PROTECTIVE EFFECTS OF LOW-INTENSITY PULSED ULTRASOUND ON MANDIBULAR CONDYLAR CARTILAGE EXPOSED TO MECHANICAL OVERLOADING

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(Received 10 September 2018; revised 4 December 2018; in final from 17 December 2018)

Abstract—The aim of this study was to assess the effect of low-intensity pulsed ultrasound (LIPUS) application on rat temporomandibular joints (TMJs) with early-stage of osteoarthritis-like conditions induced by mechanical overloading. Fifteen-week-old male Wistar rats were divided into two experimental groups and a control group (n = 10 each). Both TMJs of all rats in one experimental group were subjected to mechanical overloading for 5 d, and those in the other experimental group were exposed to LIPUS for 20 min/d after overloading. Condyles were assessed using micro-computed tomography, histology and histomorphometry. LIPUS treatment attenuated cartilage degeneration, decreased the number of osteoclastic cells and restored the expression of aggrecan after an initial decrease induced by mechanical overloading. These results indicate that LIPUS may have a protective effect on the early progression of TMJ osteoarthritis. (E-mail: fujita.mutsumi@tokushima-u.ac.jp) © 2018 World Federation for Ultrasound in Medicine & Biology. All rights reserved.

Key Words: Temporomandibular joint, Osteoarthritis, Mandibular condyle, Low-intensity pulsed ultrasound, Rats.

INTRODUCTION

Synovial joints play a key role in allowing relatively large bone movements induced by surrounding muscle forces (Widegren et al. 2000). The bone ends come together within a fibrous joint capsule. The synovium, a metabolically active tissue, covers the inner lining of this joint capsule. Each bone end within the joint is covered by a thin layer of dense connective tissue known as the articular cartilage (Warwick and Williams 1973). Ligaments, tendons and other soft tissues around the joint cavity provide stability to the joint and maintain appropriate alignment of the articulating bone ends during motion (Warwick and Williams 1973). An important facial joint, the temporomandibular joint (TMJ), is a diarthrodial synovial joint that allows a relatively wide range of movements between the temporal bone and the mandibular condyle (Rees 1954; Scapino et al. 2006). The integrity of the joint is maintained intrinsically by a fibrous capsule with ligamentous thickenings and extrinsically by accessory ligaments, both of which restrict movement at the extremes of the mandibular range of motion and, consequently, have limited influence on the mechanics of normal symmetric function (Kawai et al. 2004; Tanaka et al. 2004). Within the joint, the articular surfaces of the condyle and articular eminence are covered by a thin fibrocartilaginous layer with a very low coefficient of friction (Tanaka et al. 2004).

The most common pathology affecting TMJ is degenerative joint disease, also known as osteoarthritis (OA). The hallmarks of OA include collapse of the articular cartilage, osteophyte formation and subsequent joint space narrowing (Arends et al. 2017). TMJ-OA is characterized by degradation of the mandibular condylar cartilage caused by mechanical overloading (Kuroda et al. 2009; Leonard et al. 2003; Tanaka et al. 2008). Mechanical overloading of the mandibular condylar cartilage

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increases the expression of interleukin-1β (IL-1β) (Yoshida et al. 1997), an inflammatory cytokine closely associated with the progression of TMJ-OA (Ghassemi-Nejad et al. 2011; Kuroda et al. 2009). IL-1β markedly stimulates osteoclastic bone resorption by enhancing both osteoclast formation and function (Kusano et al. 1998). Because the fibrocartilage covering both the TMJ condyles and glenoid fossa is avascular, its cells have limited ability for self-repair (Neumann et al. 2010; Scheven et al. 2009), similar to hyaline cartilage in other synovial joints (Iwamoto et al. 2013). Therefore, once cartilage breakdown starts, TMJ-OA can be crippling, leading to various morphologic and functional deformities. This suggests the importance of suppressing cartilage degradation during the early stages of this disease. Although moderate physical exercise has been associated with delayed onset and slow progression of OA in humans (Griffin and Guilak 2005; Toyoda et al. 2003), no treatment remedy has been developed for severe OA.

