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Effects of *Clematis chinensis* Osbeck mediated by low-intensity pulsed ultrasound on transforming growth factor-β/Smad signaling in rabbit articular chondrocytes

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Abstract

Purpose *Clematis chinensis* Osbeck (CCO) is an essential herb that has been shown to promote the biological functions of cartilage cells. In this study, we aimed to explore whether and how low-intensity pulsed ultrasound (LIPUS) enhanced CCO delivery into chondrocytes and stimulated biological activity in vitro.

Methods Chondrocytes were isolated from knee articular cartilage of 2-week-old rabbits and treated with LIPUS plus CCO or recombinant transforming growth factor beta 1 (TGF-β1; 0.5 ng/mL), with or without anti-TGF-β1 antibodies (10 μg/mL), for 3 days. Cell proliferation was assessed by Cell-Counting Kit-8 assays. Immunocytochemistry, western blotting, and quantitative polymerase chain reaction were applied to detect the expression of type II collagen and some molecules in the TGF-β1 signal pathway.

Results LIPUS plus 0.1 mg/mL CCO solution promoted chondrocyte proliferation and type II collagen and TGF-β1 expression synergistically in vitro ($P < 0.05$). In addition, treatment with anti-TGF-β1 antibodies blocked this effect ($P < 0.01$), but not completely. CCO plus LIPUS also showed more enhanced effects on promoting TGF-β receptor II and Smad2 signaling and reducing Smad7 signaling than either intervention separately ($P < 0.05$).

Conclusions CCO plus LIPUS promoted extracellular matrix deposition by accelerating the TGF-β/Smad-signaling pathway in chondrocytes.

Keywords Low-intensity pulsed ultrasound · Tissue-engineered cartilage · Chondrocytes · *Clematis chinensis* Osbeck · Transforming growth factor-β/Smad signaling

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Introduction

Cartilage damage is a common and complex disease related to low-tissue metabolic activity, poor nerve distribution, and insufficient blood supply and lymphatic flow, resulting in poor structural reconstruction and functional recovery. Therefore, clinical treatment of cartilage damage has been a critical problem in the field of orthopedics [1]. In the 1970s, Green constructed artificial cartilage with chondrocytes combined with demineralized bone in vitro [2]; this method is currently one of the most promising methods for repairing articular cartilage injury.

Seed cells, scaffolds, and growth factors are the three most important elements for tissue engineering of cartilage. To obtain tissue-engineered cartilage with a normal structure and physiological function, the main challenge is constant optimization of culture systems in vitro, allowing seed cells

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to be appropriately regulated (by mechanical stimulation, growth factors, cytokines, etc.) [3]. Studies have shown that biomechanics play a vital role in the formation, physiology, pathology, and regeneration of articular cartilage, and appropriate mechanical stimulation can efficiently promote cell proliferation, differentiation, and matrix synthesis [4, 5].

The use of growth factors to promote cell proliferation and maintain the phenotype of seed cells in vitro has attracted much attention [6]. Currently, growth factors used in cartilage tissue engineering, such as transforming growth factor-β (TGF-β), bone morphogenetic protein (BMP), insulin-like growth factor, and fibroblast growth factor (FGF) [7], have yielded specific results. However, because of their high cost, unclear efficacy, and potential side effects, many growth factors are still not applicable in the clinical setting. In recent years, traditional Chinese medicine, which has the advantages of low cost, safety, and multitarget regulation, has attracted much attention for modulation of chondrocytes. Indeed, many scholars have found that Chinese medicines may have beneficial effects on promotion of cartilage cell proliferation, inhibition of apoptosis, and degradation of the cell cartilage extracellular matrix [8–10].

In our previous research, we found that *Clematis chinensis* Osbeck (CCO) was prescribed 15 of 40 times (37.5%) for the management of knee osteoarthritis (OA) [11]. We also established an articular cartilage cell-culture system of 2-week-old New Zealand rabbits and developed an effective animal model for cartilage defects [12, 13]. Moreover, we found that CCO may have growth factor-like effects, promoting chondrocyte proliferation and matrix expression in vitro. However, chondrocytes cultured in dishes exhibit low proliferation ability, high dependence on cell density, and features of aging. Some researchers have found that low-intensity pulsed ultrasound (LIPUS) may be effective for promoting cartilage repair in patients with OA [14]; however, more in-depth studies are needed to determine the biologic properties of LIPUS in OA and to provide sufficient evidence for the clinical efficacy of LIPUS in the treatment of OA [15].

Based on the biological effects of CCO plus LIPUS on promotion of cartilage cell proliferation and type II collagen expression [16], in this study, we further evaluated the effects of CCO plus LIPUS on the biological activities of chondrocytes and functions of the TGF-β/Smad-signaling pathway.

Materials and methods

Chondrocyte isolation, identification, and culture with CCO

Rabbit articular cartilage cells were isolated and cultured in vitro according to previously reported methods [12].

