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Original Article

LIPUS promotes synthesis and secretion of extracellular matrix and reduces cell apoptosis in human osteoarthritis through upregulation of SOX9 expression

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Abstract: Increasing studies have illustrated that low-intensity pulsed ultrasound (LIPUS) has a therapeutic effect in experimental animal models of osteoarthritis (OA). However, the function of the LIPUS on human chondrocytes of OA remains unclear. The aim of the current study was to observe the effect and explore the mechanism of LIPUS treatment on proliferation, apoptosis, and extracellular matrix (ECM) production of chondrocytes *in vitro*. Results showed that LIPUS stimulation at 30, 60, and 90 mW/cm^2 intensities markedly inhibited the apoptosis of chondrocytes compared with the 0 mW/cm^2 intensity; however, the effect of LIPUS stimulation at 0, 30, 60, and 90 mW/cm^2 intensities on the proliferation of chondrocytes had no significant difference. Furthermore, the mRNA and protein levels of COL2A1 and ACAN were upregulated in chondrocytes treated with LIPUS stimulation at 30, 60, and 90 mW/cm^2 intensities. The concentration of COL2A1 and ACAN in supernatants of chondrocytes in the 30, 60, and 90 mW/cm^2 groups were obviously higher than those in the 0 mW/cm^2 group. In addition, activation of SOX9 mRNA and protein expression were observed in the 30, 60, and 90 mW/cm^2 groups compared with the 0 mW/cm^2 group. In summary, our data demonstrated that LIPUS promotes ECM synthesis and secretion and reduces apoptosis of human OA by activation of SOX9, indicating LIPUS might bea promising therapy for the treatment of OA.

Keywords: LIPUS, osteoarthritis, chondrocytes, apoptosis, extracellular matrix

Introduction

Human osteoarthritis (OA) is a common inflammatory joint disease that frequently occurs in middle-aged and elderly people [1, 2]. OA seriously affects patients' quality of life and its therapy uses a significant amount of healthcare resources [3]. It is certain that OA is a multifactorial degenerative joint disease involving multiple tissues, including cartilage, subchondral bone, synovium, and associated tendons. However, the detailed mechanism of pathogenesis in OA remains elusive [4]. Articular cartilage, as a specific hyaline cartilage, is composed mainly of unique chondrocytes and extracellular matrix (ECM). Articular cartilage degeneration is a key procedure in the pathogenesis of OA. In recent years, studies have confirmed that pathophysiologic changes of articular cartilage degeneration are involved in the abnormal apoptosis of chondrocytes and progressive metabolic disorders of ECM [5]. Therefore, identifying a new therapy to inhibit chondrocyte apoptosis and to induce the production of ECM may defer the degenerative process of articular cartilage.

Low-intensity pulsed ultrasound (LIPUS) is a form of physical therapy with a strength less than 100 mW/cm^2 intensity, which has been used for a long time to accelerate the healing of bone fractures [6, 7]. It has been verified by many randomized controlled trials that LIPUS improves the repair of cartilage injury [8-10]. Previous research has shown that LIPUS treatment alleviates joint symptoms by relieving swelling and inflammation, and increasing mobility in OA patients [11]. In addition, LIPUS treatment regulates ECM synthesis in a rabbit OA model by regulating the integrin-mediated p38 MAPK and integrin-FAK-PI3K/Akt mecha-

nochemical transduction signaling pathways [12, 13]. Nevertheless, the precise functional mechanism of LIPUS treatment on chondrocytes of human OA is not fully understood.

The current research mainly focused on the effect and potential mechanism of LIPUS treatment on the proliferation, apoptosis, and ECM production of human OA chondrocytes *in vitro*. Our results demonstrated that LIPUS stimulation could reduce apoptosis and promoted ECM synthesis and secretion of human OA chondrocytes by activation of SOX9, indicating LIPUS might be a promising therapy for the treatment of OA.