Low-intensity pulsed ultrasound (LIPUS) is an acoustic radiation source with an intensity less than 100 mW/cm². Ultrasound has been widely accepted as a therapeutic, operative and diagnostic tool in the medical field. Previous in vivo studies have reported that LIPUS can promote bone healing, remodeling and regeneration and augment osteogenesis at the distraction site. It is generally accepted that LIPUS has no deleterious or carcinogenic effects. Moreover, LIPUS exposure has no thermal effects inducing biological changes in living tissues. Previously, we reported that LIPUS reduces inflammation and promotes regeneration in various conditions involving injured soft tissues, such as synovitis of the knee joint, injury of the skeletal muscle and sialadenitis (Nagata et al. 2013; Nakamura et al. 2010, 2011; Sato et al. 2015). Therefore, we hypothesized that the anti-inflammatory effects of LIPUS can help to suppress cartilage degradation during early stages of TMJ-OA. The aim of the present study was to assess the effect of LIPUS application on TMJs in the early stage of OA-like conditions induced by mechanical overloading.

METHODS

Animals

A total of 30 male Wistar rats, aged 15 wk and weighing on average 300 g, were kept individually in plastic cages with smooth walls, maintained at room temperature and average humidity. They were fed standard solid diet pellets containing 1% calcium and 0.06% phosphorus, 20 IU/g vitamin A, 2.20 IU/g vitamin D, 7 IU/g vitamin E, 20% protein, 4% fat, 3.5% fiber, 6% ash and 0.5% salt, ad libitum throughout the experiment. These conditions were maintained and monitored throughout the experiment to ensure the proper health of the rats. The animals were randomly divided into two experimental groups and a control group of 10 animals each. Both TMJs of all rats in one experimental group were subjected only to mechanical overloading for 5 d (OL group), and those in the other experimental group were exposed to LIPUS for 20 min/d after mechanical overloading (OL + LIPUS group). In the control group, TMJs were not subjected to any form of mechanical overloading, although the same anesthesia schedule was maintained. Five animals from each group were prepared and assessed using micro-computed tomography (micro-CT), and the remaining 5 animals in each group were prepared for histology and immunohistochemistry. All procedures performed in this study were approved by the Tokushima University Animal Care and Use Committee (Permit No. T27-93).

Application of mechanical stress

In the experimental groups, both TMJs were subjected to mechanical overloading by forced mouth opening using a jaw-opening device for 3 h/d for 5 consecutive d. A previous protocol of overloading TMJs was adopted, in which a custom-made spring was used to keep the maxillary and mandibular incisors 20 mm apart, applying a force of 2 N on each TMJ (Kawai et al. 2008; Izawa et al. 2016). Before and during the mouth-opening period, rats were anesthetized with intra-abdominal injections of sodium pentobarbital (Nembutal, Dainabott, Osaka, Japan) at a dose of 50 mg/kg weight.

Low-intensity pulsed ultrasound

In the OL + LIPUS group, LIPUS was applied with a modified version of the clinical device, Osteotron V (ITO Co., Tokyo, Japan) after mechanical overloading. Both TMJs were exposed to LIPUS for 20 min/d during the 5-d experimental period. The ultrasound exposure system was equipped with a circular surface transducer with a cross-sectional area of 5.0 cm². The ultrasound head exhibited a mean beam nonuniformity value of 3.6 and an effective radiating area of 4.1 cm². An ultrasound signal was transmitted at a frequency of 1.5 MHz, with a spatial average intensity of 30 mW/cm² and a pulse rate of 1:4 (2 ms on and 8 ms off).

Micro-computed tomography

Rats in all groups were then sacrificed with an overdose of anesthesia. Mandibles were resected, and the condyles were carefully cut and separated from the surrounding soft tissues and fixed in 70% ethanol overnight. They were then analyzed using high-resolution micro-CT (SkyScan 1176 scanner, Buruker, Billerica, MA) and its analysis software. Images were acquired at 50 kV and 500 µA. During scanning, samples were tightly covered with plastic wrap to prevent movement and dehydration.
Thresholding was applied to the images to segment the bone from the background. The resolution of the micro-CT images was 9 μm/pixel. The region of interest (ROI) was the posterior region of the mandibular condyle in the midsagittal section. The precision error of the micro-CT was less than 5%. For each ROI, microstructural parameters, including the bone volume (BV)-to-trabecular volume (TV) ratio (BV/TV), trabecular thickness (Tb.Th) and trabecular number (Tb.N), were analyzed.

