

In the rats treated with US, only the right knee was treated. US was applied with SONOPULS 590 (Enraf-Nonius, Rotterdam, The Netherlands). During the sonication procedure, the US head was held stationary, approximately 2 cm from the target area under water. The pulsed US was applied at a frequency of 1 MHz with the intensity of 1.5 W/cm^2, the pulse rate of 20%, and the duration of 10 minutes. The continuous US group was administered US for 10 minutes at a frequency of 1 MHz and an intensity of 1.5 W/cm^2. The CON group received sham sonication. Each group received the single US treatment session.

When euthanized, the animals were anesthetized with 2 mL/kg of 50% Zoletil and a 50% xylazine hydrochloride mixture and perfused through the heart with 200 mL of 0.9% NaCl solution followed by 4% paraformaldehyde solution. The articular cartilage was obtained 1 cm from the distal femur and proximal tibia in the knee joint without soft tissue. After collection, the samples were maintained for 24 hour post fixation period at room temperature and were then decalcified in solution (0.04 g/ml paraformaldehyde, 0.055 g/ml EDTA) for 4 weeks. The obtained samples were embedded in paraffin and sectioned at a thickness of 10 μm for immunohistochemistry. The sections were then subjected to deparaffinization and hydration, washed (3×10 minutes) in 0.01 M PBS, and incubated for 12 hours at room temperature with mouse monoclonal anti-HSP 70 (Chemicon International Inc., Temecula, CA, USA). The antibody was diluted 1:200 with a solution of Triton X-100 and normal donkey serum. After incubation in primary antibody, the sections were rinsed (3×10 minutes) in PBS, incubated for 90 minutes at room temperature with a anti-mouse IgG (Vector Laboratories Inc., Burlingame, CA, USA), and diluted to 1:25 with a solution of Triton X-100 and normal donkey serum. After incubation in the secondary antibody, the sections were rinsed (3×10 minutes) in PBS, and incubated for 1 hour at room temperature with a Vectastain Elite ABC kit (Vector Laboratories Inc., Burlingame, CA, USA). Sections were rinsed with PBS and incubated for 10 minutes in 0.04 mg 3,3′-diaminobenzidine (DAB, in 200 mL distilled water). Sections were then incubated for 1 minutes in DAB solution with 35% H_2O_2. The DAB sections were rinsed with PBS (3×10 minutes) to halt the chromogen reaction, wet-mounted onto gelatin/chromium-coated slides and allowed to air-dry overnight. After counter-staining with Toluidine Blue (Fluka Chemika, Buchs, Switzerland), the sections were dehydrated via a graded series of alcohols, soaked in xylene, and cover slipped with Clarion (Biomedia Corp., Foster City, CA, USA).

Moreover, when euthanized, the animals were anesthetized with 2 mL/kg of 50% Zoletil and a 50% xylazine hydrochloride mixture. The articular cartilage was obtained 1 cm from the distal femur and proximal tibia in the knee joint without soft tissue. After collection, the samples were washed twice and homogenized in PBS. The homogenates were centrifuged for 10 minutes at 15,000 rpm and 4 °C and collected by removing the supernatant. Total RNA was acquired from collected homogenates of articular cartilage using STAT-60, a monophasic solution of phenol and guanidine isothiocyanate (Tel-Test, Friendswood, TX, USA). RT was carried out with 3 μg of RNA using a reverse transcription system kit (AccuPower RT PreMix, Bioneer, Daejeon, Korea) with oligo(dT)$_{18}$ primers. Five μL of the RT products were then amplified using a polymerase chain reaction (PCR) kit (AccuPower PCR Premix) under the following conditions: denaturation at 94 °C for 5 minutes and 30 cycles at 94 °C for 45 seconds, 55 °C for 30 seconds, and 72 °C for 30 seconds, followed by 5 minutes of extension at 72 °C. The primers used were: 5′-GCTTTTCCTCTGCCACCTC-3′ (sense), 5′-CTCCACCCAGCCCCTTA-3′ (antisense) for Mustn 1; 5′-CCGACACGGAGAACACAC-3′ (sense), 5′-CACGACTTCCCGATACTGAC-3′ (antisense) for Sox9. PCR for β-actin was also carried out as a control for RNA quantity, as described in the previous report[13].

