Ultrasound in Medicine & Biology 51 (2025) 695−706

Contents lists available at ScienceDirect

Ultrasound in Medicine & Biology

journal homepage: www.elsevier.com/locate/ultrasmedbio

Original Contribution

Characterization of LIPUS Parameters Suitable for Hip Bone Fracture

Poornima Palanisamy[a,#], Shuai Li[a,#], Queenie Tsung-Kwan Shea[a], Michelle Meng-Chen Li[b], Wing-Hoi Cheung[b], Ling Qin[b], Simon Kwoon-Ho Chow[b,c], Yong-Ping Zheng[a,d,*]

[a] Department of Biomedical Engineering, The Hong Kong Polytechnic University, Hong Kong SAR, China
[b] Musculoskeletal Research Laboratory, Department of Orthopaedics and Traumatology, Hong Kong Special Administrative Region, The Chinese University of Hong Kong, Hong Kong SAR, China
[c] Department of Orthopaedic Surgery, Stanford University, Stanford, CA, USA
[d] Research Institute of Smart Ageing, The Hong Kong Polytechnic University, Hong Kong SAR, China

A R T I C L E I N F O

Keywords:
Low-intensity pulsed ultrasound stimulation (LIPUS)
Hip bone fractures
Animal study
Optimal parameters

A B S T R A C T

Objective: To investigate the effects of ultrasound treatment on the healing of hip bone fractures using frequencies of 0.5 MHz and 1.5 MHz with constant intensity (30 mW/cm^2) at the fractured site.
Methods: For the *ex vivo* experiments, acoustic attenuations of 0.5 MHz and 1.5 MHz ultrasound were measured and compared using different thicknesses of human cadaver and porcine tissues in a hydrophone system. For the *in vivo* experiments, 20 hip-fractured rabbits were divided into four groups, namely: control, 1.5 MHz with unchangeable intensity (positive control), 0.5 MHz with changeable intensity, and 1.5 MHz with changeable intensity. For the 0.5 and 1.5 MHz groups with changeable intensity, a constant intensity of 30 mW/cm^2 at the fracture site was achieved using a compensation method for power transmission with reference to the acoustic attenuation.
Results: The effective intensity measured using a hydrophone was substantially reduced to 6.16 mW/cm^2 from 30 mW/cm^2 in the positive control device after propagating soft tissues with a thickness of 5.0 cm, with an attenuation of approximately 6.0 dB. Meanwhile, for the 0.5 and 1.5 MHz groups, the ultrasound intensity was consistently controlled at 30 mW/cm^2 after passing through tissues with different thicknesses using the compensation method. In the *in vivo* study using a newly established hip fracture rabbit model, the best results in bone histomorphometry, mechanical properties, and histological evaluation were consistently found in the 0.5 MHz group, while the 1.5 MHz group exhibited relatively better bone healing than the positive control group.
Conclusion: The results suggest a LIPUS frequency of 0.5 MHz together with the consistent intensity of 30 mW/cm^2 at the fracture site for more effective treatment of hip bone fractures.

Introduction

Hip fractures are life-threatening injuries for older people and are treated as public health concerns in many countries [1]. The annual global incidence of hip bone fracture is currently about 1.6 million [2], which is estimated to increase to 6.26 million by 2050 [3]. Hip fractures are usually the result of impact from falling, while factors such as smoking, ethnicity, age, and medical conditions such as osteoporosis, may weaken the bones, increasing their susceptibility to fracture on impacts. The healing of hip fractures generally takes a long time whilst incurring high medical costs [4], and there is an increased risk of fracture-related complications due to the complex morphology of the hip bones. LIPUS has been reported to be a safe and effective approach to accelerate the bone healing process as it has been found to enhance bone healing at all stages, specifically, the inflammatory, reparative, and remodeling stages [5]. An FDA-approved, commercially available LIPUS device has been used in previous studies on fracture healing. However, there have been no investigation into the benefits of LIPUS for hip fractures. The effectiveness of LIPUS in cases with deeper fracture sites (femur and humerus) was found to be inferior compared with fractures in superficial bones (tibia, fibula, radius, ulna) [6−8]. Fracture sites in the hip or acetabulum are painful and take longer time to heal and can become a lifelong burden in elderly people. Since most studies have investigated superficial bone fractures using LIPUS, the demonstration of whether it is less efficacious in deeper fracture sites and how to make it more effective requires more investigation [9].

Commercial LIPUS devices use a fixed set of parameters with an ultrasound frequency of 1.5 MHz, 20% duty cycle of burst wave pattern

* Corresponding author. Department of Biomedical Engineering, The Hong Kong Polytechnic University, Hong Kong SAR, China.
E-mail address: yongping.zheng@polyu.edu.hk (Y.-P. Zheng).
# Poornima Palanisamy and Shuai Li contributed equally to this manuscript.

https://doi.org/10.1016/j.ultrasmedbio.2025.01.001
Received 5 October 2023; Revised 30 November 2024; Accepted 2 January 2025

(200 μs on and 800 μs off) with a pulse repetition frequency of 1 kHz, and spatial average temporal average (I_{SATA}) intensity of 30 mW/cm^2 [10]. During the 1930s−1950s, the usage of ultrasound intensity ranging from 5000 to 25000 mW/cm^2 for bone fracture healing caused several complications in the soft tissues, which led to the use of decreased ultrasound intensity (200 mW/cm^2) [11−13]. Subsequently, numerous human and animal studies reported that an intensity of 30 mW/cm^2 accelerated both bone formation and reduction of healing time in fresh fractures, delayed unions, and nonunions [10,14−19]. However, very few studies have investigated its use for deeper bone fractures such as the hip bone. Hence, we selected this intensity in the present study. Ultrasound is an acoustic wave that can be attenuated with some energy absorbed by soft tissues [20,21], determined by the attenuation coefficient α with unit dB/cm/MHz which is associated by the type of soft tissue and ultrasound frequency [22]. Given this factor, it seems not to be reasonable to use the same parameter setting of the commercial device for deeper fractures where various soft tissues with different thicknesses covering the bone.

In this study, a customized compensation method was applied to maintain an acoustic intensity of 30 mW/cm^2 at the fractured site by referring to other uses of deeply targeted ultrasound stimulation, which makes this as the first study to demonstrate the effect of ultrasound on hip fractures in an animal model. The objective was not to identify the best parameter but to demonstrate that LIPUS can be more effective by compensating for the attenuation caused by the soft tissues covering the fracture location, as well as by lowering the frequency from the conventionally used 1.5 MHz to 0.5 MHz. In this study, the thickness of the soft tissue was hypothesized to be the key factor affecting fracture healing using LIPUS, and the effectiveness of fracture healing may be dependent on the effective intensity (30 mW/cm^2) of the LIPUS reaching the fracture site. Hence, different thicknesses of human cadaver and porcine tissues were first used to assess the attenuation of the ultrasound after propagation through the tissues. Soft tissue immediately anterior to the superior iliac spine and acetabular rim was collected from a human cadaver, while and porcine tissues were collected from the thigh region of the pig. The use of tissue samples from a human cadaver can help to recognize actual changes in acoustic intensity in human tissues, while the porcine tissues were used to evaluate changes in attenuation in different tissue thicknesses. According to previous studies, porcine tissues have very similar properties to human tissues and have been widely utilized in studies of musculoskeletal biomechanics [23]. In the present study, a portable customized ultrasound stimulator was developed to allow adjustments of ultrasound frequency and output intensity. The output intensity of the customized stimulator could be increased to ensure an intensity of 30 mW/cm^2 after passing through a layer of soft tissue, thus compensating for ultrasound attenuation caused by the soft tissue layer. Moreover, an ultrasound frequency of 0.5 MHz was hypothesized to be more effective for healing fractures of the hip bone, as ultrasound with a lower frequency can more easily penetrate deeper into the fracture site due to its lower attenuation [20]. There is currently a lack of experimental data about the penetration characteristics of LIPUS for hip fracture healing with frequencies lower than 1.5 MHz. The results of this study on the use of frequencies of 0.5 and 1.5 MHz together with the controlled intensity at the fracture surface may provide a direct reference for the future investigation of LIPUS for the healing of deep bone fractures.

Materials and methods

LIPUS devices and stimulation pattern

Two LIPUS stimulators were used in this study; one was a commercial LIPUS stimulator (EXOGEN 4000+, Bioventus LLC, USA), while the other was our customized stimulator, as shown in Figure 1 (a−b). The customized stimulator is a portable device that includes a signal generator, a 50 W power amplifier, and removable unfocused ultrasound transducers with resonance frequencies of 0.5 and 1.5 MHz. Parameters such as frequency, duty cycle, and pulse repetition frequency (PRF) could be adjusted by controlling the signal generator. An external power of 16 V was used to drive the customized device, and the voltage was boosted to around 50 V to supply the power amplifier unit. For the stimulation pattern, 20% of the duty cycle (pulse duration of 200 μs) and PRF of 1 kHz were fixed for both devices, as shown in Figure 1 c. The LIPUS signal amplitude was fixed for the EXOGEN device, while it was adjustable for the customized LIPUS device.