Tissue preparation and histologic analysis
After the animals were sacrificed, TMJs were resected, fixed in 10% buffered paraformaldehyde and decalcified using 10% EDTA at 4°C for 8 wk. They were then embedded in paraffin and prepared for sectioning. Histologic serial sections (7 μm) were cut in the sagittal plane and were stained with hematoxylin and eosin, 0.1% Safranin-O and 0.02% fast green. The last two stains were used for the detection of cartilage and proteins, respectively. In addition, toluidine blue staining was used to detect proteoglycans in the condylar cartilage.

A modified Mankin scoring system (Xu et al. 2003) was used to assess the degree of cartilage degeneration. The Safranin-O-stained sections were used for scoring the features of cartilage disease, including changes in cellularity and structural abnormalities. Safranin-O uptake was assessed to measure glycosaminoglycan distribution and loss. Sections were blindly examined by three independent experts.

Tartrate-resistant acid phosphatase staining
Tartrate-resistant acid phosphatase (TRAP) activities were measured to identify the characteristics of osteoclast lineage cells according to the method of Minkin (1982). The staining medium consisted of naphthol AS-MX phosphate (Sigma Chemical Co., St. Louis, MO, USA) as a substrate, Fast Red Violet LB Salt (Sigma Chemical Co.) as a coupler and 50 mM sodium tartrate (Wako, Osaka, Japan). Hematoxylin was used for counterstaining. Negative staining was performed without substrate.

Immunohistochemistry
To investigate the expression of aggrecan, type II collagen (Col2a1), matrix metalloproteinase (MMP)-9 and MMP-13 in the condylar cartilage, immunohistochemical staining was performed using various primary antibodies (Immuno Biological Laboratories, Fujioka, Japan). After the sections were deparaffinized and blocked in, they were incubated overnight at 4°C in a humid atmosphere with various primary antibodies diluted in phosphate-buffered saline/0.1% bovine serum albumin. Immunostaining was performed using a Histofine simple stain kit (Nichirei, Tokyo, Japan). Briefly, after blocking endogenous peroxidase activity with 0.3% hydrogen peroxide in methanol, non-specific binding of the antibody was blocked by incubating the sections for 30 min with Non-Specific Staining Blocking Reagent (Dako, Carpinteria, CA, USA). After being washed, the sections were incubated with the corresponding secondary antibodies for 1 h at room temperature and then mounted. The sections were examined under a BioRevoBZ-9000 microscope (KEYENCE, Osaka, Japan). Negative controls were stained with non-immune immunoglobulin G.

Histometric analysis
From each animal, one sagittal section was selected from the midsagittal plane of the condyle. The condylar cartilage in each section was divided into anterior, intermediate and posterior regions. As the posterior region was subjected to damage caused by mouth opening, the numbers of aggrecan-positive, Col2a1-positive, MMP-9-positive and MMP-13-positive chondrocytes were counted under a fixed measuring frame (430 × 1470 μm) in the posterior region.

For one TRAP-stained section through the midsagittal plane of the condyle, the number of osteoclasts was also counted in the mineralized layer subjacent to the hypertrophic cell layer of the condylar cartilage. TRAP-positive cells with two or more nuclei were regarded and counted as osteoclasts.

Statistical analysis
All values are expressed as means with standard deviations. Significant differences in experimental data were analyzed using one-way analysis of variance, followed by the Turkey-Kramer test as a post hoc test to examine mean differences at a significance level of 5%.

RESULTS
All rats survived throughout the experiment with good health. There were no dropouts.