The results are expressed as the mean ± standard error (SE). All experiments were analyzed via analysis of variance, and some experiments were examined by comparing the treatment means with the controls using the Bonferroni-Dunn test. Differences were considered statistically significant when $p < 0.05$.

RESULTS

The immunoreactivity of the HSP 70 antibody was detected in the partial articular cartilage (Fig. 1 and Table 1). HSP 70 was found to be slightly expressed in the deep layer of cartilage in the CON group. In the pulsed US irradiation group, HSP 70 expression was observed in the superficial and intermediate layers. In the continuous US irradiation group, HSP 70 expression was noted in all three layers. The expression of HSP70 protein was significantly increased in the pulsed and continuous US groups ($p<0.05$). The increase in the continuous US group was more prominent than in the pulsed US group ($p<0.05$).

The mRNA levels of Mustn1 and Sox9, which are the makers of chondrogenesis, were detected (Table 2). Their expressions were increased significantly in the continuous and pulsed groups, respectively ($p<0.05$). In addition, the increase in the continuous US group was significantly more

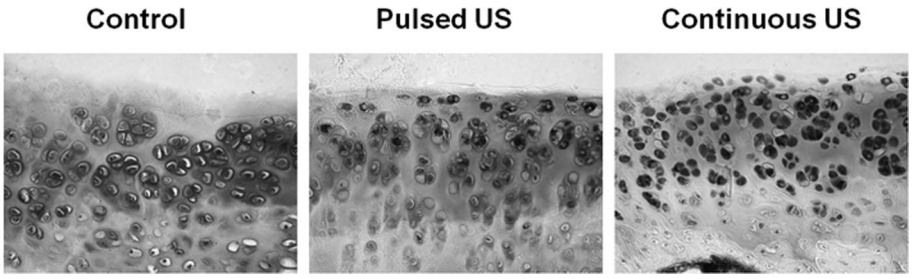

Fig. 1. Morphological results in US induced-expression of HSP 70
HSP 70 expression was detected after 10 minutes of application using immunohistochemistry in the CON (A), pulsed US (B), and continuous US (C) groups as described in the SUBJECTS AND METHODS. The ratio of expressed pixels per total pixels, was determined from densitometry relative to the CON, respectively. Each example shown is representative of three experiments.

Table 1. The effect of US on HSP 70 expression

Group	Relative expression ratio (% of CON)		
	CON	Pulsed US	Continuous US
	92.0±4.7	564.3±22.4*	833.6±40.0*§

HSP 70 expression in the CON, pulsed US, and continuous US groups was analyzed. Each example shown is representative of three experiments. The ratio of expressed pixels per total pixels for HSP 70 expression, was determined from densitometry relative to the CON, respectively. Each example shown is representative of three experiments. Statistical analysis was performed using one-way ANOVA. * $p < 0.05$ versus the CON group; §$p < 0.05$ versus the pulsed US group.

Table 2. The effects on US in Mustn1 and Sox9 expression

Group	Relative optical density (% of CON)		
	CON	Pulsed US	Continuous US
Mustn1	103.0±2.4	127.3±3.7*	149.5±5.6*§
Sox9	100.5±1.2	137.5±2.1*	152.5±1.8*§

Mustn1 and Sox9 mRNA expression in the CON, pulsed US, and continuous US groups was examined using RT-PCR. Each example shown is representative of three experiments. The levels of expression were determined by densitometry relative to β-actin, respectively. Statistical analysis was performed using one-way ANOVA. * $p < 0.05$ versus the CON group; §$p < 0.05$ versus the pulsed US group

prominent than in the pulsed group ($p<0.05$).