Acoustic intensity measurement in human cadaver and porcine tissues

The aim of this measurement was to investigate the relationship between acoustic intensity and tissue thickness, as well as the intensity patterns at different distances from the transducer surface. In this study, acoustic intensity, i.e., I_{SATA} was measured in human cadaver and porcine tissues of different thicknesses (ranging from 1 cm to 5 cm). This range was chosen because the average trochanteric soft tissue thickness in hip fracture cases is 2.9 ± 1.1 cm in men [24] and 4.0 ± 1.6 cm in women [25]. The ethical approval was granted to use the human cadaveric tissue and was not required by the Joint CUHK-NTEC CREC (The Chinese University of Hong Kong-New Territories East Cluster Clinical Research Ethics Committee) for this study. A schematic diagram of the experimental setup is shown in Figure 2. A water bath (length, 60 cm; width, 35 cm; height, 30 cm) was filled with degassed water to a height of 25 cm. A 25 mm diameter unfocused ultrasound transducer with an operating frequency of 0.5 MHz and 1.5 MHz was used. The diameter of the commercial LIPUS device was also 25 mm. The tissue sample was placed on top of the transducer, and a rugged needle-type hydrophone (HNR-1000, Onda Corporation, USA) was mounted immediately above the tissue. The distance between the transducer surface and the hydrophone was approximately 5.3 cm and was kept constant for all the measurements. During the measurements, the transducer was connected to the output of the laboratory-constructed and commercial LIPUS devices. The output of the hydrophone was connected to the data acquisition card (NI 5112, National Instruments, USA). The movement of the hydrophone was controlled by an XYZ motor controller to enable scanning of the whole tissue; this was operated using a custom-made program designed in LabVIEW (LabVIEW 2015 SP1, National Instruments, USA). The scanning range was set to 0−25 mm with a step size of 0.5 mm that generated a color map of a 50*50 matrix. The voltage recorded by the hydrophone was converted into acoustic intensity using the standard calibration factor V^2cm^2/W provided by the manufacturer of the hydrophone. The I_{SATA} values were then calculated by drawing a circle with a diameter of 22 mm (to avoid data points in the low-intensity region at the edge of the transducer based on acoustic distribution pattern) using the custom script code in MATLAB r2016b (Math Works Inc., Natick, MA, USA). The color map generated by MATLAB shows the distribution pattern of the acoustic field, and the circle denotes the effective region of the transducer during ultrasound stimulation.

The size of the animal used in this animal study was smaller than that of human subjects, and the average soft tissue thickness covering the fracture location in the rabbit model was around 1 to 2 cm. Therefore, we also conducted additional scanning to map the acoustic intensity at distances of 1, 1.25, 1.5, 1.75, and 2 cm from the transducer surface for both 0.5 and 1.5 MHz transducers, using the scanning parameters as described above.

Animal model

Twenty adult New Zealand white rabbits (1.6−2 kg) were used in this study. All the surgical and experimental procedures were approved

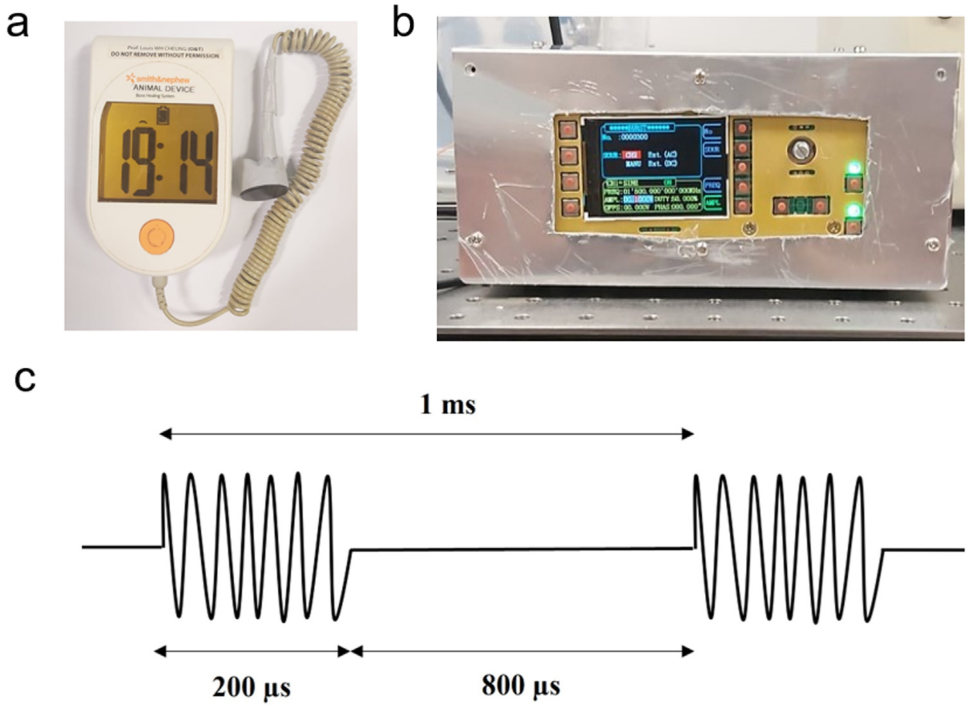

Figure 1. (a) Commercially available LIPUS device, Exogen 4000$^+$ ultrasound bone healing system. (b) Customized LIPUS device. (c) LIPUS wave pattern used for stimulation in both devices.

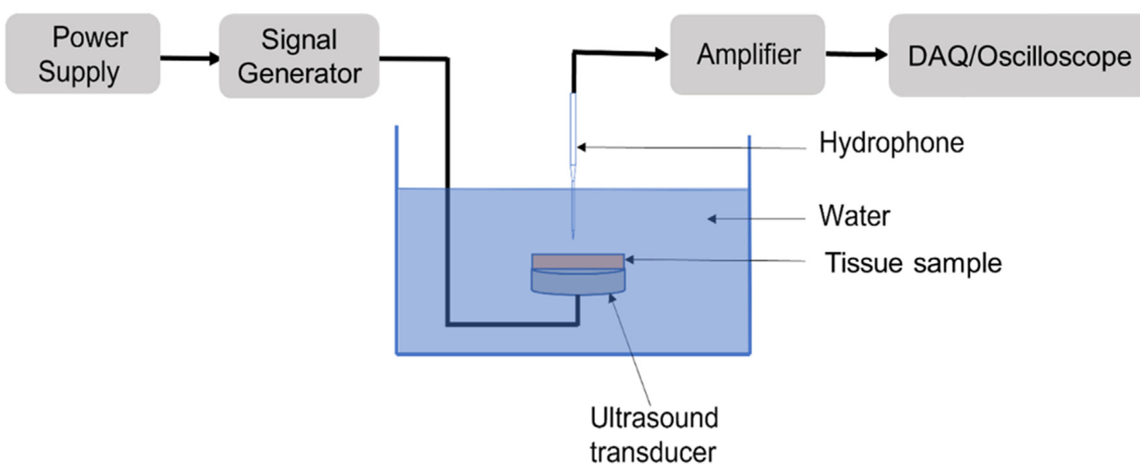

Figure 2. Experimental setup for measurement of ultrasound intensity. A rechargeable battery was used as the power supply to operate the EXOGEN and the customized stimulator. The hydrophone was mounted perpendicular to the tissue sample to receive the ultrasound signal, which was amplified and collected by a data acquisition card (DAQ) or an oscilloscope.

by the Animal Experimentation Ethics Committee (AEEC, Reference 20-051-MIS) of The Chinese University of Hong Kong. The rabbits were randomly allocated to four groups, namely: the control group, the positive control (PC) group (1.5 MHz with intensity fixed at 30 mW/cm^2), the 0.5 MHz group, and the 1.5 MHz group.

Surgical procedures for the fracture model

All the animals received general anesthesia using a mixture of ketamine (50 mg/kg) and xylazine (10 mg/kg) *via* intramuscular injection. The surgical site was shaved and aseptically prepared for the procedure. An incision was made between the biceps femoris and semimembranosus muscles with a scalpel blade (surgical blade, size 23) to expose the subtrochanteric region. Then an air-powered sagittal oscillating saw (Air pen drive, DePuy Synthes, Germany) with a blade thickness of 1 mm was used to create an open osteotomy at the subtrochanteric region, as shown in Figure 3. Five holes were drilled with a diameter of 1.5 mm to fix the 3D-printed titanium implant (U3DP, The Hong Kong Polytechnic University, Hong Kong) using five self-tapping cortical screws (Biortho Co Ltd, Jiangsu, China). The surgical site was then cleaned using sterile saline, and the incision was sutured layer by layer (Mersilk, W580, Ethicon, USA). The animals were given an intramuscular injection of 0.1 ml of analgesic at the fracture site twice daily for three consecutive days after the surgery. A postoperative radiograph was taken immediately after the surgery to confirm the success of the fracture and followed up by weekly monitoring of the healing process (Xpert-80, Kubtec, Stratford, USA).