Micro-computed tomography
Micro-CT images revealed severe subchondral trabecular bone loss in the OL group, but not in the control group (Fig. 1). In contrast, treatment with LIPUS after overloading reduced the amount of subchondral trabecular bone resorption in the OL + LIPUS group. Furthermore, BV/TV, Tb.Th and Tb.N in different areas of the condylar subchondral bone were significantly lower in the OL group than in the control group ($p < 0.01$), whereas all parameters were significantly higher in the OL + LIPUS group than in the OL group ($p < 0.05$ or $p < 0.01$). Although Tb.Th and Tb.N were higher in the
control group than in the OL + LIPUS group, the differences were not significant.

**Histology**

Hematoxylin and eosin-stained sections of mandibular condyles from the control group revealed that the surface of the condylar cartilage was smooth, with obviously distinguished cell layers (Fig. 2). In contrast, the mandibular condyles from the OL group exhibited marked OA-like lesions, including a decrease in the thickness of the cartilage layer, irregularities in chondrocyte alignment and hyalinization of the cartilage matrix in the cartilage layers. However, there was markedly less damage in the condylar cartilage from the OL + LIPUS group than in that from the OL group.

Safranin-O-stained sections revealed that proteoglycans were abundant in the deep layer of the mandibular condylar cartilage derived from the control group (Fig. 3a). However, the amount of proteoglycans was significantly decreased in the samples derived from the OL group. Modified Mankin scores confirmed that overloading caused significant \( p < 0.01 \) changes in the structural characteristics that paralleled the progression of OA (Fig. 3c). LIPUS exposure after overloading...
significantly \((p < 0.01)\) decreased the modified Mankin score relative to that for the OL group, although the scores were still significantly lower in the control group than in the OL + LIPUS group \((p < 0.01)\). Attenuated toluidine blue staining was observed in condylar cartilage from the OL group; this reduction in staining was restricted after LIPUS exposure (Fig. 3b). The significantly reduced amount of proteoglycans in the condylar cartilage \((p < 0.01)\) after overloading was significantly \((p < 0.05)\) restored to the level observed in the control group after LIPUS exposure (Fig. 3d).

**Number of TRAP-positive osteoclasts**

In the control group, the number of TRAP-positive osteoclasts in the mineralized layer of the condylar cartilage was 5.6 ± 0.9 (Fig. 4a, b). This number was 13.2 ± 3.0 after mechanical overloading and 10.0 ± 1.6 after LIPUS exposure. Thus, the number of TRAP-positive osteoclasts was significantly smaller in the control group than in the OL \((p < 0.01)\) and OL + LIPUS \((p < 0.05)\) groups.

**Immunohistochemical analysis**

Immunohistochemistry showed more MMP-9 and MMP-13-positive cells and fewer Col2 a1- and aggrecan-positive cells in the OL group than in the control group (Fig. 5a–d). LIPUS exposure downregulated the numbers of MMP-9- and MMP-13-positive cells and upregulated the numbers of Col2a1- and aggrecan-positive cells in condylar cartilage from the OL + LIPUS group. These data indicate that LIPUS exposure downregulates expression of the cartilage destruction factor.

The numbers of Col2a1- and aggrecan-positive cells were significantly \((p < 0.01)\) larger in the control group than in the OL and OL + LIPUS groups (Fig. 5a, b). The OL group exhibited significantly more MMP-9- and MMP-13-positive cells than did the control \((p < 0.01)\) and OL + LIPUS \((p < 0.05\) or \(p < 0.01)\) groups.

**DISCUSSION**

Osteoarthritis, the most common joint disease, is characterized by joint pain and stiffness with subsequent disability. The prevalence rate for symptomatic knee OA in individuals \(\geq 60\) y ranges from 10% to 13% (Zhang and Jordan 2010). OA is also the most common joint pathology affecting TMJ; among patients with TMJ disorders, 11% exhibited symptoms of TMJ-OA (Mejersjö and Hollender 1984). Management strategies for TMJ-OA may be divided into non-invasive and invasive strategies, and for end-stage disease, salvage modalities must be considered (Tanaka et al. 2008). Although conventional treatment modalities for OA are pharmacological as well as non-pharmacological (regulation of daily activities, education and exercise), no remedy has been able to revive cartilage degeneration. Therefore, ultrasound therapy aimed at tissue repair and regeneration has garnered considerable attention.

