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A Feasibility of Low Intensity Ultrasound Stimulation for Treatment or Prevention of Osteoporosis and Its-Related Fracture

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1. Introduction

Osteoporosis is characterized by low bone mass and deterioration of bone architecture, resulting in increased bone fragility and risk for bone fracture. This disease is associated with significant morbidity and mortality and has become a major public health concern. Osteoporosis and its-related fractures are an important public health concern; increasing in physical and/or psychological problems (depression, chronic disabling pain, fear and anxiety) as well as difficulty of the activities of daily life (NIH Consensus Development Panel on Osteoporosis Prevention Diagnosis and Therapy, 2001; Totosy de Zepetnek et al., 2009; WHO (World Health Organization), 2004). In addition, they cause increase in morbidity and mortality, and decrease in functional mobility and thereby reduction in quality of life (QOL) (Tosteson et al., 2008). Also, directly/indirectly financial expenditures for treating and caring of osteoporosis and its-related fracture are increasing over time.

Pharmacological interventions are widely used to treat and prevent osteoporosis and its-related fracture clinically. However, such interventions can be accompanied with undesirable side effects. The long-term estrogen replacement therapy may increase in a risk of breast or ovarian cancer or venous thromboembolism (Grady et al., 2004; Nelson et al., 2002; Noller, 2002; Schairer et al., 2000). The bisphosphonates may cause osteonecrosis of the jaw, a syndrome of myalgias and arthralgias, and gastrointestinal intolerance (Khosla et al., 2007; Lewiecki, 2010; Wysowski,Chang, 2005), and induce osteopetrosis in a child (Marini, 2003; Whyte et al., 2003; Whyte et al., 2008). Calcium and vitamin D supplementation might not be effective for reduction of osteoporotic bone fracture (Porthouse et al., 2005). Furthermore, inadequate vitamin D supplementation may increase in vascular calcification (Tang et al., 2006;Zittermann et al., 2007). Therefore, alternatives to pharmacological interventions are required for reduction of the adverse side effects.

Mechanical signals are the most important one of extrinsic factors for regulating bone homeostasis (Dufour et al., 2007;Judex et al., 2009). The relation between mechanical signal

and bone homeostasis is elucidated by the mechanostat theory and the daily stress stimulus theory (Frost, 1987, 2003, 2004;Qin et al., 1996;Qin et al., 1998). The former describes that a net bone is regulated by the strain or deformation applied to the skeleton. In the latter theory, a net bone is modulated by a daily stress stimulus (considering both the magnitude as well as the number of cycles on loading) applied to the skeleton. Therefore, when strain applied on skeleton is bigger than target strain, the daily stress stimulus is bigger than some target stimulus, or a low magnitude and high cycle number, a net bone can be increased. Based on these rationales, external biophysical stimuli have been suggested as alternatives to pharmacological therapies. Ultrasound stimulation is one such promising stimulus (Monici et al., 2007;Perry et al., 2009;Rubin et al., 2001b).

Ultrasound is a high-frequency non-audible acoustic pressure wave with mechanical energy and can be transmitted at osteoporotic sites through biological tissues. It has been applied clinically for diagnosis or operation (Rubin et al., 2001a). Several *in-vivo* studies showed its therapeutic potential. LIUS could improve the defective or damaged bone healing; enhancement of the mechanical properties on the healing callus, bone bridging, nonunion fractures healing and distraction osteogenesis and reduction of healing time (Eberson, 2003;Pilla et al., 1990). Furthermore, in vitro cellular studies supported these in-vivo results (Kokubu et al., 1999;Li et al., 2003;Monici et al., 2007;Naruse et al., 2000;Unsworth et al., 2007;Yang et al., 2005) . LIUS could regulate bone cells; enhancing osteoblast formation and function and suppressing osteoclast formation and function.

