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Mitigation of articular cartilage degeneration and subchondral bone sclerosis in osteoarthritis progression using low-intensity ultrasound stimulation

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Abstract

The purpose of this study was to evaluate the effect of low-intensity ultrasound on articular cartilage and subchondral bone alterations in joints under normal and functional disuse conditions during osteoarthritis (OA) progression. Sixty 5-month-old female Sprague-Dawley rats were randomly assigned to six groups (n=10/group): Age-match group, OA group, OA+ultrasound (US) group, Hindlimbs suspension (HLS) group, HLS+OA group, and HLS+OA+US group. The surgical anterior cruciate ligament was used to induce OA in the right knee joints. After two weeks of OA induction, low-intensity ultrasound generated by a 3 MHz transducer with 20% pulse duty cycle and 30 mW/cm² acoustic intensity was conducted in right knee joints for 20 mins a day, five days a week, and a total of 6 weeks. Then, the right tibias were harvested for Micro-CT, histological and mechanical analysis. Micro-CT results indicated that the thickness and sulfated glycosaminoglycan (sGAG) content of cartilage decreased, but the thickness of the subchondral cortical bone plate and the formation of subchondral trabecular bone increased in the OA group under the normal joint use condition. Furthermore, histological results demonstrated that chondrocyte density and arrangement in cartilage corrupted and the underlying subchondral bone increased during OA progression. These changes were accompanied by reductions of mechanical parameters in OA cartilages. However, less OA symptoms were observed in HLS+OA group under the joint disuse condition. The cartilage degeneration and subchondral bone sclerosis were alleviated in the US treatment group, especially under normal joint use condition. In conclusion, low-intensity ultrasound could improve cartilage degeneration and subchondral sclerosis during OA progression. Also, it could provide a promising strategy for future clinical treatment for OA patients.

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Competing interest statement

The authors have no conflicts of interest to disclose.

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Keywords

Osteoarthritis; Articular Cartilage; Subchondral bone; Joint disuse; Low-intensity Ultrasound; Acoustic Radiation Force

Introduction

Osteoarthritis (OA) is one of the most common degenerative joint disorders. It is characterized by the gradual but progressive loss of articular cartilage (AC), sclerosis of subchondral bone and formation of osteophyte (Joseph et al. 2006). It most commonly affects the knee, hip, spine, and hand joints (Yuqing et al. 2010). OA is affecting more than 27 million people in the US. The incidence of OA is likely to increase with the increasing proportion of older adults and the growing obesity epidemic and is projected to affect 67 million people in the United States by 2030 (Hootman et al. 2006; Lawrence et al. 2008; Zhen et al. 2013). OA is difficult to cure as there is no effective treatment, by the time X-ray radiographs of OA joints in the clinic show joint space narrowing and osteophyte formation, it has come to the middle or late stage of OA (Yan et al. 2012). Moreover, AC cannot be truly repaired once it is damaged because it does not have blood vessels, lymphatic, and nerves. Therefore, nutrients are transported to the AC by diffusion from the synovial fluid or the vascularized subchondral bone (Blanco et al. 2013; Pearle et al. 2005). Existing therapies, such as injection of the drug in synovial space, joint disuse, joint replacement surgery and arthroscopic surgical intervention can alleviate pain and mitigate deformity, but cannot slow AC degradation and OA progression (Buckwalter et al. 1998; Pearle et al. 2005). Moreover, although joint disuse can alleviate pain caused by OA, it has been demonstrated that prolonged joint disuse or joint immobilization can cause joint atrophy and AC degeneration. Progressive thinning of AC and the degradation of extracellular matrix (ECM) has been reported in both immobilized patients and unload animal models (Guilak et al. 2004; Guilak et al. 2011; Trudel et al. 2005; Vanwanseele et al. 2002; Sun. 2010).

Previous studies have made significant progress in clarifying molecular mechanisms underlying the pathogenesis of AC degeneration in OA. Numerous animal or human studies have shown that the balances of anabolic and catabolic activities in chondrocytes are disrupted to adapt to the changes to the biomechanical or biological environments in OA (Martin et al. 2001; Boumediene K et al. 1998; Baugé C et al. 2012). Additionally, the catabolic activities increased, and chondrocytes were stimulated to express hypertrophy and terminal differentiation (Martin et al. 2001; Boumediene K et al. 1998; Baugé C et al. 2012). These shifts are accompanied by calcification of ECM, loss of negatively charged sulfated glycosaminoglycan (sGAG), and disruption of the collagen network in AC. Consequently, the surface of AC became rougher, and the thickness of AC became thinner during the progression of OA (Tummala et al. 2011; Eckstein et al. 2011).

