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● *Original Contribution*

FOCUSED LOW-INTENSITY PULSED ULTRASOUND AFFECTS EXTRACELLULAR MATRIX DEGRADATION *VIA* DECREASING CHONDROCYTE APOPTOSIS AND INFLAMMATORY MEDIATORS IN A SURGICALLY INDUCED OSTEOARTHRITIC RABBIT MODEL

LANG JIA,*† JINYUN CHEN,* YAN WANG,* YU ZHANG,* and WENZHI CHEN*

*State Key Laboratory of Ultrasound Engineering in Medicine Co-Founded by Chongqing and the Ministry of Science and Technology, College of Biomedical Engineering, Chongqing Medical University, Chongqing, China; and †Department of Rehabilitation Medicine, the Second Affiliated Hospital, Chongqing Medical University, Chongqing, China

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Abstract—We investigated whether focused low-intensity pulsed ultrasound (FLIPUS) affects extracellular matrix (ECM) production in osteoarthritic (OA) rabbits by decreasing chondrocyte apoptosis and pro-inflammatory mediators. An OA model using New Zealand White rabbits (N = 30) and 30 normal rabbits were randomized into three groups (2-, 4- and 8-wk groups; n = 10 knees each). A knee from each rabbit was randomly selected to receive FLIPUS and the other knee received a sham treatment as a control. Another 30 normal rabbits were blank controls. We measured ECM degradation, joint effusion volume and levels of prostaglandin E2 and nitric oxide. Also, ratios of chondrocyte proliferation and apoptosis were calculated. Compared with sham stimulation, FLIPUS attenuated release of type II collagen and proteoglycans and reduced chondrocyte apoptosis as well as total joint effusion volume and significantly alleviated OA-induced accretion of prostaglandin E2 and nitric oxide in the synovial fluid. FLIPUS application promoted ECM production in OA through down regulation inflammatory mediators, joint effusion volume and chondrocyte apoptosis. (E-mail: chenwz@cqmu.edu.cn) © **2015 World Federation for Ultrasound in Medicine & Biology.**

Key Words: **Osteoarthritis, Focused low-intensity pulsed ultrasound, Extracellular matrix, Proliferation, Apoptosis.**

INTRODUCTION

Osteoarthritis (OA), the most common of all arthritides, is a heterogeneous disease characterized by synovial joint failure (Hunter et al. 2014). Recent estimates suggest that osteoarthritis of the knee affects approximately 250 million people globally (Murray et al. 2013). Although the precise pathologic mechanisms responsible for OA remain unclear, improper functioning of chondrocytes is believed to be pivotal in the pathology of OA *via* the induction of an imbalance of extracellular matrix (ECM) anabolism and catabolism (Park et al. 2005). Pro-inflammatory mediators such as prostaglandin E2 (PGE2) and nitric oxide (NO) are believed to alter the balance of ECM degradation and repair (Sellam and Berenbaum 2010). Nitric oxide and prostaglandins often are simultaneously increased in synovial fluid from arthritic patients (Sellam and Berenbaum 2010). PGE2 and NO may influence the synthesis of one another, thereby modulating cellular responses. PGE2 decreases proteoglycans (PG) synthesis and enhances the degradation of both aggrecan and type II collagen (COL II; Attur et al. 2008). Nitric oxide has been proposed as a contributor to the OA pathologic process by (i) inducing synthesis of matrix metalloproteases (Murrell et al. 1995) and depolymerizing hyaluronan (Stefanovic-Racic et al. 1993), (ii) inhibiting PG and collagen synthesis (Lotz 1999) and (iii) inhibiting chondrocyte proliferation and inducing chondrocyte apoptosis (Blanco et al. 1995; Lotz 1999).

Although various management techniques are available for the treatment of OA, there are presently no therapies that modify the onset or progression of OA-induced structural damage (Matthews and Hunter 2011; Zhang et al. 2010). Ultrasound (US) treatment has been used as a non-invasive modality for management of OA over the past 60 y because it relieves pain through thermal

Address correspondence to: Wenzhi Chen, State Key Laboratory of Ultrasound Engineering in Medicine Co-Founded by Chongqing and the Ministry of Science and Technology, College of Biomedical Engineering, Chongqing Medical University, Chongqing 400016, China. E-mail: chenwz@cqmu.edu.cn

and non-thermal modalities (mechanical effects; Ter Haar 1999). Traditional unfocused US technology is beneficial with regard to cartilage metabolism. In basic science studies, US stimulation of cartilage increased expression of COL II or PG (Tien et al. 2008), promoted cartilage healing, induced chondrocyte proliferation (Cook et al. 2001; Wiltink et al. 1995; Korstjens et al. 2008) and enhanced chondrocyte differentiation (Ebisawa et al. 2004). However, unfocused US did not alter apoptosis (Zeng et al. 2012), and a recent study confirmed that stimulation of COL II and PG synthesis by US was not due to increased chondrocyte proliferation (Tien et al. 2008). To date, how US modifies expression of COL II and PG in OA cartilage has not been concluded.

To study these events, appropriate US selection is paramount. Although pressure waves propagated by US transfer mechanical energy into tissue (Gleizal et al. 2006; Reher et al. 1997), energy from unfocused US can diffuse and destroy adjacent structures (Jung et al. 2015). Previous work suggests that focused low-intensity pulsed ultrasound (FLIPUS) improves re-ossification by enhancing cell proliferation in calvarial defect sites in rats (Jung et al. 2015). However, few applications of FLIPUS have been published that describe healing cartilage.

Therefore, we used histologic assays and magnetic resonance imaging (MRI) in a surgically induced OA rabbit model to investigate whether FLIPUS increases expression of COL II and PG *via* decreasing joint effusion volume, pro-inflammatory mediators and cell apoptosis and inducing cell proliferation.

MATERIALS AND METHODS

Ethics statement

The experimental and animal care protocols were approved by the Committee on the Ethics of Animal Experiments at Chongqing Medical University (Chongqing, China). In addition, this study was performed in accordance with the recommendations described in the "Guide for the Care and Use of Laboratory Animals" from the Ministry of Science and Technology of the People's Republic of China. All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize animal suffering during the course of this study.

Rabbit patients and OA model construction

Sixty male New Zealand White rabbits (11–12-mo old, 3.0–3.5 kg) were obtained from the Animal Center at Chongqing Medical University. All animals were housed in individual cages with a 12:12-h light–dark cycle in 20–25°C and were given a standard laboratory diet and drinking water *ad libitum*.