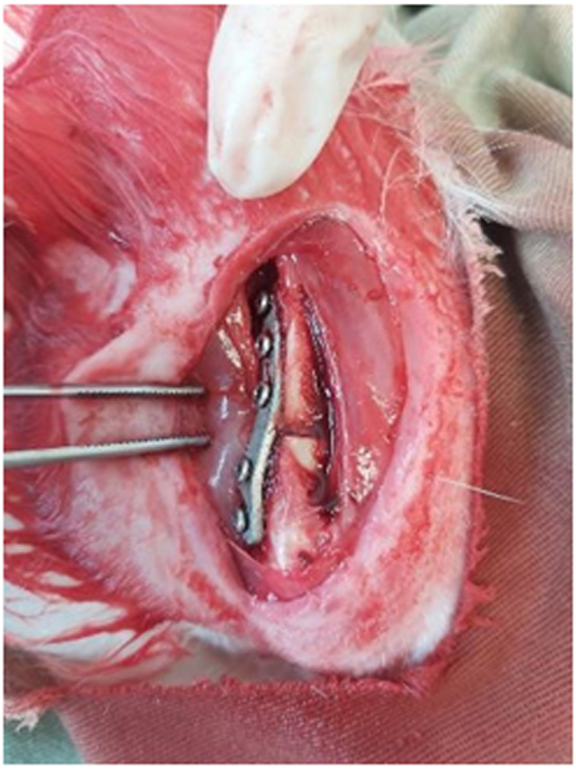

Figure 3. Creation of a subtrochanteric fracture on the left side of the rabbit limb.

LIPUS treatment strategy

LIPUS treatments were given to the animals on the day following surgery. These rabbits received general anaesthesia during treatment and were placed in a supine position with the ultrasound treatment probe positioned directly above the fracture site (Figure 4). The position of the fracture site was marked on the rabbit's skin surface for accurate placement of the LIPUS probe to ensure exact delivery of the ultrasound signal over the surgical site. Treatment was given for 20 mins/day for a total of six weeks. Based on the measurement obtained during the acoustic intensity assessments after passing through different depths of porcine tissue, the input voltage was adjusted to deliver a consistent acoustic intensity of 30 mW/cm^2 for the 0.5 MHz and 1.5 MHz groups at the fracture surface. A portable ultrasonography device was used to measure the thickness of the soft tissue. During the measurement, the portable ultrasonography device was placed in the same area as that used for the LIPUS treatment, as shown in Figure 5a. The thickness of the soft tissue was determined according to the ultrasound image, as shown in Figure 5b. The thickness was measured weekly, and the output of the customized LIPUS device was adjusted to ensure a sufficient and consistent intensity for treatment.

Radiographic examination

A plain radiograph was taken immediately after surgery and every week thereafter using a KUBTEC X-ray machine (KUBTEC XPERT-80, Stratford, USA) to monitor the stability of the bone fixation and to observe the fracture healing. The radiographs were taken under the same conditions using a standardized protocol. The initial osteotomy site was noted for identification of the newly formed bone.

Micro-CT analysis

After six weeks of treatment, all the animals were euthanized, and the femoral bones were harvested. The soft tissues surrounding the harvested femora and the metal implants were first removed after thawing in 0.9% saline at room temperature for 1 hour. Micro-CT scanning was performed using a multi-slice high-resolution peripheral quantitative

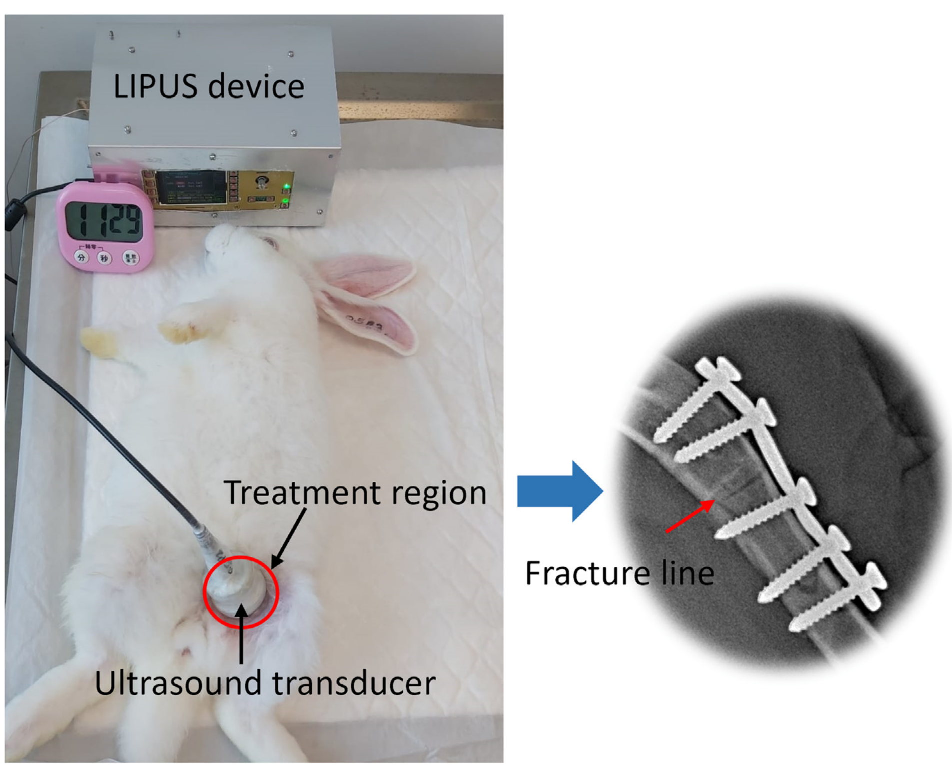

Figure 4. Representative image showing LIPUS treatment.

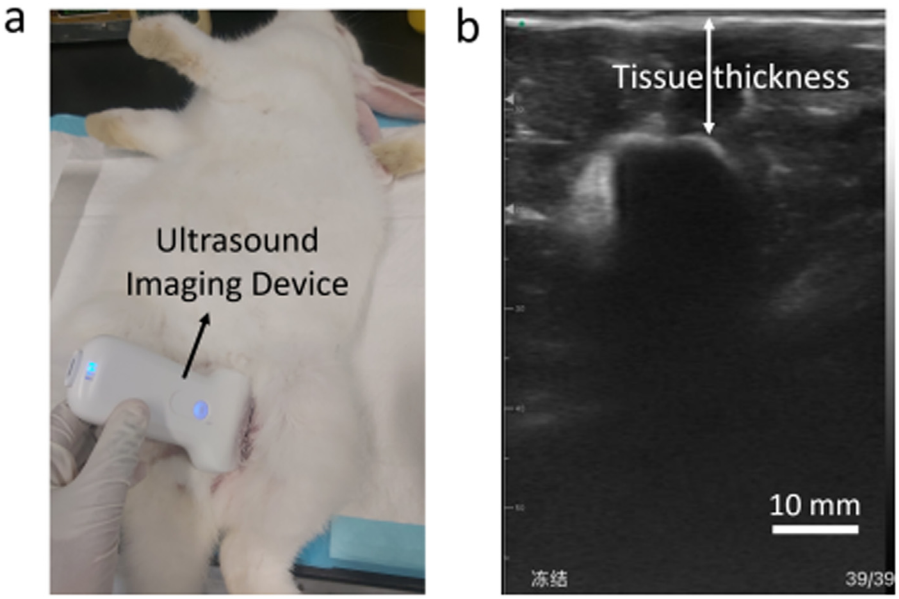

Figure 5. Ultrasonography for the measurement of tissue thickness. (a) Position of the rabbit for measurement of soft tissue thickness. (b) Recorded image showing the thickness of the soft tissue.

computed tomography (HR-pQCT, XtremeCT, Scanco Medical, Bruttisellen, Switzerland) operating at 60 kV, according to the protocol for animal studies [26]. The bones were fixed into a carbon-fiber sample holder. The subtrochanteric fracture site was scanned, resulting in 1316 slices, followed by the selection of the region of interest (ROI) and segmentation using Scanco analytical software v5.08b (Scanco Medical AG). Following the three-dimensional evaluation, the volumetric bone mineral density (BMD), bone volume (BV, mm^3), tissue volume (TV, mm^3), bone volume fraction (BV/TV, %), trabecular number (Tb.N), trabecular thickness (Tb.Th, mm), and trabecular spacing (Tb.Sp, mm) were generated for comparison. These microarchitectural parameters, such as Tb.N, Tb.Th, and Tb.Sp, help to provide information about the mechanical properties of the bone.

Mechanical testing

A standard four-point bending test was used to evaluate the mechanical properties of the fractured femora [27]. Samples were stored in saline at -80°C and were thawed at room temperature for one hour. In the four-point bending test, the femur was placed perpendicular to the

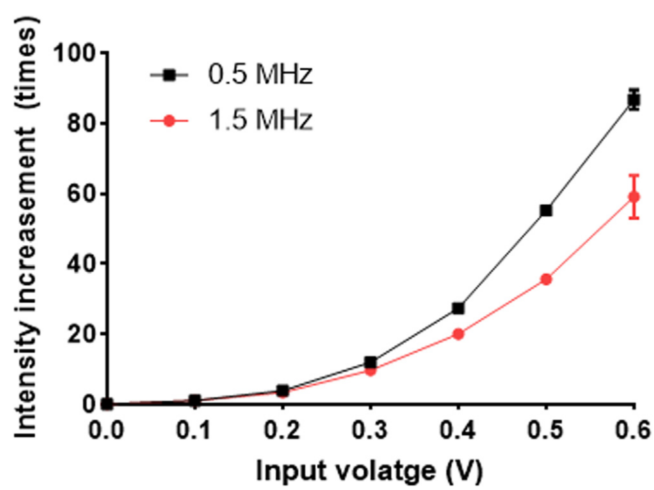

Figure 7. Increase in intensity with different input voltages.

