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● *Original Contribution*

THERAPEUTIC EFFECTS OF LOW-INTENSITY PULSED ULTRASOUND ON OSTEOPOROSIS IN OVARIECTOMIZED RATS: INTENSITY-DEPENDENT STUDY

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Abstract—This study investigated the effects of low-intensity pulsed ultrasound (LIPUS) of different spatial-average-temporal-average intensity (I_{SATA}) ranging from 15–150 mW/cm^2 on the treatment of osteoporosis in ovariectomized rats. Healthy 3-mo-old female Sprague–Dawley rats were randomly divided into nine groups (n = 12 per group): sham-ovariectomy (OVX) control group, OVX control group and OVX groups treated with LIPUS at seven different intensities (I_{SATA}: 15, 30, 50, 75, 100, 125 and 150 mW/cm^2, respectively). LIPUS was applied to bilateral femurs 12 wk post-OVX for 20 min/d for 6 wk. Micro-computed tomography, biomechanical tests, serum biochemical analysis and grip strength tests were performed to evaluate the therapeutic effects of LIPUS at different intensities. Results revealed that LIPUS intensity yielded strong correlations with bone mineral density and bone microstructure (R^2 = 0.57–0.83) and bone mechanical strength (R^2 = 0.80–0.97), and that the intensity of 150 mW/cm^2, instead of the 30 mW/cm^2 widely used in bone fracture healing, was most effective in maintaining bone mass among all the LIPUS signals between 15 and 150 mW/cm^2. This suggests that higher ultrasound intensity (*i.e.*, 150 mW/cm^2) may be more effective than lower intensity in mitigation of osteopenia and osteoporosis. (E-mail: tda@fudan.edu.cn)

Key Words: **Low-intensity pulsed ultrasound, Osteoporosis, Ovariectomy, Therapeutic effect, Different intensity.**

INTRODUCTION

Osteoporosis is a major public health concern worldwide in modern society. It is characterized mainly by reduced bone mineral density (BMD) and bone microstructure deterioration, leading to enhanced bone fragility and increased fracture risk (Bouxsein 2005; Felsenberg and Boonen 2005; Diab and Watts 2013; Cosman et al. 2014; Drake et al. 2015). On one hand, osteoporosis is common in people aged ≥50 y, especially postmenopausal women. In the United States, it was estimated that 16% of men and 29.9% of women 50+ y were affected based on the expanded National Bone Health Alliance diagnostic criteria (Wright et al. 2017). In the European Union, approximately 22 million women and 5.5 million men had osteoporosis in 2010 (Hernlund et al. 2013). Globally, osteoporosis causes approximately 9 million fractures annually, which brings substantial pain, long-term disability and even early death to patients, imposing a huge economic burden on both family and society (Johnell and Kanis 2006; Bliuc et al. 2013; Frost et al. 2013; Drake et al. 2015). Moreover, with the progressively aging population, the number of people living with osteoporosis is set to grow dramatically, suggesting that osteoporosis will assume even greater significance for health care systems worldwide. On the other hand, skeletal unloading resulting from prolonged spaceflight or being bedridden/inactive invariably results in regional bone loss at load-bearing sites (Lang et al. 2006; LeBlanc et al. 2007; Androjna et al. 2011; Thomsen et al. 2012). It can disrupt the balance between bone formation and bone resorption, deteriorate bone microstructure and lead to fractures (Thomsen et al. 2012). Currently, bone loss in space represents a critical issue for manned deep-space exploration. Therefore, immediate and cost-efficient

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treatment of osteoporosis and early prevention of bone loss are of great significance.

Current pharmacologic therapies for osteoporosis, although accepted by many doctors and patients, are not perfect with respect to efficacy, cost and long-term safety issues. For example, estrogen treatment has long-term non-skeletal adverse effects, including an increased risk of cardiovascular disease and invasive breast cancer (Rossouw et al. 2002). The most commonly prescribed antiresorptive agents, such as bisphosphonates, calcitonin and denosumab, focus on reducing bone resorption but rarely enhance bone formation (Khajuria et al. 2011; Cosman et al. 2014). Therefore, these antiresorptive agents can be challenging in the older senior, and their antifracture efficacy may be less than desired. Likewise, parathyroid hormone (PTH), the major anabolic therapy for osteoporosis, promotes bone formation first but subsequently stimulates bone resorption; thus, the osteoanabolic effect is significant during the initial 6−12 mo of PTH therapy but tends to wane thereafter (Jilka 2007; Augustine and Horwitz 2013). Meanwhile, widespread clinical use of PTH is limited because of the rebound bone loss on discontinuation, the inconvenient administration through subcutaneous injection and the associated high cost (Cosman et al. 2014; Drake et al. 2015; Makras et al. 2015). In addition, physical exercise has been proposed as a promising non-pharmacologic attempt to avoid the disadvantages of drug therapy, yet with a small statistically significant effect, potential risk of falling and fracture and no agreement on the proper type, intensity and duration of the training programs corresponding to individual differences in age, sex, bone status, sites and so forth (Howe et al. 2011; Gomez-Cabello et al. 2012).

Considering the limitations and possible side effects of the aforementioned methods, researchers are looking for potential physical therapies in osteoporosis management. One such representative strategy is low-intensity pulsed ultrasound (LIPUS), a form of mechanical stimulation that can be transmitted into living tissues and induce biological effects, as osteocytes are mechanosensitive (Klein-Nulend et al. 2013). Many *in vitro* and *in vivo* studies (Azuma et al. 2001; Hantes et al. 2004; Unsworth et al. 2007; Fung et al. 2012; Manaka et al. 2015) and clinical trials (Gebauer et al. 2005; Rutten et al. 2008; Schofer et al. 2010; Zura et al. 2015a, 2015b) have reported the positive role that LIPUS could play in bone healing. LIPUS may induce micromechanical stimulation of bone cells, thus promoting osteoblast differentiation (Manaka et al. 2015) and increasing mineralization and bone regeneration (Unsworth et al. 2007). Animal studies on the effects of LIPUS on fracture healing have reported enhanced biomechanical properties of the remodeled bone and acceleration of the healing process (Hantes et al. 2004; Fung et al. 2012). LIPUS actively influences all stages involved in fracture healing, such as inflammatory reaction, soft callus formation, bone formation and bone remodeling, in rats with artificial femoral fractures (Azuma et al. 2001). Furthermore, the clinical application of LIPUS improved the healing rate of fresh fractures (Zura et al. 2015b), non-unions (Gebauer et al. 2005; Zura et al. 2015a) and delayed unions (Rutten et al. 2008; Schofer et al. 2010). Osteoporosis results when bone resorption exceeds bone formation; therefore, the key to anti-osteoporosis treatments lies in stimulating bone formation or modulating bone remodeling in ways that improve bone strength. Because bone healing and treatment of osteoporosis share some similar physiologic mechanisms, it is reasonable to assume that using LIPUS is a potential feasible approach to treatment of osteoporosis.

Furthermore, as the U.S. Food and Drug Administration (FDA) has already approved LIPUS treatment for fresh fractures and established non-unions, parallel approval for osteoporosis can therefore be expected. It is also worth mentioning that LIPUS has the potential to provide local treatment (Ferreri et al. 2011; Torstrick and Guldberg 2014) at skeletal sites at risk, which represents a unique advantage over current systemic agents, as bone loss may be more prominent in the hip, spine and wrist for people ≥50 y old (Trajanoska et al. 2018) and in the load-bearing bones for astronauts (Thomsen et al. 2012). With the advantages of being non-invasive, radiation-free, relatively inexpensive, portable and convenient, LIPUS stimulation is an attractive candidate for osteoporosis and deserves considerably more research attention.

