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The effects of therapeutic ultrasound stimulation on the inflammation cytokine in rat articular chondrocytes

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Objective: The aim of this study was to investigate the effect of therapeutic ultrasound (US) of cell viability and inflammatory cytokine in rat articular chondrocyte cultures stimulated with lipopolysaccharide (LPS).
Design: One group pretest-posttest design.
Methods: Cultured chondrocytes were treated with US and/or LPS and assessed for viability, Tumor necrosis factor (TNF)-α and Interleukin (IL)-1 production.
Results: Oxidative stress was induced in rat chondrocytes with LPS. The cell viability was decreased in chondrocytes after treatment with LPS. The viability revealed that low-intensity pulsed ultrasound (LIPUS) exerted no significant cytotoxicity in the rat chondrocyte. LIPUS inhibited decreased cell viability in the presence of LPS (30 μg/ml) in a intensity dependent pattern at LIPUS ($p < 0.05$). TNF-α production in the presence of LPS was also inhibited in a dose dependent manner ($p < 0.05$ from 30 mW/cm^2). IL-1 production in the presence of LPS was inhibited as well ($p < 0.05$ from 7.5 mW/cm^2).
Conclusions: Our results demonstrate that US was the anti-inflammatory effect of chondrocytes. LIPUS may exert its anti inflammatory effects through inhibition of TNF-α and IL-1 synthesis. These results suggest that US have potential for use as a pain relief and reduce the articular destruction.

Key Words: Chondrocytes, Inflammatory cytokine, Ultrasound

Introduction

Osteoarthritis (OA) is one of the most common a degenerative joint disease, considered to be one of the major public health problems in the world [1]. The risk of mobility impairments caused by knee OA alone is greater than due to any other medical condition in people over 65 years of age [2]. Also, It prevalence after age 65 is increasing in incidence as a chronic condition widespread, and it is likely to rise further with the obesity epidemic [3]. Women are affected more than men [4], and the most common OA site in, hips, knees, spine, and hands [5,6]. Patients predominant symptoms are joint pain, stiffness and swelling in the early. Joint pain typically worsened with weight bearing, muscle weakness, and functional activity [7,8].

Joint inflammation is a well recognized feature of OA, notably in the early stage [9]. The histological pathology reflects the result of joint inflammation and cartilage erosion. Since the initial stages of OA involve increased proteinase, inflammation cytokines, and other inflammatory mediators by chondrocytes [10]. OA related between the increased concentration of catabolic enzymes and inflammatory mediators such as cyclo-oxygenase 2, nitric oxide, interleukin 1 (IL-1), and tumor necrosis factor α (TNF-α) in synovial flu-

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ids and joint tissue [11]. Current OA drugs treatment used to manage OA are failed to efficacy or were related with side effects. Non-steroidal anti-inflammatory drugs are widely used but their prolonged consumption is associated with serious adverse side effects such as cardiovascular risk and gastrointestinal ulcerations [12]. The need for effective treatment modalities with fewer side effects has prompted OA patients to consider complementary approaches to control pain as well as to improve function and quality of life.

The most common types of electrotherapy are ultrasound (US), a nondestructive modality in which mechanical energy is transmitted transcutaneously as high-frequency acoustical pressure waves into biological tissues [13]. Therapeutic US provides an immediate mechanical stimulation for cell proliferation, angiogenesis, and enhancement of mineralization in vitro [14-16]. Low intensity pulsed US relieve pain and inflammation in OA patients or rheumatoid arthritis and delay its progression, but not yet studies have so far failed to prove convincingly that it anti-inflammatory related works in lipopolysaccharide (LPS) induced chondrocyte oxidative stress. So our research has focused on the chondrocyte as the cellular inflammation mediator of OA pathogenesis.

This study investigated the effect of therapeutic US in inflammatory cytokine such as TNF-α and IL-1, which are implicated in OA by using rat primary cultured chondrocyte exposed to an LPS-induced stress.