Briefly, cartilage slices were first harvested under sterile conditions from the knees of 2-week-old New Zealand rabbits, after sacrifice by an overdose of pentobarbital sodium. Tissues were placed in phosphate-buffered saline (PBS), washed three times, and cut into 1-mm^3 pieces. Subsequently, cartilage slices were dissociated with 0.2% collagenase type II in low-glucose Dulbecco's modified Eagle's medium (LG-DMEM) for 12 h. Chondrocytes were isolated through centrifugation (1500 rpm, 8 min, room temperature) and then suspended in LG-DMEM containing 20% (v/v) fetal bovine serum and 1% (v/v) antibiotics. All cultures were maintained in an incubator with an atmosphere containing 5% CO_2 at 37 °C. Cells were passaged after reaching 70–80% confluence (1:2 to 1:3). Chondrocytes in the logarithmic growth phase at passage 2 were prepared for further studies after cell cycle synchronization by serum starvation.

For culture of chondrocytes in 0.1 mg/mL CCO solution (the optimal concentration, as demonstrated in the previous studies [16]), CCO extract (extraction rate: 30:1; Aojing Tech Int., Xi'an, China) was dissolved in LG-DMEM into stock solution (10 mg/mL) before filtering for sterilization. The CCO solution was then diluted to 0.1 mg/mL and applied to chondrocytes seeded in 96-well plates (500 cells/well, $n = 6$ wells) for 24, 48, or 72 h. Cell-Counting Kit-8 (CCK-8) assays were then used to detect the relative cell number.

Lipus

As illustrated in Fig. 1, an ultrasonic signal source (33250A; Agilent, Santa Clara, USA), power amplifier (Skyworks, Woburn, USA), and 1-MHz ultrasonic transducer (Wuxi Medical Equipment Factory, Wuxi, China) were provided by Nanjing University Institute of Acoustics (Nanjing, China). Chondrocytes were seeded in 60-mm dishes (5000 cells/dish, $n = 6$ dishes). The petri dish was filled with culture medium and sealed with Parafilm M (0.13 mm; Bemis, Oshkosh, USA), which was stretched 200–300% to be thin enough (0.03–0.04 mm), so that acoustic standing waves could be avoided as much as possible. Right before the experiment, the petri dish was placed 5 cm upon the ultrasonic probe, and LIPUS exposures were applied for 20 min with various acoustic peak negative amplitudes (0.126, 0.157, or 0.25 MPa). For the negative control, no LIPUS was applied. The in situ peak negative pressure of the transmitter was calibrated using a needle hydrophone (TNU001A; NTR Systems, Inc., Seattle, WA) with a 30-dB preamplifier (HPA30; NTR Systems, Inc., Seattle, WA). The LIPUS conditions were as follows: sinusoidal pulse frequency, 1 MHz; pulse repetition frequency, 1 kHz; pulsewidth, 200 μs; and pulse length, 50 cycle. LIPUS was applied at 9:00 AM every day, with 20 min of application per day for 3 days. Subsequently, CCK-8 assays were used to detect the OD at 450 nm.

Fig. 1 Schematic diagram of the experimental system
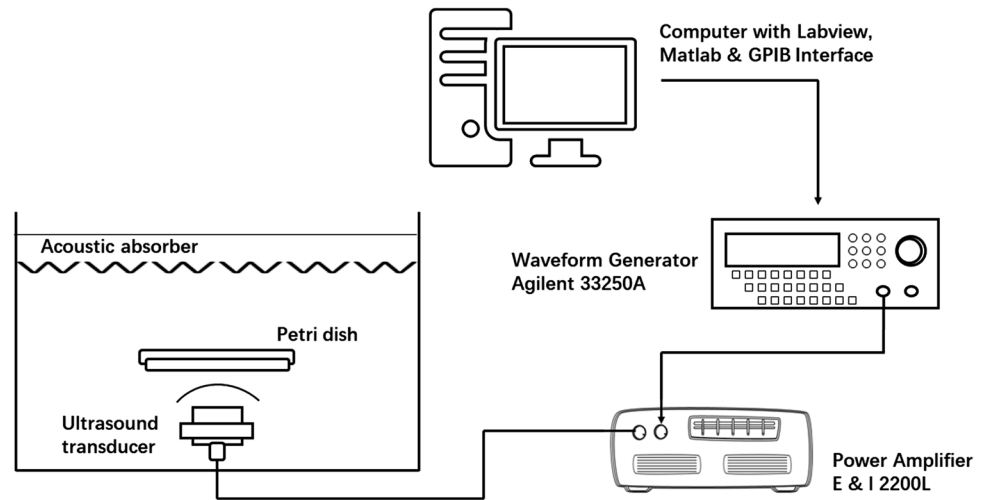
Application of CCO + LIPUS

Chondrocytes were seeded into 60-mm cell-culture dishes (6×10^5 cells/dish). Cells were then treated for 3 days in a control (no LIPUS), CCO, LIPUS, or LIPUS plus CCO group ($n = 6$ dishes per group), using the optimal ultrasonic parameters. Each group was treated for 3 days. The proliferation of chondrocytes was measured by CCK-8 assays, and the expression of type II collagen was detected by immunocytochemistry (ICC), western blotting, and fluorescence quantitative polymerase chain reaction (qPCR). The expression of TGF-β1 was detected by western blotting and qPCR.

Application of anti-TGF-β1 antibodies

Chondrocytes were left untreated (control) or treated with 0.5 ng/mL TGF-β1 (Peprotech, Rocky Hill, USA), LIPUS plus CCO, or LIPUS plus CCO plus anti-TGF-β1 antibodies (10 µg/mL anti-TGF-β1 antibodies; Abcam, Cambridge, UK), with six dishes per group. According to the manufacturer's instructions for the antibody (cat. no. ab64715), 0.5 ng/mL TGF-β1 was inactivated with 1.2–4 µg/mL antibody. ICC, western blotting, and qPCR were applied to detect the expression of type II collagen, and western blotting and qPCR were used to detect the expression of TGFβ1 receptor II (TGFβRII), Smad2, Smad7, and Smurf2.