However, in recent years, the underlying subchondral bone has drawn more and more attention in the OA pathology. Recent animal or human studies have indicated that both AC and subchondral bone are responsible for OA progression (Sharma et al. 2013; Lacourt et al. 2012; Yuan et al. 2014). Studies indicated that the progressive loss of AC failed to distribute

the mechanical load, and the load transmitted to the underlying subchondral bone increased. Meanwhile, the abnormal subchondral bone could induce more severe AC degeneration. It has been demonstrated in animal or human studies that the bone turnover in subchondral bone increased during OA progression (Jaiprakash et al. 2012; Zhen et al. 2013; Zhen et al. 2014). These studies found that osteoblasts in subchondral bone in OA expressed a high level of alkaline phosphatase (ALP), growth factors like insulin growth factor (IGF-1), IGF-2 and transforming growth factor- β (TGF- β) (Sharma et al. 2013; Lacourt et al 2012; Yuan et al. 2014; Jaiprakash et al. 2012; Zhen et al. 2013; Zhen et al. 2014). Therefore, the increased remodeling of subchondral bone induced the subchondral cortical bone plate to become thicker (McErlain et al. 2008).

In spite of numerous existing pharmaceutical interventions, low-intensity pulsed ultrasound (US) still represents an advantageous minimally invasive strategy in the maintenance of AC. Previous studies have provided evidence that the application of low-intensity pulsed US can attenuate the progressive degeneration of AC and promote the AC repair (Zhang et al. 2002; Zhang et al. 2003; Korstjens et al. 2008; Naito et al. 2010; Cheng et al. 2014). Low-intensity pulsed US, as a form of mechanical energy, can generate acoustic pressure waves in the form of acoustic radiation force (Khanna et al. 2009; Yang et al. 2014). It has been demonstrated that low-intensity pulsed US, generated by ultrasonic pressure waves, can stimulate the AC anabolism by enhancing chondrocytes proliferation and production of matrix molecules including proteoglycans (PG) and collagen, promoting mRNA expression of type II collagen and aggrecan of AC (Zhang et al. 2002; Zhang et al. 2003; Korstjens et al. 2008; Naito et al. 2010; Schumann et al. 2006; Cui et al. 2007; Park et al. 2007; Cheng et al. 2014).

Although numerous *in-vitro* and *in-vivo* studies have investigated the effect of low-intensity pulsed US on AC repair in OA, this treatment for OA can not only focus on AC. These studies also need to consider the subchondral bone, because the crosstalk between AC and subchondral bone in OA progression have demonstrated that both AC and subchondral bone contribute to OA development (Sharma et al. 2013; Lacourt et al. 2012; Yuan et al. 2014; Jaiprakash et al. 2012; Zhen et al. 2013; Zhen et al. 2014). Furthermore, whether low-intensity acoustic radiation force could mitigate subchondral sclerosis during the period of treatment or not is unclear. Moreover, recent US treatment studies in OA mainly investigated AC regeneration under a normal joint use condition. Few studies investigate whether low-intensity acoustic radiation force is effective for mitigating AC degeneration and subchondral bone sclerosis in OA under a joint disuse condition. Therefore, the objective of this study is to evaluate the effect of low-intensity acoustic radiation force on the AC and subchondral bone morphology, content, microstructure, and mechanical properties in joints under normal use or disuse condition during OA progression.

Materials and Methods

Animal models

The Institutional Animal Care and Use Committee of Stony Brook University (IACUC) approved the experimental surgery procedures and study protocol. Sixty 5-month-old female Sprague-Dawley rats (Charles River, Wilmington, MA, USA) were randomly assigned to six groups (n=10/group, average weight: 282 $\pm$ 8 g): (1) Age-match group, (2) OA group, (3) OA

+US group, (4) Hindlimbs suspension (HLS) group, (5) HLS+OA group, and (6) HLS+OA +US group. All animals were housed in individual cages with a 12:12-h light-dark cycle in 20–25°C and were allowed access to a standard laboratory diet and drinking water. In group 2, 3, 5, and 6, the surgical anterior cruciate ligament (ACL) was used to induce mechanical instability-associated OA on the right knees under general anesthesia. After surgery, animal weight, wound healing, activity, and food consumption were monitored daily. The rats were allowed to move around to induce OA development in the following two weeks. After two weeks of OA induction, animals in groups 3, 4, 5, and 6 were given US treatment and HLS for six weeks, respectively. In HLS groups, the tails of rats were suspended with surgical tape with the body in a head-down position. The angle formed between the torso of the rats and the floor of the cage was kept at 30°, the hind-limbs were prevented from touching the ground of the cage at all time during the experiment, but free movement of forelimbs was allowed. All animals in HLS groups were checked twice a day to monitor their health conditions and suspension statuses.

Low-intensity ultrasound treatment for ACL-induced rat OA model

After two weeks of OA induction, the right knees of animals in US treatment groups (groups 3, 6) received ultrasound treatment for 20 min per day, five days a week. The US unit, Sonicator 740 (Mettler Electronics Corp, Anaheim, CA, USA) was used in this study. The transducer frequency was 3 MHz, and the pulse duty cycle was 20%. The acoustic intensity transmitted to the cartilage in right knees was 30 mW/cm². US treatment was conducted for six weeks. The rats were placed in a supine position, and the knees were angled approximately 120° at the flexion position to make sure the transducer was perpendicular to the knees and close to the surface skin of the medial tibial plateau to target the cartilage position. Kendall™ ultrasound transmission gel (Covidien Ltd, Dublin, Republic of Ireland) was applied in the surface skin to facilitate acoustic energy transmission through the surface skinhead to the inner tissue. The US device setup for animal treatment was shown in Fig. 1. After the study, the animals were sacrificed through cervical dislocation method. Right tibias were harvested and stored in phosphate buffered saline (PBS) at 4° C for subsequent analysis.