Research on LIPUS treatment of osteoporosis, however, is still at an early stage, with only a few reported positive effects on osteoporotic animals (Carvalho and Cliquet Junior 2004; Wu et al. 2009; Ferreri et al. 2011; Lim et al. 2011; Uddin and Qin 2015) and even some conflicting results (Warden et al. 2001a, 2001b; Leung et al. 2004). For example, using LIPUS with almost the same parameters—frequency 1.5 or 1.0 MHz, duty cycle 20%, pulse repetition frequency (PRF) 1.0 kHz, intensity 30 mW/cm^2 (spatial average temporal average intensity, I_{SATA}), daily exposure 20 min—Wu et al. (2009), Carvalho and Cliquet Junior (2004) and Lim et al. (2011) found that LIPUS prevented bone loss in ovariectomized (OVX) rats, whereas Warden et al. (2001a) found no stimulatory effects of LIPUS in a similar model, Unfortunately, positive effects on animal models have not translated into similar benefits in human bones (Warden et al. 2001b; Leung et al. 2004; Zhang et al. 2017), In a clinical trial, patients with calcaneal osteoporosis caused by spinal cord injury received LIPUS treatment (frequency 1.0 MHz, duty cycle 3.3%, PRF 3.3 kHz, I_{SATA}

30 mW/cm^2, daily exposure 20 min) over 6 wk, but obtained no positive effects (Warden et al. 2001b). Reasons for the controversy revolve around many aspects, such as different skeletal sites, baseline bone mass, pathogenic factors of osteoporosis and, especially, the LIPUS parameters (*e.g.,* center frequency, duty cycle, intensity and PRF), suggesting the necessity for optimization of the treatment regimen. But currently the vast majority of related studies have been conducted using the commercial system Exogen (Bioventus LLC, Durham, NC, USA) or similar devices with the typical parameters: frequency 1.5 or 1.0 MHz, duty cycle 20%, PRF 1.0 kHz, I_{SATA} 30 mW/cm^2, daily exposure 20 min. Despite being approved by the FDA, the Exogen device and the widely used intensity, 30 mW/cm^2, are actually for fracture healing and might not be optimal for osteoporosis treatment. More *in vivo* research is warranted to confirm the efficacy of LIPUS in osteoporosis; in particular, investigation of the effects of different ultrasound intensities and design of dedicated experimental setups for osteoporosis will contribute.

In this study, we employed LIPUS of different intensity ranging from 15−150 mW/cm^2 to treat ovariectomy-induced osteoporosis in rats using a self-developed LIPUS device designed for multiple experimental purposes regarding ultrasonic bone therapy. Micro-computed tomography (μCT), biomechanical tests, serum biochemical analysis and grip strength tests were performed to evaluate the therapeutic effects of LIPUS of different intensities.

METHODS

Experimental design and animal model

In total, 108 healthy 3-mo-old female Sprague−Dawley rats (269.1 ± 17.7 g) were obtained from the Laboratory Animal Breeding and Research Center of Xi'an Jiao Tong University (Xi'an, China). They were housed in separate cages, with 6 animals per cage, under controlled temperature (22 ± 2°C), humidity (60 ± 5%) and light:dark cycle (12 h:12 h), and had access to chow diet and distilled water *ad libitum*. During the experimental period, rat activities were monitored daily, and weights were recorded weekly to guarantee their health status. The animal care and experimental protocol of this study were approved by the Animal Ethical Committee of Shaanxi Normal University, and conducted according to the *Guide for the Care and Use of Laboratory Animals* published by the U.S. National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Osteoporosis models were established by bilateral ovariectomy, except for the 12 rats randomly selected among the total of 108 to undergo sham operation and form the sham-OVX control group. Before surgery, rats were anesthetized by intraperitoneal injection of 3% pentobarbital (50 mg/kg), and both ovaries were removed through a bilateral dorsal incision to produce OVX groups. Sham operations were performed under the same conditions under which the ovaries were exposed, and part of the surrounding fatty tissue was removed, leaving the ovaries intact. The validity of osteoporosis models was examined by comparison of BMD and serum estradiol (E2) between the sham-OVX group and 12 randomly selected OVX rats. BMD of bilateral femurs and lumbar spine was measured *in vivo* by dual-energy X-ray absorptiometry (DXA; InAlyzer, MEDIKORS Inc., Seungnam, Korea), and serum E2 levels were measured with an enzyme-linked immunosorbent assay (ELISA) kit (Cayman Chemical, MI, USA). Measurements were carried out every 2 wk from 8 wk post-ovariectomy until a significant decline in BMD (>10%) and serum E2 (>50%) were observed in OVX rats compared with the sham-OVX group at 12 wk post-ovariectomy.

Thereafter, LIPUS treatment began. Depending on whether sham surgery or ovariectomy was performed, rats were randomly assigned to nine groups with n = 12 per group: (1) sham control (sham-C), (2) ovariectomized control (OVX-C), (3) OVX + 15 mW/cm^2, (4) OVX + 30 mW/cm^2, (5) OVX + 50 mW/cm^2, (6) OVX +7 5 mW/cm^2, (7) OVX + 100 mW/cm^2, (8) OVX + 125 mW/cm^2, (9) OVX + 150 mW/cm^2. Group 1 was the sham ovariectomy control group, group 2 was the ovariectomy control group and groups 3−9 were OVX groups treated with LIPUS at seven different intensities (I_{SATA}): 15, 30, 50, 75, 100, 125 and 150 mW/cm^2, respectively.

After 6 wk of LIPUS treatment, all rats were deprived of food but allowed water overnight and then were euthanized after anesthesia overdose. Blood samples were collected and immediately centrifuged at 1500*g* (20 min, 4°C) for the separation of serum, which was stored at −80°C until serum biochemical analysis. Bilateral femurs were harvested, carefully cleaned of the attached soft tissue, wrapped in saline-soaked gauze on ice and then stored at −20°C. One femur from each animal was randomly selected for μCT analysis, while the contralateral bone was submitted to biomechanical testing.

LIPUS treatment

The self-developed LIPUS device (designed and manufactured by Bone Ultrasound Electronic Engineering Lab of Fudan University, Shanghai, China) for animal treatment is illustrated in Figure 1a. The device was specially designed to meet multiple experimental purposes in terms of ultrasonic bone therapy. It has eight channels, which can be controlled separately and connected to different types of transducers, allowing up to eight treatment sites simultaneously. Ultrasound parameters, including intensity, frequency, PRF, duty cycle and

treatment duration, are manually adjustable to a certain extent through a capacitive touchscreen. In particular, the intensity is regulated by changing the voltage and calibrated by a fast-response (<3 s), high-resolution (1 mW) ultrasound power meter (UPM-DT-1000 PA, Ohmic Instruments, MO, USA). The principle underlying intensity measurement is the radiant force method, and the intensity (W/cm^2) of a transducer is determined by measuring the total power output and dividing by the active cross-sectional area of the transducer (Padilla et al. 2014). Accurate calibrations were carried out twice a week in the treatment phase to ensure that these devices worked as desired.

Except for intensity, other parameters had the typical values (frequency 1.5 MHz, PRF 1.0 kHz, duty cycle 20%, daily exposure 20 min). During treatment, rats wearing flexibel tights were gently fixed to the holder (Fig. 1b). Bilateral femurs of each rat in treated groups were exposed to corresponding LIPUS stimulation 6 d/wk for 6 wk. The treatment lasted 6 wk because the average bone remodeling period in the rat is roughly 40 d (Thompson et al. 1995), and the participating students were allowed a 1-d break every week. A type of plane circular transducer (Fig. 1a, Suzhou Acoustic Origin Transducer Co., Ltd, Suzhou, China), 25 mm in diameter, was used for this intervention. To guarantee the transmission of ultrasound between the transducer and the skin surface, fur was shaved from the femur regions twice a wk, and ultrasonic coupling gel was applied between the transducer and the contact skin during LIPUS exposure (Fig. 1b). The two control groups were treated with the same regimen except that the LIPUS devices were left turned off.