Materials and methods

Isolation and identification of primary human OA chondrocytes

The study was authorized by the Ethics Committee of Chongqing Medical University (Chongqing, China). Written informed consents were received from the patients prior to the start of the study. A total of eleven OA patients age 65-82 years were randomly selected for the study with mean age of 71.2 ± 4.8 years. The patients had not received chemotherapy prior to admission. Human articular cartilages were collected from non-weight-bearing areas of the knee joint in OA patients who underwent knee replacement surgery. The Outerbridge classification was applied to grade the articular cartilages [14]. Subsequently, the cartilage tissues were treated with small fragments of approximately 1 mm^3 using ophthalmic scissors. The fragments were added to 0.25% trypsin reagent (Sigma, St. Louis, MO, USA) and digested for 30 min at 37°C. 0.2% type II collagenase (Sigma) was added to digest tissue for 10 h at 37°C to obtain chondrocytes. The chondrocytes were filtered using a 200-mesh sieve (Sigma) and washed five times with phosphate buffered saline (PBS). Finally, the chondrocytes were seeded in a 25 cm^2 cell culture flask (Corning, USA) at a cell density of 2×10^5 per ml at 37°C in 95% air with 5% CO_2 atmosphere, and conventionally cultivated using Dulbecco's Modified Eagle Medium plus Ham's F-12 medium (DMEM/F12, HyClone, Logan, UT, USA) and 20% fetal bovine serum (FBS, HyClone). The growth status of chondrocytes was observed using the inverted microscope Leica DM IL LED (Leica, Wetzlar, Germany), and the cells were treated with fresh medium every 2 days.

Toluidine blue staining

The second generation of chondrocytes was seeded on glass slides (Corning, USA) at a density of 1×10^3 cells and cultured for 24 h. PBS solution was applied to wash the slides for two times, and 4% paraformaldehyde (Boster, Wuhan, Hubei, China) was used for fixing at 37°C for 30 min. After that, the slides were washed two times with PBS and stained with toluidine blue (Biotopped Science & Technology Co., Haidian, Beijing, China) at 37°C for 45 min, according to the specific protocol. Finally, the slides were washed with PBS until the background was clear, dehydrated with 75% ethyl alcohol, and observed using an inverted microscope Leica DM IL LED (Leica).

Immunocytochemical staining

The chondrocytes seeded on the slides were fixed in the way referred to for toluidine blue staining. To make the chondrocytes permeable, 0.2% Triton X-100 (Sigma) was added to the slides at 37°C for 15 min. Then, the chondrocytes were treated with 3% H_2O_2-methanol solution (Sigma) at 37°C for 10 min to inactivate endogenous enzymes, and washed with PBS two times. 200 μl of normal goat serum (Abcam, Chongqing, China) was added to the slides to block antigen at 37°C for 15 min. After that, 100 μl of COL2A1 antibody at 1:100 dilution (Abcam) was added to the slides at 4°C for overnight. The slides were rewarmed at 37°C for 10 min and washed with PBS two times. Subsequently, 150 μl of HRP-conjugated specific secondary antibodies at 1:200 dilution (Abcam) was added on the slides at 37°C for 1 h. Finally, the slides were washed three times with PBS and dyed using hematoxylin (Sigma) and diaminobenzidine (Sigma). The staining images were viewed using an inverted microscope Leica DM IL LED (Leica).

LIPUS stimulation for chondrocytes

The chondrocytes were seeded in a six-well plate at a density of 1×10^3 cells/well and randomly divided into four groups with LIPUS stimulation at different intensities, including 0, 30, 60, and 90 mW/cm^2. The instrument of LIPUS

Table 1. Primer sequences for quantitative real-time polymerase chain reaction

Gene	Sequences (5'-3')	Production size
GAPDH	F: GAAGGTGAAGGTCGGAGTC	226 bp
	R: GAAGATGGTGATGGGATTTC	
COL2A1	F: CAGGTGAACCTGGACGAGAG	103 bp
	R: CCCACAGCACCAGTCTCAC	
ACAN	F: GGAATGATGTTCCCTGCAAT	258 bp
	R: GTCTGCGTTTGTAGGTGGTG	
SOX9	F: TGCTCGGGCACTTATTGG	304 bp
	R: TCCTCAGGCTTTGCGATTT	

GAPDH: glyceraldehyde-3-phosphate dehydrogenase; COL2A1: collagen type II alpha 1 chain; ACAN: aggrecan; SOX9: SRY-box transcription factor 9; F: forward; R: reverse.

stimulation was provided by the Life Sciences Institute of Chongqing Medical University (Chongqing, China). LIPUS stimulation was performed through the bottom of the six-well plate and a layer of coupling agent was placed between the transducer and the plate under the following conditions: on-off ratio of 20%, frequency at 1.5 MHz, stimulation time (10:00-10:20 am) at 20 min/d × 7 d.