To construct the OA model, the medial collateral ligament, the complete medial meniscus and both cruciate ligaments were excised from both knees of 30 rabbits under general anaesthesia (3% pentobarbital, 1 mL/kg) as previously described (Hulth et al. 1970). Activity, weight, food consumption, rectal temperature and wound healing were monitored daily during post-operative week 1. After post-operative week 1, rabbits were induced to move for 30 min daily for 5 d per wk for 7 wk to promote OA development.

Intervention

After 4 wk of OA induction, 30 rabbits were randomized into three treatment groups (2-, 4- and 8-wk groups; n = 10 knees each). One knee was selected randomly to receive FLIPUS for 20 min, once daily, 5 d/wk (Monday–Friday), and the other knee received a sham treatment without energy output as a control. Additionally, 30 normal rabbits were randomized and grouped into three groups (2-, 4- and 8-wk groups; n = 10 knees each) as blank controls (Fig. 1). All treatment was standardized with a device that placed the rabbits in a supine position and the knee was angled approximately 120° at the flexion position. The US probe was closed to the surface skin of medial femoral condyle, and the targeted tissue was cartilage of the medial femoral condyle (Fig. 2). A Model CZG200 Ultrasound Therapeutic Device for Arthritis (Chongqing Haifu Medical Technology Co. Ltd., Chongqing, China) with an ultrasonic transducer diameter of 25 mm, radius of curvature of 28 mm, frequency of 0.6 MHz, pulse repetition frequency of 300 Hz, spatial and temporal average intensity (I_{sta}) of 120 mW/cm^2 and a duty cycle of 20% was used. The ellipsoid-shaped acoustic focus was 0.25 mm in diameter and 0.54 mm in length, measured at the full width at half-maximum of the acoustic intensity (Yoo et al. 2011). Rabbits in all groups were sacrificed by air embolization at 2, 4 and 8 wk after each intervention.

Measurement of joint effusion volume via *MRI*

Quantification of knee effusion volume was performed with T2-FI3 D-we-sag images using a 1.5-T superconducting magnet (Siemens; Siemens Electrical Apparatus LTD, Berlin, Germany) at 2, 4 and 8 wk immediately after the intervention as previously described (Ostergaard et al. 1995). MRI procedures were as follows: flex loop small coil, sagittal scanning repetition time: 19 ms; echo times: 9.5 ms; flip angle: 40°; slice thickness: 1 mm; field of view read: 160 mm; field of view phase: 100%; scanning matrix: 512 × 512; voxel size: 0.6 × 0.6 × 1.0 mm; number of excitations: 1; total acquisition time: 3 min 25 s. Three-dimensional reconstruction was performed in the rabbit OA model with knee effusion, which exhibited hyper-intensity on the T2-FI3 D-we-sag sequence. The joint effusions of hyper-intense areas in each slice were outlined and the

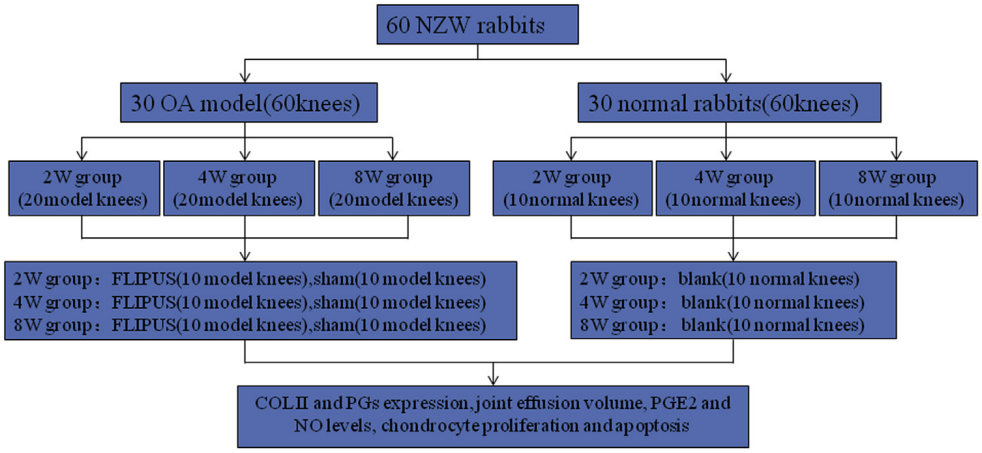

Fig. 1. Illustration of animal use during the study.

areas automatically calculated. The total volumes of effusion were calculated by summation of the slices respectively *via* software (HIFU TPS, Chongqing Haifu Medical Technology Co. Ltd.).

Measurement of PGE2 and NO concentrations

After 2, 4 and 8 wk of treatment, knee effusions from all experimental animals were collected percutaneously. PGE2 and NO were measured in knee effusion with an enzyme immunoassay and nitrile reductase using a PGE2 kit and an NO kit purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China; batch #H099; A012). Assays were performed according to the manufacturer's instructions. Briefly, standard PGE2 (960 pg/mL) was used to produce a dilution series. The micro-plate was prepared with Calibrator Diluent, zero standard and sample according to the manufacturer's instructions. After 1 h incubation at room temperature on a micro-plate shaker, PGE2 conjugate (50 μL) was added to each well, and samples were incubated for 2 h. Wells then were washed with washing buffer (400 μL), residual buffer was aspirated and the plate was blotted. Substrate solution was added to each well (100 μL) and incubated for 10 min at room temperature. Stop solution (50 μL) was added to each well. Finally, the optical density of each well was measured within 10 min using a micro-plate reader (λ = 450 nm). Nitric oxide was measured by quantifying nitrite, a stable product of NO, using a fluorometric reagent, 2,3-diaminonaphthalene. First, 100 μl of each sample was transferred to 96-well plates and incubated with 10 μl fresh 2,3-diaminonaphthalene solution (500 μg/mL in 0.62 N HCl) for 10 min at room temperature. Reactions were terminated with 5 μl of 2.8 N NaOH. Formation of 2,3-diaminonaphthotriazole was measured using a fluorescent multi-well plate reader (BIO-TEK, Winooski, VT, USA) with excitation/emission at 365/450 nm. Nitrite concentration was calculated from a $NaNO_2$ standard curve.