Methods

Chondrocyte isolation and culture

A modified method for harvesting chondrocytes was used as previously [17]. Chondrocytes were isolated from the rat articular cartilage of 3 week old male Sprague- Dawley rats. Cartilage was removed from animals that were subsequently euthanized by an overdose of anesthesia. The cartilage was cut into thin slices, washed with sterilized phosphate-buffered saline, and soaked in 5% penicillin- streptomycin (Sigma, St. Louis, MO, USA) for 15 min. The cartilage slices were washed with PBS to remove residual antibiotic solution and digested with 0.02% type II collagenase (Sigma) in Dulbecco's Modified Eagle's Medium (HyClone, Logan, UT, USA) for 2 h in a 37°C water bath. The digested cartilage was collected and centrifuged. The pellet was resuspended in Dulbecco's modified Eagle medium (DMEM) and filtered through 70-μ m nylon mesh. The resultant chondrocytes were cultured in DMEM supplement with 10% fetal bovine serum and 1% penicillin- streptomycin in a 5% CO_2 incubator at 37°C. All experiments were performed when cells reached confluence within the first passage.

Ultrasound treatment

For Healing Stimulation, US Device (Exogen 4000+, Smith & Nephew Inc., London, UK) at a frequency of 1.5 MHz was applied to the chondrocytes after 72h in culture. The US parameters included a spatial-average temporal-average output intensity of 0, 7.5, 15, 30, 60, 120, 200 mW/cm^2. The frequency was 1.5 MHz with a 200-μs tone burst repeated at 1.0 kHz. Each 6-well plate of the low-intensity pulsed ultrasound (LIPUS) group was placed on an ultrasonic transducer [18]. After the plate cover had been removed, an anti-reflection chamber was placed in each well while taking care to avoid producing air bubbles. LIPUS was applied to the chondrocytes after 24 hours in culture through the bottom of the culture dish that had been placed between the LIPUS transducer and the dish. LIPUS was administered for 20 minutes every day in a span of this experiment. The control plates were handled in the same manner without LIPUS. Thereafter, the cultured tissues and their supernatant medium were harvested at 3, 7, and 12 days.

Cell viability

Cell viability was analyzed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole (MTT) assay. Tetrazolium salts such as MTT are metabolic by mitochondroal dehydrogenases to form a blue formazen dye and are there for useful for the measurement of cell viability. The cell were gently washed with Hanks' balanced salt solution (HBSS, Sigma Chemical Co., St Louis, MO, USA), and exposed to rat primary chondrocyte. After washing the cells, cultures medium containing 0.5 mg/ml of MTT was added to each well. The cells were incubated for 2 hr at 37°C, the supernatant was removed and the formed formazen crystals in viable cells were solubilized with 110 μl of dimethyl sulfoxide. A 100 μl aliquot of each sample was then translated to 96 well plates and the absorbance of each well was measured at 550 nm with ELISA reader (Bio-Rad Instruments Inc., Winooski, VT, USA). Data were expressed as a percentage of control measure in the absence of rat primary chondrocyte [19].

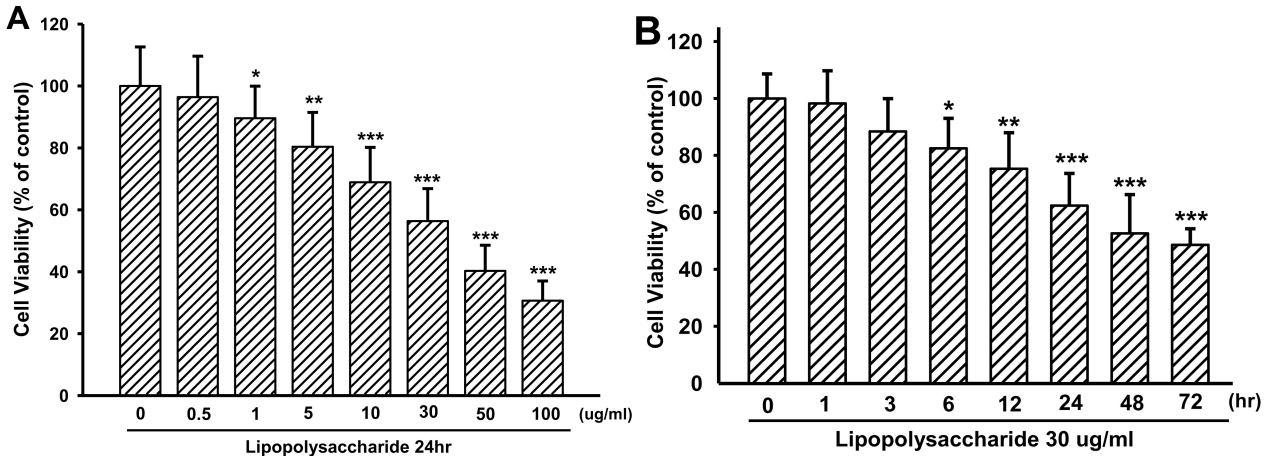

Figure 1. Dose-(A) and time-(B) dependent effects of lipopolysaccharide (LPS) on cell viability in rat chondrocyte. Values are mean (SD) of three independent experiments. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ compared to the control.