Micro-CT scan and analysis for cartilage and subchondral bone in right tibia plateau

A high-resolution Micro-CT scanner (μCT-40, SCANCO Medical AG, Bassersdorf, Switzerland) was adopted in this study. Contrast agent Hexabrix 320 (Mallinckrodt, Hazelwood, MO, USA) was used to enhance cartilage x-ray attenuation to visualize cartilage in the tibial plateau. Before Micro-CT scan, the tibias were immersed in ionic CT contrast agent (40% Hexabrix 320 contrast agent +60% PBS) at 37°C water bath for 30 mins. The contrast agent concentration and the incubation time used here have been explored and demonstrated in previous studies (Xie et al. 2009; Xie et al. 2010). After the tibias were taken out from the contrast agent, samples were patted dry and immediately scanned by Micro-CT at 55 kVp, 110 μA, 300-ms integration time, and a voxel size of 15μm in a 16mm scanning tube. During the scan, all of the tibia samples were consistently secured so that transverse axis was aligned with the vertical axis of the Micro-CT scanning tube to obtain the sagittal section imaging of the tibias. Approximately 400 slices (6 mm) of each tibia

were scanned. All the samples were immersed in PBS at 4°C overnight to get rid of the contrast agent after the scan.

Following the scan, the Micro-CT images were reconstructed and manually contoured to analyze cartilage thickness, subchondral cortical bone plate thickness and trabecular bone microstructure within the central region of medial compartment in the right tibial plateau, which is the thickest cartilage area and weight bearing area (Hamann et al. 2014). The region of interest was shown in the red rectangular region in the 3D reconstruction of right tibial plateau shown in Fig. 2(A). Because US transducers were close to the surface skin of the medial side of the knee joints, the effect of acoustic intensity was primarily on the cartilage in the medial side. The region of interest to analyze cartilage and subchondral bone was chosen in the central region of the medial compartment in this study.

After Micro-CT reconstruction, sagittal images of the right tibial plateau were analyzed by the μ CT-40 software (SCANCO Medical AG, Bassersdorf, Switzerland). The cartilage and corresponding underlying subchondral bone in 2D sagittal Micro-CT images were used to calculate Micro-CT parameters in the region of interest in medial compartment was shown in Fig. 2(B). The right tibial plateaus were segmented into cartilage, subchondral cortical bone, and trabecular bone slice by slice in the region of interest based on the threshold. Segmented sagittal cartilage and subchondral bone were then evaluated slice by slice to calculate cartilage thickness, cartilage X-ray attenuation, and the corresponding underlying subchondral cortical bone plate thickness and trabecular bone microstructures such as trabecular bone volume fraction (%), bone volume/total volume, BV/TV), connectivity density (mm^{-3} , Conn.D), trabecular thickness (mm, Tb.Th), trabecular separation (mm, Tb.Sp), trabecular number (mm^{-1} , Tb.N), trabecular space (mm, Tb.Sp), and bone mineral density (mmHg/cc, BMD).

Histological analysis

After the Micro-CT scan, the right tibial plateau samples were fixed in 10% neutral buffered formalin solution overnight and decalcified in 10% EDTA (pH=7.4) (Sigma-Aldrich, St. Louis, MO, USA) for three weeks. After decalcification, samples were dehydrated and embedded in sagittal orientation in paraffin to correspond to the sagittal slices of the Micro-CT images. Following embedding, samples were cut at 5 μ m thicknesses by Leica microtome (Leica Biosystems Inc. Buffalo Grove, IL, USA), and then stained with 0.04% toluidine blue (Sigma-Aldrich, St. Louis, MO, USA) and 0.02% fast green (Sigma-Aldrich, St. Louis, MO, USA). With the optical Zeiss microscope (Carl Zeiss Inc, Thornwood, NY, USA), digital images of each section in the central area of medial cartilage in right tibial plateau were captured at a 100 μ m resolution under the controlled ambient light. To count the chondrocyte density in the region of interest, the region in a rectangle 100 μ m wide and 300 μ m long for each section was chosen, and the chondrocytes with visible nuclei were calculated. The average number of chondrocytes in ten consecutive sections was considered as the chondrocyte density. To evaluate the sGAG optical density, the histological images were exported to Matlab (MathWorks, Natick, MA, USA), and the cartilage was segmented from the subchondral bone. The region used for chondrocyte density was chosen to calculate sGAG optical density. A blue content parameter (Bc) representing the sGAG optical density

was determined by non-linear weighing of the fractional intensity of the blue component (Martin et al. 1999; Xie et al. 2010; Li et al. 2015). The blue content of a region was calculated as the average Bc value for all pixels in that region.