Micro-computed tomography

To scan the distal portions of femurs, a high-resolution μCT system (Y.cheetah, YXLON International GmbH, Hamburg, Germany) with an isotropic voxel resolution of 18 μm was employed at a voltage of 80 kV, current of 62.5 μA, total rotation angle of 180˚ and rotation step of 0.4˚. Three-dimensional image reconstruction and data processing were completed with VG Studio MAX 2.2 (Volume Graphics GmbH, Heidelberg, Germany), a software for the analysis and visualization of computed tomography data. A volume of interest (VOI; Fig. 2) was selected for the evaluation of trabecular bone micro-architecture, which was 2 mm long and 0.3 mm proximal to the lowest end of the growth plate of the distal femur, excluding the cortical shell and growth plate. To achieve better delineation of the trabecular bone and exclusion of cortical bone, contour lines were manually drawn every 6−10 slices. Representative 3-D images of bone microstructure in the VOI were obtained and trabecular bone parameters were evaluated: trabecular BMD (mg HA/cm^3), trabecular bone volume fraction (bone volume/tissue volume [BV/TV, %]), bone surface/bone volume (BS/BV, 1/mm), trabecular number (Tb.N, 1/mm), trabecular thickness (Tb.Th, μm) and trabecular separation (Tb.Sp, μm). BMD was determined as the ratio of bone mineral content in the specific site to the

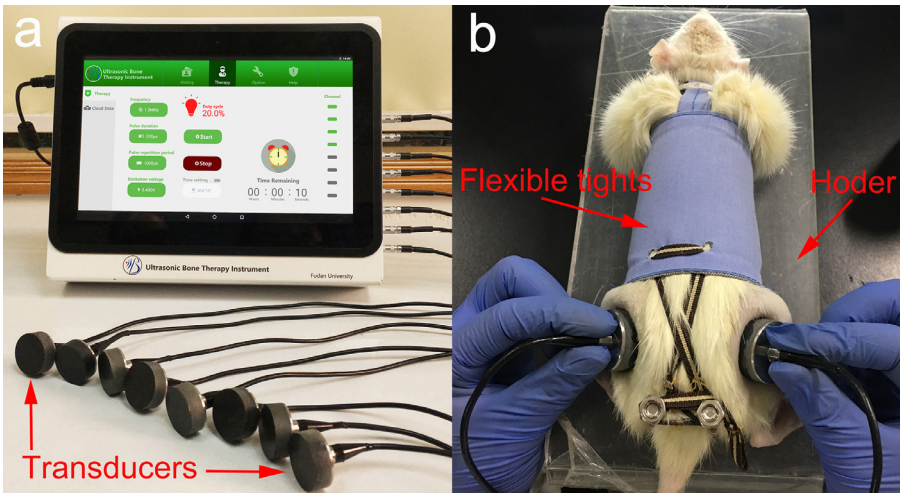

Fig. 1. (a) A self-developed LIPUS device that meets multiple experimental purposes with respect to ultrasonic bone therapy. It has eight channels, and ultrasound parameters (including intensity, frequency, pulse repetition frequency, duty cycle and treatment duration) are adjustable manually to a certain extent through a capacitive touchscreen. (b) Rats wearing flexible tights were gently fixed to the holder. Bilateral femurs received corresponding LIPUS stimulation using plane circular transducers (25 mm in diameter). Fur was shaved from the femur regions twice a week, and ultrasonic coupling gel was applied between the transducer and the contact skin during LIPUS exposure. LIPUS = low-intensity pulsed ultrasound.

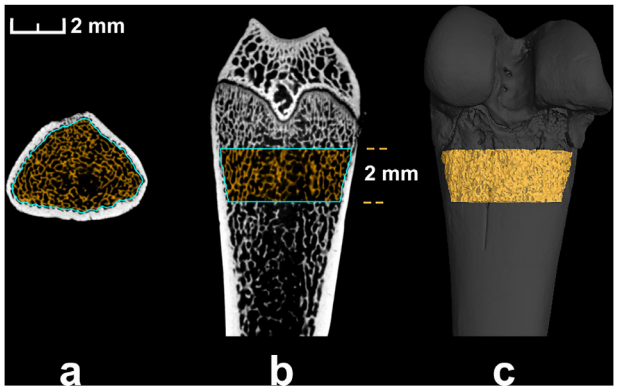

Fig. 2. Schematic of a VOI selected for the evaluation of trabecular bone microarchitecture. Shaded in yellow, the VOI was 2 mm long and 0.3 mm proximal to the lowest end of the growth plate of the distal femur, excluding the cortical shell and growth plate. (a) Cross-sectional plane of VOI. (b) A vertical plane of the distal femur. (c) Three-dimensional reconstruction of the distal femur. VOI = volume of interest.

total volume measured (Kanis 2002); BV/TV was defined as the ratio of the segmented bone volume to the total volume measured; BS/TV was defined as the ratio of the segmented bone surface to the total volume measured; Tb.N was defined as the average number of trabeculae per unit length; Tb.Th was defined as the mean thickness of trabeculae; Tb.Sp was defined as the mean distance between trabeculae (Bouxsein et al. 2010). Trabecular bone microarchitecture parameters were assessed using direct 3-D methods. Osteoporosis resulted in reduced bone mass and the deterioration of bone microstructure, thus BMD, BV/TV, Tb.N and Tb.Th would reduce, while BS/BV and Tb.Sp would increase.

Biomechanical tests

Three-point bending tests were undertaken to evaluate the biomechanical properties of the femur with MTS 858 material testing systems (MTS System Corp., MN, USA). Frozen femurs were slowly thawed at room temperature before the test and continuously moistened with isotonic saline solution. The thawed femur, with its anterior aspect facing up, was placed horizontally on two fixed supports (15-mm span). Then a vertical load, with a constant displacement rate of 2 mm/min, was applied at the midpoint of the femur until fracture occurred. The material testing systems automatically recorded the load-displacement curve throughout the loading process. And three major structural variables were calculated according to the curve: (i) maximum load (N)—the maximum force that the sample withstood during the test; (ii) stiffness (N/mm)—the slope of the load-displacement curve in the linear/elastic region responding to bone stiffness; (iii) energy absorption (mJ)—integration of area under the curve until fracture representing the energies absorbed during elastic and plastic deformation (Hogan et al. 2000).

Serum biochemical analysis

Serum bone-specific alkaline phosphatase (bALP) level is generally accepted as a marker of osteoblast activity and bone formation, while serum tartrate-resistant acid phosphatase 5b (TRACP-5b) level is generally considered to be a marker of osteoclast activity and bone resorption. Both indices were quantified using commercial ELISA kits (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China) according to the manufacturer's protocols.

Grip stength tests

As osteoporosis was associated with a poor physical performance that might increase the risk of falling, grip strength, one of the direct reflections of physical function for a biological individual, was tested. A digital grip-strength meter (YLS-13 A, Jinan Yiyan Technology and Development Co., Ltd., Shangdong, China) was utilized to estimate hindlimb grip strength of rats. After the two forelimbs were wrapped with tape, the rat to be tested was gently placed on the grid of the dynamometer, allowing its hindlimbs to grab the metal pull bar. Then, the experimenter horizontally pulled the rat backward by grabbing its tail, until the rat withdrew its paws from the dynamometer. The instrument automatically recorded the maximum grip strength (in grams), which represented the motor function of the tested rat. The final value was the average of three attempts for each animal. Throughout the entire experiment, grip strength tests were executed at three times: before ovariectomy, 12 wk post-ovariectomy (before LIPUS treatment) and at the end of LIPUS treatment. An experimenter with no knowledge of group allocation blindly accomplished the tests.