Thus, LIUS may be useful for treatment or prevention of osteoporosis and its-related fracture. However, there are arguments about the effects of LIUS on osteoporotic bone. Carvalho and Cliquet (Carvalho,Cliquet Jr, 2004) and Perry et al. (Perry et al., 2009) suggested that LIUS might be beneficial in osteoporotic bone, but not in Warden *et al.*(Warden et al., 2001). The reasons of differences in the effects of osteoporotic bone were unclear. These arguments may be attributable to the several intrinsic and extrinsic limitations of experimental and analytic methodologies. For examples, bone architecture is heterogeneous and variable individually, but the previous studies did not consider those. For evaluation of the effects of LIUS on osteoporotic bones, histomorphometric analysis was widely performed. However, it has several limitations such as analysis of a few fields of view and impossibility of longitudinal analysis of identical specimen, but there were lacks of longitudinal studies j23. Moreover, bone adaptation with an identical bone is variable from location to location24, but there was no study on the effects of LIUS application considering irradiation location/direction of LIUS. Recently, *in-vivo* micro computed tomography (micro-CT) technique is widely used to investigate the longitudinal changes in 3D bone microarchitecture with overcoming these limitations. Finally, there was no study on longitudinal changes in mechanical strength of osteoporotic bone and on prediction of bone fracture risks after LIUS treatment. Finite element (FE) analysis is widely used to evaluate longitudinal changes in bone mechanical or behavior characteristics and predict bone fracture risks.

This study aimed to address such limitations in the previous studies and determine whether LIUS therapy cans effective for treatment or prevention of osteoporosis and its-related fracture based on in-vivo micro-CT technology and FE analysis.

2. Materials and method

2.1 Animal preparation

Eight 14-week-old virginal ICR mice (weighing approximately 24.0 ± 0.7 g) were ovariectomized (OVX) to induce osteoporosis. Osteoporosis was confirmed at 3 weeks after

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OVX by changes in the bone biomechanical characteristics (52.2% decrease in bone volume fraction (BV/TV, Fig. 1) (van der Jagt et al., 2009) and 16.8% decreased in effective structural modulus relative to before OVX). All procedures were performed under a protocol approved by the Yonsei University Animal Care Committee (YWC-P107).
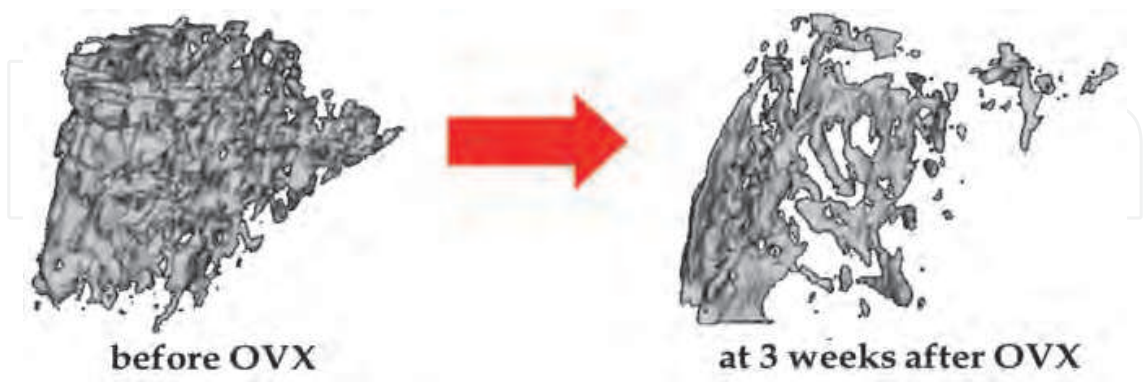