Cell cycle analysis

After applying LIPUS stimulation for 7 d, the chondrocytes in the four groups were treated with 0.25% trypsin solution and then collected for cell cycle analysis. The cells were washed with PBS for two times and fixed with 70% ethanol (Sigma) at 4°C overnight. After filtration using a 300-mesh nylon sieve (Sigma), a total of 10^6 cells were reacted with 50 μg/ml propidium iodide (KeyGen Biotech, Nanjing, Jiangsu, China) for 30 min in the dark at 37°C, according to the specific protocols. Finally, the cells were detected using flow cytometer (FACSCalibur; BD Biosciences, CA, USA), and the proliferation index was calculated according to the formula: (S + G2/M)/(G0/G1 + S + G2/M)*100%.

Cell apoptosis assay

After applying LIPUS stimulation for 7 d, the chondrocytes in the four groups were separated with 0.25% trypsin solution and then used for apoptosis analysis. The cells were washed with PBS twice for two times and reacted with 4 μl of annexin V-fluorescein isothiocyanate (KeyGen Biotech) and 2.5 μl of propidium iodide for 10 min in the dark at 37°C, according to the specific processes. The apoptotic cells were probed and analyzed using flow cytometer (FACSCalibur; BD Biosciences, CA, USA).

Quantitative real-time polymerase chain reaction (qRT-PCR)

A total of 10^6 chondrocytes in each group were collected, and total RNA was isolated using a centrifugal column-type ultrapure extract total RNA kit (BioTeke, Haidian, Beijing, China), according to the specific protocols. Then the RNA was reverse-transcribed to complementary DNA (cDNA) using an All-in-One First-Strand cDNA Synthesis Kit (GeneCopoeia, Guangzhou, Guangdong, China). After that, cDNA was performed to qPCR reaction using an All-in-One qPCR Mix (GeneCopoeia) on the iQ5 RT-PCR instrument (Bio-Rad Laboratories, Hercules, CA, USA), according to the specific protocol. The sequences of the primers were synthesized from GeneCopoeia (**Table 1**). The relative expression levels of collagen type II alpha 1 chain (COL2A1), aggrecan (ACAN), and SRY-box transcription factor 9 (SOX9) were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression and were calculated using the $2^{-\Delta\Delta Ct}$ method.

Western blotting analysis

A total of 10^6 chondrocytes in each group were collected, and COL2A1, ACAN, and SOX9 expression was determined using western blotting analysis. The cells were washed with PBS for two times and the residual liquid was sucked clean with different specifications of pipette tips alternately. The RIPA lysis buffer (Life Technologies, Carlsbad, CA, USA) with 1% PMSF protease inhibitors (Beyotime Biotechnology, Shanghai, China) was used to separate total protein from chondrocytes. The BCA protein kit (Beyotime Biotechnology) was applied to quantify protein concentration. Commensurate protein samples (28 μg each) were segregated by 10% sodium dodecyl sulfate (SDS) polyacrylamide gels and were transferred onto polyvinylidene difluoride (PVDF) membranes. Then membranes were incubated with 5% bovine serum albumin (Beyotime Biotechnology) for 1 h 37°C and reacted with rabbit anti-human against ACAN antibody at 1:250 dilution (Santa Cruz Biotechnology, Inc., Dallas, TX, USA), COL2A1 antibody at 1:300 dilution (Abcam),