Assessment of COL II and PCNA

Cartilaginous tissues were prepared in 5-μm sections to measure COL II and PCNA expression by immunohistochemistry. Commercial kits for immunohistochemistry assessment of COL II and PCNA were purchased from Novus Biologicals (Littleton, CO, USA; batch #NBP2-33343) and Abcam (Cambridge, MA, USA; batch

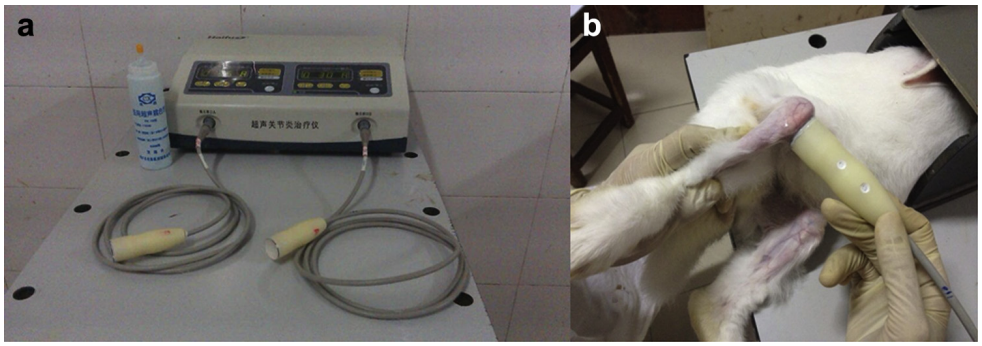

Fig. 2. (a) Model CZG200 Ultrasound Therapeutic Device for Arthritis; (b) US probe was close to the skin surface of the medial femoral condyle, and targeted tissue was cartilage of the medial femoral condyle. US = ultrasound.

#ab80576). An immunohistochemical SP assay was performed according to the manufacturer's instructions. Briefly, the selected sections were de-paraffinized in xylene and then dehydrated in graded alcohol. Endogenous peroxidases were blocked by incubating the sections with 3% H_2O_2 for 10 min at room temperature. After microwave retrieval and washing, non-specific staining was blocked by incubating the sections with BSA for 20 min at 37°C. Monoclonal antibodies for COL II or PCNA were primary antibodies (diluted 1:100 in PBS). Then, slides with primary antibodies against COL II or PCNA were incubated overnight at 4°C. The following day, sections were washed and incubated with secondary antibodies (biotin-labeled anti-rabbit IgG) for 30 min at 37°C. After washing, slides were incubated with horseradish-labeled avidin for 40 min at 37°C. Next, sections were incubated for approximately 5 min in xylene, followed by rinsing in tap water. After counterstaining with hematoxylin and washing, slides were dehydrated in graded alcohol, cleared in dimethyl benzene and finally mounted in DPX. Digital images were captured by an optical microscope (200× magnification). PCNA-positive cell expression and COL II-positive expression appeared brown. The average optical density of COL II expression and ratios of PCNA-positive cells were measured using Image-Pro Plus 6.0 software (Media Cybernetics limited of America).

TUNEL assay for apoptosis

The degree of chondrocyte apoptosis was measured by TUNEL (Roche Co., Buffalo, NY, USA). Cartilaginous tissue was prepared in 5-μm sections and stained with TUNEL according to the manufacturer's instructions. Briefly, the selected sections were de-paraffinized in xylene and then dehydrated in graded alcohol at room temperature. After washing with 0.85% NaCl and phosphate buffered saline (PBS), the sections were fixed in paraformaldehyde. Next, 100 μL of 20 μg/mL Proteinase K was added to each slide to cover the tissue section, and samples were incubated for 10 min at room temperature. After washing in PBS, tissue sections were fixed again in paraformaldehyde and equilibrated with equilibration buffer. Thirty μL of terminal deoxynucleotidyl transferase (rTDT) incubation buffer was added to each slide and samples were protected from light. The tailing reaction was performed at 37°C for 60 min in a humidified chamber. The reaction was terminated by immersing slides in 2× SSC at room temperature. After washing, sections were stained with propidium iodide and slides were washed again, dehydrated in graded alcohol and finally mounted in distyrene-plasticizer-xylene (DPX). Images of tissue sections were captured by an optical microscope (200× magnification). Apoptotic cells appeared brown and were obtained with Image-Pro Plus 6.0 software.

Alcian blue–periodic acid Schiff staining

Cartilaginous tissues were prepared in 5-μm sections and stained with alcian blue–periodic acid Schiff staining (Amresco, Solon, OH, USA) to measure PG in cartilage ECM. Staining was performed according to the manufacturer's instructions. Briefly, cartilaginous tissues sections were stained with 1% Alcian blue in 3% aqueous acetic acid, pH 2.2, for 5 min and washed in running tap water for 10 min before immersion in 1% aqueous periodic acid for 5 min. After washing in distilled H_2O for 10 min, sections were stained with periodic acid-Schiff for 15 min and washed in running tap water for 10 min before nuclei were lightly stained with hematoxylin and slides were visualized. Alcian blue stains acidic mucopolysaccharides blue, and PAS stains neutral mucopolysaccharides magenta, whereas mixtures of acid and neutral mucopolysaccharides stain purple. The images of the tissue sections were captured with an optical microscope (200× magnification), and the average optical density of PG expression was measured.

Statistics

Statistical analysis was done with the SPSS 19.0 statistical software program (Armonk, NY: IBM Corp.). Measurements from the assessment of each group were expressed as means ± standard deviation. Before multiple comparisons, we tested whether the data were normally distributed and the variances were equal. If so, a one-way analysis of variance with least significant difference testing was used. If not, the non-parametric Kruskal-Wallis test was applied. We analyzed differences between FLIPUS versus sham control, FLIPUS versus blank control and sham control versus blank control. The Bonferroni correction was applied to the alpha level of significance (0.05) according to number of comparisons conducted; therefore, for any of these comparisons to be considered significant, an alpha level of 0.02 (0.05/3) was used.

RESULTS

Knee effusion volumes

As shown in MRI imaging and 3-D reconstruction of knee effusion imaging (Figs. 3 and 4), knee effusion volumes were significantly decreased after FLIPUS ($p < 0.02$) compared with sham controls; however, effusion volumes increased compared with blanks ($p = 0.000$) at 2, 4, and 8 wk after the intervention. Despite variations in data of knee effusion volumes after FLIPUS over time, the difference was not statistically significant ($p > 0.02$). Data show that FLIPUS decreased knee effusion volumes and maintained these volumes at a relatively low level over time (Table 1).