Fig. 1. Changes in trabecular bone structure over time induced by OVX

2.2 Application of LIUS

The right tibiae of each mouse were treated using LIUS (US group), whereas the left tibiae were not treated and served as an internal control (CON group). LIUS was composed of a pulse width of 200 μs containing 1.5MHz sine waves, with a repeated frequency of 1.0 kHz with a spatial-averaged temporal-averaged intensity of 30 mW/cm^2 (Warden, 2001;Warden et al., 2001). Application of LIUS was continued for 6 weeks and consisted of 20min/day and 5days/week. Before the application of LIUS, its output characteristics were measured by hydrophonic scanning. The mice were immobilized using a customized restrainer (David et al., 2003) and both tibiae were submerged in warm (35–40°C) water in a customized tank for the application of LIUS (Fig. 2) (Warden et al., 2001).
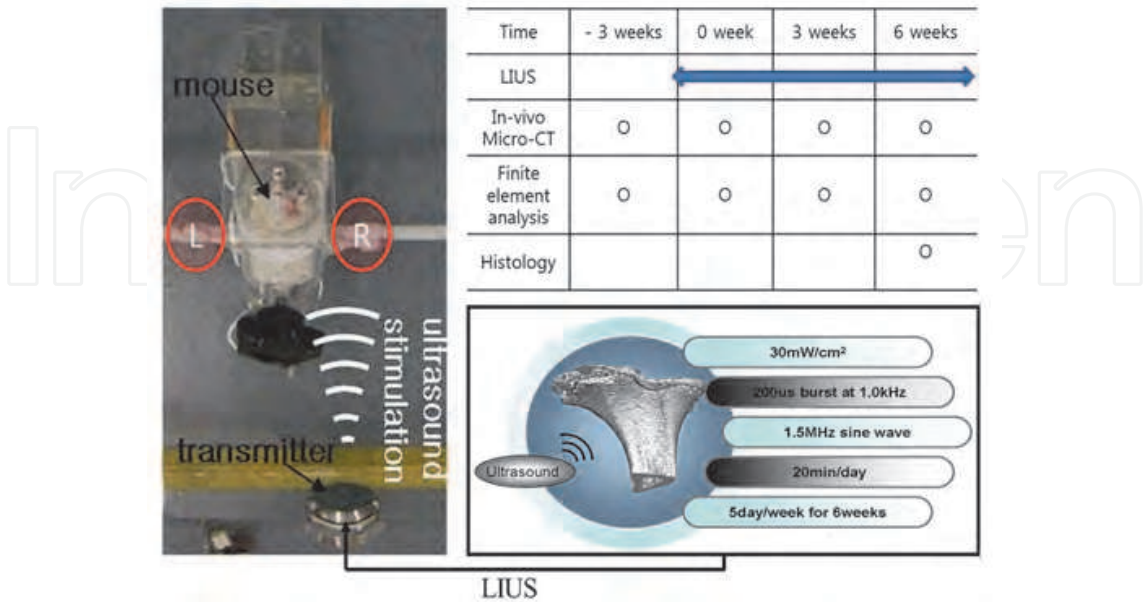

Fig. 2. Experiment setup, LIUS application, in vivo micro-CT scanning, finite element analysis, histology