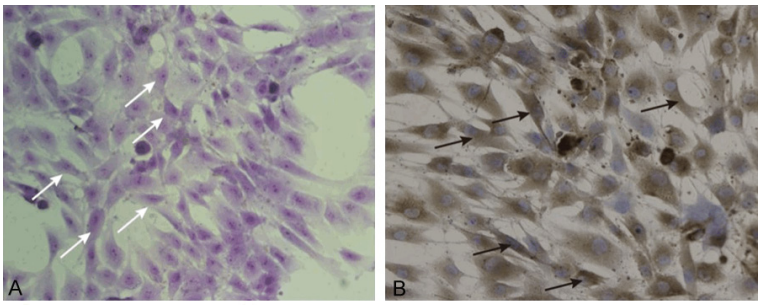

Figure 1. Identification of human OA (osteoarthritis) chondrocytes. A. Toluidine blue staining revealed that the cytoplasm of OA chondrocytes was mainly dyed with blue-violet and metachromatic granules were interspersed among or around the cells (× 400). White arrow: chondrocytes. B. Immunohistochemical staining for COL2A1 (collagen type II alpha 1 chain) showed the cytoplasm of OA chondrocytes was positively stained with brown (× 400). Black arrow: COL2A1.

and SOX9 antibody at 1:750 dilution (Santa Cruz Biotechnology) overnight at 4°C. After that, TBST buffer was used to wash the membranes for two times, and HRP-conjugated specific secondary antibody at 1:1000 dilution (Beyotime Biotechnology) was added on the membranes for 2 h at 37°C. After that, the membranes were washed using TBST buffer two times and treated with a chemiluminescent reagent (Beyotime Biotechnology) and images were obtained through a specific chromogenic reaction. Relative protein expression was evaluated using Image-Pro plus software 6.0 (Media Cybernetics Inc, Silver Springs, MD, USA), compared the density ratio of beta actin (β-Actin) gene expression.

Enzyme-linked immunosorbent assay (ELISA)

The supernatants of chondrocytes in each group were obtained after LIPUS stimulation for 7 d. Then supernatants were centrifuged and diluted to the appropriate concentration, according to the specific protocol. The levels of COL2A1 and ACAN in the supernatants were analyzed using ELISA kits (Colorful Gene, Wuhan, Hubei, China). The experiments were conducted three times according to instructions.

Statistical analysis

Measurement data were presented as mean ± standard deviation (SD), and the SPSS 19.0 software (IBM, Armonk, NY, USA) was performed to conduct statistical analysis. One-way ANOVA was used to compare the statistical significance of differences between the four groups. *P* value <0.05 was considered significant.

Results

Identification of human OA chondrocytes

After attachment to the culture dish, human OA chondrocytes were in polygonal or elongated shapes. The cells were prone to aggregate and grew to complete confluence within 14 to 17 d. Toluidine blue staining indicated that the cytoplasm of the OA chondrocytes was mainly stained with blue-violet, and metachromatic granules were interspersed among or around the cells (**Figure 1A**). Immunohistochemical staining for COL2A1 indicated that the cytoplasm of OA chondrocytes was positively dyed with brown (**Figure 1B**).

Effect of LIPUS stimulation on chondrocytes proliferation

To investigate whether LIPUS treatment had an effect on the proliferation of chondrocytes, flow cytometry was performed to detect the cell proliferation index after LIPUS stimulation at four different intensities (0, 30, 60, and 90 mW/cm^2). As shown in **Figure 2A** and **2C**, there was no significant difference in cell proliferation indexes in the four groups (18.61 ± 0.59%, 18.16 ± 0.99%, 17.65 ± 0.67%, and 17.74 ± 0.92%).

Effect of LIPUS stimulation on chondrocytes apoptosis

Flow cytometry was used to detect apoptosis of chondrocytes after LIPUS stimulation at four different intensities (0, 30, 60, and 90 mW/cm^2). The apoptosis rates of chondrocytes in the 30, 60, and 90 mW/cm^2 groups were 17.48 ± 1.06%, 19.07 ± 0.68%, and 19.32 ± 0.88%, respectively, which were notably lower than that in the 0 mW/cm^2 group (22.34 ± 0.94%) (**Figure 2B** and **2D**, $P < 0.05$). However, there was no significant difference of cell apoptosis rates between the 30, 60, and 90 mW/cm^2 groups.