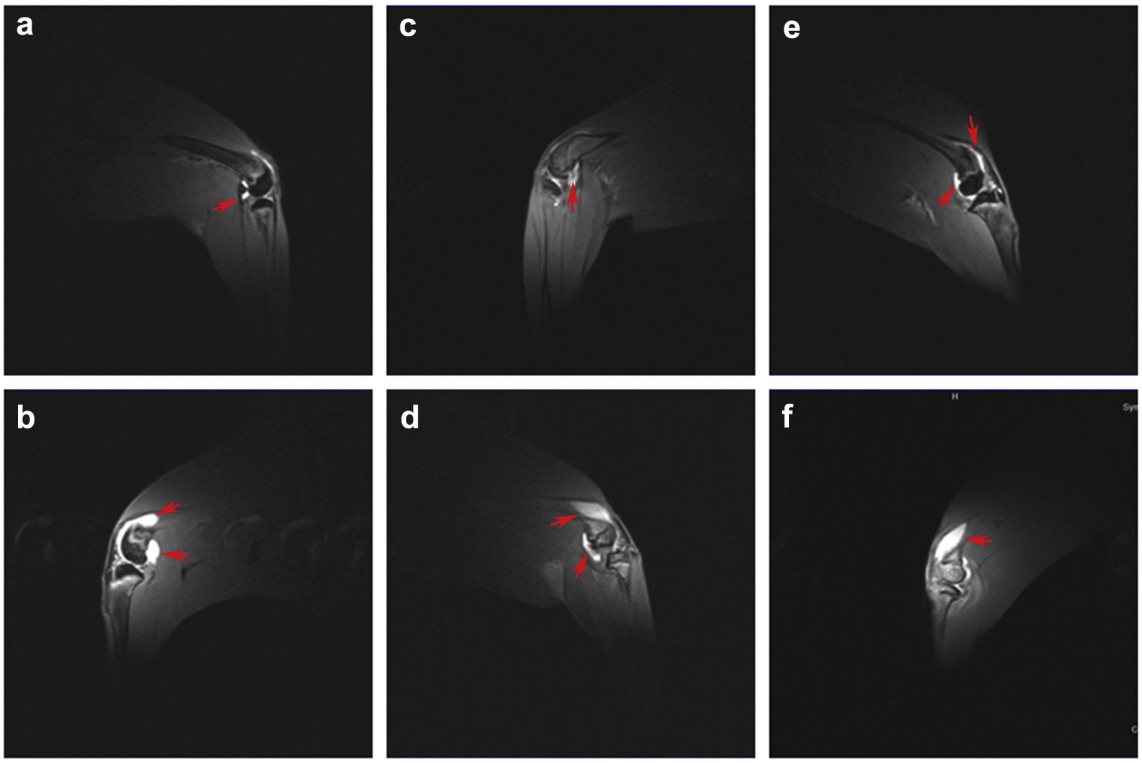

Fig. 3. Knee effusion volume in all groups; *red arrows* indicate joint effusion. (a) FLIPUS intervention at 2 wk; (b) Sham intervention at 2 wk; (c) FLIPUS intervention at 4 wk; (d) Sham intervention at 4 wk; e: FLIPUS intervention at 8 wk; f: Sham intervention at 8 wk (n = 10 knees each). FLIPUS = focused low-intensity pulsed ultrasound.

PGE2 and NO concentrations in synovial fluid

PGE2 and NO concentrations in synovial fluid were significantly decreased after FLIPUS compared with sham controls at 2, 4, and 8 wk after intervention ($p = 0.000$); however, they were significantly increased compared with blanks ($p = 0.000$).

Despite variations in data for PGE2 and NO concentrations after FLIPUS over time, differences were not statistically significant between the 4-wk group and the 8-wk group ($p > 0.02$). However, PGE2 and NO concentrations in controls increased significantly over time ($p < 0.02$). Thus, FLIPUS decreased PGE2 and NO and kept them low over time (Table 2).

COL II and PG in ECM

COL II-positive appeared brown and PG-positive expression in blue and magenta (Fig. 5). COL II and PG expressions were significantly increased after FLIPUS stimulation compared with sham controls ($p < 0.02$) at 2, 4, and 8 wk, but these significantly decreased compared with blanks ($p = 0.000$; Table 3).

Chondrocyte proliferation and apoptosis

Immunohistochemistry revealed PCNA-positive cells and apoptosis in the articular cartilage of all animals (Fig. 6). The ratio of chondrocyte proliferation after FLIPUS stimulation was significantly greater than those of sham controls and blanks at 2 wk ($p < 0.01$). However, no significant changes were seen after FLIPUS stimulation and in sham controls at 4 and 8 wk ($p > 0.02$; Table 4).

Apoptotic chondrocytes after FLIPUS stimulation were significantly less than in sham controls at 2, 4, and 8 wk ($p < 0.01$), but significantly more apoptotic chondrocytes were observed in shams compared with blanks ($p < 0.01$; Table 4).

These data show that chondrocytes proliferate rapidly and that apoptosis decreased after FLIPUS at early disease stages (2 wk). Differences in apoptosis between the 4- and 8-wk groups after FLIPUS treatment were not statistically significant ($p > 0.02$), indicating that FLIPUS treatment kept apoptosis low over time.

DISCUSSION

Ultrasound has been confirmed to be a tolerable and effective physiotherapy for OA (Tascioglu et al. 2010) and can relieve the severity of surgically induced cartilage degeneration (Zeng et al. 2012). In a previous study, unfocused US was shown to increase expression of PG or COL II (Parvizi et al. 1999; Wang et al. 2010) and promote healing of cartilage (Xia et al. 2015). However,