CCK-8 assays

Chondrocytes were cultured in 96-well plates with 100 µL DMEM, and 10 µL CCK-8 reagent (Dojindo, Kumamoto, Japan) was added to each well. After incubation with shaking at 37 °C in an atmosphere containing 5% CO_2 for 3 h,

the OD was determined by microplate reader (PerkinElmer, Waltham, USA) at 450 nm.

Envision ICC

After washing with PBS twice, chondrocytes were fixed with 4% paraformaldehyde for 30 min. Cells were then incubated with 3% H_2O_2 for 10 min to remove endogenous peroxidase activity, followed by sequential incubation with antigen retrieval buffers. After washing with PBS three times, mouse anti-collagen, type II, monoclonal antibody (Millipore, Massachusetts, USA) was diluted 1:100 and added to cells. Cells were incubated for 60 min at room temperature, washed with PBS three times, and incubated with 50 µL polymer reinforcing agent (agent A). Cells were washed twice, and 50 µL secondary antibody and enzyme-labeled anti-mouse/rabbit polymer (agent B) were added successively for 30 min at room temperature. Cells were then washed with PBS twice, and the chromogenic reaction for detection of collagen type II was visualized using 100 µL diaminobenzene, following by counterstaining with hematoxylin. Finally, cells were gradually dehydrated in a graded series of ethanol concentrations and sealed with neutral gum. An upright microscope was used to capture images at 200× magnification.

Western blotting

Total proteins were extracted with a mixture of RIPA lysis buffer (containing 1 mM phenylmethylsulfonyl fluoride; Beyotime, Shanghai, China). BCA protein assays (Thermo, Waltham, USA) were applied to estimate the protein concentrations, and proteins were then denatured at 95 °C for 5 min with 4× sodium dodecyl sulfate polyacrylamide gel electrophoresis loading buffer (Bio-Rad, Minneapolis, USA). Proteins (40 µg) were separated on 6–10% polyacrylamide minigels and transferred to 0.22-µm polyvinylidene difluoride

membranes (Millipore, Massachusetts, USA). For immunoblotting, membranes were blocked with 5% nonfat dry milk in Tris-buffered saline (TBS) for 2 h at room temperature and then incubated at 4 °C overnight with primary antibodies [mouse anti-collagen, type II, monoclonal antibody (Millipore, Massachusetts, USA); mouse anti-β-actin monoclonal antibody (Abcam, Cambridge, UK); rabbit anti-TGF-β1 polyclone antibody (Abcam, Cambridge, UK); rabbit anti-Smad2 and TGFβR II polyclone antibody (biorbyt, Cambridge, UK)] diluted in 5% bovine serum albumin (Roche, Basel, Switzerland). The membranes were washed three times for 5 min each in TBST (Tris-buffered saline Tween) and incubated with secondary antibodies (goat anti-mouse secondary antibody (Abcam, Cambridge, UK); goat anti-rabbit secondary antibody (Bioworld, Louis Park, USA)] for 2 h at room temperature. Proteins were visualized with BeyoECL Plus (Thermo, Waltham, USA) and imaged using an Image Quant LAS 4000 mini ultrasensitive chemical luminescence tomograph (GE Healthcare, Pittsburgh, USA). The expression relative to that of β-actin was analyzed using the Adobe Photoshop CS5 software.

qPCR

Total RNA was extracted from chondrocytes with RNAiso Plus (Takara, Shiga, Japan) according to the manufacturer's instructions. RNA purity and concentration were determined by measurement of the OD at 260 and 280 nm (Eppendorf, Hamburg, Germany). Approximately 500 ng total RNA was used as a template to reverse transcribe into cDNAs with PrimeScript RT Master Mix (RR036A; Takara, Shiga, Japan). qPCR was then performed with SYBR Premix Ex Tag (RR820A; Takara, Shiga, Japan). Two-step qRT-PCR was performed with initial denaturation at 95 °C for 30 s, 40 cycles of denaturation at 95 °C for 5 s, and annealing at 60 °C for 34 s (ABI 7500; Applied Biosystems, Foster City, CA, USA). The primers used for PCR are shown in Table 1, and primer specificity was confirmed by analyzing dissociation curves for each primer pair. Relative gene expression levels were calculated by the $2^{-\Delta\Delta CT}$ method after normalizing to

glyceraldehyde-3-phosphate dehydrogenase gene expression. Each gene was analyzed in triplicate to reduce randomization errors.

Statistical analysis

All results were analyzed by SPSS.17.0 and expressed as means ± standard deviations. Statistical significance was determined by one-way analysis of variance followed by Dunnett's post hoc test and least significant difference (LSD) tests. Differences were deemed statistically significant when the P value was less than or equal to 0.05.