2.3 Bone structural parameters analysis

Both tibiae of each mouse were scanned at 0, 3 and 6 weeks after application of LIUS, as shown in Fig.1, using an in vivo micro-computed tomography (CT; Skyscan 1076, SKYSCAN N.V., Aartselaar, Belgium) at a voxel resolution with 18 μm in each axis under anesthesia induced by ketamine (1.5 ml/kg, Huons, Seoul, Korea) and xylazine (0.5 ml/kg, Bayer Korea, Seoul, Korea). The volume of interest (VOI) was determined as following; trabecular bone corresponding to the proximal tibia was selected from a 1.8-mm length of bone, located 0.54 mm below the growth plate, and cortical bone corresponding to the diaphyseal tibia was selected from a 0.9 mm length of bone, located 2.88 mm below the growth plate (Fig. 3). To investigate changes in 3D structural characteristics, structural parameters for the trabecular and cortical bone of both tibiae were measured and calculated by using micro-CT images and CT-AN 1.8 software (Skyscan). For the entire trabecular bone, the BV/TV (%), trabecular thickness (Tb.Th, mm), trabecular number (Tb.N, mm-1), trabecular separation (Tb.Sp, mm), structure model index (SMI), and trabecular bone pattern factor (Tb.Pf, mm-1) were measured and calculated. Additionally, to determine whether the LIUS irradiation location/direction affected in detail, the two-dimensional (2D) cross-sectional images of the trabecular bone were subdivided into five regions of interest (ROIs, Fig. 3). The ROIs as they correspond to the maximum selectable diameter in the medullary cavity were 0.5 mm in diameter. The ROI locations are shown in Fig. 3, corresponding to the direction of LIUS application. For the entire cortical bone, cross-section thickness (Cs.Th, mm) and mean polar moment inertia (MMI, mm⁴) were measured and calculated. Additionally, the 2D cross-sectional micro-CT images of the cortical bone were subdivided into four ROIs, to determine whether the LIUS irradiation location/direction affected in detail, as described above (Fig. 3).
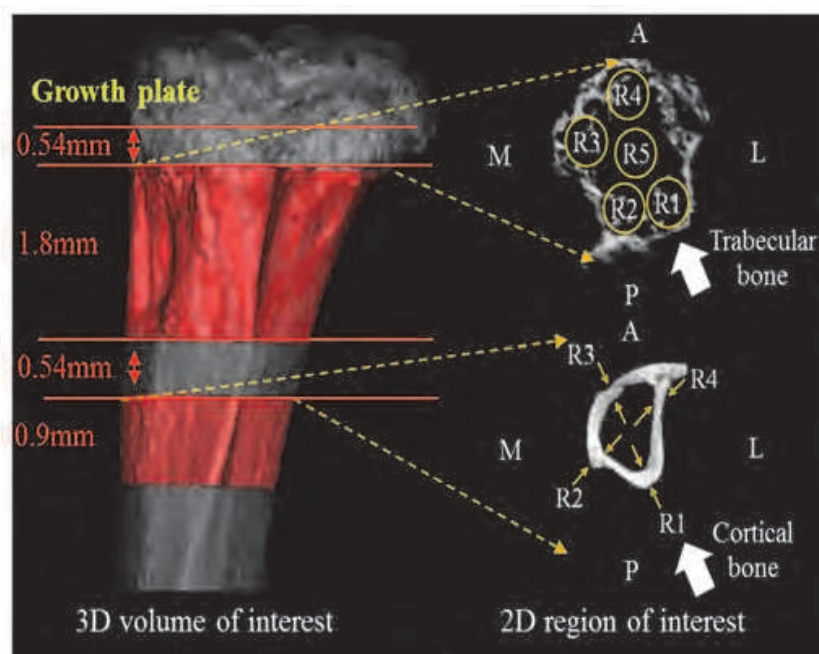

Fig. 3. Location of the 3 dimensional (3D) volume of interest (VOI) and the 2D regions of interests, R1: region 1, R2: region 2, R3: region 3, R4: region 4, R5: region 5, A: anterior, P: posterior, M: medial, L: lateral, white arrow: LIUS. (Figure was modified in Journal of Orthopaedic Research, 29(2011), 116-125)

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2.4 Elastic tissue modulus analysis

The elastic tissue modulus, which are related to bone quality, was determined form Hounsfield units, for each element calculated by equation (1) (Rho et al., 1995) using Mimics 12.3 software (Materialise, Leuven, Belgium). To quantitatively evaluate the degree of improvement in bone quality achieved using LIUS, its distributions were used.

$$E = 5.54 \times \rho - 326$$
$$(\rho = 0.916 \times HU + 114)$$

$$(1)$$

where ρ is the density, HU is Hounsfield Unit, and E is elastic tissue modulus.

2.5 Effective structural modulus analysis

Binary images of the tibia in each mouse were converted from μ-CT images using BIONIX (CANTIBio, Suwon, Korea). Then 3D tetrahedral FE models with 18 μm mesh size were generated using a mass-compensated thresholding technique (Ulrich et al., 1998). Material property of bone (Young's modulus: 12.5 GPa and Poisson's ratio: 0.3) (Kinney et al., 2000;Woo et al., 2009), which was assumed to be isotropic and perfectly elastic, was assigned into the FE models. To analyze FE models, a compressive displacement of an uniaxial 0.5% strain as displacement boundary conditions was applied to the FE models (Woo et al., 2009). All FE analyses were performed using the commercial FE software package ABAQUS 6.4 (HKS, Pawtucket, RI, USA).