Statistical analysis

Results are expressed as the mean ± standard deviation. Before LIPUS treatment, differences between the sham-C group and OVX rats were tested using the independent sample *t*-test. At the end of LIPUS intervention, statistical relationships among all nine groups were evaluated by one-way analysis of variance (ANOVA), and Tukey's *post hoc* test was used for pairwise multiple comparisons if one-way ANOVA revealed statistical significance. Differences were considered significant at $p < 0.05$ and $p < 0.001$. Correlations between LIPUS intensity and bone parameters were calculated based on Pearson's product−moment correlation coefficient. The correlation is generally considered strong with a Pearson's coefficient (r value) >0.7 (Moore et al. 2013). All

statistical analyses were accomplished using GraphPad Prism software (Version 6.01, GraphPad Software Inc., San Diego, CA, USA).

RESULTS

Effects of ovariectomy on animals

Ovariectomy had significant effects on experimental animals. At 12 wk post-surgery, areal BMD measured by DXA declined 11.9% ($p < 0.05$) in the right femur, 12.3% ($p < 0.05$) in the left femur and 14.3% ($p < 0.05$) in the lumbar spine, and serum E2 declined 58.1% ($p < 0.001$) in OVX-C rats compared with sham-C rats (Table 1). Together these results suggested significant bone loss and confirmed that we had successfully established osteoporosis rat models.

Significant alterations in weight were detected from 2 wk after ovariectomy (Fig. 3a). Weight of OVX rats increased 14.3% ($p < 0.001$) compared with that of sham-C rats at 12 wk post-ovariectomy (Fig. 3a and Table 1), which additionally proved the success of ovariectomy. Moreover, weight increase slowed in OVX rats subjected to LIPUS treatment (Fig. 3b), although each OVX group continued to weigh significantly more than the sham-C group.

Effect of LIPUS intensities on bone density and microstructure

Representative 3-D μCT images of trabecular bone microarchitecture determined by the VOI for all groups are provided in Figure 4. OVX rats had less bone mass and notable degradation of trabecular microarchitecture compared with the sham-C group, which was also evidenced by significant decreases in BMD, BV/TV, Tb.N and Tb.Th (−72.0%, −78.4%, −74.5% and −15.1%, respectively; $p < 0.001$ for all) and significant increases in BS/BV and Tb.Sp (+26.3% and +439.8%, respectively; $p < 0.001$ for both) in OVX-C compared with sham-C rats (Fig. 5).

Six weeks of LIPUS exposure ameliorated ovariectomy-induced trabecular bone loss in an intensity-dependent manner, according to quantitative μCT analysis of various bone parameters (Fig. 5). In comparison with the OVX-C group, the groups treated with 75, 100, 125 and 150 mW/cm^2 LIPUS had significantly increased BMD (Fig. 5a) and Tb.Th (Fig. 5e) ($p < 0.05$ or $p < 0.001$), whereas all treated groups had markedly ($p < 0.05$ or $p < 0.001$) increased BV/TV (Fig. 5b) and reduced Tb.Sp (Fig. 5f). As for BS/BV (Fig. 5c), only 15 mW/cm^2 had no apparent therapeutic effect on OVX rats; and for Tb.N (Fig. 5d), both 30 and 100 mW/cm^2 were excluded from significantly effective intensities. Overall, LIPUS treatment significantly increased bone mass for the OVX rats, and LIPUS intensity yielded strong positive correlations with BMD ($R^2 = 0.82$, $p < 0.05$), BV/TV ($R^2 = 0.74$, $p < 0.05$), Tb.N ($R^2 = 0.61$, $p < 0.05$) and Tb.Th ($R^2 = 0.83$, $p < 0.05$) and strong negative correlations with BS/BV ($R^2 = 0.81$, $p < 0.05$) and Tb.Sp ($R^2 = 0.57$, $p < 0.05$), as outlined in Table 2.

In addition, BV/TV was significantly higher in rats subjected to + 150 mW/cm^2 LIPUS than that in the rats subjected to 15, 30, 50 and 100 mW/cm^2 LIPUS (+42.5%, +52.7%, +44.6% and +39.5%, respectively; $p < 0.05$ for all; Fig. 5b). Similarly, significant enhancement of Tb.N was observed in OVX + 150 mW/cm^2 rats compared with OVX + 50 mW/cm^2 rats (+36.0%; $p < 0.05$; Fig. 5d). In contrast, 150 mW/cm^2 decreased Tb.Sp more successfully than 15, 30 and 100 mW/cm^2 (−32.9%, −32.9% and −33.9%, respectively; $p < 0.05$ for all; Fig. 5f). Meanwhile, significant suppression of BS/BV was noted in the 100, 125 and 150 mW/cm^2 groups compared with the OVX + 30 mW/cm^2 group (−7.1%, −6.9% and −6.6%, respectively; $p < 0.05$ for all; Fig. 5c).

Effect of LIPUS intensities on bone biomechanical properties

The structural properties of femurs determined with three-point bending tests and Pearson correlations between femur biomechanical parameters and LIPUS intensity are outlined in Table 3. As expected, OVX resulted in significantly lower maximum load, stiffness and energy absorption compared with sham control group (−21.5%, −36.4% and −27.2%, respectively; $p < 0.001$ for all). However, LIPUS exposure might mitigate this decline. Maximum load was significantly ($p < 0.05$ or $p < 0.001$) promoted in OVX rats exposed to LIPUS

Table 1. Effects of ovariectomy on bone mineral density, serum estradiol and weight 12 wk post-surgery

Group	Bone mineral density			Serum estradiol	Weight
	Right femur	Left femur	Lumbar spine		
Ovariectomy control Sham control	−11.9%*	−12.3%*	−14.3%*	−58.1%†	+14.3%†

* $p < 0.05$ versus sham control (independent sample t-test).
† $p < 0.001$ versus sham control (independent sample t-test).

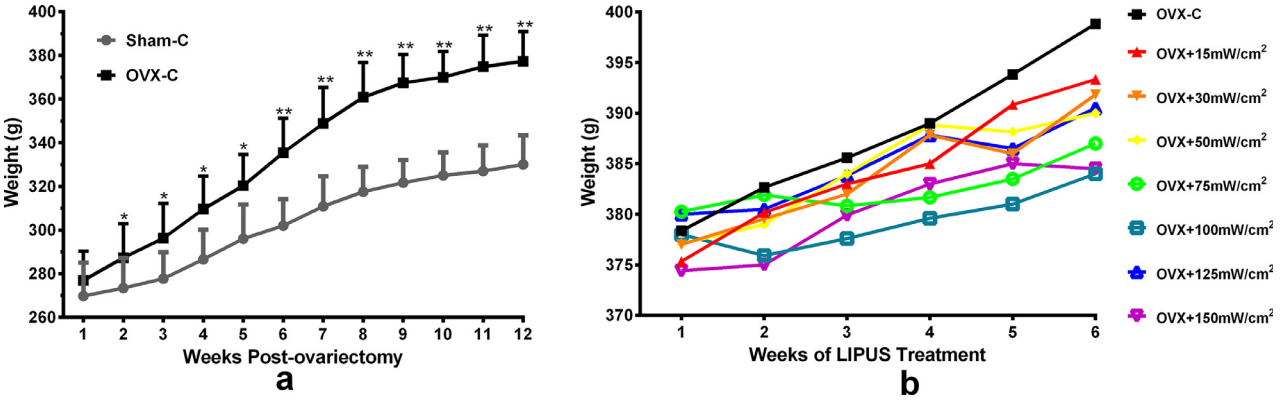

Fig. 3. Record of weight. (a) Weekly weight of sham-C and OVX control rats from 1–12 wk post-ovariectomy. Values are reported as the mean ± standard deviation. (b) Mean weights of all OVX groups during LIPUS treatment. *$p < 0.05$, **$p < 0.001$, versus sham control (independent sample t-test). LIPUS = low-intensity pulsed ultrasound; OVX = ovariectomized; OVX-C = ovariectomized control; sham-C = sham control.