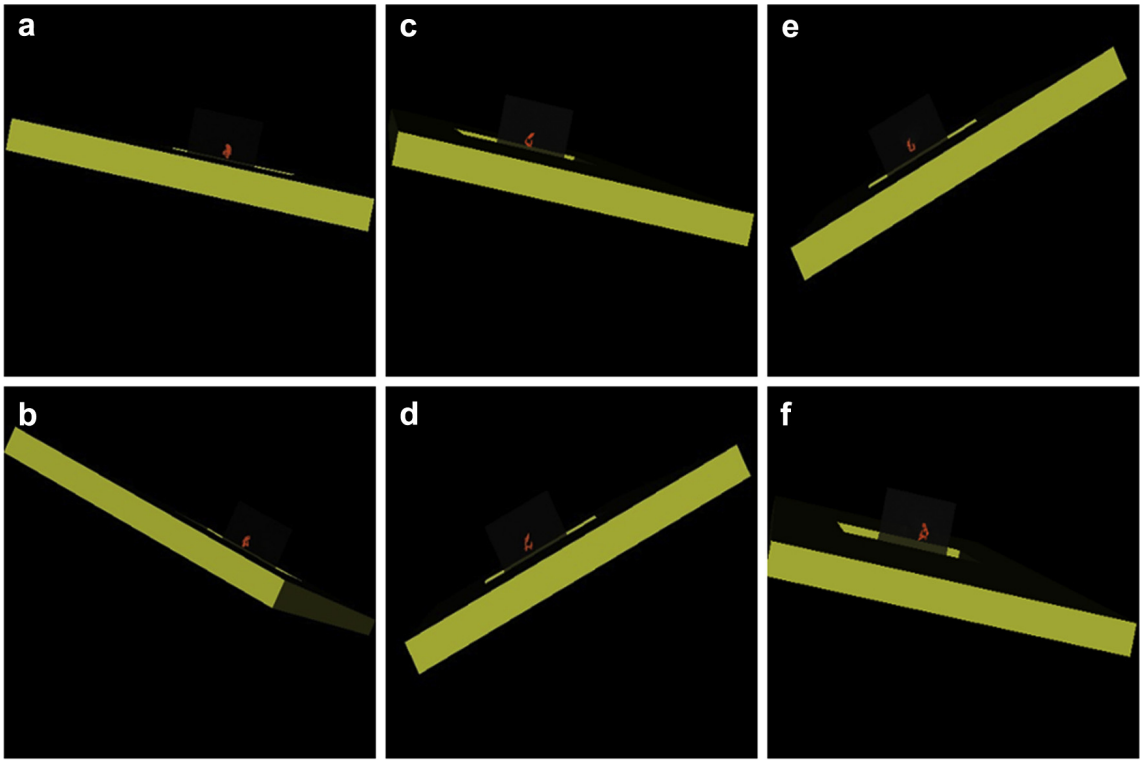

Fig. 4. Three-dimensional reconstruction of knee effusion imaging; *red imaging* indicates knee effusion. (a) FLIPUS intervention at 2 wk; (b) Sham intervention at 2 wk; (c) FLIPUS intervention at 4 wk; (d) Sham intervention at 4 wk; (e) FLIPUS intervention at 8 wk; (f) Sham intervention at 8 wk (n = 10 knees each). FLIPUS = focused low-intensity pulsed ultrasound.

unfocused US did not modify cell apoptosis and proliferation (Min and Woo 2006; Zeng et al. 2012). Recently, a FLIPUS technique was applied to heal bone defects (Jung et al. 2015). However, few investigations of the application of this technique to healing cartilage have been conducted. Thus, we studied whether FLIPUS affects COL II and PG production by down-regulating chondrocyte apoptosis, reducing pro-inflammatory mediators and up-regulating cell proliferation. We found that FLIPUS significantly increased ECM production mainly through decreasing cell apoptosis and lowering inflammatory mediators.

Table 1. Comparison of knee effusion volume (mL) among all groups after intervention

Group	N	FLIPUS side	Control side	Blank side
2 wk	10	0.76 ± 0.46*§	1.15 ± 0.70¶	0.28 ± 0.06
4 wk	10	0.68 ± 0.42†‖	1.34 ± 0.55#	0.27 ± 0.07
8 wk	10	0.86 ± 0.43‡	1.70 ± 0.68	0.27 ± 0.07

FLIPUS = focused low-intensity pulsed ultrasound.
* Compared with control side and blank side in 2-wk group, p = 0.008 and 0.000, respectively.
† Compared with control side and blank side in 4-wk group, p = 0.000 and 0.000, respectively.
‡ Compared with control side and blank side in 8-wk group, p = 0.002 and 0.000, respectively.
§ Compared with 4-wk group and 8 wk group in FLIPUS side, p = 0.524 and 0.073, respectively.
‖ Compared with 8-wk group in FLIPUS side, p = 0.232.
¶ Compared with 4-wk group and 8-wk group in control side, p = 0.686 and 0.613, respectively.
# Compared with 8-wk group in control side, p = 0.366.

From a physical point of view, US effects depend on frequency, intensity, duty cycle and time of insonification. First, the US attenuation coefficient in soft tissue is proportional to a frequency in the range of 0.5–13 MHz. Low-frequency US waves have greater depth of penetration. Ultrasound at 1 MHz is absorbed primarily by tissues at a depth of 3–5 cm (Gann 1991) and articular cartilage is shaded by the patella just below the skin. Clement and Hynynen (2002) reported that US at 0.74 MHz could propagate through the skull, so a frequency less than this is recommended for articular cartilage shaded by bone and soft tissue. Second, low-intensity US (100–400 mW/cm^2) can induce the expression of COL II, PG and aggrecan; decrease expression of metalloproteinases-1; and induce tissue inhibitors of matrix metalloproteinases-2 in human or animal chondrocytes (Choi et al. 2006; Korstjens et al. 2008; Min and Woo 2006; Nishikori et al. 2002). Third, US application of about 20–30 min daily to accelerate fracture healing and for treatment of delays or nonunions and for bone lengthening has been

Table 2. Comparisons of concentration of prostaglandin E2 (pg/mL) and nitric oxide (μmol/L) in the synovial fluid after intervention

		PGE2			NO		
Group	N	FLIPUS	Control	Blank	FLIPUS	Control	Blank
2 wk	10	51.55 ± 10.45*§	66.50 ± 8.15¶	22.50 ± 0.95	40.40 ± 9.3**§§	54.59 ± 5.71¶¶	29.17 ± 3.76
4 wk	10	56.66 ± 9.01†‖	81.94 ± 7.41#	23.55 ± 1.26	52.15 ± 8.20††‖‖	103.57 ± 24.26##	28.61 ± 5.71
8 wk	10	56.50 ± 12.01‡	138.35 ± 27.01	22.17 ± 0.61	59.56 ± 7.78‡‡	180.00 ± 20.11	31.94 ± 3.00

PGE2 = prostaglandin E2; NO = nitric oxide; FLIPUS = focused low-intensity pulsed ultrasound.
PGE2 concentration:
* Compared with control side and blank side in 2-wk group, $p = 0.000$ and 0.000, respectively.
† Compared with control side and blank side in 4-wk group, $p = 0.000$ and 0.000, respectively.
‡ Compared with control side and blank side in 8-wk group, $p = 0.000$ and 0.000, respectively.
§ Compared with 4-wk group and 8-wk group in FLIPUS side, $p = 0.289$ and 0.304, respectively.
‖ Compared with 8-wk group in FLIPUS side, $p = 0.974$.
¶ Compared with 4-wk group and 8-wk group in control side, $p = 0.048$ and 0.000, respectively.
# Compared with 8-wk group in control side, $p = 0.000$.
NO concentration:
** Compared with control side and blank side in 2 wk group, $p = 0.001$ and 0.000, respectively.
†† Compared with control side and blank side in 4 wk group, $P = 0.000$ and 0.000, respectively.
‡‡ Compared with control side and blank side in 8 wk group, $P = 0.000$ and 0.000, respectively.
§§ Compared with 4-wk group and 8-wk group in FLIPUS side, $p = 0.004$ and 0.000, respectively.
‖‖ Compared with 8-wk group in FLIPUS side, $p = 0.058$.
¶¶ Compared with 4-wk group and 8-wk group in control side, $p = 0.000$ and 0.000, respectively.
Compared with 8-wk group in control side, $p = 0.000$.