Mechanical indentation test

Because the region of interest of cartilage was selected at the central area of the medial compartment in the right tibial plateau in Micro-CT and histological analysis, the thickness of cartilage can be obtained. The central area was also used to evaluate viscoelastic properties of cartilage by a stress-relaxation test. Tibia plateau samples were mounted in a sample holder, and the surface of the tibial plateau was kept wet during indentation test. Stress-relaxation tests were performed immediately using a non-porous 1 mm diameter spherical-ended indenter. After preloading with 0.002N, stress-relaxation tests were performed. 10% strain was applied to 400s using ElectroForce Mechanical Test Instruments BOSE (TA Instruments, New Castle, DE, USA). Data were recorded at 2 Hz during the whole measurement time. The stress-relaxation curve was shown in Fig. 3. Instantaneous moduli E_{inst} (MPa) and equilibrium moduli E_{eq} (MPa) were obtained using the following equation (Desrochers et al. 2012):

$$E_i = \frac{3F(1-\nu^2)}{4\sqrt{Rd^3}}$$

$$E_i(t) = E_{i,inst} e^{-\frac{t}{\tau}} + E_{i,eq}$$

E_i is the indentation modulus. F was indentation force, and it could be obtained from the stress-relaxation curve. A Poisson's ratio ν of 0.3 was used for the modulus calculation (Wang et al. 2006). R was the radius of spherical-ended indenter, and d was the indentation depth.

$E_i(t)$ equation is the time-dependent response for a three-element model for spherical indentation. $E_{i,inst}$ is the instantaneous moduli. $E_{i,eq}$ is the equilibrium moduli. t is the

relaxation time, τ is the longest relaxation time constant, and could be computed via a least squares nonlinear regression based on the three-element spring-dashpot model.

Statistical analysis

All the results were reported as mean $\pm$ SD for all of the analyses. Cartilage thickness, cartilage X-ray attenuation, subchondral cortical bone plate thickness, subchondral trabecular bone microstructure, mineral density and cartilage mechanical testing data were analyzed by one-way analysis of variance (ANOVA) with Tukey's pairwise multiple comparison tests in GraphPad Prism software (Version 5.01, GraphPad Software Inc., San Diego, CA, USA). The level of significant differences between two groups were identified by using a p-value of less than 0.05.

Results

Articular Cartilage thickness and X-ray attenuation

Fig. 4(A) and 4(B) displayed cartilage thickness and X-ray attenuation in the central area of the medial compartment in the right tibial plateau in different groups respectively. Cartilage thickness significantly decreased by 9% (from $157\pm 7\mu\text{m}$ to $143\pm 5\mu\text{m}$, $p<0.01$), and cartilage X-ray attenuation significantly increased by 15% (from $192\pm 10\text{mg HA/ccm}$ to $220\pm 14\text{mg HA/ccm}$, $p<0.01$) in the progressive OA compared with the control group under normal joint use condition. However, low-intensity US can mitigate cartilage X-ray attenuation compared with OA group under the normal joint use condition (from $220\pm 14\text{mg HA/ccm}$ to $201\pm 10\text{mg HA/ccm}$, $p<0.05$), but there were no significant differences in cartilage thickness between the OA group and the US treatment group. In all HLS groups, cartilage thickness under joint disuse condition didn't have significant changes, and the mean thickness in all HLS groups was about $152\pm 6\mu\text{m}$. The cartilage X-ray attenuation in the HLS+OA group increased from $193\pm 6\text{mg HA/ccm}$ to $209\pm 8\text{mg HA/ccm}$ compared with the HLS group ($p<0.01$), and it decreased to $201\pm 7\text{mg HA/ccm}$ in the HLS+OA+US treatment group.

Subchondral cortical bone plate thickness

The thickness of subchondral cortical bone beneath the corresponding cartilage in the central area of the medial compartment in the right tibial plateau was shown in Fig. 5. According to the results in Fig.5, in the OA group under normal joint use or disuse condition, the thickness of subchondral cortical bone plate significantly increased (from $157\pm 9\mu\text{m}$ to $172\pm 8\mu\text{m}$ and from $152\pm 7\mu\text{m}$ to $161\pm 9\mu\text{m}$, respectively, $p<0.05$). However, in the US treatment group under normal joint or functional disuse condition, subchondral cortical bone plate thickness had a decreasing trend compared to OA group or HLS+OA group (from $172\pm 8\mu\text{m}$ to $160\pm 7\mu\text{m}$ under normal joint use condition, $p<0.05$ and from $161\pm 9\mu\text{m}$ to $157\pm 7\mu\text{m}$ under joint disuse condition).