Previously, several studies reported the efficacy of LIPUS exposure in pathologic chondrocytes derived from diseased knee cartilage. Uddin et al. (2016) described the potential of LIPUS therapy to prevent cartilage destruction through mechanical stimulation, thereby inhibiting the catabolic action of IL-1\(\beta\) and stimulating chondrocyte migration, proliferation and differentiation. Nishida et al. (2017) also stated that CCN protein 2 (CCN2) production with chondrocyte differentiation was regulated by mitogen-activated protein kinase (MAPK) pathways activated by LIPUS-induced Ca\(^{2+}\) influx. Furthermore, we investigated the effects of LIPUS exposure on IL-1\(\beta\)-induced cyclooxygenase-2 (COX-2) expression in cultured chondrocytes derived from porcine mandibular condyles and emphasized that LIPUS exposure inhibited IL-1\(\beta\)-induced COX-2 expression via the integrin \(\beta 1\) receptor, followed by the phosphorylation of extracellular signal-related kinase (ERK) 1/2 (Iwabuchi et al. 2014). However, the effects of LIPUS on mandibular condylar cartilage in patients with TMJ-OA induced by mechanical overloading in vivo remain unclear. To the best of our knowledge, this
Fig. 3. Histochemically stained sections of mandibular condylar cartilage from experimental and control rats. (a) Safra-
nin-O and fast green staining. (b) Toluidine blue staining. (c) Comparison of proteoglycan staining results among the
three groups. (d) Histologic grading according to modified Mankin scores for the mandibular condylar cartilage obtained
from the three groups. Data are expressed as means and standard deviations. *$p < 0.05$, **$p < 0.01$ per Tukey–Kramer
tests ($n = 5$ for each group). Bars = 100 μm. LIPUS = low-intensity pulsed ultrasound; OL = mechanical overloading.
is the first study investigating the effects of LIPUS on the early progression of TMJ-OA induced by mechanical overloading.

In the present study, exposure of overloaded condylar cartilages to LIPUS attenuated cartilage degradation, reduced the number of osteoclastic cells and reduced the expression of MMP-9 and MMP-13. In addition, the amount of proteoglycans in condylar cartilage treated with LIPUS after forced mouth opening was restored to the same level observed in the control group. These results imply that inhibitory effects of LIPUS on osteoclastic bone resorption and condylar cartilage degradation in patients with TMJ-OA are induced by mechanical overloading. Jia et al. (2016) evaluated the efficacy of focused LIPUS therapy as a non-invasive modality for knee OA in a double-blind placebo-controlled trial including 106 patients with bilateral knee OA. The authors found that LIPUS is a safe and effective modality for pain relief and improvement of knee joint function in OA patients. Similarly, the present study indicates that LIPUS is a non-invasive treatment modality that prevents mandibular condylar resorption and cartilage degradation during the early progression of TMJ-OA.

Because of its limited capacity for regeneration, articular cartilage requires structural and metabolic support after traumatic and/or chronic damage. However, available techniques for cartilage regeneration have either been invasive or ineffective. Recently, Yilmaz et al. (2017) conducted a randomized controlled trial of LIPUS treatment in a rat model of knee OA and found that extracorporeal therapy and LIPUS exposure have systemic proliferative and regenerative effects on cartilage and tissue. Our results revealed that treatment with LIPUS upregulated the expression of aggrecan and type II collagen, which was reduced after mechanical overloading. However, the levels were still significantly lower than those in the control group. Micro-CT revealed that BV/TV, Tb.Th and Tb.N were significantly greater for mandibular condyles treated with LIPUS.
after mechanical overloading than for overloaded condyles not treated with LIPUS; moreover, Tb.Th and Tb.N were almost the same as for untreated condyles. Furthermore, the modified Mankin score for the mandibular condyles from the OL + LIPUS group was significantly lower than that for the condyles from the OL group, although the score for the condyles from the OL + LIPUS group was significantly higher than that for the condyles from the control group. These results indicate that LIPUS contributes, to some extent, to condylar cartilage repair and regeneration in patients with TMJ-OA induced by mechanical overloading. Further studies are needed to identify the maximal effects of LIPUS on cartilage regeneration.