Results

LIPUS (0.157 MPa) promoted chondrocyte proliferation

Within 72 h, 0.1 mg/mL CCO promoted the proliferation of chondrocytes in vitro (Fig. 2a), similar to our previous results [16]. LIPUS promoted the proliferation of chondrocytes through mechanical stimulation, and this effect was closely related to the stimulus intensity. Our results showed that 0.157 MPa LIPUS promoted the proliferation of cartilage cells more dramatically than 0.126 or 0.250 MPa LIPUS (Fig. 2b).
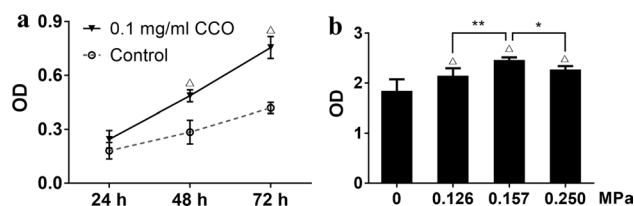
Fig. 2 Effects of 0.1 mg/mL *Clematis chinensis* Osbeck (CCO) or 0.157 MPa low-intensity pulsed ultrasound (LIPUS) on chondrocyte proliferation ($n = 6$). $^{\triangle}P < 0.01$ vs control, $^*P < 0.05$, $^{**}P < 0.01$. **a** Effects of CCO on cell proliferation over time. **b** Effects of different LIPUS intensities on cell proliferation

Table 1 Nucleotide primers used for RT-PCR

Primer	Forward	Reverse
Collagen II	5′-CTCAAGTCCCTCAACAACCAG-3′	5′-CTATCCAGTAGTCACCGCTCTTC-3′
TGF-β1	5′-AATACAAAGAAAGTCGGCACAG-3′	5′-GACGGTAAATGACAACCACTCA-3′
TGFβR II	5′-TCTGCCTCTCCAAAGTGTATCA-3′	5′-CGGACTGCTGGTGGTGTATT-3′
Smad2-like	5′-GAGAAAGCCATCACCACTCA-3′	5′-TGGGACACCTGAAGACGAC-3′
Smad7	5′-GGGCTTTCAGATTCCCAACT-3′	5′-GCGTCTTCTCCTCCCAGTAT-3′
Smurf2	5′-TTGCGGACTTGGAAAGATAGA-3′	5′-CCAGAACCACTTGACCACATT-3′
GAPDH	5′-ATGGTGAAGGTCGGAGTGAAC-3′	5′-GTGGGTGGAATCATACTGGAAC-3′

LIPUS mediated the effects of CCO on promotion of proliferation and type II collagen expression in articular cartilage cells

CCK-8 assays indicated that proliferation of chondrocytes in each group was significantly different ($P < 0.001$). When using optimized parameters for CCO, LIPUS, and LIPUS plus CCO, cell proliferation was promoted compared with that in the control group ($P < 0.01$). LIPUS plus CCO showed increased promoting effects compared with CCO or LIPUS alone ($P < 0.01$); however, the combined effect was less than the sum of the two (Fig. 3a).

Three days after the intervention, ICC staining showed that type II collagen expression (brown granules) was significantly increased in the CCO, LIPUS, and LIPUS plus CCO groups compared with that in the control group (Fig. 3b). The average OD (MD) values of ICC images, as analyzed using Image-Pro Plus 6.0, were significantly different ($P < 0.001$) among groups. The optimized parameters for CCO, LIPUS, and LIPUS plus CCO all significantly promoted collagen type II expression compared with the control group ($P = 0.015$, $P = 0.010$, and $P < 0.000$, respectively).

However, when using the LSD method, there were no significant differences between the CCO and LIPUS groups ($P = 0.831$), although the relative expression levels in the LIPUS plus CCO group were significantly higher than those in the CCO and LIPUS groups ($P < 0.001$, Fig. 3c).

Three days after treatment, western blot analyses showed that the expression levels of type II collagen differed significantly among groups ($P < 0.001$). Type II collagen expression in the CCO, LIPUS, and LIPUS plus CCO groups was significantly higher than that in the control group ($P < 0.001$). Moreover, type II collagen expression in the LIPUS group was higher than that in the CCO group ($P < 0.001$) and was lower than that in the LIPUS plus CCO group ($P = 0.001$, Fig. 3d).