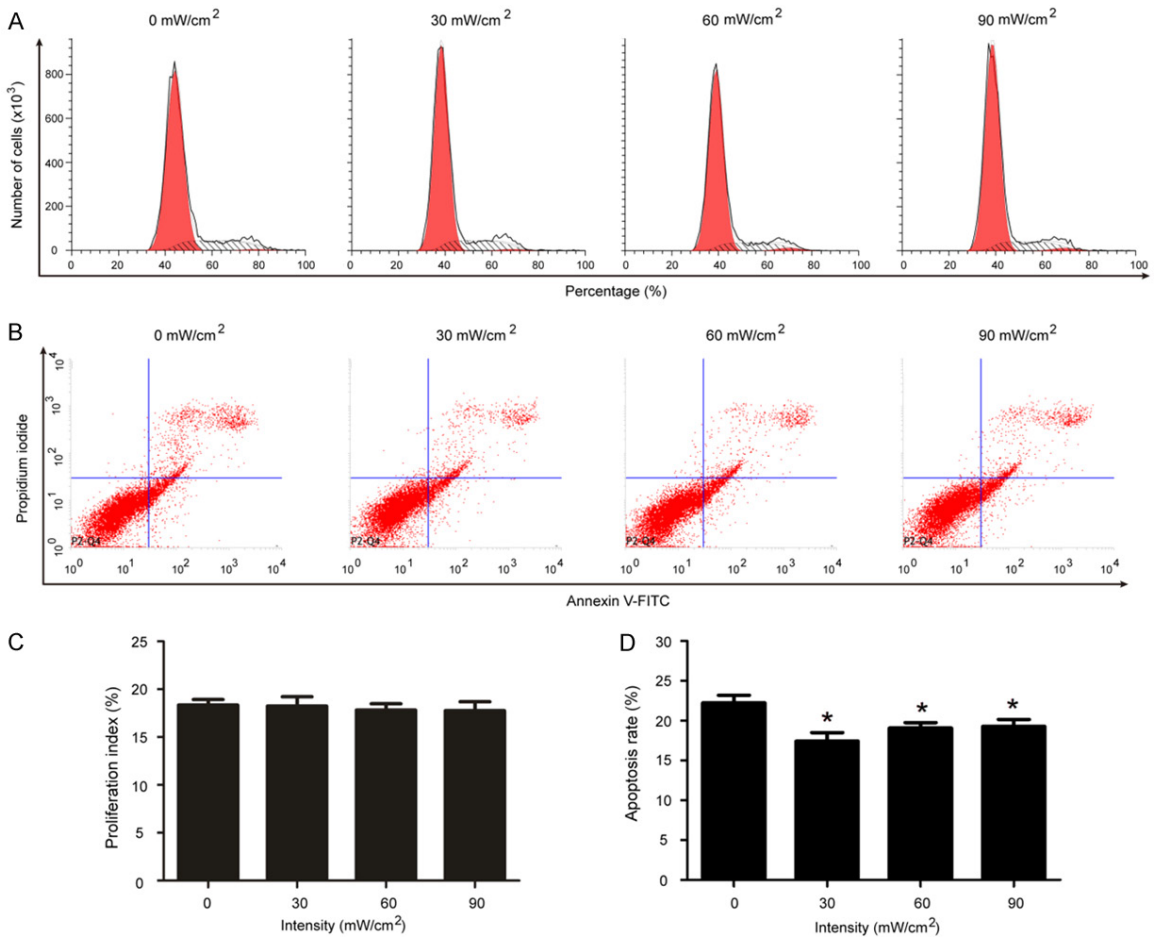

Figure 2. The effect of low-intensity pulsed ultrasound (LIPUS) stimulation on chondrocyte proliferation and apoptosis. A. Cell cycle analysis of chondrocyte proliferation index by flow cytometry after LIPUS stimulation at four different intensities (0, 30, 60, and 90 mW/cm^2). B. Apoptosis analysis of chondrocytes by flow cytometry after LIPUS stimulation at 0, 30, 60, and 90 mW/cm^2 intensities. C. Histogram of the human OA chondrocyte proliferation index after LIPUS stimulation. D. Histogram of the human OA chondrocyte apoptosis rate after LIPUS stimulation. $^*P < 0.05$ compared with the 0 mW/cm^2 group.