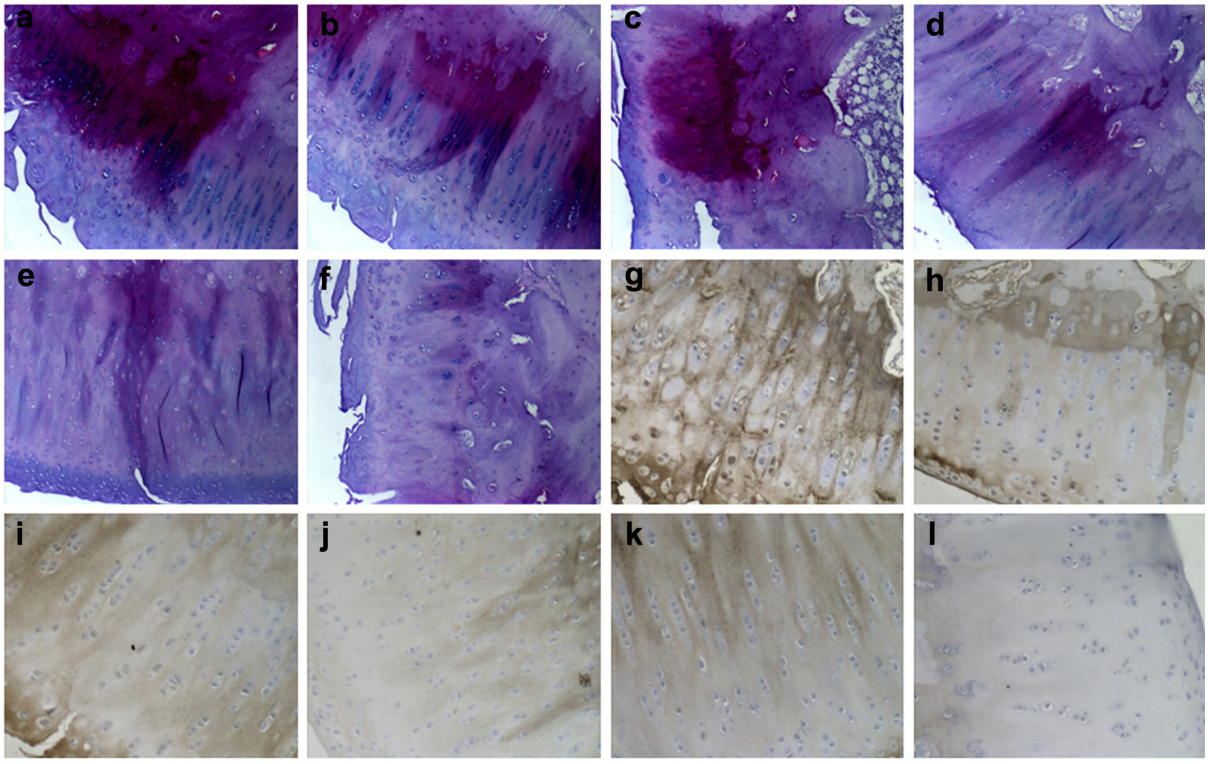

Fig. 5. AB stains acidic mucopolysaccharides blue, and PAS stains neutral mucopolysaccharides magenta, whereas mixtures of acidic and neutral mucopolysaccharides stain purple in the each group, ×200 magnification. (a) FLIPUS intervention at 2 wk; (b) Sham intervention at 2 wk; (c) FLIPUS intervention at 4 wk; (d) Sham intervention at 4 wk; (e) FLIPUS intervention at 8 wk; (f) Sham intervention at 8 wk. COL II expression was brown in each group, ×200 magnification. (g) FLIPUS intervention at 2 wk; (h) Sham intervention at 2 wk; (i) FLIPUS intervention at 4 wk; (j) Sham intervention at 4 wk; (k) FLIPUS intervention at 8 wk; (l) Sham intervention at 8 wk (n = 10 knees each).
AB = alcian blue; PAS = periodic acid Schiff; FLIPUS = focused low-intensity pulsed ultrasound.

Table 3. Comparison of Type II collagen and proteoglycans expression after intervention

		AOD values of COL II			AOD values of PG		
Group	N	FLIPUS	Control	Blank	FLIPUS	Control	Blank
2 wk	10	0.13 ± 0.02*§	0.07 ± 0.01¶	0.17 ± 0.03	0.15 ± 0.01**§§	0.06 ± 0.01¶¶	0.19 ± 0.01
4 wk	10	0.06 ± 0.01†‖	0.03 ± 0.01#	0.17 ± 0.03	0.08 ± 0.01††‖‖	0.03 ± 0.01##	0.20 ± 0.004
8 wk	10	0.03 ± 0.01‡	0.01 ± 0.01	0.18 ± 0.03	0.04 ± 0.01‡‡	0.01 ± 0.003	0.19 ± 0.004

AOD = average optical density; PG = proteoglycans; COL II = type II collagen; FLIPUS = focused low-intensity pulsed ultrasound.
AOD values of COL II:
* Compared with control side and blank side in 2-wk group, $p = 0.000$ and 0.000, respectively.
† Compared with control side and blank side in 4-wk group, $p = 0.018$ and 0.000, respectively.
‡ Compared with control side and blank side in 8-wk group, $p = 0.009$ and 0.000, respectively.
§ Compared with 4-wk group and 8-wk group in FLIPUS side, $p = 0.000$ and 0.000, respectively.
‖ Compared with 8-wk group in FLIPUS side, $p = 0.000$.
¶ Compared with 4-wk group and 8-wk group in control side, $p = 0.000$ and 0.000, respectively.
# Compared with 8-wk group in control side, $p = 0.000$.
AOD values of PG:
** Compared with control side and blank side in 2-wk group, $p = 0.000$ and 0.000, respectively.
†† Compared with control side and blank side in 4-wk group, $p = 0.000$ and 0.000, respectively.
‡‡ Compared with control side and blank side in 8-wk group, $p = 0.000$ and 0.000, respectively.
§§ Compared with 4-wk group and 8-wk group in FLIPUS side, $p = 0.000$ and 0.000, respectively.
‖‖ Compared with 8-wk group in FLIPUS side, $p = 0.000$.
¶¶ Compared with 4-wk group and 8-wk group in control side, $p = 0.000$ and 0.000, respectively.
Compared with 8-wk group in control side, $p = 0.000$.