The relative mRNA expression levels of type II collagen, as determined by qRT-PCR, were similar to those of protein expression determined by western blotting (Fig. 3e). That is, LIPUS and CCO both enhanced type II collagen mRNA expression ($P = 0.001$ versus the control group). Higher type II collagen mRNA levels were detected in the LIPUS plus CCO group than in the CCO or LIPUS groups ($P = 0.002$ and $P = 0.001$, respectively). However, there were no
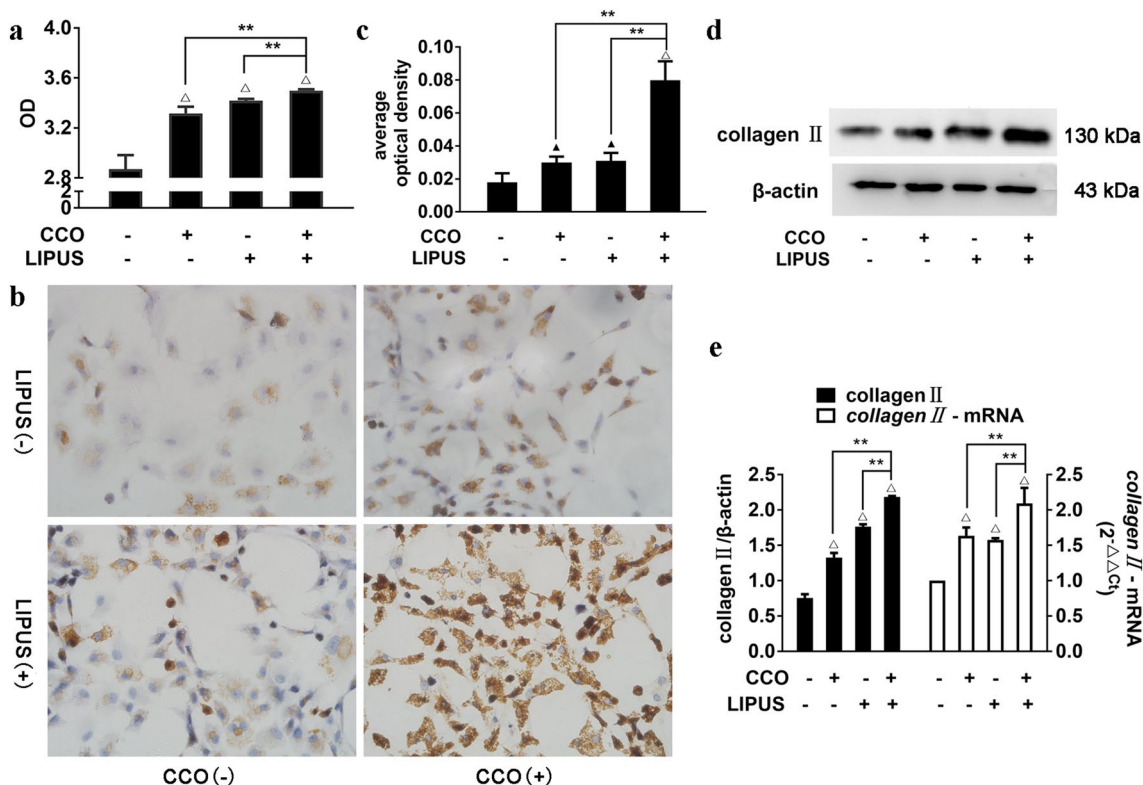
Fig. 3 Effects of LIPUS plus CCO on the proliferation of and collagen II expression in rabbit articular chondrocytes ($n = 6$). ▲$P < 0.05$ vs control, △$P < 0.01$ vs control, **$P < 0.01$. **a** Effects of CCO, LIPUS, and LIPUS plus CCO on rabbit articular chondrocyte proliferation. **b** Effects of CCO, LIPUS, and LIPUS plus CCO on type II collagen expression (brown granules) in chondrocytes. **c** MD analysis by Image-Pro Plus 6.0. **d** Western blot analysis of collagen protein expression in the CCO, LIPUS, LIPUS plus CCO, and control groups. **e** Quantification of type II collagen expression at the protein and mRNA levels

significant differences between the CCO and LIPUS groups ($P = 0.568$).

LIPUS plus CCO promoted TGF-β1 expression in chondrocytes

Three days after intervention, TGF-β1 protein expression was analyzed by western blotting. Notably, CCO, LIPUS, and LIPUS plus CCO increased TGF-β1 expression in chondrocytes compared with that in the control group ($P < 0.001$). TGF-β1 expression was higher in the LIPUS group than in the CCO group ($P = 0.001$), but was lower in the LIPUS group than in the LIPUS plus CCO group ($P = 0.005$, Fig. 4a). Changes in mRNA levels were consistent with changes in protein levels. Specifically, TGF-β1 expression was increased in the LIPUS plus CCO group ($P = 0.001$ versus control, $P = 0.029$ versus CCO, and $P = 0.033$ versus LIPUS); however, there were no differences between the CCO and LIPUS groups ($P = 0.933$, Fig. 4b).

Type II collagen expression promoted by LIPUS plus CCO mimicked the effects of TGF-β1 protein and was blocked by anti-TGF-β1 neutralizing antibodies

ICC results showed that TGF-β1 and LIPUS plus CCO both increased type II collagen expression compared with that in the control group ($P < 0.001$), and the effects of LIPUS plus CCO were stronger than those of TGF-β1 ($P < 0.001$). However, after addition of anti-TGF-β1 neutralization antibodies, the expression of type II collagen in the LIPUS plus CCO group was reduced ($P < 0.001$), but still remained higher than that in the control group ($P = 0.015$; Fig. 5a, b). Western blot analysis showed the same results as those for ICC analysis, indicating that LIPUS plus COO had TGF-β1-like effects and enhanced collagen expression (Fig. 5c, d). Analysis of mRNA levels showed that LIPUS plus CCO also enhanced type II collagen expression compared with TGF-β1 ($P = 0.005$). These effects were blocked by anti-TGF-β1 neutralizing antibodies, although the expression of

type II collagen remained higher than that in the control group ($P < 0.001$, Fig. 4d).