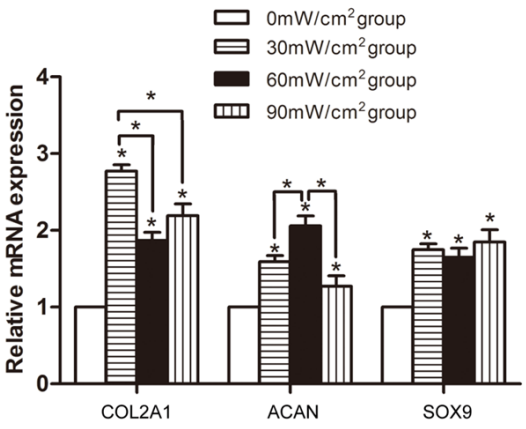

Figure 3. Relative mRNA expression levels of COL2A1, ACAN (aggrecan), and SOX9 (SRY-box transcription factor 9) of OA chondrocytes after LIPUS stimulation at 0, 30, 60, and 90 mW/cm^2 intensities. $^*P < 0.05$ compared with the 0 mW/cm^2 group.

Effect of LIPUS stimulation on chondrocyte extracellular matrix synthesis and secretion

After treatment with LIPUS stimulation for 7 d, the mRNA and protein expression levels of COL2A1 and ACAN in chondrocytes were detected. The results revealed that the mRNA expression of COL2A1 and ACAN was significantly upregulated in the 30, 60, and 90 mW/cm^2 groups compared with the 0 mW/cm^2 group (**Figure 3**, $P < 0.05$). The COL2A1 mRNA had the highest level in the 30 mW/cm^2 group, while ACAN mRNA had the highest level in the 60 mW/cm^2 group. Simultaneously, the protein levels of COL2A1 and ACAN were also notably increased in the 30, 60, and 90 mW/cm^2 groups compared to the 0 mW/cm^2 group (**Figure 4A** and **4B**, $P < 0.05$). In addition, there were no significant differences of COL2A1 and ACAN

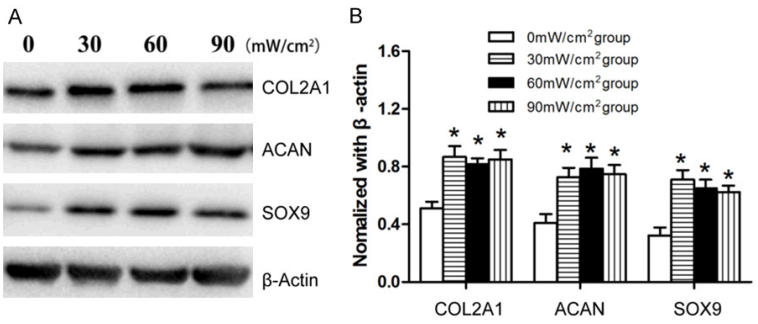

Figure 4. The effect of LIPUS stimulation on the protein expression of COL2A1, ACAN, and SOX9 of chondrocytes. A. Western blotting analysis of COL2A1, ACAN, and SOX9 expression in OA chondrocytes after LIPUS stimulation at 0, 30, 60, and 90 mW/cm^2 intensities. B. The protein levels of COL2A1, ACAN and SOX9 in the 30, 60, and 90 mW/cm^2 groups were significantly increased compared with the 0 mW/cm^2 group. $^*P < 0.05$ compared with the 0 mW/cm^2 group.

Table 2. Concentration of COL2A1 and ACAN (n = 3, ng/ml)

Groups (mW/cm^2)	COL2A1	ACAN
0	6.45 ± 1.09	7.34 ± 0.72
30	8.91 ± 0.80*	10.55 ± 0.96*
60	8.84 ± 0.60*	10.74 ± 0.61*
90	9.15 ± 0.74*	11.03 ± 1.08*

$^*P < 0.05$ compared with the 0 mW/cm^2 group.

protein expression between the 30, 60, and 90 mW/cm^2 groups. Furthermore, ELISA assay was used to investigate the effect of LIPUS on the secretory function of chondrocytes. As shown in **Table 2**, the concentrations of COL2A1 and ACAN in the 30, 60, and 90 mW/cm^2 groups were obviously increased compared with the 0 mW/cm^2 group ($P < 0.05$). However, there were no significant differences of COL2A1 and ACAN concentration between the 30, 60, and 90 mW/cm^2 groups.