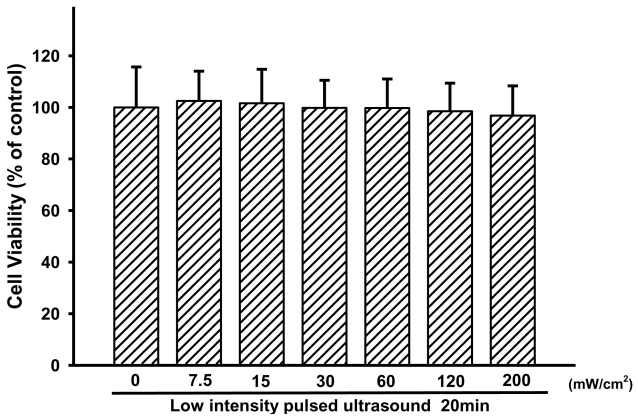

Figure 2. The effects of ultrasound (US) on cell viability in rat chondrocyte. Values are mean (SD) of three independent experiments.

Enzyme-linked immunosorbent assay

TNF-α and IL-1 levels in culture media were determined by the quantikine human immunoassay (R&D System Inc., Minneapolis, MN, USA). Which employ the quantitative sandwich enzyme linked immunoassay (ELISA) technique. The sensitivity limit of both assays was about 20 pg/ml, the linear assay range was between 100 and 2,000 pg/ml. Aliquots of 100 μl from control or LPS-treated cultures were analyzed according to the manufactures instruction.

Data analysis

Data analysis was performed with PASW Statistics 18.0 (IBM Co., Armonk, NY, USA). All of the data were expressed as mean (SD). The difference between two mean values was analyzed by student t-test. Values of $p<0.05$ were considered as significantly different.

Results

To examine effect of LPS on rat chondrocytes, the cell viability of the chondrocytes in the presence of LPS time and dose was determined. The dependence of the inhibitory effect of LPS on the LPS concentration (0 to 100 μ g/ml) was examined. Figure 1A shows that LPS at 0.5 μ g/ml (24 hr) did not inhibit cell viability. However when added at levels over 1 μ g/ml, LPS caused significant inhibition of cell viability (10.42 % decrease vs. control; $p<0.05$) (Figure 1A). Figure 1B shows that cell viability occurred at a significantly reduced rate after a 6 hr incubation period with LPS 30 μ g/ml (17.52 % decrease vs. control; $p<0.05$). This level of inhibition was maintained over 72 hr incubation periods (Figure 1B). Thus, 24 hr incubation period and 30 μ g/ml was used (Figure 1). Figure 2 shows that cell viability revealed that US exerted no significant cytotoxicity in the rat chondrocyte. This results showed that the TNF-α and IL-1 levels by US (0-200 mW/cm^2) in the rat chondrocyte. As shown in Figure 3 the absorbances of TNF-α (A) and IL-1 (B) in 30 μ M LPS-treated chondrocytes over 24 h increased approximately 580% and 990%, respectively, compared with non-treated chondrocytes. LIPUS intensity dependently suppressed the TNF-α and IL-1 production induced by LPS to approximately 72.85% and 57.10% at the highest concentration.