Targeting the TGF-β/Smad signal pathway in chondrocytes by LIPUS plus CCO

At both the protein and mRNA levels (except *Smad2* mRNA levels, $P = 0.247$ for TGF-β1 versus the control), CCO, TGF-β1, and LIPUS plus CCO enhanced TGFβRII and Smad2 expression in chondrocytes in vitro ($P < 0.05$). The effects of TGF-β1 were weaker than those of the LIPUS plus CCO group, although TGF-β1 induced higher TGFβRII expression than CCO alone. In contrast, Smad2 expression in the CCO group was higher than that in the TGF-β1 group (Fig. 6a–d). Notably, CCO, TGF-β1, and LIPUS plus CCO downregulated *Smad7* and *Smurf2* mRNAs ($P < 0.01$ versus the control). *Smad7* expression was lower in the LIPUS plus CCO group than in the CCO or TGF-β1 groups ($P = 0.000$ and $P = 0.028$, respectively), but was lower in the TGF-β1 group than in the CCO group ($P = 0.004$). There were no significant differences among the CCO, TGF-β1, and LIPUS plus CCO groups ($P = 0.624$, $P = 0.257$, and $P = 0.497$, respectively; Fig. 6e).

Discussion

Arthrodial cartilage is composed of chondrocytes and extracellular matrix, although chondrocytes account for less than 2% of the volume of this tissue [17]. In addition, the number and vitality of chondrocytes are reduced with aging. Therefore, researchers have aimed to determine how to promote the proliferation and vitality of chondrocytes to prevent and treat OA, with a focus on developing a mechanical environment similar to that encountered in vivo. Moreover, growth factors, such as TGF-β, BMP, and FGF, also regulate cell proliferation, differentiation, and metabolism, which are important for cartilage growth and repair [18].

Cartilage damage is defined as "Bi Syndrome" in Chinese medicine. Many studies have shown that cartilage damage
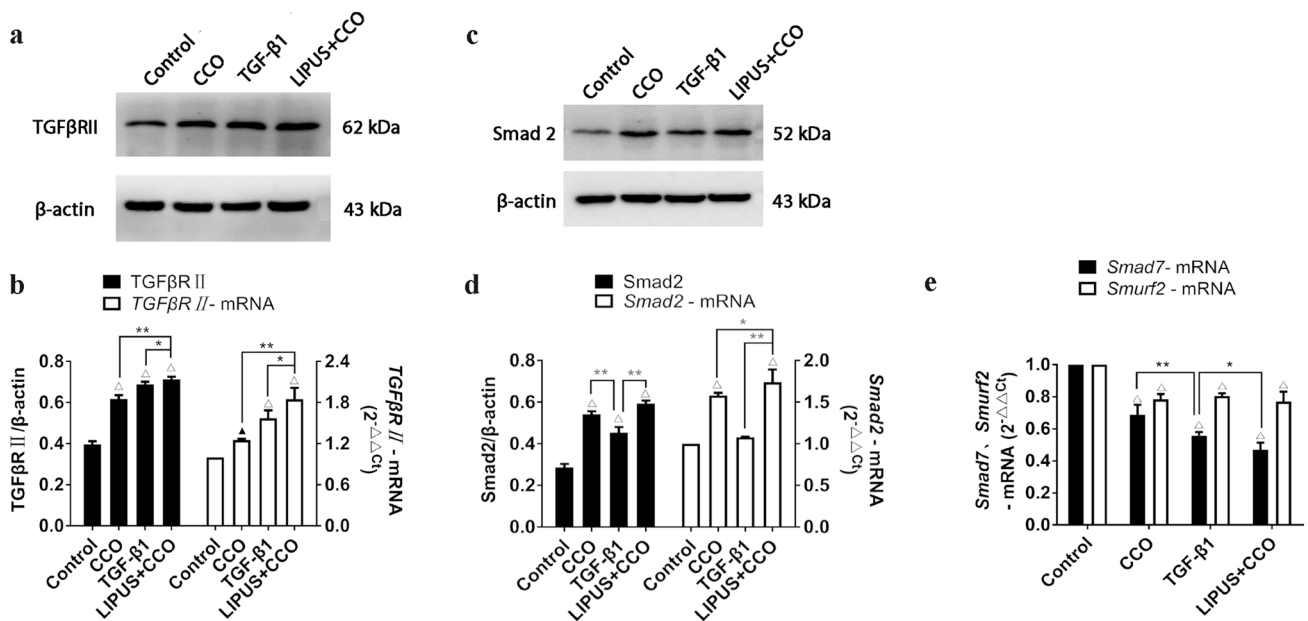
Fig. 4 Effects of LIPUS plus CCO on TGF-β1 expression in rabbit articular chondrocytes ($n = 6$). ▲$P < 0.05$ vs control, △$P < 0.01$ vs control, *$P < 0.05$, **$P < 0.01$. **a** Western blot analysis of TGF-β1 protein expression in the CCO, LIPUS, LIPUS plus CCO, and control groups. **b** Quantification of protein and mRNA expression of TGF-β1
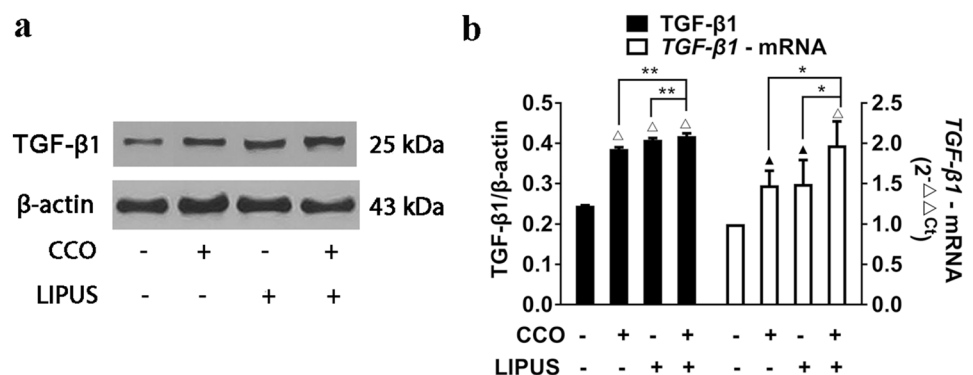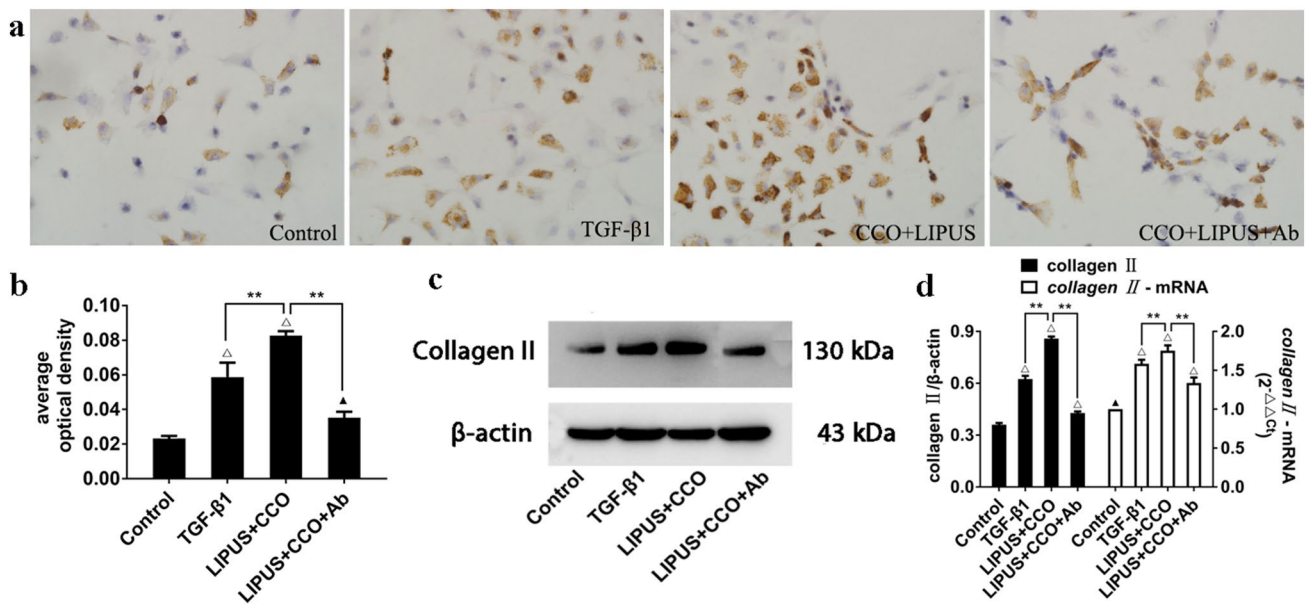
Fig. 5 Effects of LIPUS plus CCO on type II collagen expression in rabbit articular chondrocytes following treatment with anti-TGF-β1 neutralizing antibodies (Ab, *n* = 6). ▲*P* < 0.05 vs control, △*P* < 0.01 vs control, **P < 0.01. **a** Type II collagen (brown granules) in chondrocytes. **b** MD analysis by Image-Pro Plus 6.0. **c** Western blot analysis of type II collagen expression in the TGF-β1, LIPUS plus CCO, LIPUS plus CCO plus Ab, and control groups. **d** Quantification of type II collagen protein and mRNA expression