Subchondral trabecular bone microstructure

The data for the subchondral trabecular bone microstructure and mineral density were shown in Fig. 6(A-F). Under normal joint use condition, trabecular bone formation increased in the OA group and microstructure parameters, such as BV/TV, Conn-Dens, Tb.N, Tb.Th, and BMD significantly increased (by 48% ($p<0.01$), 21% ($p<0.01$), 134% ($p<0.01$), 42%

($p<0.05$), and 14% ($p<0.05$), respectively), Tb.Sp significantly decreased by 31% ($p<0.05$). The bone formation in the US treatment group was less than the OA group, and it showed a significant 19% ($p<0.05$) decrease in BV/TV and 35% ($p<0.05$) in Tb.N in the OA+US group. Under joint disuse condition, subchondral trabecular bone formation in HLS group was less compared to the age-matched group. Subchondral trabecular bone microstructure showed a decreased tendency in BV/TV, Conn-Dens, Tb.N, and Tb.Th (decreased by 25% ($p<0.05$), 17% ($p<0.05$), 28% ($p<0.05$), and 36%, respectively). However, the bone formation increased in the HLS+OA group compared with the HLS group. Bone formation increased by 45% ($p<0.05$), 13% ($p<0.05$), 34% ($p<0.05$), 63% ($p<0.05$) and 13% ($p<0.05$) in BV/TV, Conn-Dens, Tb.N, Tb.Th, and BMD respectively. The subchondral trabecular bone microstructure was not significantly affected by the US treatment in the HLS+OA+US group compared with the HLS+OA group.

Histological images and analysis

Fig. 7(A) shows the toluidine blue and fast green histological images of the central area of the medial compartment in the right tibia plateau in different groups. As shown in Fig. 7(A), the cartilage area was stained with blue; the underlying subchondral bone was stained in green; and the nuclei of chondrocytes were stained in dark blue. Cartilage surface was smooth, and chondrocytes in the superficial layer of cartilage were parallel to the surface of cartilage and perpendicular to the surface of cartilage in the deep layer in the age-matched group. Compared with the age-matched group, the thickness of cartilage decreased. The surface became rough, and the degenerative changes were observed. Chondrocyte density decreased, causing cell arrangement disruption in the OA group. However, the underlying subchondral bone increased, and more bone formed beneath the corresponding cartilage area. Although cartilage thickness didn't significantly decrease in the OA+US group, and the number of chondrocytes was larger than OA group, the chondrocytes aggregated together in the different layers of cartilage. The cartilage in OA+US group did not maintain the arrangement of chondrocytes. Also, the intensity of toluidine blue decreased in the superficial layer compared with the age-matched group. Under joint disuse condition, cartilage surfaces were smooth and almost the same, but the irregular and loose cell distribution were observed in the superficial layer of cartilage. Moreover, chondrocyte density showed a decreased trend in the HLS+OA group compared with HLS group, especially in the superficial layer of cartilage.

The data for the chondrocyte density in the region of interest area in the central area of the medial compartment in the right tibial plateau was shown in Fig. 7(B). Compared with chondrocyte density in the age-matched group, it significantly decreased by 49% in the OA group (from $43\pm7/\mu\text{m}^2$ to $22\pm4/\mu\text{m}^2$, $p<0.01$). However, it increased from $22\pm4/\mu\text{m}^2$ to $37\pm5/\mu\text{m}^2$ in the OA+US treatment group ($p<0.01$). Under joint disuse condition, compared with the HLS group, chondrocyte density slightly decreased from $35\pm3/\mu\text{m}^2$ to $31\pm4/\mu\text{m}^2$ in the HLS+OA group and decreased to $33\pm4/\mu\text{m}^2$ in the HLS+OA+US treatment group respectively. Moreover, the data showed that chondrocyte density in the HLS group and the HLS+OA group also significantly decreased by 19% and 27% compared with an age-matched group ($p<0.05$).

For the sGAG optical density results (Fig. 7(C)), sGAG content in cartilage significantly decreased in the OA group compared with the age-matched group. sGAG content decreased from 127 ± 7 to 93 ± 3 ($p < 0.01$). However, US mitigated sGAG content degeneration, increasing it from 93 ± 3 to 120 ± 10 in the OA+US group ($p < 0.01$). Besides, sGAG content decreased from 127 ± 7 to 119 ± 10 in the HLS group compared with the age-matched group ($p < 0.05$). However, there was not an obvious sGAG content difference in all of the HLS groups. sGAG content slightly decreased from 119 ± 10 to 112 ± 13 in the HLS+OA group, then slightly increased to 117 ± 13 in the HLS+OA+US group.

Articular cartilage viscoelastic mechanical properties

The cartilage viscoelastic mechanical parameters, instantaneous moduli (E_{inst}) and equilibrium moduli (E_{eq}), were shown in Fig.8 (A) and Fig.8 (B) respectively. E_{inst} and E_{eq} reduced from 389 ± 51 KPa to 239 ± 19 KPa ($p < 0.01$), and from 164 ± 13 KPa to 97 ± 4 KPa ($p < 0.01$) respectively in the OA group compared with the age-matched group. Also, the parameters increased up to 304 ± 29 KPa ($p < 0.05$) and to 140 ± 28 KPa in the OA+US group respectively ($p < 0.05$). Under the joint disuse condition, E_{inst} and E_{eq} decreased from 389 ± 51 KPa to 293 ± 23 KPa ($p < 0.05$) and from 164 ± 13 KPa to 149 ± 24 KPa in the HLS group compared to the age-matched group. Also, E_{inst} showed a significant decrease in the HLS+OA and the HLS+OA+US groups compared with the age-matched group (from 389 ± 51 KPa to 269 ± 21 KPa, and from 389 ± 51 KPa to 287 ± 16 KPa, $p < 0.05$). However, E_{inst} slightly decreased by 8% and 2% in the HLS+OA group and the HLS+OA+US group compared with the HLS group separately. Also, E_{eq} showed a slight decrease tendency in the HLS+OA group, as it decreased to 117 ± 23 KPa ($p < 0.05$ vs. age-matched group).