Cartilage is a highly mechanoresponsive tissue. Chondrocytes function as mechanosensors and undergo a series of complex changes, including proliferation and metabolic alteration, as targets of external biomechanical and biochemical stimuli (Uddin et al. 2016). Therefore, mechanical stimulation such as LIPUS exposure may inhibit the catabolic action of IL-1β and stimulate chondrocyte migration, proliferation and differentiation. IL-1β is a known inflammatory mediator that acts through the nuclear factor-kB pathway to induce the expression of several genes that are upregulated in cartilage affected by OA, such as IL-1β, IL-6, IL-8 and MMP-13 (Goldring 2012). Several reports suggest that LIPUS exposure activates integrins on the cell membrane that act as mechanoreceptors to promote the attachment of various focal adhesion adaptor proteins (Lal et al. 2007; Uddin et al. 2016). We also found that LIPUS exposure caused significant upregulation of phosphorylated focal adhesion kinase (FAK) in knee synovial joint cells, and that inhibition of FAK phosphorylation led to significant downregulation of MAPK phosphorylation (Sato et al. 2014; Tanaka et al. 2015). Because the MAPK pathway is considered a general pathway involved in cell proliferation (Cowan and Storey 2003), the biological effects of LIPUS exposure on chondrocytes may be particularly promoted by the integrin/FAK/MAPK pathways. Further studies should evaluate in detail the mechanisms underlying the effects of LIPUS on articular cartilage affected by TMJ-OA.

Despite the evident protective effect of LIPUS reported in the present study, caution should be taken when applying these findings to humans. Unlike humans, in whom clinical OA is generally diagnosed as irreversible (Shirakura et al. 2010), the induced OA-like lesions in the TMJs of rats in this study decreased after forced mouth opening ceased, without site-specific changes. This could be due to the fact that rats were free to bite in this model. Also, it is difficult to extract mandibular condylar chondrocytes from rat condyles because of their small size. Finally, masticatory patterns during chewing

Fig. 5. Immunohistochemical staining for aggrecan, type II collagen and matrix metalloproteinase (MMP)-9 and MMP-13 in mandibular condylar cartilage samples from experimental and control rats. (a) Aggrecan. (b) Collagen type II (Col2 a1). (c) MMP-9. (d) MMP-13. *p < 0.05, **p < 0.01 per Tukey-Kramer tests (n = 5 for each group). Bars = 100 μm. LIPUS = low-intensity pulsed ultrasound; OL = mechanical overloading.
obviously differ among animal species (Langenbach and van Eijden 2001). In rats, all masticatory muscles can contract symmetrically, whereas a separation in time is present in forward and backward pulling muscles, resulting in addition of a retraction and protraction of the jaw to the hinge movement (Langenbach and van Eijden 2001). Meanwhile, in humans and pigs, the activities of working side temporalis, balancing side masseter and medial pterygoid muscles can be clearly separated in time, resulting in a smaller or larger transverse components in the jaw movement (Langenbach and van Eijden 2001). This might explain the difference in loading conditions in the TMJ between humans and rats (El-Bialy and Kaur 2018).

CONCLUSIONS

The results of this study suggest that LIPUS treatment attenuates cartilage degeneration, downregulates chondrocyte differentiation and protects the early progression of TMJ-OA induced by mechanical overloading. Moreover, it restores the expression of aggrecan after an initial decrease induced by mechanical overloading. Therefore, LIPUS exposure may represent an effective treatment strategy for TMJ-OA induced by mechanical overloading. These findings may have important implications for future ultrasound research, particularly in terms of the management of TMJ-OA.

Acknowledgments—This research was supported by a Grant-in-Aid (26293436 [E.T.]) for Science Research from the Ministry of Education, Culture, Sports, Science and Technology. Japan. We are grateful to Tsukasa Kurahashi, Nobuyasu Yamanaka and Atsushi Chuma for providing the ultrasound devices and technical assistance during the experiments.

Conflict of interest disclosure—E.T. has received research funding from ITO Company, Ltd. All of the other authors state that they have no conflicts of interest.

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