Fig. 6 Effects of LIPUS plus CCO on TGFβ/Smad2 signaling in rabbit chondrocytes (*n* = 6). ▲*P* < 0.05 vs control, △*P* < 0.01 vs control, *P < 0.05, **P < 0.01. **a** Western blot analysis of TGFβRII protein expression in the CCO, TGF-β1, LIPUS plus CCO, and control groups. **b** Quantification of TGFβRII protein and mRNA expression. **c** Western blot analysis of Smad2 protein expression in the CCO, TGF-β1, LIPUS plus CCO, and control groups. **d** Quantification of Smad2 protein and mRNA expression in each group. **e** *Smad7* and *Smurf2* mRNA expression, as determined by qRT-PCR

can be mitigated by the therapy known as "removing wind and dampness, getting rid of arthralgia" [19]. CCO, one key herb known to have this function, is clinically used to treat OA and has been shown to be effective for protecting arthrodial cartilage [20]. Previously, our team showed that CCO extract effectively improved rabbit chondrocyte

proliferation in vitro. Further evaluation showed that many studies on cartilage defects were based on "Wolff's law," which indicated that proper mechanical stimulation could help maintain normal cell phenotypes and improve the synthesis of matrix components, including type II collagen and proteoglycans [21]. LIPUS has also been shown to promote the metabolism of chondrocytes and matrix components [22]. However, the effects of LIPUS on cell proliferation have not been established due to variations in the parameters used in different studies.