Discussion

The crosstalk between articular cartilage and underlying subchondral bone has drawn more and more attention in OA pathology in recent years (Sharma et al. 2013; Lacourt et al. 2012; Yuan et al. 2014; Jaiprakash et al. 2012; Zhen et al. 2013; Zhen et al. 2014). Articular cartilage and subchondral bone need to be considered as a whole therapeutic unit during OA treatment. Previous studies have demonstrated that low-intensity acoustic radiation force, generated by US, has obvious advantages in promoting cartilage regeneration due to its minimally invasive and nonradiative properties (Zhang et al. 2002; Zhang et al. 2003; Korstjens et al. 2008; Naito et al. 2010; Cheng et al. 2014; Schumann et al. 2006; Cui et al. 2007; Park et al. 2007). However, the relationships between cartilage and subchondral bone during low-intensity acoustic radiation force therapy for OA are still unclear. Therefore, we mainly observed the morphology, histology and mechanical changes of cartilage and subchondral bone during OA progression in US treatment effect under normal joint use or joint disuse condition in this study.

Our Micro-CT and histological data showed that cartilage thickness and sGAG significantly decreased in ACL-induced mechanical instability OA model after eight weeks. sGAG is negatively charged under physiologic conditions and has been considered to play a major role in cartilage hemostasis. The interaction between negatively charged sGAG and ionic interstitial fluid in extracellular matrix provides articular cartilage with a swelling pressure,

which is resisted by the shear and tension forces caused by collagen network in cartilage OA (Buckwalter et al. 1998; Xie et al. 2010). Previous studies have demonstrated that sGAG loss is one of earliest signs of OA (Buckwalter et al. 1998; Xie et al. 2010; *Pengling et al. 2015*; Fan et al. 2015). The loss of sGAG could increase the permeability of the extracellular matrix to induce the contrast agent ions to diffuse into the inner cartilage based on the charge distribution theory (Yan et al. 2012; Xie et al. 2010; *Pengling et al. 2015*; Fan et al. 2015). Thus, the X-ray attenuation of cartilage increases in OA progression. The increased X-ray attenuation of cartilage in the OA group in our study (increased by 15.09% in OA group, $p<0.01$) was consistent with previous studies. The continuous sGAG loss was associated with cartilage morphological changes in the ACL-induced rat OA model because knee joints were in an ambulatory status and the joints space became narrow after ACL surgery. Thus, the frictions between joints induced by normal wear and tear in daily activities increased, which could make the thickness of cartilage decrease in the normal joint use condition. However, when the joint hindlimbs were exerted suspension two weeks after ACL surgery, it alleviated the wear and tear, and frictions between joints. The elimination of these factors could be the reason why we didn't observe significantly decrease of cartilage thickness in the HLS+OA compared with the HLS group.

Chondrocytes are only one type of cell in articular cartilage and only make up 2% of the volume of articular cartilage (Pearle et al. 2005). They originate from mesenchymal stem cells (MSCs) and are highly specialized, metabolically active cells. Chondrocytes vary in shape, size, and number in different layers of articular cartilage. Chondrocytes could synthesize macromolecules, such as sGAG, PG, and collagen, and maintain the turnover of extracellular matrix in cartilage. Chondrocytes have exhibited apoptosis, a very low proliferative and synthetic activities in cartilage degeneration in OA in previous studies (*Parvizi et al. 1999*; Zhang et al. 2002; Korstjens et al. 2008; Cheng et al. 2014; Kourí et al. 2000). The low chondrocyte density and corrupted distribution in cartilage in the OA group in our study have been observed. This phenomenon was strongly related to sGAG content and cartilage X-ray attenuation.

Moreover, chondrocyte density and sGAG content also decreased under joint disuse condition in the HLS group (decreased by 19%, $p<0.01$ and 7%, respectively). The reason is that moderate mechanical stress generated by normal joint loading and movement is critical to the hemostasis of chondrocytes (Martin et al. 2001). Mechanical unload induced by joint hindlimb suspension caused chondrocyte atrophy, and thus chondrocyte density and sGAG content decreased. There was a slight reduction but not significant change in cartilage thickness in the HLS group compared with the age-matched group in this study. There is no consensus about cartilage thickness and chondrocyte numbers in cartilage after joint unloading in previous studies related to morphology and content variation in cartilage after joint unloading (Palmoski et al. 1980; Basso et al. 2006; Tomiya et al. 2009; Luan et al. 2015). Previous studies showed decreased thickness and chondrocytes in cartilage in joint unload condition (Palmoski et al. 1980; Basso et al. 2006; Tomiya et al. 2009; Luan et al. 2015, Nomura et al. 2017), while other indicated unchanged thickness and chondrocytes (Hagiwara et al. 2009; Nomura et al. 2017). These controversial results could result from the period, and the patterns of the joint unload model as well as the area of cartilage analyzed.