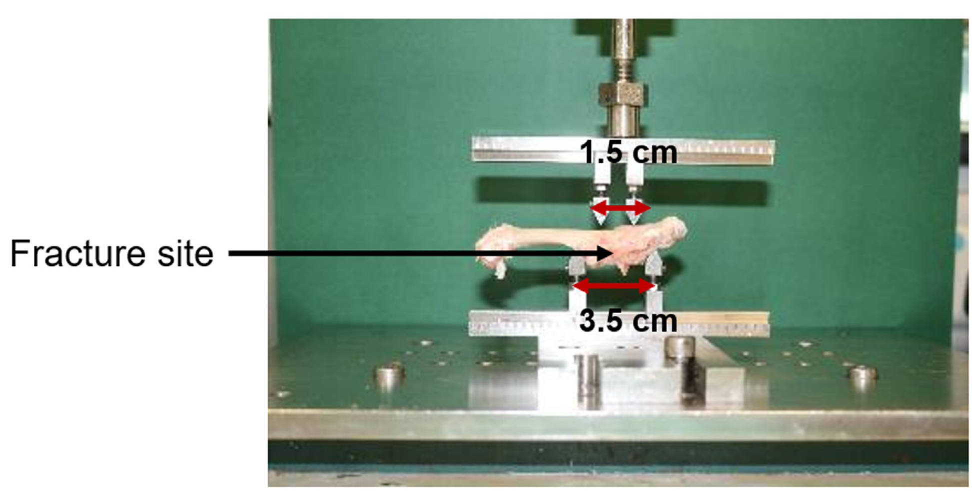

Figure 6. Experimental setup for the four-point bending test.

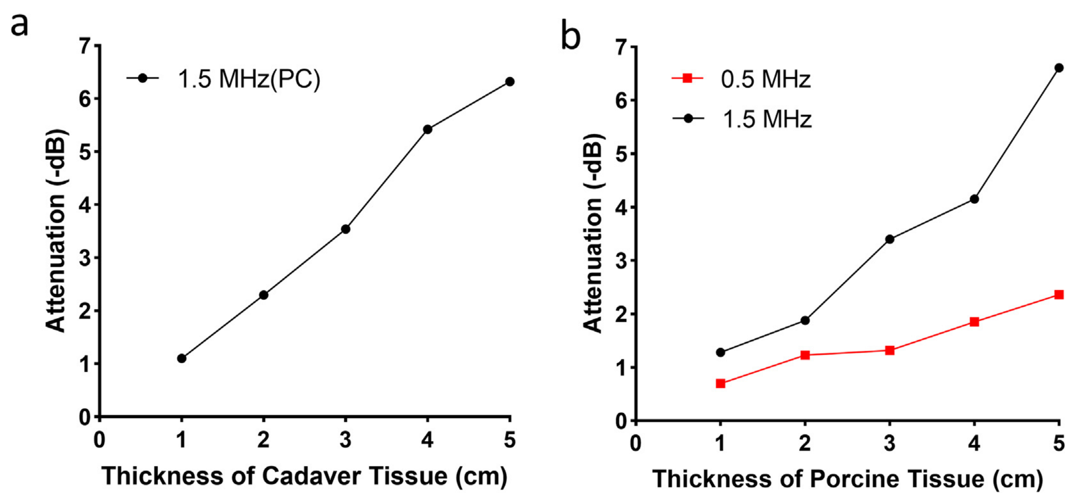

Figure 8. Attenuation of different ultrasound frequencies after propagation through tissue samples of different thicknesses. (a) Cadaver tissue using a PC device (1.5 MHz) and (b) Porcine tissue using a customized LIPUS device with transducers of 0.5 and 1.5 MHz.

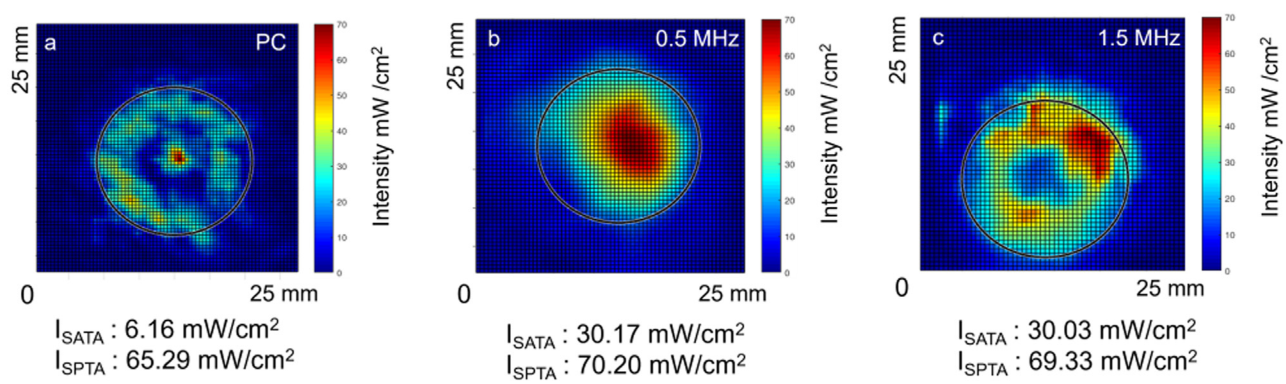

Figure 9. Color maps of harvested intensity from a PC device and the customized stimulator with a tissue thickness of 5 cm. (a) PC (1.5 MHz), (b) 0.5 MHz, and (c) 1.5 MHz groups.

blades, after which the fractured region was located in the middle of the blades (Figure 6), with lowering at a constant displacement rate of 5 mm/min (H25KS, Hounsfield Test Equipment Ltd, Redhill, Surrey, UK). Using the load−displacement curve, the ultimate load (N), stiffness (N/mm), and energy to failure (J) were evaluated using the built-in software.

Histological examination

The harvested bone samples were fixed in 4% neutral buffered formalin for one day and decalcified in 10% formic acid for six weeks, followed by sequential dehydration. The samples were then cut into two halves along the mid-sagittal plane using a thin blade and were embedded in paraffin wax and sectioned at a thickness of 5 µm (1130 Biocut microtome, Reichert-Jung GmbH, Nussloch, Germany) [28]. The bone sections were stained with hematoxylin and eosin (H&E) and examined under a microscope (Q500MC, Leica Cambridge Ltd., Cambridge, UK).

Statistical analysis

The collected data were processed using SPSS (version 20, IBM Corp., Armonk, NY, USA). All values are expressed as the mean and standard deviation. An independent sample t-test and one-way ANOVA were used to compare two or multiple groups, respectively. The *p*-value was set to <0.05, denoting a statistically significant difference between groups.

Table 1
Weekly measurements of tissue thickness and adjustment of the input signal for a consistent acoustic intensity of 30 mW/cm^2

Time (week)	Measured thickness (cm) in 1.5 MHz group	Weight (kg)	Input voltage (V)	Measured thickness (cm) in 0.5 MHz group	Weight (kg)	Input voltage (V)
Pre-operation	0.84 ± 0.05	1.67 ± 0.25	Null	0.88 ± 0.08	1.74 ± 0.26	Null
1	0.88 ± 0.04	1.62 ± 0.22	0.36	0.92 ± 0.08	1.8 ± 0.29	0.26
2	1.00 ± 0.00	1.78 ± 0.26	0.36	0.98 ± 0.08	1.9 ± 0.32	0.26
3	1.18 ± 0.13	1.92 ± 0.22	0.37	1.06 ± 0.08	2.07 ± 0.39	0.27
4	1.4 ± 0.23	2.1 ± 0.19	0.38	1.12 ± 0.13	2.2 ± 0.36	0.28
5	1.58 ± 0.27	2.28 ± 0.26	0.40	1.28 ± 0.17	2.3 ± 0.32	0.29
6	1.74 ± 0.26	2.54 ± 0.16	0.41	1.48 ± 0.109	2.4 ± 0.32	0.30

Results

The relationship between the stimulating intensities and input voltages

The acoustic intensities measured under different input voltages using the customized LIPUS device at resonating frequencies of 0.5 and 1.5 MHz are shown in Figure 7. The intensity increased linearly as the input voltage increased in both the 0.5 and 1.5 MHz groups. The results shown in Figure 7 were used as a reference for the adjustment of the input voltage to ensure a consistent intensity of 30 mW/cm^2 at the fracture site, with the consideration of tissue attenuation.