2.6 Histomorphometric analysis

At the end of experiment, mice were sacrificed through cervical dislocation. Both tibiae were extracted, and surrounding tissues (skin, muscle, and tendons) were removed. To perform histology, routine procedures were followed. The first, the tibiae were fixed for 3 days in 10% neutral buffered formalin, treated with 10% formic acid for 1 h. The second, the fixed tibiae were decalcified with a 10% ethylenediaminetetraacetic acid solution and then embedded in paraffin. Then, each tibia was cut at a 4 micrometer sections (4-μm-thick) through the long axis in the sagittal plane with a microtome (Microm, Walldorf, Germany). Finally, Masson's trichrome (MT) stain was performed to visualize. Analyses were performed using a microscope (Olympus BX50, Tokyo, Japan) to evaluate new bone formation (blue: mature mineralization, red: uncompleted mineralization (osteoid)) and osteocytes. To quantity osteocyte, the number of osteocytes in a square (200 × 200 μm) was counted.

2.7 Statistical analysis

The structural parameters and effective structural modulus were compared using ANOVA with a mixed factorial design and repeated measures. A paired t-test was performed to compare the number of osteocytes and elastic tissue modulus between the US and CON groups. All descriptive data are represented as mean±standard error. All statistical analyses were performed with the SPSS 12.0 (Chicago, IL, USA). p Values <0.05 were considered significant.

3. Results

3.1 Structural changes

During experiment, there were no significant differences in the structural parameters of the trabecular and cortical bone in the US group over time (Fig. 4 ~ 7, p<0.05). However, in the

CON group, the BV/TVs and Tb.Ns significantly decreased over time, whereas the SMIs and Tb.Pfs significantly increased (p<0.05, Fig. 4). The BV/TVs on R1, R4 and R5 in trabecular bone and the Cs.Ths of all regions in cortical bone did not significantly change over time in both the US and CON groups (Fig. 5 and Fig. 7, p>0.05).
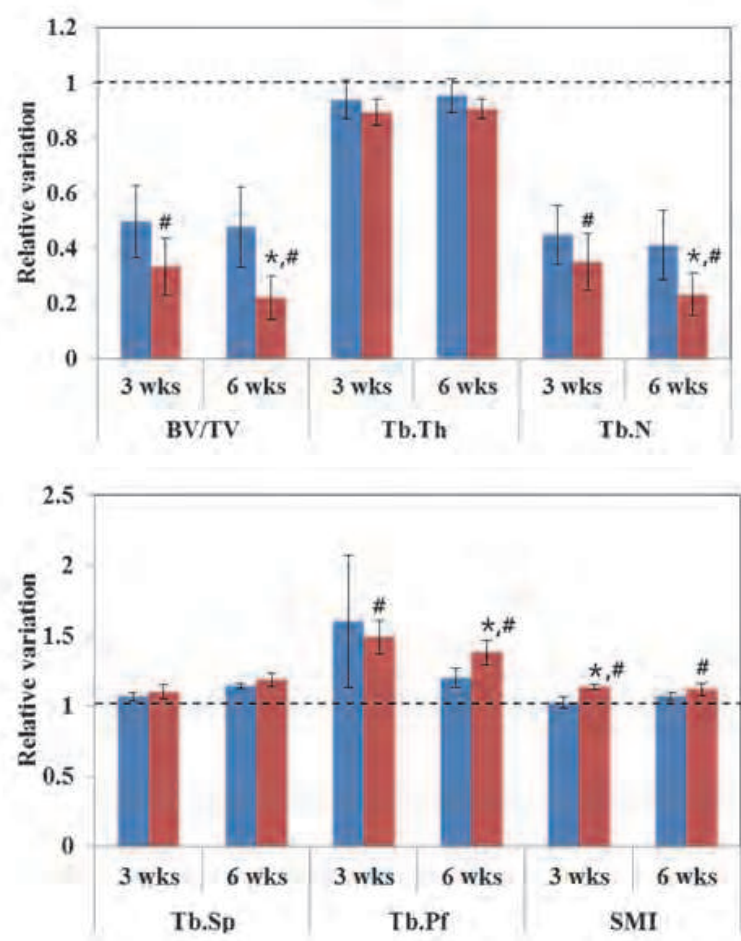