intensities ≥75 mW/cm^2. Analogously, stiffness increased markedly ($p < 0.05$ or $p < 0.001$) in OVX rats exposed to LIPUS intensities ≥100 mW/cm^2. For energy absorption, significant ($p < 0.05$ or $p < 0.001$) enhancement was achieved in the groups subjected to 75, 100, 125 and 150 mW/cm^2 LIPUS compared with the OVX-C group. Overall, LIPUS treatment maintained bone mechanical integrity for the OVX rats, and LIPUS intensity yielded strong positive correlations with maximum load ($R^2 = 0.97$, $p < 0.001$), stiffness ($R^2 = 0.92$, $p < 0.001$) and energy absorption ($R^2 = 0.80$, $p < 0.05$), as outlined in Table 3.

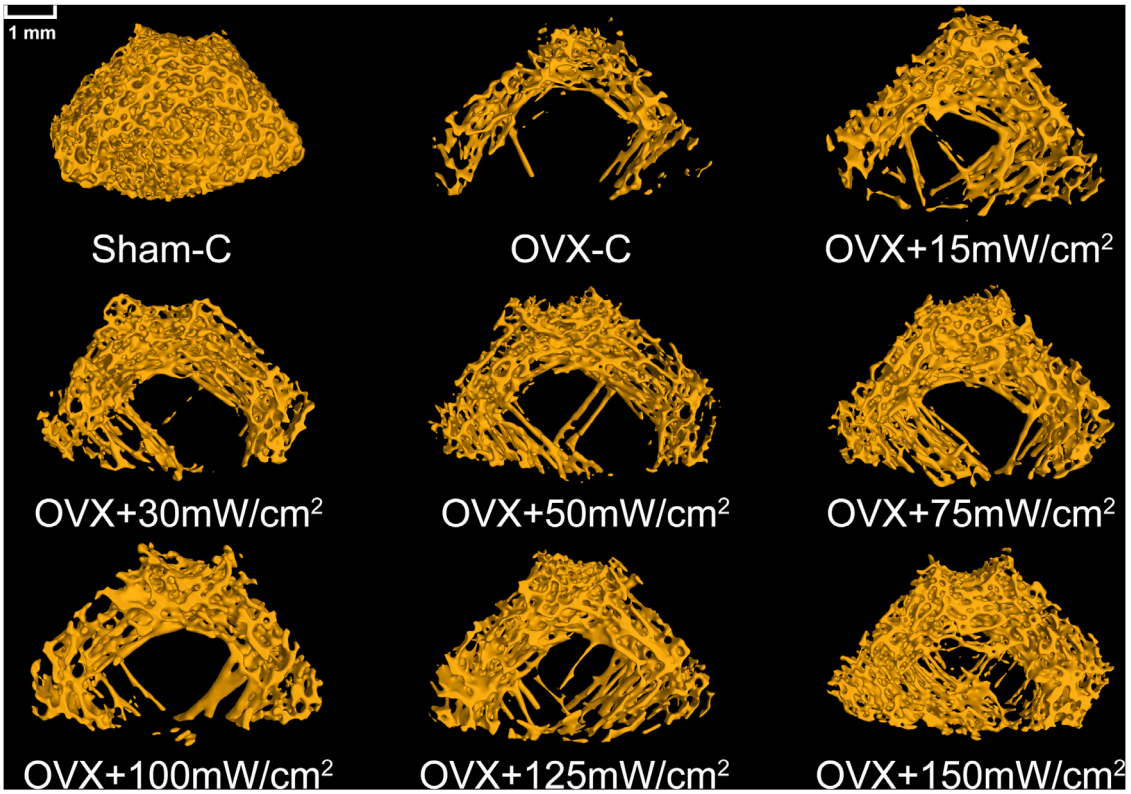

Fig. 4. Representative 3-D micro-computed tomography images of trabecular bone microarchitecture determined by the VOI for all groups. OVX = ovariectomized; OVX-C = ovariectomized control; VOI = volume of interest; sham-C = sham control.