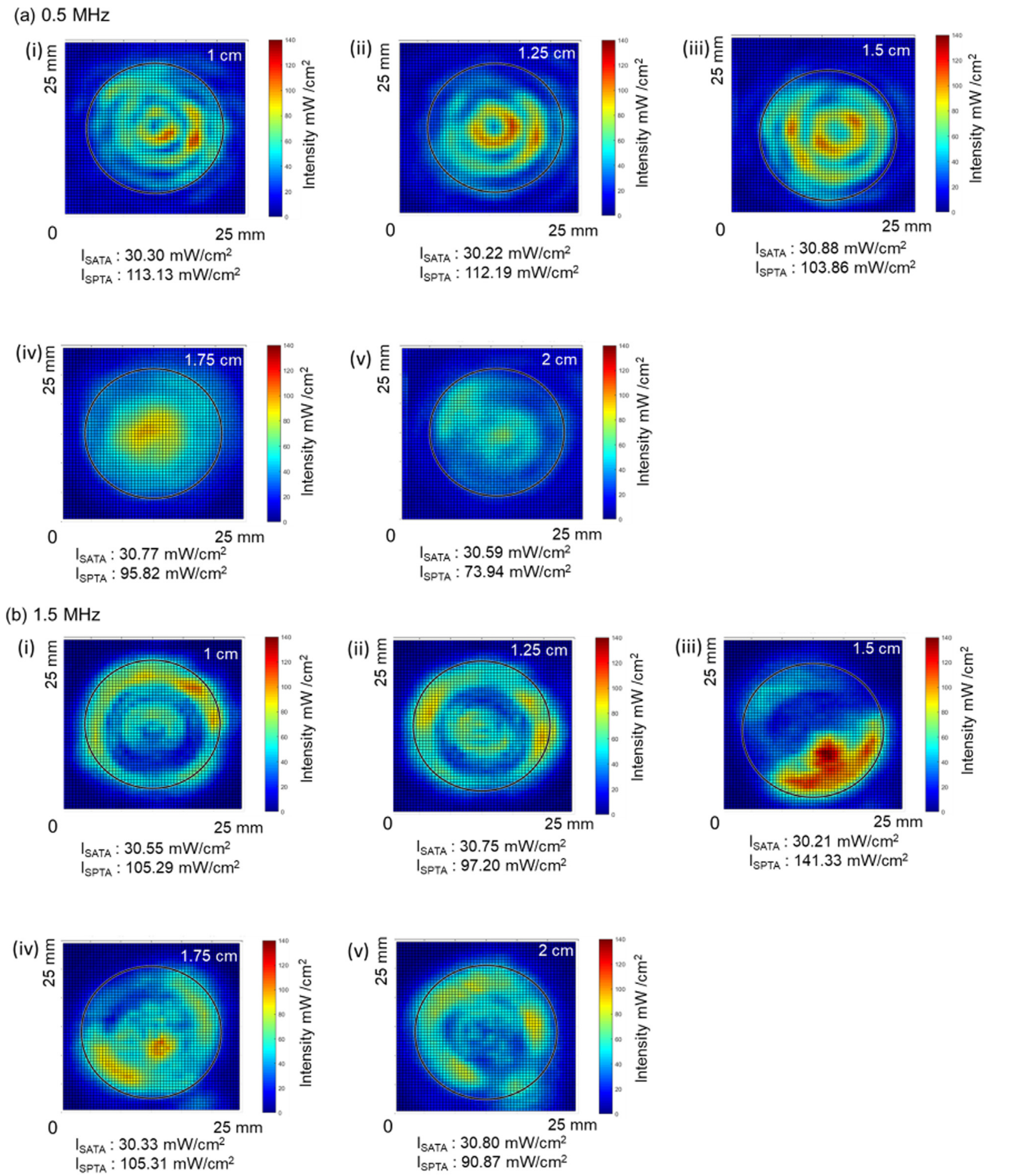

Figure 10. Color maps of consistent acoustic intensity achieved by adjusting the input voltages at distances of 1, 1.25, 1.5, 1.75, and 2 cm. (a) 0.5 MHz and (b) 1.5 MHz.

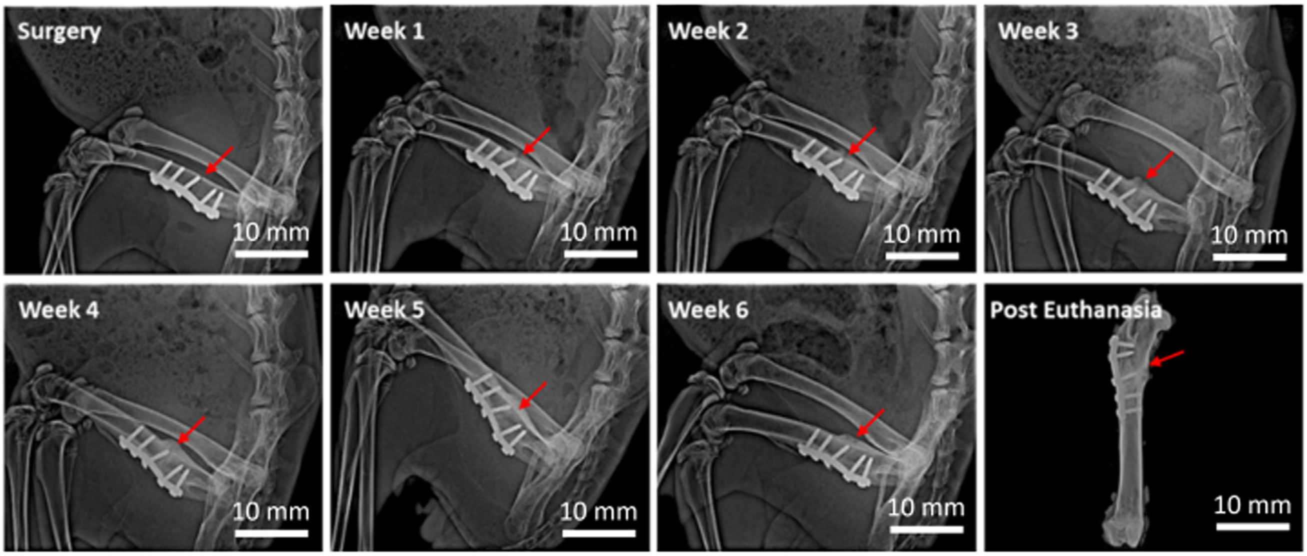

Figure 11. Stages of fracture healing in one representative X-ray analysis of the left femoral bone of a rabbit after surgery, at weeks 1−6, and after euthanasia. The red arrows indicate the location of the fracture.

Acoustic intensity measurement using human cadaver and porcine tissues

As shown in Figure 8, the degree of attenuation generally increased as the thickness of tissue samples increased for all ultrasound frequencies. At a frequency of 1.5 MHz, the attenuation was 6.0 dB (acoustic intensity decreased by 75%) after propagation through tissue with a thickness of 5.0 cm in both cadaver and porcine tissues. In contrast, attenuation of approximately 2.0 dB was found with 0.5 MHz after passing the same tissue layer (Fig. 8b). The color maps in Figure 9 show the distribution of ultrasound intensity from the commercially available unit and the customized stimulator after propagating through the tissue with a thickness of 5.0 cm. The acoustic intensity measured from the commercial device was dramatically reduced to 6.16 mW/cm^2 (Fig. 9a). In contrast, the customized stimulator could deliver 30.03 mW/cm^2 for 1.5 MHz (Fig. 9b) and 30.17 mW/cm^2 for 0.5 MHz (Fig. 9c) after adjustment of the power transmission. However, the custom transducer operating at 1.5 MHz used a compensation method for power transmission, and the attenuation value was the same as that obtained with the PC device. The color map generated by the 0.5 MHz transducer exhibited a more concentrated pattern of acoustic distribution than that obtained using the 1.5 MHz transducer, as the acoustic intensity is stronger in the focal zone of the ultrasound beam at 0.5 MHz. These measurements indicate that there will be a better therapeutic outcome using the effective region of the transducer, and this can be achieved by using the compensation method to deliver the ultrasound intensity at the desired stimulation site through the tissues.

Measurement of soft tissue thickness and the adjustment of the input voltage for healing

The measurement of tissue thickness was conducted weekly using a palm-size ultrasound imaging device, with the values shown in Table 1. The thickness of the soft tissue increased continuously after the operation, which is possibly the result of the normal growth of the animal. Meanwhile, the amplitude of the driving signal (input voltage) was adjusted to maintain a consistent acoustic intensity of 30 mW/cm^2 in both the 0.5 and 1.5 MHz groups. The average thickness of the soft tissue covering the fracture site in the rabbit model was in the range of 1−2 cm at different weeks after the creation of the fracture. The acoustic intensity measured from the transducer surface at 1, 1.25, 1.5, 1.75, and 2 cm for 0.5 MHz generated I_{SATA} values of 30.30, 30.22, 30.88, 30.77, and 30.59 mW/cm^2, respectively, as shown in Figure 10a, while 1.5 MHz generated I_{SATA} values of 30.55, 30.75, 30.21, 30.33, and 30.80 mW/cm^2, respectively, as shown in Figure 10b. The corresponding spatial peak temporal average intensity (I_{SPTA}) values for the aforementioned distances for 0.5 MHz were 113.13, 112.19, 103.86, 95.82, and 73.94 mW/cm^2, respectively, and for 1.5 MHz were 105.29, 97.20, 141.33, 105.31, and 90.87 mW/cm^{2,} respectively. The beam patterns for both the frequencies exhibited some variations at different distances. However, the 0.5 MHz transducer still exhibited a more concentrated pattern of intensity, with ring patterns at some distances, which may contribute to a better therapeutic effect in comparison with 1.5 MHz.