Furthermore, these structure and content changes in cartilage degeneration in OA progression were responsible for the mechanical properties of cartilage. We mainly observed and evaluated 10% stress-relaxation response of cartilage in this study. E_{inst} and E_{eq} decreased by 39% and 41% in the OA group respectively. It is well known that PGs (5–10% wet weight) and collagen (10–20% wet weight) are the main components of the ECM in cartilage (Buckwalter et al. 1998; Pearle et al. 2005). Almost 90% of Collagen in the ECM is type II collagen, and it forms a firm collagen network in articular cartilage. PGs primarily exist as aggregates formed by a high density of negatively charged sGAG (Buckwalter et al. 1998; Pearle et al. 2005). The interaction between negatively charged sGAG and ionic interstitial fluid in the ECM provides articular cartilage with a swelling pressure, which is resisted by the shear and tension forces caused by collagen network. The mechanical balance between sGAG and collagen in ECM is responsible for the stable micromechanical environment for chondrocytes as well as the structural integrity and mechanical behavior of articular cartilage. During OA progression, sGAG is lost from the ECM in the superficial layer of AC, and thus triggered the degradation of chondrocytes due to the change in the micromechanical environment. Therefore, the ability of chondrocytes to synthesize sGAG and collagen in ECM decreased, and collagen network in the middle and deep layer was corrupted. In this case, it is hard to maintain the mechanical properties of cartilage. Some earlier studies have also demonstrated reduced E_{inst} and E_{eq} in degraded cartilage in OA (Desrochers et al. 2012; Wang et al. 2006). Also, chondrocyte density, sGAG content, E_{inst} and E_{eq} decreased in the HLS group compared with the age-matched group. We speculate that mechanical unload could alter the static and osmotic pressures in the cartilage and lead to less fluid flow of interstitial fluid as well as fewer nutrients transported from synovial fluid to cartilage. Thus, chondrocyte activities and proliferation decreased. Consequently, sGAG content, as well as E_{inst} and E_{eq} , decreased.

The cartilage degeneration in OA progression was accompanied by the ossification of the underlying subchondral bone. The subchondral cortical bone plate thickness, trabecular bone mineral density, BV/TV and other microstructure data obtained from Micro-CT results in the study have demonstrated more bone formation in the corresponding subchondral bone area. These results are in agreement with prior studies (Sharma et al. 2013; Lacourt et al. 2012; Yuan et al. 2014; Jaiprakash et al. 2012; Zhen et al. 2013; Zhen et al. 2014). The abnormal bone formation and changes in the subchondral bone diminished structural support for the overlying cartilage. Also, the abnormal bone formation reduced the cartilage's ability to dissipate the load in OA progression, and worsened its degeneration in turn. After joint unloading, less bone formed in the subchondral bone areas, suggesting that mechanical unload could cause subchondral bone loss and alleviate the subchondral bone ossification in OA progression. We assumed that less bone formation and expansion in the subchondral bone under the joint disuse condition may slow cartilage regeneration after ACL induced OA progression, which might be another explanation for less OA symptoms after joint unload.

The low-intensity US has been proposed to promote cartilage regeneration in many previous studies, including ones in our lab (Zhang et al. 2002; Zhang et al. 2003; Korstjens et al. 2008; Naito et al. 2010; Cheng et al. 2014; Schumann et al. 2006; Cui et al. 2007; Park et al. 2007). It can produce acoustic radiation force in the form of mechanical stress and pressures, which causes acoustic streaming. Acoustic streaming can displace ions and small molecules

to induce a small-scale eddying of fluids near a vibrating tissue, thereby affecting diffusion rate, intramedullary pressure, membrane permeability and mechanotransduction in tissues (Sarvazyan et al. 2010). Moreover, the thermal effect of low-intensity US is substantially lower compared with high-intensity US, and it has been regarded as an anabolic stimulator for tissue healing and regeneration in daily practice in the recent studies (Phenix et al. 2014; Zhang et al. 2012). Because cartilage is in the fluid environment with synovial fluid, these acoustic streaming effects can also occur, and accelerate cartilage regeneration. Our US treatment has also shown that it can mitigate cartilage degeneration after ACL induced OA. The improvement of OA can be attributed to an increase in chondrocyte density, sGAG content, and improvement of the cartilage mechanical parameters, especially under joint normal use condition. However, US has limited effect on cartilage thickness, which may be because alterations in biochemical composition preceded structural changes. Moreover, US has limited effect on cartilage and subchondral bone in OA progression under joint disuse condition. One reason for the limited effect could be that less severe OA symptoms were developed after ACL induced OA during the six weeks suspension due to less wear and tear between joints under joint disuse condition. Also, there could be less subchondral bone formation after joint unload, which may alleviate cartilage degeneration compared with the normal joint use condition. However, the relationship between cartilage and subchondral bone, and the mechanism of US treatment for OA under joint disuse condition, still need further investigations.