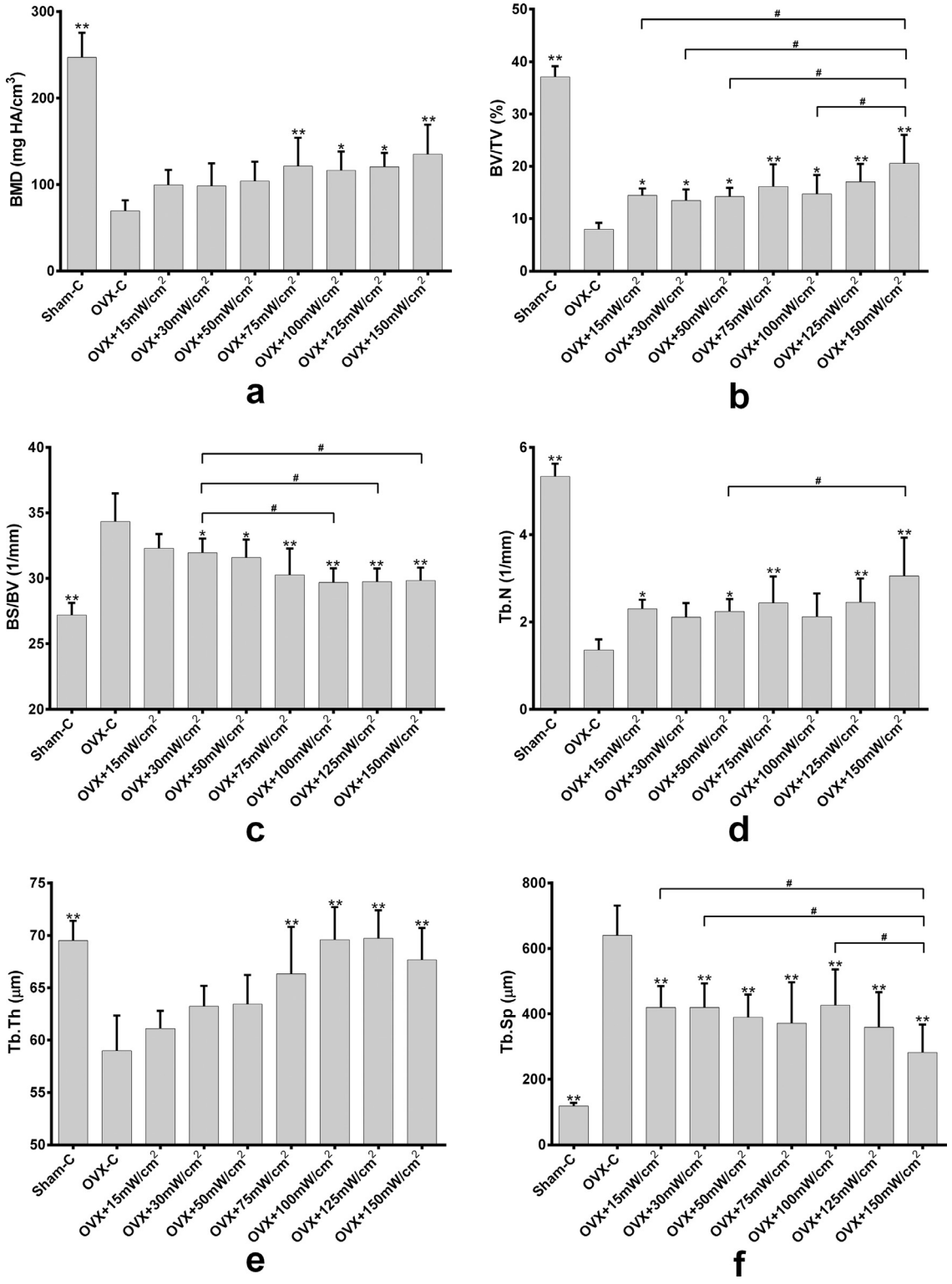

Fig. 5. Results of micro-computed tomography analysis of trabecular bone in the distal femur region determined by the VOI. (a) BMD, (b) BV/TV, (c) BS/BV, (d) Tb.N, (e) Tb.Th, (f) Tb.Sp. Values are reported as the mean ± standard deviation. $^{*}p < 0.05$, $^{**}p < 0.001$, versus OVX-C (Tukey's multiple comparison tests); $^{\#}p < 0.05$ between two specified groups (Tukey's multiple comparison tests). BMD = bone mineral density; BV/TV = bone volume per tissue volume; BS/BV = bone surface per bone volume; Tb.N = trabecular number; Tb.Th = trabecular thickness; Tb.Sp = trabecular separation; OVX = ovariectomized; OVX-C = ovariectomized control; sham-C = sham control; VOI = volume of interest.

Table 2. Pearson correlations between trabecular microstructural parameters and LIPUS intensity

	BMD	BV/TV	BS/BV	Tb.N	Tb.Th	Tb.Sp
R^2	0.82	0.74	0.81	0.60	0.83	0.57
p	0.002	0.006	0.002	0.023	0.002	0.030

LIPUS = low-intensity pulsed ultrasound; BMD = bone mineral density; BV/TV = bone volume per tissue volume; BS/BV = bone surface per bone volume; Tb.N = trabecular number; Tb.Th = trabecular thickness; Tb.Sp = trabecular separation.

Effect of LIPUS intensities on serum indices

Measurements of serum bALP (bone formation marker) and TRACP-5b (bone resorption marker) levels are illustrated in Figure 6. Serum bALP level (Fig. 6a) in the OVX-C group was significantly lower than that in the sham-C group (−30.0%, $p < 0.001$), but was significantly ($p < 0.05$ or $p < 0.001$) stimulated by LIPUS at intensities ≥50 mW/cm^2. At the same time, serum TRACP-5b level (Fig. 6b) in the OVX-C group was significantly higher than that in the sham-C group (+38.0%, $p < 0.05$), but the growth trend was significantly ($p < 0.05$ or $p < 0.001$) suppressed by LIPUS at intensities ≥50 mW/cm^2.

Effect of LIPUS intensities on grip strength

Hindlimb grip strength of rats at three different time points is illustrated in Figure 7. Comparisons were made between the same time points for all groups. Compared with the sham-C group, OVX rats exhibited no significant difference in grip strength before OVX surgery, but 12 wk post-ovariectomy, OVX-C rats underwent osteoporosis and had significantly decreased grip strength (−7.3%, $p < 0.05$); a similar situation continued after 6 wk of LIPUS treatment in the OVX-C group (−6.3%, $p < 0.05$). Nevertheless, grip strength in all LIPUS-treated groups was increased compared with that of the OVX-C group ($p < 0.05$ or $p < 0.001$), indicating that LIPUS contributed to developing the rats' maximum physical potential even without regular training programs.

Moreover, both the OVX + 100 mW/cm^2 and OVX + 150 mW/cm^2 groups exhibited significantly enhanced therapeutic effects on hindlimb grip strength compared with the OVX + 15 mW/cm^2, OVX + 30 mW/cm^2 and OVX + 50 mW/cm^2 groups and even sham-C groups (OVX + 100 mW/cm^2: +9.1% $p < 0.001$, +7.6% $p < 0.05$, +7.0% $p < 0.05$ and +9.7% $p < 0.001$, respectively; OVX+150 mW/cm^2: +8.9% $p < 0.001$, +7.4% $p < 0.05$, +6.8% $p < 0.05$ and +9.5% $p < 0.001$, respectively; Fig. 7). The grip strength in the OVX + 125 mW/cm^2 group was significantly higher than that in the OVX + 15 mW/cm^2, OVX + 30 mW/cm^2 and sham-C groups (+7.8%, +6.4% and +8.5%, respectively; $p < 0.05$ for all; Fig. 7).

DISCUSSION

Osteoporosis is a musculoskeletal disease in which bones become weak and are more likely to break. It is a gradual change that is often overlooked by patients until fracture. Although management of osteoporosis and its associated consequences for men and women irrespective of age is necessary to improve quality of life and reduce the economic burden on society, postmenopausal osteoporosis, which is the major type of osteoporosis in humans, deserves considerably more attention. To better comprehend the biological mechanism associated with postmenopausal osteoporosis, the FDA recommends the OVX rat model to simulate bone loss induced by estrogen deficiency (Thompson et al. 1995). Because osteoporosis is more likely to develop if an individual did not

Table 3. Femur structural properties determined *via* three-point bending test and Pearson correlation between femur biomechanical parameters and LIPUS intensity

Group	Maximum load (N)	Stiffness (N/mm)	Energy absorption (mJ)
Sham control	110.5 ± 6.4†	391.5 ± 32.3†	108.1 ± 10.8†
OVX control	86.8 ± 8.7	249.2 ± 29.7	78.87 ± 11.4
OVX + 15 mW/cm^2	88.9 ± 6.9	268.2 ± 28.9	80.8 ± 11.7
OVX + 30 mW/cm^2	87.9 ± 6.8	260.4 ± 29.6	89.8 ± 11.4
OVX + 50 mW/cm^2	93.4 ± 6.5	289.6 ± 27.3	96.5 ± 10.8*
OVX + 75 mW/cm^2	97.8 ± 6.5*	279.7 ± 32.1	94.7 ± 10.3
OVX + 100 mW/cm^2	98.9 ± 6.9*	301.1 ± 30.2*	102.6 ± 17.7†
OVX + 125 mW/cm^2	101.4 ± 8.0*	311.6 ± 32.8†	98.9 ± 9.5*
OVX + 150 mW/cm^2	105.4 ± 7.9†	326.0 ± 28.6†	102.4 ± 10.0†
Pearson correlation with intensity			
R^2	0.97	0.92	0.80
p	<0.0001	0.0001	0.003

LIPUS = low-intensity pulsed ultrasound; OVX = ovariectomy.
Values of femur biomechanical parameters are reported as mean ± standard deviation.
* $p < 0.05$ versus OVX control (Tukey's multiple comparison tests).
† $p < 0.001$ versus OVX control (Tukey's multiple comparison tests).