Radiographic evaluation

A plain X-ray with the right lateral recumbent position was taken weekly to record the progress of bone formation, as shown in Figure 11. A progression of consolidation of the fracture gap was observed throughout the experimental period. The callus width was not measured in the current study, as the fracture in the diaphyseal region would not have significant callus formation.

Micro-CT analysis

At the end of the experiment, a complete bone union could be seen in the high-resolution micro-CT images of all the groups. Qualitatively, representative three-dimensional reconstructed image showed greater callus volumes in all treatment groups of PC, 0.5 MHz, and 1.5 MHz compared to the control group. The 0.5 MHz group exhibited the largest apparent callus volume compared to the other groups (Fig. 12a). Bone densitometry was performed by using the Micro-CT built-in software. Significantly higher BV/TV and BMD values were detected in the 0.5 MHz group compared with the control group (Fig .12b−c) (p<0.05); and an apparent trend of higher Tb.Th and Tb.N and lower Tb.Sp were shown in the quantitative analysis (Fig. 12d −f). This indicated greater bone regeneration at the fracture site of the 0.5 MHz group compared to the other groups. The higher BMD rate in the 0.5 MHz group indicated that LIPUS could help to accelerate bone mineral density if the acoustic intensity was delivered consistently at the fracture site. The values of Tb.N and Tb.Th were similar in the PC and 1.5 MHz groups. However, no statistical significance detected between the Control, PC, and 1.5Hz groups with regard to all the parameters.

Mechanical test

The results of the four-point bending test after six weeks of treatment indicated that the values of ultimate load, stiffness, and energy to failure

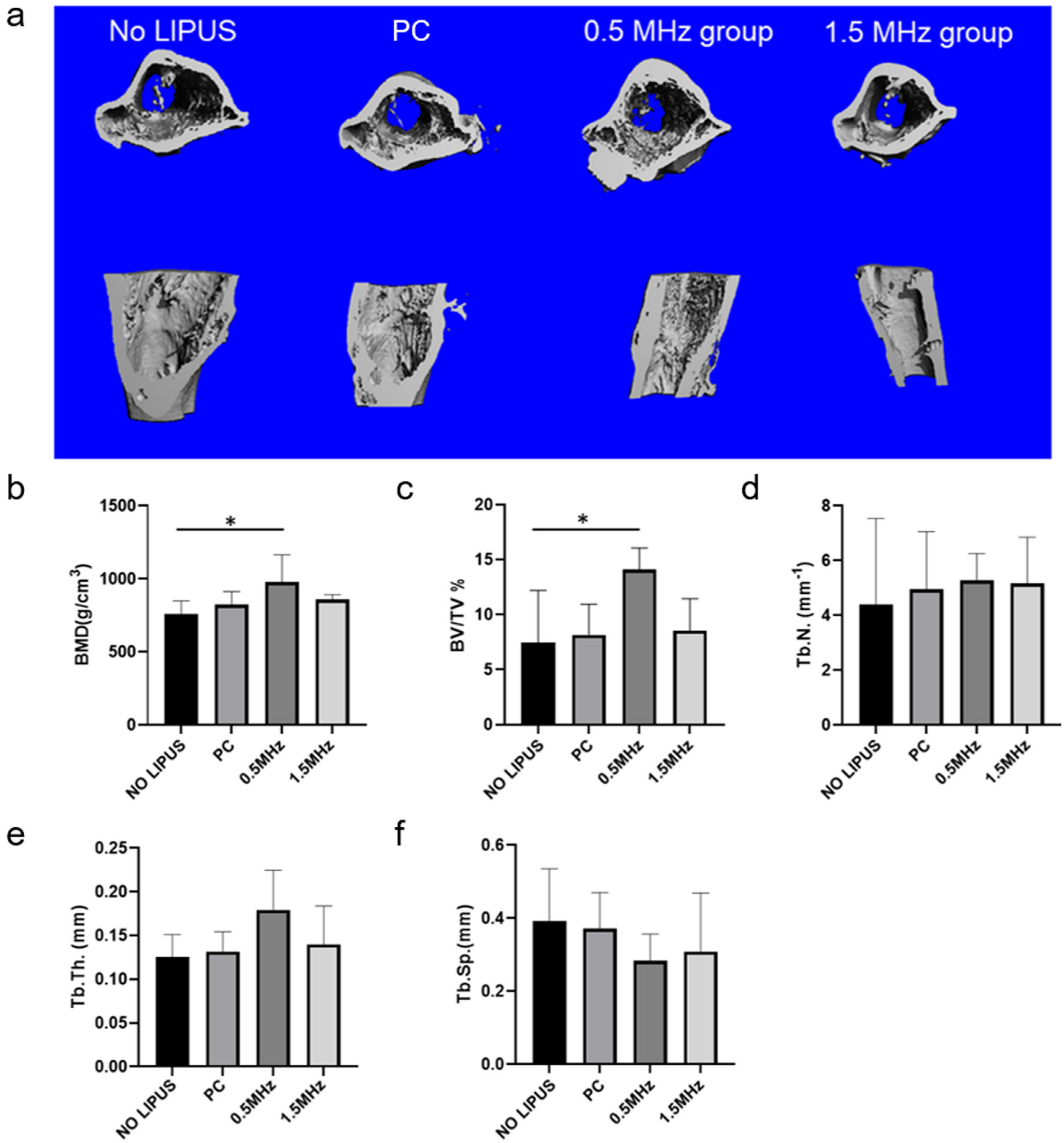

Figure 12. Micro-CT analysis of (a) representative 3D-reconstructed images of the fracture callus. (b-f) Statistical comparison of the micro-CT data, including (b) BMD, (c) BV/TV, (d) Tb.N, (e) Tb.Th, and (f) Tb.Sp. *$p<0.05$.

were higher in the treatment groups compared with the control group (Fig. 13a–c). Specifically, significant increase in values were observed in the 0.5 MHz group ($p<0.001$) and the 1.5 MHz group ($p<0.05$). Compared with the PC group, relatively higher values of ultimate load, stiffness, and energy to failure were detected in the 1.5 MHz group.

Histological examination

Histological evaluation (Fig. 14a–b) indicated significantly higher osteocyte number in the treatment groups compared with the control group ($p<0.05$). The highest value was found in the 0.5 MHz group, and more osteocytes were observed in the 0.5 MHz and 1.5 MHz groups compared with the PC group.

Discussion

This study aimed to achieve improved therapeutic effects by adjustment of the LIPUS signal to produce a constant acoustic intensity of stimulation at deeper fracture sites, with compensation for the attenuation caused by soft tissues. We also characterized the attenuation of LIPUS signals in human and animal soft tissues to provide a reference for the compensation. According to the measured acoustic intensity and calculated attenuation, the intensity was reduced by 41% (2.3 dB) (Fig. 8) in the commercial LIPUS device after passing through a 2 cm tissue layer, which is the maximum depth of the soft tissue layer covering the fractured bone in the present animal model during the treatment period. The improved bone histomorphometry and mechanical testing results

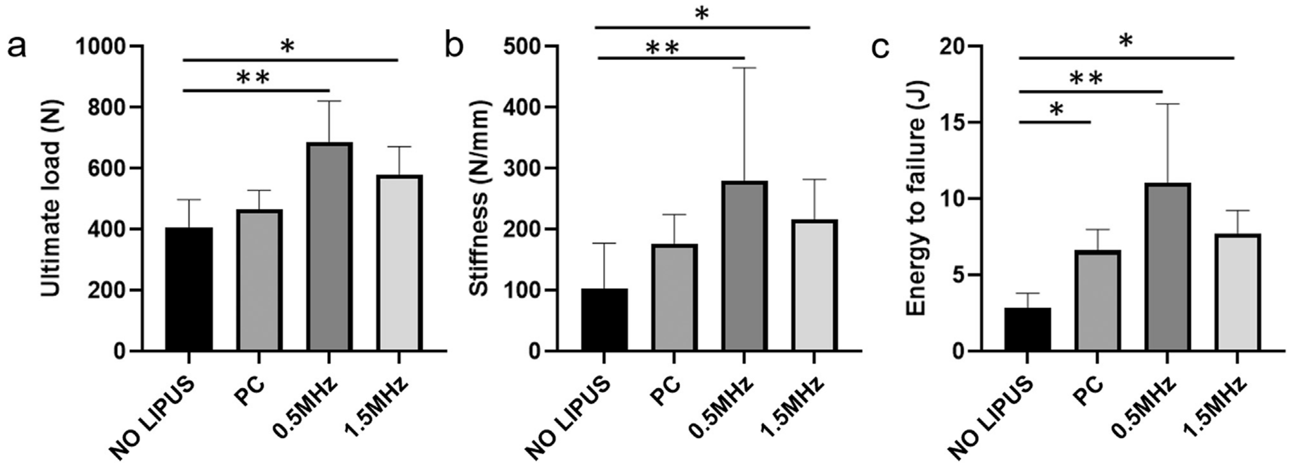

Figure 13. Mechanical properties, including (a) ultimate load, (b) stiffness, and (c) energy to failure were tested in all groups. *$p<0.05$, **$p<0.001$.

obtained in the animal experiments demonstrated the usefulness of the PC device under these conditions, despite the lack of optimization due to the reduction in energy reaching the bone. Therefore, considering that the soft tissue covering the hip joint is much thicker in human subjects, it is predicted that the PC device may not be effective enough.