Recent studies have suggested that ultrasound promotes drug delivery to cells via thermal effects, mechanical effects, convective transport, and cavitation effects [23]; among these effects, ultrasonic cavitation is thought to be the physical basis for increasing membrane permeability [24]. In the present study, we found that LIPUS promoted the proliferation of chondrocytes with or without CCO; in addition, when combined with CCO, the effects of LIPUS were further improved. Thus, our results indicated that LIPUS plus CCO promoted the proliferation of chondrocytes, likely through a mechanical effect mediated by LIPUS. In our experiments, the petri dish was filled with culture medium and sealed with parafilm. Although the acoustic field inside the petri dish cannot be scanned directly, the thickness of the parafilm should be thin enough to avoid the formation of acoustic standing waves as much as possible. In this study, ICC staining and western blotting showed that CCO plus LIPUS for 20 min/day efficiently maintained the production of collagen type II in vitro. Moreover, this treatment maintained the stability of the cell phenotype, and the combined effects were stronger than the effects of either single interventions alone. Accordingly, we concluded that LIPUS plus CCO cooperatively promoted type II collagen production in chondrocytes to maintain the phenotype of chondrocytes.

The TGF-β/Smad-signaling pathway, which is mainly composed of TGF-β superfamily members, TGF-β receptors, and Smad proteins, plays a vital role in chondrocyte biology during cartilage injury. TGF-β proteins activate downstream signals by binding to TGF-β receptors [25]. TGF-β1 is the most common TGF-β protein and has been shown to be involved in chondrocyte metabolism and fracture healing. To date, eight TGF-β receptors have been discovered [26]; among these receptors, TGFβRI, TGFβRII, and TGFβRIII, have been extensively studied and have been shown to share high homology. Smad proteins, as critical factors in TGF-β signaling, have also been extensively studied [27]. There are three types of Smad proteins with different structures and functions. First, Smad1, -2, -3, -5, and -8 are involved in receptor activation; Smad1/5/8 transduce BMP signals, whereas Smad2/3 transduce TGF-β signals. Second, Smad4 can combine with any activated Smad to form dimers and regulate current signals. Finally, Smad6/7 antagonize other Smads and form a negative feedback loop control to regulate

TGF-β signals [28]. The E3 ubiquitin ligase/Smad ubiquitin regulatory factor (Smurf) family, including Smurf1 and Smurf2, also mediates proteasomal degradation and regulates TGF-β signal transduction; Smurf2 has been shown to have critical roles in regulating cartilage cells.

TGF-β1 signaling transduced by Smads can promote chondrocyte proliferation, differentiation, and maturity, and modulate extracellular matrix synthesis and cell hypertrophy inhibition, thereby regulating the growth and reconstruction of cartilage [29]. In this study, we found that LIPUS plus CCO promoted the proliferation of rabbit articular chondrocytes, maintained type II collagen levels, and had stronger effects than either of the two interventions alone. A previous study showed that CCO promoted the expression of *TGF-β1* mRNA in rabbit articular chondrocytes, and our findings further demonstrated that LIPUS plus CCO significantly promoted the expression of TGF-β1. In addition, via the mechanical and ultrasonic effects of LIPUS, the influence of CCO was improved, generating a better cellular microenvironment and altering cell membrane permeability. We also showed that a specific concentration of exogenous TGF-β1 enhanced the proliferation and expression of type II collagen, thus efficiently maintaining the phenotype of chondrocytes. CCO, LIPUS, and LIPUS plus CCO all stimulated this effect, and the addition of anti-TGF-β1 neutralizing antibodies blocked these changes; thus, these findings indicated that TGF-β1 played a vital role in the synergistic effects of LIPUS plus CCO.

The effects of endogenous TGF-β1 induced by LIPUS plus CCO were weaker than those of exogenous TGF-β1, although the ability to promote type II collagen expression was obviously higher. We suspect that endogenous TGF-β1 rather than exogenous TGF-β1 was stimulated by LIPUS plus CCO, directly targeting adjacent cartilage cell receptors and transducing signals. However, CCO, as a Chinese herb, has multiple targets; thus, when coupled with the mechanical and acoustic effects of LIPUS, which can effectively stimulate or inhibit functional molecules downstream of TGF-β1 in chondrocytes, CCO may improve the sensitivity of corresponding receptors.

In our experiments, we found that CCO, TGF-β1, and LIPUS plus CCO significantly increased the expression of TGFβRII and Smad2 in vitro. These findings may explain the enhanced activation of the Smad2 signaling pathway, thereby promoting TGF-β signaling. TGF-β1 had weaker effects than LIPUS plus CCO, indicating that LIPUS plus CCO promoted cell proliferation and extracellular matrix synthesis by regulating the expression of TGFβRII and Smad2. This may be beneficial for repairing cartilage damage. We also found that the three interventions mentioned above inhibited Smad7 and Smurf2 expression, which could have effects as negative feedback factors. However, when a sufficient amount of anti-TGF-β1 neutralizing antibodies was

added, the biological effects of LIPUS plus CCO on cartilage cells were still higher than those of the control group. Thus, we suggest that there may be other signaling pathways regulating this process, although the specific pathways have not yet been elucidated. As such, further studies are needed to fully elucidate the mechanisms involved in this process.

Conclusion

LIPUS plus CCO treatment mimicked the effects of TGF-β1 on chondrocytes, thereby enhancing TGF-β/Smad signaling and promoting chondrogenesis in vitro.

Compliance with ethical standards

Ethical statements All experimental procedures were approved by the institutional and local committee on the care and use of animals of Nanjing University of Chinese Medicine.

Conflict of Interest The authors declare that they have no conflicts of interest.

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