Our study has several limitations. First, OA exhibited different symptoms at early, mild, moderate, and late stage. Our study mainly observed the effect of 6-weeks ultrasound treatment on cartilage and subchondral bone two weeks after the ACL induced OA model, and in a total of 8 weeks for OA progression. Earlier studies showed that two weeks to 8 weeks could be mild to moderate stage of OA. Therefore, a different time point of US treatment needs to be investigated to see if US is effective for the late stage of OA development. Secondly, ACL-induced OA model can only represent a kind of post-trauma OA in the study, and cannot represent spontaneous OA caused by aging. Also, an alternative animal OA model could be built to elucidate the effect of US on cartilage and subchondral bone during normal joint use or disuse condition in OA progression. Third, this study mainly checks phenotypes and mechanical changes of cartilage and subchondral bone in US treatment on OA. Biomarkers (PGs, collagen II, collagen I, aggrecanases (ADAMTS), collagenase (MMP13)) should be studied and evaluated after US treatment to clarify more details related with the cellular and molecular mechanism involved in US treatment for OA under normal joint use or disuse condition.

Conclusions

In summary, low-intensity ultrasound and its associated acoustic radiation force can not only mitigate articular cartilage degeneration, but also alleviate subchondral bone sclerosis during OA progression under normal joint use condition. Also, low-intensity ultrasound might have an effect on cartilage and the subchondral bone unit under joint disuse condition. This study could provide a minimally invasive and nonradiative therapy strategy for OA patients in clinical research.

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Fig. 1.

The ultrasound device setup for animal treatment. The rat was in the supine position, and the acoustic gel was applied to cover the surface skin of right knee joints. Transducer was perpendicular to the right knee joints to target the cartilage area.

Fig. 2.

The 3D reconstruction (A) and 2D sagittal slices (B) of the tibial plateau of the right knee in the sagittal view (Scale bar=1.0mm). (A) The area in the red rectangle box showed the region of interest of 60 slices in a total of 900 μm in the medial compartment to analyze the cartilage and subchondral bone. (B) Cartilage in the red box, the underlying subchondral cortical bone in the yellow box, and trabecular bone in the blue box were contoured to evaluate Micro-CT parameters.

Fig. 3.

Representative stress-relaxation curve of cartilage in the region of interest in the medial compartment of the right tibial plateau. Mechanical testing was conducted by stress-relaxation method using indentation, 10% strain was applied for 400s.

Fig. 4.

Micro-CT results of articular cartilage in the region of interest in the medial compartment of the right tibial plateau in different groups. (mean±SD, **p<0.01 vs indicated group, *p<0.05 vs indicated group). (A) Average articular cartilage thickness in the central area of the medial compartment in the right tibial plateau. (mean±SD, **p<0.01 vs age-match group) (B) Average articular cartilage X-ray attenuation in the central area of the medial compartment in the right tibial plateau. (mean±SD, **p<0.01 vs indicated group, *p<0.05 vs indicated group).

Fig. 5. Average Subchondral cortical bone plate thickness in the region of interest beneath medial cartilage of right tibial plateau in different groups. (mean±SD, *p<0.05 vs. indicated group).

Fig. 6.

Micro-CT results of subchondral trabecular bone microstructure and mineral density in the region of interest beneath subchondral cortical bone of the right tibial plateau in different groups. (A-E) shows mean+SD values for 2D subchondral trabecular bone microstructure BV/TV (%), Conn-Dens (mm⁻³), Tb.N (mm⁻¹), Tb.Th (mm), Tb.Sp (mm) (mean±SD, **p<0.01 vs indicated group, *p<0.05 vs indicated group, #p<0.05 vs Age-matched group). (F) shows mean+SD values for 2D subchondral trabecular bone mineral density (mean+SD, *p<0.05 vs. indicated group).

Fig. 7.

(A) The toluidine blue and fast green histological images of cartilage in the center area of the the medial compartment in the right tibial plateau in different groups (Scale bar=100 μm). (B) Average chondrocyte density in the region of interest in the right tibial plateau in different groups. (mean±SD, **p<0.01 vs indicated group, #p<0.05 vs Age-matched group). (C) Average sGAG optical density in the region of interest of medial compartment in the right tibial plateau in different groups. (mean±SD, **p<0.01 vs indicated group, #p<0.05 vs Age-matched group).

Fig. 8.

(A) Average E_{inst} of cartilage of the central area in the medial compartment of the right tibial plateau in different groups. (mean $\pm$ SD, ** p <0.01 vs indicated group, * p <0.05 vs indicated group, # p <0.05 vs Age-matched group). (B) Average E_{eq} of the cartilage of central area in the medial compartment of the right tibial plateau in different groups. (mean $\pm$ SD, ** p <0.01 vs indicated group, * p <0.05 vs indicated group, # p <0.05 vs Age-matched group).