The 1.5 MHz group demonstrated slightly but consistently better performances for all the parameters when a consistent intensity of 30 mW/cm^2 was maintained at the fracture site compared with the PC group. The major finding of this study is that maintenance of the ultrasound intensity to be constant at 30 mW/cm^2 at the fracture surface, instead of at the transducer surface, is important, and this would be more important when the thickness of tissue is increased. To the authors' knowledge, this is the first study to indicate the enhanced fracture healing effects of constant LIPUS intensity at the fractured bone surface. A rabbit model was used in the *in vivo* experiments where the degree of acoustic attenuation determined from the porcine tissue measurements was used to compensate for the attenuation caused by the tissues covering the rabbit hip region. The ultrasound transmitting power was adjusted according to the tissue thickness measured before each treatment session to achieve a constant acoustic intensity of 30 mW/cm^2 at the surface of the fractured bone. There is a possibility that there may be some differences in the acoustic attenuation between rabbit and porcine tissues, and this might lead to the actual acoustic intensity applied by the transducers being slightly different from 30 mW/cm^2. Since this uncertainty would affect all the three groups of ultrasound stimulation, it should not influence the observed effects and the major findings of this study. Nevertheless, it would be worthwhile to measure the acoustic attenuation and other acoustic parameters in rabbit tissues in future studies. In the present study, we did not investigate the possible side effects of thermal effects that may have occurred during the treatment. However, many

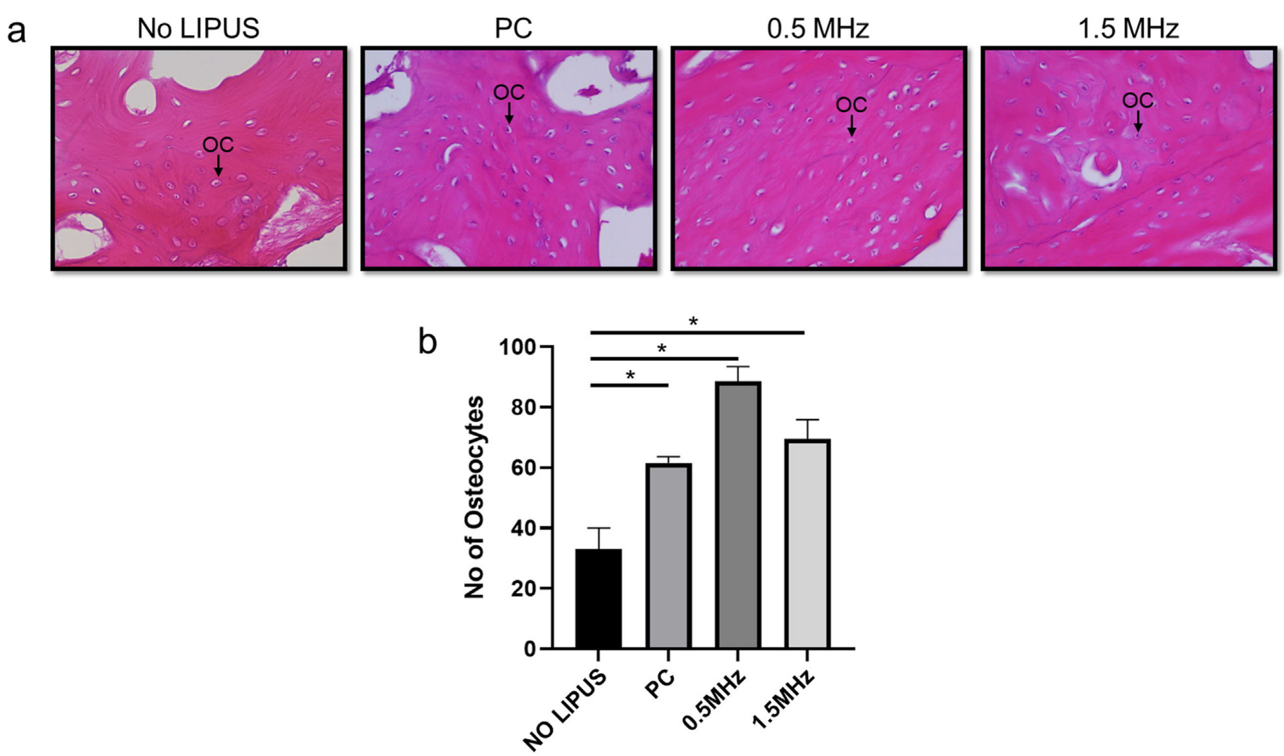

Figure 14. Histological assessments in all groups. (a) Representative images of haematoxylin and eosin (H&E) staining of the targeted bone region for quantification of osteocytes (OC), and (b) Quantification of osteocyte numbers. *$p<0.05$.

studies have demonstrated the safety of low intensities for treatment. Complications, such as necrosis, cessation of bone healing, and the formation of fibrous tissue [12,29], are so serious that thermal effects should be investigated in future studies before the proposed compensation method is implemented in human trials. Furthermore, it was found here that the ultrasound frequency of 0.5 MHz was more effective in the fracture healing of hip bone, together with a consistent intensity of 30 mW/cm^2 at the fractured bone surface. Several previous studies that used 0.5 MHz and 1.5 MHz revealed that 1.5 MHz was more effective for fracture healing [30,31]. However, the results of this study are the reverse of those of previous reports. One potential reason is that the previous studies only used ultrasound treatment to heal the fracture for approximately three weeks, which might not have been sufficient for fracture healing. Another primary reason may be linked to morphological changes in the bone during the healing stages. Hard callus is generally formatted and covered at the fractured bone. Although the acoustic intensity at the bone surface is the same, a lower ultrasound frequency may have better penetration through the hard callus and reach deeper region for treatment. Hence, ultrasound at a frequency of 0.5 MHz, with lower attenuation in the healing bone, may have a better capacity to reach the deeper parts of the bone. This is probably the primary reason why the 0.5 MHz group exhibited superior performances in this study, even though for both the 0.5 MHz and 1.5 MHz groups, the frequencies were maintained with constant intensities at the fracture site. Furthermore, the comparison of the effects of stimulation with the longer 0.5 MHz and shorter 1.5 MHz wavelengths in the callus and fracture gap during healing requires further investigation. A further point for additional investigation is whether ultrasound with frequencies lower than 0.5 MHz can provide an even better performance. Moreover, this proof-of-concept pre-clinical study confirmed that the attenuation of LIPUS caused by soft tissues can be solved by adjustment of the frequency and acoustic energy, and LIPUS can be delivered to a deeper fracture site with the same effective dose to enhance bone regeneration. As the current study used a small animal model, the findings of this study should be verified in a greater number of larger animals as well as human subjects.

In addition to the ultrasound frequency, the distribution of the acoustic field may also affect the therapeutic effects in relation to bone healing. As shown in Figure 9, the pattern of the acoustic field generated by the 0.5 MHz transducer exhibited a more concentrated intensity at the central region, whereas the 1.5 MHz transducer exhibited a ring link pattern, i.e., the energy was more distributed across the region of interest. In our *in vitro* study, we acquired the intensity map after the ultrasound beam had passed through a 5 cm layer of porcine tissue to mimic the ultrasound beam reaching the surface of fractured bone, as well as deeper into the fracture location. The average trochanteric soft tissue thickness of hip fracture cases is 2.9 ± 1.1 cm in men [24] and 4.0 ± 1.6 cm in women [25]. The focal distance of the 0.5 MHz transducer was 5.2 cm, and that of 1.5 MHz was 15.6 cm. Within the thickness range of trochanteric tissue, the 0.5 MHz transducer appears to have a more concentrated intensity at the central region, while the 1.5 MHz transducer may have a more scattered pattern. Therefore, it is important to control the intensity distribution in future human studies by using suitable dimensions for unfocused or focused transducers. For the *in vivo* study using the rabbit model, it was noted that the average thickness of the soft tissue covering the fracture location was between 1 and 2 cm during the entire experimental period. We also conducted scanning to map the acoustic intensity at distances of 1, 1.25, 1.5, 1.75, and 2 cm from the transducer surface for both the 0.5 MHz and 1.5 MHz transducers (shown in Fig. 10). While the I_{SATA} values did not change markedly among the scans, the intensity distribution patterns were significantly different for both transducers when the distance changed. This phenomenon is consistent with the theory regarding near-field intensity mapping. Considering that the region of interest had a diameter of 2.2 cm, and the dimension of the fracture region in the animal model could increase up to 1.6 cm due to callus formation, the overall therapeutic effect of ultrasound should rather be related to I_{SATA}, instead of I_{SPTA}. Hence, it would be worthwhile to investigate the therapeutic effects of different stimulating patterns. Furthermore, the exposure conditions differed slightly between the *in vitro* and *in vivo* experiments due to changes in the offset distance and lack of standoff to compensate for the tissue attenuation, which may have resulted in variable treatment effects. Therefore, all these factors should be systematically investigated in future studies.