recommended (Busse et al. 2009). Finally, pulsed US with a 20% duty cycle is often chosen to promote orderly collagen deposition to increase tissue tensile strength in healing (Byl et al. 1992, 1993). In addition, *in vitro* studies have supported the effects of pulsed US in inducing chondrocyte proliferation and increasing content of 35 S-sulfate in the extracellular matrix of cartilage grafts of normal and diseased joints, which could promote the synthesis of cartilage matrix (Ingber 2006; Korstjens et al. 2004, 2008; Nolte et al. 2001; Parvizi et al. 1999, 2002). Meanwhile, in *in vivo* experiments, pulsed US was considered to be beneficial to cartilage health in animal models of cartilage injury (Cook et al. 2001; Cui et al. 2006; Gurkan et al. 2010; Huang et al. 1997, 1999; Naito et al. 2010). Based on systematic review and meta-analysis, pulsed US is more effective for relieving pain and improving function in clinical trials (Zeng et al. 2014). To avoid diffusion of US energy, we used focused low-intensity pulsed US with a spatial and temporal average intensity (I_{sta}) of 120 mW/cm^2, a frequency of 0.6 MHz, a pulse repetition frequency of 300 Hz and a 20% duty cycle, for 20 min a day.

The potential biological actions of US arise from thermal and non-thermal effects. Thermal effects of US are closely related to frequency, intensity, the attenuation coefficient of tissue, pulsing modes and duration of insonification. Atkins and Duck (2003) reported that maximal temperature increases in tissue-mimicking materials were 0.21°C after 10 min of exposure in the Doppler imaging mode (20% duty cycle, 3 MHz and 290 mW/cm^2). Pulsed US waves of 0.6 MHz and an intensity of 120 mW/cm^2 were lower than those of Doppler imaging in our present study. When pulsed US waves propagate within the tissue, most of the thermal energy generated by absorption of US energy may be taken away by blood perfusion. Therefore, the thermal effect of US was nearly negligible in this study. Also, at least 100–400 W/cm^2 at 0.6 MHz is required to establish cavitation (Zhou and Guo 2001), and the cavitation effect of US was nearly negligible in this study as well. In summary, the main biological effect of US in our experiment is a mechanical effect.

Also, here we observed that the steady time-average acoustic radiation pressure generated by US applied on the surface of the cartilage is equal to ISTA/(rho × c), where rho is the density of the tissue medium and c is the speed of sound in the medium (Wu and Nyborg 2008). Given ISTA = 0.12 W/cm^2, and c = 1500 m/s, we can estimate the time-average radiation pressure applied on the surface of the cartilage to be ~60,000 Pa. Considering the 20% duty cycle, the temporal peak acoustic radiation pressure can reach 60,000 Pa/20% = 300,000 Pa.

The most striking effect of FLIPUS is the reduction of knee effusion volume, which is in line with Park et al. (2005) previous study. Some ultrasonic effects can reduce effusion volumes. First, plasma is prevented from leaking into the synovial cavity because sonication can stabilize the basement membranes of blood vessels. Secondly, osmotic pressure is reduced *via* hindering protein extravasation. Third, ultrasonic US alleviates inflammation as evidenced by the reduction of PG in both previous studies and our work, and synovial cells have been shown to disaggregate in previous work (Park et al. 2005).

In our study, FLIPUS could be attenuating the release of PGE2 and NO and keeping them low over time. Low-intensity pulse US (1.5 MHz, 30 mW/cm^2) significantly increased the diffusion rate of Gd-DTPA into joint fluid

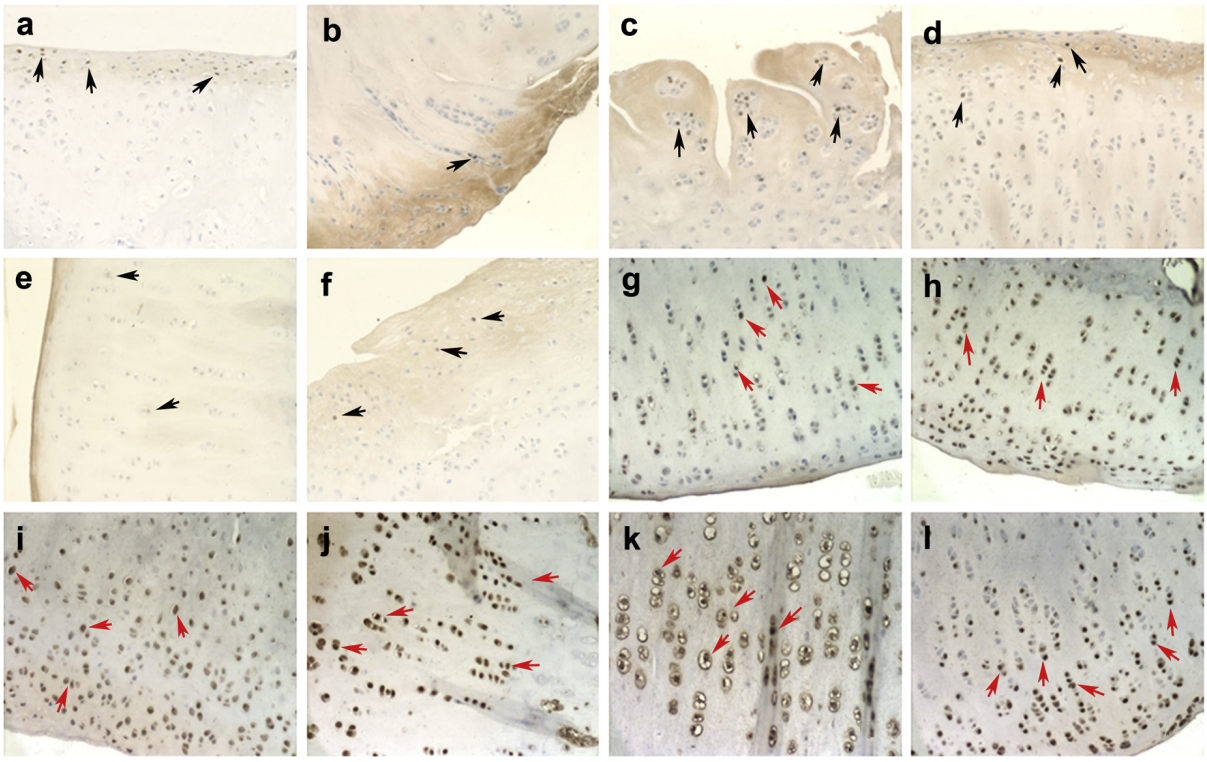

Fig. 6. PCNA expression in the each group, ×200 magnification. *Blank arrows* indicate positive cells. (a) FLIPUS intervention at 2 wk; (b) Sham intervention at 2 wk; (c) FLIPUS intervention at 4 wk; (d) Sham intervention at 4 wk; (e) FLIPUS intervention at 8 wk; (f) Sham intervention at 8 wk. Apoptosis in the each group, ×200 magnification. *Red arrows* indicate apoptotic cells. (g) FLIPUS intervention at 2 wk; (h) Sham intervention at 2 wk; (i) FLIPUS intervention at 4 wk; (j) Sham intervention at 4 wk; (k) FLIPUS intervention at 8 wk; (l) Sham intervention at 8 wk (n = 10 knees each). FLIPUS = focused low-intensity pulsed ultrasound.

in previous research (Weishaupt et al. 2001). Because Gd-DTPA molecular weight is larger (393.35 Da) than PGE2 (352.47 Da) and NO (30.01 Da), low-intensity pulse US should also increase PGE2 and NO diffusion into joint fluid. Joint fluid volume was reduced in our work, and we hypothesize that this was attributable to enhanced absorption of PGE2 and NO into the synovium facilitated by ultrasonic waves.