DISCUSSION

The aim of the present study was to investigate the effects of pulsed and continuous US on articular cartilage. Morrisette et al. demonstrated that 1 MHz US irradiation applied to the L4-L5 zygapophyseal joints induced a rise in temperature in the deep tissues[14].

Moreover, Loyola-Sánchez et al. reported that the low-intensity US therapy was favorable for repair of injured cartilage in people with mild to moderate knee osteoarthritis[7]. Clinically, US exerts both thermal and mechanical effects[15]. The thermal effect involves increases in tissue temperature and metabolism, the tensile force of soft tissue, and nerve conduction velocity as well as vasodilation and blood flow elevation, analgesia, and wound healing stimulation[16, 17]. Additionally, the mechanical effect involves the stimulation of fracture healing via the micromassage effect[18]. However, its effect on *in vivo* tissue levels is still only partly known.

First, we examined the expression of HSP 70 in tibial articular cartilage after pulsed and continuous US irradiation. The function of HSP 70 acts a chaperone protein for decrease of cell injury to help the adjustment of proper protein folding combined with degenerative enzyme proteins as well as intracellular homeostasis[13, 19]. HSP 70 is activated spontaneously in the early period of pathological conditions. However, it is incapable of providing protection against degeneration of cartilage. Thus, application of US to increase HSP 70 expression is needed to prevent the pathological progression in articular cartilage and induce the repair mechanism[20].

According to our results, expression of HSP 70 was noted in the superficial and intermediate layers in the pulsed US irradiation group. In the continuous US irradiation group, HSP 70 expression was observed in all three layers. HSP 70 expression in the pulsed US group showed a more significant increase in the superficial layer, whereas in the continuous group, HSP 70 expression was found to show a greater increase in the intermediate layer. It was demonstrated that the pulsed and continuous US modes induced enhanced endochondral ossification in vitro[21]. El-Bialy et al. demonstrated that continuous US irradiation increased bone healing in early stages, whereas pulsed US irradiation increased bone healing in later stages[22]. These facts suggest that pulsed and continuous US could increase the expression of HSP 70 by mechanical and thermal effects, respectively. However, there have been a few reports regarding the effects of US according to pulsation; this issue may require more study.

In addition, the results of this study showed that US irradiation stimulated an increase in chondrogenesis-related mRNA alterations in articular cartilage tissues. In the present study, we confirmed that the application of US increased

Sox9 and Mustn1 mRNA expression. Sox9 and Mustn1 are chondrogenic marker genes. Sox9 is an essential mediator of chondrocyte-specific protein during cartilaginous development and stimulates the early phase of chondrogenesis[23]. Especially, Sox9 induces chondrocyte differentiation and endochondral bone formation by binding to CREB[24, 25]. In addition, Mustn1 is a co-activator of a transcriptional complex that is involved in the proliferation and differentiation of chondrocytes, cartilage development, and bone regeneration[26]. The important role of Sox9 during chondrocyte differentiation induces expression of Mustn1. These are then responsible for the proper position to explore which is responsible for the transcriptional activation of Collagen II in multiple chondrogenic and osteogenic tissues[12]. Moreover, during Sox9-mediated gene regulation, HSP 70 plays an important role in the formation of multi-protein complexes[27].

In conclusion, that pulsed and continuous US increase chondrogenesis via the increase in HSP 70 and mRNA levels of chondrogenesis-related gene expressions in rat articular cartilage. Although this study has the limitation that it was performed in a normal animal model and needs more investigation concerning clinical aspects in humans, it would contribute to the suggestion of the evidence that the biological effects of US in the articular cartilage would provide a therapeutic effect on the activation in the intracellular metabolism and extracellular matrix. In addition, the present results not only demonstrate the therapeutic mechanism of US from a basic viewpoint, but also indicate the potential of physical therapy as a non-pharmacological and nonoperative method for articular cartilage repair. It would be needed to perform experiments in the various injured models and environments for support of evidence based practice.

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