Conclusions

The findings of this study demonstrated that a constant intensity of 30 mW/cm^2 at the fractured bone surface was achievable by adjustment of the transmitting power according to the thickness of the soft tissue and attenuation for different frequencies, thus making LIPUS treatment possible for fractures at deeper locations such as the hip region. The results showed that 1.5 MHz LIPUS maintaining a constant intensity of 30 mW/cm^2 at the fracture location performed better than 1.5 MHz with a fixed transmitting intensity of 30 mW/cm^2. In addition, LIPUS with 0.5 MHz achieved significantly better results than the no-LIPUS group but not significantly better than 1.5 MHz, even though both the 0.5 and 1.5 MHz groups used a constant intensity of 30 mW/cm^2 at the fracture location. More investigations should be conducted to elucidate the mechanism responsible for the better performance of the lower frequency LIPUS. The differences in the distribution of acoustic intensity within the ROI in the ultrasound beam for the transducers with different frequencies also requires further investigation. In addition, studies on human subjects need to be conducted for further confirmation of the findings of this study prior to use in clinical applications.

Conflict of interest

All the authors confirmed no known conflicts of interest are associated with this publication and there has been no significant financial support for this study.

Data availability statement

The data of this study are available from the corresponding author upon reasonable request.

Acknowledgments

This project was substantially supported by a grant from the Research Grants Council of the Hong Kong Special Administrative Region, China (Ref No. T13-402/17-N, AoEM-40220). Study design (PP, SL, SKHC), data collection (PP, SL, MMCL), data analysis (PP, SL), writing (PP, SL, SKHC, QTSS, MMCL), supervision (YPZ, LQ, LWHC). All the authors have read and approved the final submitted manuscript.

References

[1] Rapp K, Büchele G, Dreinhöfer K, Bücking B, Becker C, Benzinger P. Epidemiology of hip fractures. Zeitschrift für Gerontologie und Geriatrie 2019;52(1):10–6.
[2] Johnell O, Kanis J. An estimate of the worldwide prevalence and disability associated with osteoporotic fractures. Osteoporos Int 2006;17(12):1726–33.
[3] Kannus P, Parkkari J, Sievänen H, Heinonen A, Vuori I, Järvinen M. Epidemiology of hip fractures. Bone 1996;18(1):S57–63.
[4] Tai T-W, Lin Y-H, Chang C-J, Wu C-H. The economic burden of hip fractures in Asia. Osteoporos Sarcopenia 2021;7(2):45.
[5] Harrison A, Lin S, Pounder N, Mikuni-Takagaki Y. Mode & mechanism of low intensity pulsed ultrasound (LIPUS) in fracture repair. Ultrasonics 2016;70:45–52.
[6] Palanisamy P, Alam M, Li S, Chow SK, Zheng YP. Low-intensity pulsed ultrasound stimulation for bone fractures healing: a review. J Ultrasound Med 2022;41(3):547–63.
[7] Leighton R, Watson JT, Giannoudis P, Papakostidis C, Harrison A, Steen RG. Healing of fracture nonunions treated with low-intensity pulsed ultrasound (LIPUS): A systematic review and meta-analysis. Injury 2017;48(7):1339–47.
[8] Gebauer D, Mayr E, Orthner E, Ryaby JP. Low-intensity pulsed ultrasound: effects on nonunions. Ultrasound Med Bio 2005;31(10):1391–402.

[9] Watanabe Y, Matsushita T, Bhandari M, Zdero R, Schemitsch EH. Ultrasound for fracture healing: current evidence. J Ortho Trauma 2010;24:S56–61.
[10] Haglin JM, Jain S, Eltorai AE, Daniels AH. Bone growth stimulation: a critical analysis review. JBJS Rev 2017;5(8):e8.
[11] Pohlman R, Richter R, Parow E. Über die Ausbreitung und Absorption des Ultraschalls im menschlichen Gewebe und seine therapeutische Wirkung an Ischias und Plexusneuralgie. DMW-Deutsche Medizinische Wochenschrift 1939;65(07):251–4.
[12] Bender L, Janes J, Herrick J. Histologic studies following exposure of bone to ultrasound. Arch Phys Med Rehab 1954;35(9):555–9.
[13] Shiro I. Study on the ultrasonic irradiation in orthopedic surgery. Hirosaki Med J 1964;16:242–53.
[14] Heckman JD, Ryaby JP, McCabe J, Frey JJ, Kilcoyne RF. Acceleration of tibial fracture-healing by non-invasive, low-intensity pulsed ultrasound. JBJS 1994;76(1):26–34.
[15] Kristiansen TK, Ryaby JP, McCABE J, Frey JJ, Roe LR. Accelerated healing of distal radial fractures with the use of specific, low-intensity ultrasound. A multicenter, prospective, randomized, double-blind, placebo-controlled study. JBJS 1997;79(7):961–73.
[16] Lou S, Lv H, Li Z, Zhang L, Tang P. The effects of low-intensity pulsed ultrasound on fresh fracture: a meta-analysis. Medicine 2017;96(39):e8181.
[17] Schofer MD, Block JE, Aigner J, Schmelz A. Improved healing response in delayed unions of the tibia with low-intensity pulsed ultrasound: results of a randomized sham-controlled trial. BMC Musculoskeletal Disord 2010;11:1–6.
[18] Romano CL, Romano D, Logoluso N. Low-intensity pulsed ultrasound for the treatment of bone delayed union or nonunion: a review. Ultrasound Med Bio 2009;35(4):529–36.
[19] Nolte P, Maas M, Roolker L, Marti R, Albers G, Biostatistician A. Effect of low-intensity ultrasound on bone healing in osteotomies of the lower extremity: a randomised trial. Nolte PA, thesis. Nonunions−Surgery and low-intensity ultrasound treatment. The Netherlands: Universiteit van Amsterdam. Thela Thesis Publishers Amsterdam; 2002. p. 96–106.
[20] Makaruk H, Sacewicz T. Effects of plyometric training on maximal power output and jumping ability. Human Movement 2010;11(1):17–22.
[21] Wolbarst AB. Physics of radiology. United State: McGraw-Hill/Appleton & Lange; 1993.
[22] Wells P. Absorption and dispersion of ultrasound in biological tissue. Ultrasound Med Bio 1975;1(4):369–76.
[23] Cone SG, Warren PB, Fisher MB. Rise of the pigs: utilization of the porcine model to study musculoskeletal biomechanics and tissue engineering during skeletal growth. Tissue Eng Part C: Methods 2017;23(11):763–80.
[24] Nielson CM, Bouxsein ML, Freitas SS, Ensrud KE, Orwoll ES, Group OFiMR. Trochanteric soft tissue thickness and hip fracture in older men. J Clin Endocrinol Metab 2009;94(2):491–6.
[25] Bouxsein ML, Szulc P, Munoz F, Thrall E, Sornay-Rendu E, Delmas PD. Contribution of trochanteric soft tissues to fall force estimates, the factor of risk, and prediction of hip fracture risk. J Bone Mineral Res 2007;22(6):825–31.
[26] Xie X-H, Wang X-L, Zhang G, Liu Z, Yao D, Hung L-K, et al. Impaired bone healing in rabbits with steroid-induced osteonecrosis. J Bone Joint Surg Br Vol 2011;93(4):558–65.
[27] Wong RMY, Choy VMH, Li J, Li TK, Chim YN, Li MCM, et al. Fibrinolysis as a target to enhance osteoporotic fracture healing by vibration therapy in a metaphyseal fracture model. Bone Joint Res 2021;10(1):41–50.
[28] Choy MHV, Wong RMY, Li MC, Wang BY, Liu XD, Lee W, et al. Can we enhance osteoporotic metaphyseal fracture healing through enhancing ultrastructural and functional changes of osteocytes in cortical bone with low-magnitude high-frequency vibration? FASEB J 2020;34(3):4234–52.
[29] ARDAN JR NI, JANES JM, Herrick J. Ultrasonic energy and surgically produced defects in bone. JBJS 1957;39(2):394–402.
[30] Wang SJ, Lewallen DG, Bolander ME, Chao EY, Ilstrup DM, Greenleaf JF. Low intensity ultrasound treatment increases strength in a rat femoral fracture model. J Ortho Res 1994;12(1):40–7.
[31] Lai C-H, Chuang C-C, Li JK-J, Chen S-C, Chang WH-S. Effects of ultrasound on osteotomy healing in a rabbit fracture model. Ultrasound Med Bio 2011;37(10):1635–43.