LIPUS stimulation activated SOX9 expression in chondrocytes

To investigate the potential mechanism of LIPUS stimulation on chondrocytes, qRT-PCR and western blotting analysis were applied to determine the mRNA and protein expression of SOX9. The results revealed that the activation of SOX9 mRNA (**Figure 3**, $P < 0.05$) and protein (**Figure 4A** and **4B**, $P < 0.05$) expression was observed in the 30, 60, and 90 mW/cm^2 groups compared with the 0 mW/cm^2 group. However, there were no significant differences of SOX9 mRNA and protein expression between the 30, 60, and 90 mW/cm^2 groups.

Discussion

In this study, we aimed to investigate the effect of LIPUS stimulation on human OA chondrocytes. First, toluidine blue and immunohistochemical staining confirmed that human OA chondrocytes were established successfully *in vitro.* It was reported that culture of chondrocytes is accompanied by cell dedifferentiation after the fourth generation [15]. Therefore, we selected the second generation of chondrocytes for cell experiments *in vitro* to avoid the influence of cell dedifferentiation. Chondrocytes, as the specific cells in articular cartilage, are of vital importance to keep the anabolic-catabolic metabolism balances of ECM and normal microenvironment of articular cartilage [16]. ECM can offer a framework and attachment points for the chondrocytes and control cell adhesion, migration, viability, differentiation, and death [17]. Previous research has demonstrated that the apoptosis rate of human OA chondrocytes is obviously increased compared to normal human chondrocytes [18]. Excessive apoptosis of OA chondrocytes causes a decrease in ECM synthesis, which aggravates the degeneration of articular cartilage and promotes the progression of OA. LIPUS can recover the repair of cartilage injury as a therapy for the treatment of OA [8-10]. Whether LIPUS can exert a similar effect in human OA chondrocytes and its potential mechanism is still unclear. Therefore, we further determined the proliferation and apoptosis of human OA chondrocytes after LIPUS stimulation at different intensities. Results showed that cell apoptosis rates were significantly decreased after LIPUS stimulation at 30, 60, and 90 mW/cm^2. However, the proliferation index of chondrocytes had not significantly changed after LIPUS stimulation, which was inconsistent with the previous study. We speculated that this inconformity might be due to differences in cell types and experimental methods.

COL2A1 and ACAN are the two major macromolecular components of ECM, which play key role in maintaining the normal structure and function of articular cartilage [19, 20]. To detect the

effect of LIPUS stimulation on the synthesis and secretion of chondrocytes, the mRNA and protein levels of COL2A1 and ACAN were assessed. The results of qRT-PCR and western blotting revealed that the mRNA and protein levels of COL2A1 and ACAN in the 30, 60, and 90 mW/cm^2 groups were notably higher than those in the 0 mW/cm^2 group. Simultaneously, ELISA assay showed that the concentration of COL2A1 and ACAN in the 30, 60, and 90 mW/cm^2 groups were increased compared with the 0 mW/cm^2 group. These results indicated that LIPUS really promoted chondrocyte ECM synthesis and secretion mainly related to the suppression of apoptosis instead of the promotion of proliferation in chondrocytes.

SOX9 is the key transcription factor for COL2A1 synthesis and chondrogenesis, which can maintain chondrocyte lineage commitment, induce cell survival, and transcriptionally activate the genes for many cartilage-specific structural components [21]. It has been reported that LIPUS treatment facilitates the ECM synthesis of degenerative human nucleus pulposus cells, in part by regulating the expression of SOX9 [22]. Therefore, we speculated whether LIPUS stimulation regulated SOX9 expression in chondrocytes. Results from qRT-PCR and western blotting showed that the mRNA and protein levels of SOX9 were obviously upregulated after LIPUS stimulation. The data suggested that activation of SOX9 might be involved with the chondrocyte-protective effect of LIPUS stimulation on human OA chondrocytes. The shortcoming of the present study is that the accurate mechanism involving the effect of LIPUS stimulation on chondrocytes had not been rigorously investigated. Therefore, we will continue to explore the molecular mechanism in further research.

In conclusion, our data demonstrated that LIPUS stimulation reduced the apoptosis and promoted the ECM synthesis and secretion of human OA chondrocytes *in vitro*; its mechanism might be the activation of SOX9. Our study provided a new basis for LIPUS as an effective therapy for OA treatment.

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Disclosure of conflict of interest

None.

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