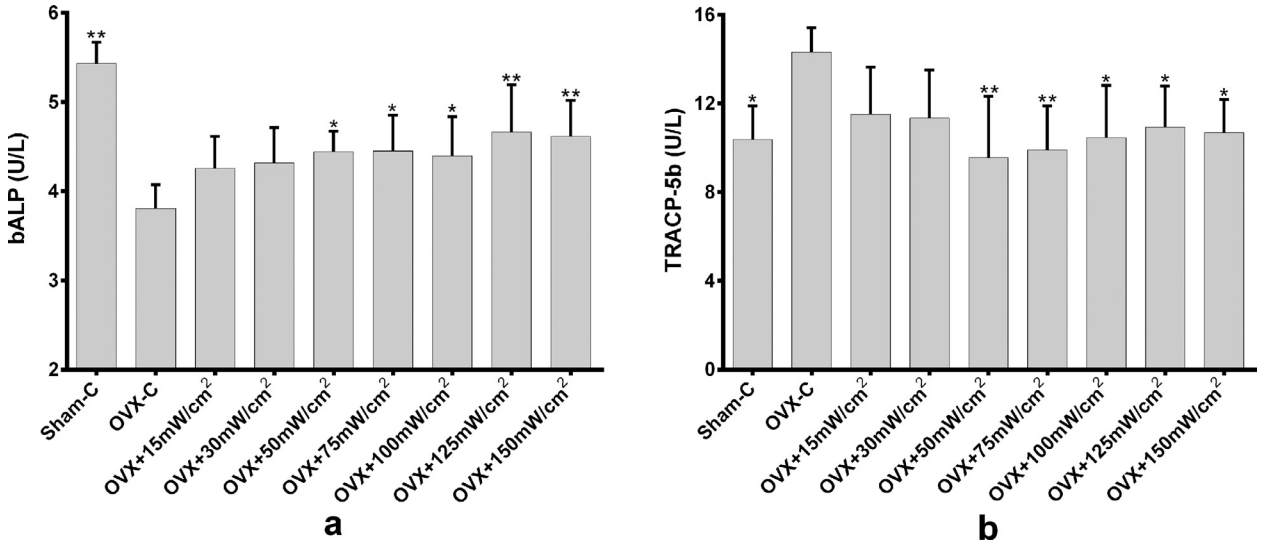

Fig. 6. Measurements of serum (a) bALP (bone formation marker) and (b) TRACP-5b (bone resorption marker) levels are reported as the mean ± standard deviation. *$p < 0.05$, **$p < 0.001$, versus OVX-C (Tukey's multiple comparison tests). bALP = bone-specific alkaline phosphatase; TRACP-5b = tartrate-resistant acid phosphatase 5b; OVX = ovariectomized; OVX-C = ovariectomized control; sham-C = sham control.

reach optimal peak bone mass during the bone-building years, to better simulate actual cases, female Sprague–Dawley rats are commonly ovariectomized at 12 wk of age, when they are sexually mature and have attained peak bone mass for the whole body, femur and tibias (Sengupta et al. 2005). The effects of estrogen deficiency on the rate of bone loss are site dependent. For example, a significant decrease in trabecular bone volume is observed 2 wk after ovariectomy compared with the sham control in the proximal tibia (Wronski

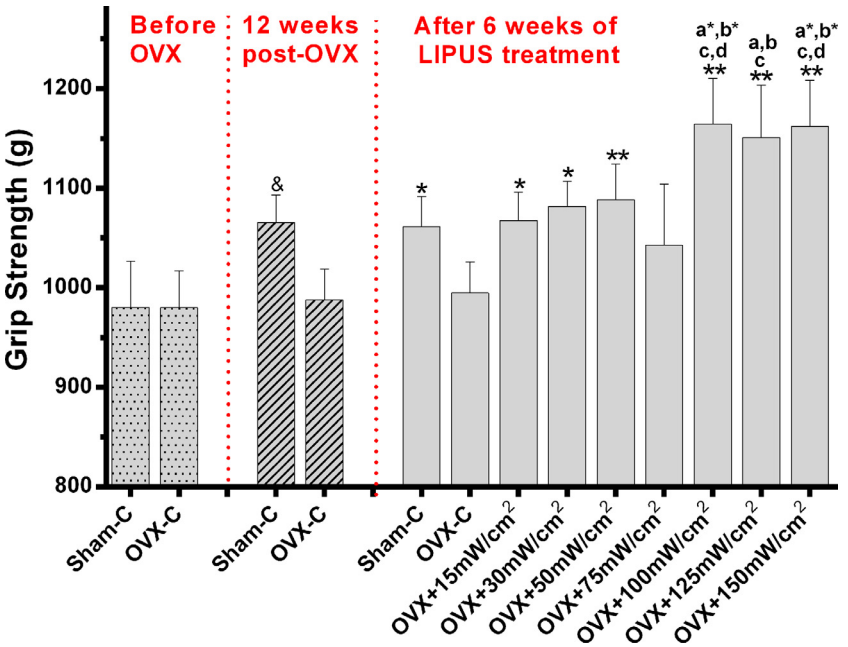

Fig. 7. Hindlimb grip strength of rats at three different time points. Comparisons were made between the same time points for all groups. Values are reported as the mean ± standard deviation. $^{\&}p < 0.05$ versus sham-C 12 wk post-OVX (independent sample *t*-test). *$p < 0.05$, **$p < 0.001$, versus OVX-C; $^{a}p < 0.05$, $^{a*}p < 0.001$, versus sham-C; $^{b}p < 0.05$, $^{b*}p < 0.001$, versus OVX + 15 mW/cm^2; $^{c}p < 0.05$, $^{c*}p < 0.001$, versus OVX + 30 mW/cm^2; $^{d}p < 0.05$, $^{d*}p < 0.001$, versus OVX + 50 mW/cm^2 after 6 wk of LIPUS treatment (Tukey's multiple comparison tests). LIPUS = low-intensity pulsed ultrasound; OVX = ovariectomized; OVX-C = ovariectomized control; sham-C = sham control.

et al. 1988), 30 d after ovariectomy at the femoral neck (Li et al. 1997), 60 d after ovariectomy in the lumbar vertebrae (Wronski et al. 1989) and 12 wk after ovariectomy in the maxilla (Du et al. 2015). Therefore, in our study, 3-mo-old female Sprague-Dawley rats were subjected to OVX and 3 mo was allowed after surgery for induction of systemic osteoporosis, which was confirmed by measurement of BMD and serum E2. The declines in BMD and serum E2 in our models revealed the same trend observed in a former study (Kang et al. 2015), in which areal BMD of the right femur and the fourth lumbar vertebrae measured by DXA declined 18.8% and 21.6%, respectively, and serum E2 declined more than 80% in OVX rats compared with the sham group 16 wk after surgery.

Although a few *in vivo* experiments have verified that LIPUS attenuates the decrease in bone mass and the deterioration of bone microarchitecture in OVX rats (Carvalho and Cliquet Junior 2004; Wu et al. 2009; Ferreri et al. 2011; Lim et al. 2011; Uddin and Qin 2015), few studies exploring the optimal dose of LIPUS needed to achieve greater benefits for treatment of osteoporosis have been performed. As LIPUS intensity was indeed reported to affect the results of *in vitro* experiments with respect to osteoblasts (Li et al. 2002; Angle et al. 2011), it would make sense to examine *in vivo* whether the effects of LIPUS are intensity dependent and to determine the optimal intensity. Generally, I_{SATA} values <150 mW/cm^2 have been considered the intensity spectrum of LIPUS in clinical musculoskeletal applications (Perry et al. 2009). The 15 mW/cm^2 value was chosen as representative of values lower than 30 mW/cm^2. Hence, to investigate the relationship between LIPUS intensity and the therapeutic effects of LIPUS on ovariectomy-induced osteoporosis in rats, seven different intensities were selected in the range 15−150 mW/cm^2, including the widely used 30 mW/cm^2. Meanwhile, previous studies using 150 mW/cm^2 to treat rat non-fractured ulna (Perry et al. 2009) ulna and rat fractures (Fung et al. 2012) reported no safety risks. Within the intensity spectrum of therapeutic ultrasound, the intensities investigated in our experiment are believed to be safe for animals and humans.