Interestingly, we observed chondrocyte proliferation and apoptosis simultaneously. FLIPUS stimulated increases in proliferating cells at early time points (2 wk) compared with sham intervention, which was not compatible with a previous *in vivo* study (Yang et al. 2014). The US waves of 1.5 MHz frequency and a peak intensity of 30 mW/cm^2 have been applied previously (Yang et al. 2014) and the US could barely propagate through the patella; energy from unfocused US can diffuse, which reduces the effectiveness of US. There were no significant differences in proliferation between FLIPUS and sham interventions at 4 and 8 wk, and there were fewer proliferating cells in the sham group at 2 wk as compared with 4 and 8 wk. Chondrocyte proliferation that increased cell content may have represented a cellular reaction to cartilage destruction and FLIPUS may have accelerated this normal process. At later stages of OA (4 or 8 wk), persistent cartilage destruction increased apoptotic chondrocytes and reduced proliferating cells.

After FLIPUS stimulation, COL II and PG expression significantly increased ($p < 0.02$). Chondrocytes secrete COL II and PG, and the ECM is composed primarily of COL II and large networks of PG that contain glycosaminoglycan to protect chondrocytes, including hyaluronic acid and chitosan. ECM as it pertains to cell survival is likely to contribute to cartilage apoptosis (Wang et al. 2006), and degradation of COL II may be correlated with chondrocyte apoptosis or the loss of a survival signal (Yang et al. 1991). Abnormal chondrocyte apoptosis can not only affect the development of cartilage, but also lead to OA. Also, aberrant production of inflammatory mediators by chondrocytes could result in OA and ECM degradation (Borzi et al. 2002). Nitric oxide regulates cartilage degradation by causing de-differentiation and apoptosis of chondrocytes *via* activation of ERK1/2 and p38 (Kim et al. 2002), and PGE2 has been implicated in cartilage erosion (Wang et al. 2010) and inflammation associated with OA. Our data

Table 4. Comparisons of proliferation and apoptosis percentage after intervention

		Proliferation of chondrocyte (%)			Apoptosis of chondrocyte (%)		
Group	N	FLIPUS	Control	Blank	FLIPUS	Control	Blank
2 wk	10	21.10 ± 1.52*§	4.20 ± 1.93¶	0.31 ± 0.20	27.82 ± 5.25**§§	41.74 ± 3.43¶¶	1.49 ± 1.38
4 wk	10	14.50 ± 7.56†‖	11.44 ± 5.79#	0.28 ± 0.19	41.40 ± 6.89††‖‖	49.76 ± 7.05##	1.29 ± 1.07
8 wk	10	9.95 ± 4.47‡	9.30 ± 5.08	0.31 ± 0.18	45.54 ± 5.16‡‡	56.83 ± 5.32	1.38 ± 1.42

FLIPUS = focused low-intensity pulsed ultrasound.
Proliferation percentage of chondrocyte (%):
* Compared with control side and blank side in 2-wk group, $p = 0.008$ and 0.000 respectively.
† Compared with control side and blank side in 4-wk group, $p = 0.225$ and 0.000 respectively.
‡ Compared with control side and blank side in 8-wk group, $p = 0.713$ and 0.000 respectively.
§ Compared with 4-wk group and 8-wk group in FLIPUS side, $p = 0.008$ and 0.000 respectively.
‖ Compared with 8-wk group in FLIPUS side, $p = 0.058$.
¶ Compared with 4-wk group and 8-wk group in control side, $p = 0.002$ and 0.019 respectively.
# Compared with 8-wk group in control side, $p = 0.306$.
Apoptosis percentage of chondrocyte (%):
** Compared with control side and blank side in 2-wk group, $p = 0.000$ and 0.000 respectively.
†† Compared with control side and blank side in 4-wk group, $p = 0.002$ and 0.000 respectively.
‡‡ Compared with control side and blank side in 8-wk group, $p = 0.000$ and 0.000 respectively.
§§ Compared with 4-wk group and 8-wk group in FLIPUS side, $p = 0.000$ and 0.000 respectively.
‖‖ Compared with 8-wk group in FLIPUS side, $p = 0.123$.
¶¶ Compared with 4-wk group and 8-wk group in control side, $p = 0.003$ and 0.000 respectively.
Compared with 8-wk group in control side, $p = 0.043$.

show that FLIPUS decreased NO and PGE2 and kept them low over time, which may decrease apoptosis and prevent further degradation of the ECM. Based on our present study, we suggest that FLIPUS prevented COL II and PG degradation by down-regulation of apoptosis and reduction of inflammatory mediators in OA chondrocytes.

Our study has several limitations. First, this surgically induced OA model cannot duplicate the natural course of OA in humans, as OA's structural changes take place over decades; therefore, alternative animal models of OA, such as the spontaneous OA model, may be more suitable for future studies. Second, mechanotransduction mechanisms of chondrocytes are uncertain and warrant investigation. Third, OA biomarkers (BMP-2, CTX-II and COMP) may be promising for predicting the prognosis and progress of OA and these should be studied after FLIPUS intervention.

CONCLUSION

On the basis of the present study, we determined FLIPUS to be an effective therapeutic modality for the reduction of the COL II and PG. Moreover, it became clear that FLIPUS reduced joint effusion volume, chondrocyte apoptosis and levels of PGE2 and NO more vigorously than did sham intervention. However, it was found that proliferating cells are normally increasing only at early time points after OA induction. In summary, reduction of apoptotic cells is due to FLIPUS down-regulation levels of PGE2 and NO, which gave rise to a better reservation of ECM. Our findings further revealed the mechanism underlying the effects of FLIPUS on chondrocytes and provide some basis for the management of OA therapy using FLIPUS.

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