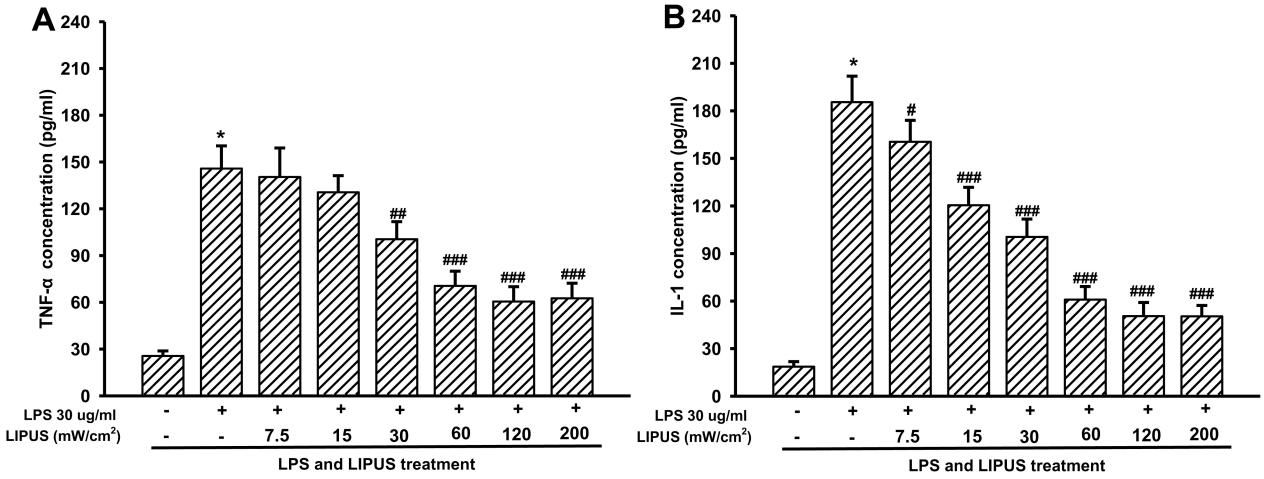

Figure 3. Effect of ultrasound on tumor necrosis factor (TNF)- α (A) and interleukin (IL)-1 (B) production in rat chondrocyte. Values are mean (SD) of three independent experiments. $^{*}p<0.05$ compared to the control. $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ compared to the LPS-treated group. LPS: lipopolysaccharide, LIPUS: low-intensity pulsed ultrasound.

Discussion

OA of the hands, hips, and knees are mostly the result of a slow and degenerative joint process [20]. Inflammation and degradation of the articular cartilage is at the characteristic of OA [21,22]. The causes of cartilage destruction in OA include cell death, degradation of the extracellular matrix, production of inflammatory cytokines, and activation differentiation of remaining cartilage cells [23,24]. Among these destruction factors, joint inflammation of varying severity is present in osteoarthritic patients. US therapy is one of the most common electrical therapeutic methods that have been used to be effective in the treatment of many diseases [25-27]. The present study investigated the effect of low intensity pulsed US in pro-inflammatory cytokine (TNF-α and IL-1), which are implicated in OA by using LPS-induced oxidative stress rat chondrocytes.

The pathogenesis of OA such as free radical and oxidative stress has been suggested to be important factors involved in OA [28]. Chondrocyte survival is essential for ensuring ongoing homeostatic maintenance of cartilage and repair to damaged cartilage after injury [29]. In our study, cell proliferation occurred at a significantly decreased on the LPS does- and time- dependent. Excessive oxidative stress causes damage to DNA, protein, and lipid and induces concomitant cellular damage [30]. Oxidative stress plays an important role in the pathology of OA [31]. Cell proliferation showed at a significantly increased viability rate after US treatment. LIPUS increases cell membrane permeability either directly by mechanical deformation of the cell membrane, or indirectly by an electrical effect caused by cell deformation [32,33].

Pathologically, synovitis leads to the secretion of pro-inflammatory cytokines such as TNF, IL-1 or IL-6. This cytokine imbalance in the synovial fluid leads to the induction of proteinases (aggrecanase or metalloproteinases) with followed by an inflammatory reaction and cartilage degradation the fluid has contact with the subchondral bone [23]. Cytokines acting individually or in networks, profoundly influence cellular responses in joint tissues, both anabolic and catabolic activities [34]. Inflammatory mediator products are released and are postulated to activate the synovium and to cause synovitis [9,35]. Moreover, the major inflammatory cytokines TNF-α and IL-1 are highly expressed in OA. It is well established as a key mediator in the progression of cartilage degeneration. We also found that treatment with variable US intensity significantly reduced secretion of inflammatory cytokines in response to stimulation with LPS in rat chondrocyte. These cytokines downregulate the enzymatic antioxidant defenses in chondrocytes, resulting in the transient accumulation of LPS [36,37]. Inflammatory mediator can directly sensitize nociceptors and increase neuronal excitation and sensitivity [38]. Therefore, cytokine level downregulation is important to pain control.

Our study evaluated the chondrocyte protective effects of LIPUS on anti-inflammatory process include the inhibition of pro-inflammatory mediators (TNF-α, and IL-6) release. Our results provide a scientific rationale of including LIPUS

in therapeutic interventions for OA patients.

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