Our results indicate that the mechanical and biological effects of LIPUS are highly related to the exposure intensity. In detail, for 15 mW/cm^2, as a low-level representative despite causing slightly significant increases in some parameters derived from μCT analysis, there was no biomechanical significance to prove reduced fracture risk and functional bone restoration. Likewise, 30 mW/cm^2, the intensity widely used to accelerate fracture healing, resulted in markedly higher BV/TV and lower Tb.Sp values without biomechanical significance. These results are consistent with Lim et al. (2011), who also observed significantly higher BV/TV in the LIPUS-treated tibias of OVX rats with the same parameters, including I_{SATA}, frequency, PRF, duty cycle, daily exposure and treatment period. However, these minor effects of 30 mW/cm^2 on intact osteoporotic bone conflicted with results in a fractured bone, as 30 mW/cm^2 was generally accepted to have a significantly positive influence on fracture healing (Padilla et al. 2014; Harrison et al. 2016). Interestingly, our results indicate that therapeutic effects were enhanced with increasing intensity. This trend was in accordance with other research conducted by Ferreri et al. (2011), who addressed the effects of three different intensities: 5, 30 and 100 mW/cm^2. In Ferreri et al.'s experiment, LIPUS intervention was applied at the L4/L5 vertebrae immediately after ovariectomy, and the results suggested that the bone's response was sensitive to ultrasound signal intensity when 100 mW/cm^2 represented the best alternative to mitigate bone loss. What may be an improvement in our experiment compared with Ferreri et al.'s study was that we applied LIPUS treatment 12 wk post-ovariectomy when the significantly substantial bone loss was detected, which is closer to the clinical setting in which treatment is typically introduced after diagnosis of osteoporosis. What's more, we extended the range of sound intensities with seven gradients, exploring the connections between therapeutic effect and LIPUS intensity in more detail.

Clinically, BMD values of individuals with and without bone fractures may overlap substantially; thus, mechanical properties of the bones are also the important in predicting fracture risk, and biomechanical analysis of bone is necessary for determining the integrity of bone function (Ulrich et al. 1999). In the present study, maximum load, stiffness and energy absorption were all stimulated by LIPUS only at intensities $\geq$100 mW/cm^2. However, BV/TV was increased in all LIPUS-treated groups compared with OVX rats, indicating that although lower intensities are able to enhance bone mass, they might fail to maintain bone mechanical integrity.

By combining the results from μCT and biomechanical testing, we observed that LIPUS at intensities $\geq$100 mW/cm^2 had beneficial effects on both bone microarchitecture and mechanical properties and 150 mW/cm^2 had the best effects, indicating that increasing intensity indeed enhances the therapeutic effects of LIPUS. Meanwhile, we also found 30 mW/cm^2, an intensity proved to be effective for fracture healing, had little effect on osteoporosis. The reasons revolve around many aspects, such as different skeletal sites, bone size scales, acoustic properties of the tissues involved and different ultrasound fields derived from different parameters or transducers. One possible explanation might be attributed to the different levels of ultrasound attenuation

between bone fracture and intact osteoporotic bone. When ultrasound propagates into the intact bone, 25%−40% of ultrasound energy is reflected at the soft tissue−bone interface (Warden et al. 1999), and around 72% of the remaining energy is attenuated in the first 2 mm of propagation in the cortex (Pichardo et al. 2011). The mechanical stimulus provided by LIPUS may influence only the outer cell layer of the cortex, and the remaining energy might be insufficient to induce a biological response in the bone cells in the trabecular region. Therefore, 30 mW/cm^2 might be insufficient because of strong attenuation in the intact osteoporotic bone and increasing intensity may help. In comparison, when ultrasound is applied to fractured bone, the signals do not need to propagate through the intact cortex. Ultrasound only had to pass through the soft tissue layer to reach the targeted cortical bone region and to induce biological reactions, such as enhancement of angiogenesis and blood flow and promotion of endochondral ossification, and thus play a positive role in the dynamic process of fracture healing (Zhang et al. 2017).

Grip strength is one of the direct reflections of physical function for a biological individual. We noted significantly decreased grip strength in OVX rats 12 wk after ovariectomy, indicating that osteoporosis is usually associated with reduced physical function and ability to exercise. Moderate physical activities are always recommended to preserve BMD and enhance bone and muscle strength; thus, poor exercise ability accompanied by osteoporosis might, in turn, lead to insufficient exercise, finally resulting in worse osteoporosis and increased fracture risk. However, in our study, LIPUS irradiation was able to reverse the reduction in grip strength in OVX rats to a level similar to or higher than that of the sham control group. LIPUS does this by stimulating bone mass and microarchitecture by improving the function of related muscles, as the improved muscle function was directly evidenced by higher hindlimb grip strength. As reported, the decline in muscle function could result in a deterioration of bone health (Szulc et al. 2005), and mechanical loading is a key mechanism linking bone and muscle with a central promoting role of physical activity (Tagliaferri et al. 2015). Thus, LIPUS may act as a type of mechanical loading to promote muscle function and further to stimulate bone mass and microarchitecture.

Osteoporosis results from an imbalance in the regulation of two sub-processes of bone remodeling: bone resorption and bone formation. Osteoblasts and osteoclasts play leading parts in regulating bone remodeling and maintaining the balance between bone formation and resorption. bALP, synthesized and secreted by osteoblasts, is a specific marker of bone formation, and TRACP-5b, produced by osteoclasts, is well known as a bone resorption marker. In the present study, serum bALP level significantly decreased and serum TRACP-5b level significantly increased in OVX rats, following the same trend reported by Liu et al. (2018) 12 wk post-ovariectomy (Liu et al. 2018). LIPUS at an intensity $\geq$50 mW/cm^2 significantly enhanced bALP and reduced TRACP-5b compared with the OVX group, indicating LIPUS has a dual therapeutic effect on promoting bone formation and suppressing bone resorption. In addition, serum bALP level was strongly positively correlated with LIPUS intensity ($R^2 = 0.7$, $p < 0.05$), but no correlation was observed between TRACP-5b and intensity. The correlation between bALP and intensity was similar to that in an *in vitro* study in which ALP activity was more enhanced with higher-intensity LIPUS in 5-d-treated cells (Angle et al. 2011).

One limitation of the present study was that the experiments were conducted on rats instead of humans. Considering the different bone size and acoustic field properties, the extrapolation of conclusions drawn from rats to human bones should be done with care. Another limitation was that only a single time point of assessment at the end of LIPUS treatment was chosen. The treatment lasted 6 wk in our experiment, as the average bone remodeling period in rats is roughly 40 d (Thompson et al. 1995). Future studies will extend treatment duration to two or more complete remodeling cycles and take advantage of *in vivo* detection technology to assess the effect of LIPUS at more time points. In addition, our results indicated that 150 mW/cm^2, the highest intensity investigated in the current experiment, had the optimal therapeutic effect. We cannot rule out the possibility that even higher intensities will be better, which is a desirable assumption for future work.

CONCLUSIONS

We found that LIPUS could partially attenuate the decrease in bone mass and deterioration of bone microarchitecture caused by estrogen deficiency by accelerating bone formation and suppressing bone resorption, and LIPUS treatment may stimulate bone mass and microarchitecture by improving the function of related muscles. Intensity is a crucial factor in the therapeutic effects of LIPUS. We found that LIPUS intensities in the range 15 to 150 mW/cm^2 were strongly correlated with BMD and bone microstructure ($R^2 = 0.57-0.83$) and bone mechanical strength ($R^2 = 0.80-0.97$). With no known contraindications to LIPUS, we believe that LIPUS irradiation holds a latent anabolic therapeutic capacity to mitigate adverse changes in bone and thus should be explored further as a prominent alternative for the treatment of osteoporosis.

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