Fig. 4. Results of the structural parameter analysis for trabecular bone (mean ± standard error of relative variations), *significant difference between the US and CON groups (p<0.05), # significant difference in each group over time (p<0.05), blue bar: US group, red bar: CON group, dashed line: 1 at 0 week (Figures were modified in Journal of Orthopaedic Research, 29(2011), 116-125)

The relative variations were determined by calculating the degree of changes relative to the base line value, at 0 week (1 at 0 week). At week 3, the relative variation of the SMI in the US group was significantly smaller than that in the CON group (Fig. 4, p<0.05). However, there were no significant differences between the US and CON group for the other structural parameters (Fig.4, p>0.05). The relative variations of BV/TV, Tb.N and Tb.Pf in the US group were significantly bigger at 6 weeks after LIUS treatment than those in the CON group (Fig.4, p<0.05). However, there were no significant differences of the other structural parameters, Tb.Th, Tb.Sp and SMI, between the two groups (Fig.4, p>0.05). At week 3, the relative variation of the BV/TV in any of the five ROIs between groups did not shown significant differences (Fig.5, p>0.05). However, after 6 weeks of LIUS treatment, the relative variation of BV/TV in R1 was significantly bigger than those in the CON group (Fig.5,

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p<0.05), whereas there were no significant differences between groups in other regions, R2, R3, R4 and R5 (Fig.5, p>0.05).
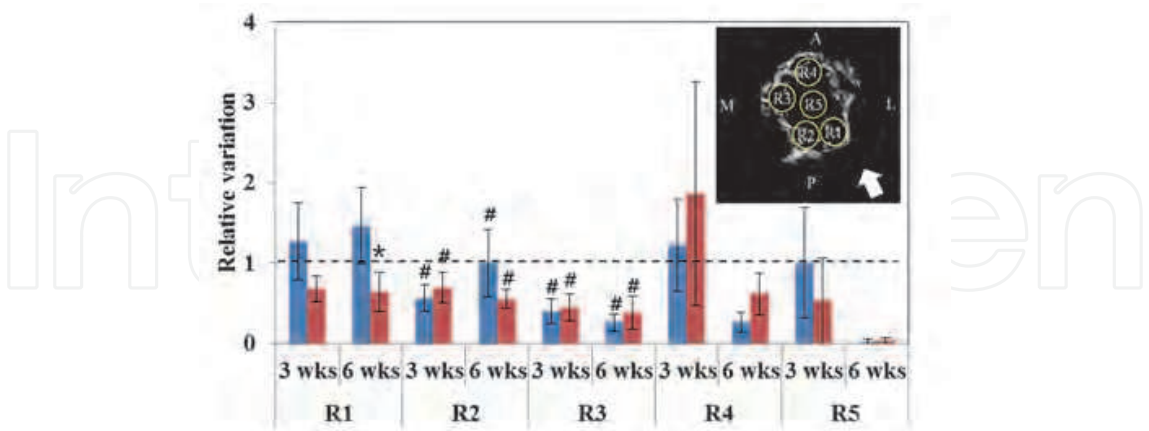

Fig. 5. Results of the BV/TV in five regions of interest for trabecular bone (mean ± standard error of relative variations), *significant difference between the US and CON groups (p<0.05), # significant difference in each group over time (p<0.05), blue bar: US group, red bar: CON group, dashed line: 1 at 0 week, A: anterior, P: posterior, M: medial, L: lateral, white arrow: LIUS (Figure was modified in Journal of Orthopaedic Research, 29(2011), 116-125)

The relative variations of the Cs.Th and MMI for cortical bone were significant differences between the US and CON groups following 3 weeks of treatment (Fig.6, p>0.05). However, in the US group after 6 weeks of LIUS treatment, the relative variation in the MMI was significantly higher than that in the CON group (Fig.6, p<0.05), whereas there were no differences of the relative variation in the Cs.Th between groups (Fig.6, p>0.05). In the regional analysis, at 3 weeks after LIUS, the relative variation in Cs.Th in R2 was significantly increased compared with the CON group (Fig. 7, p<0.05). However, there were no significant differences of Cs.Th in the other regions between groups (Fig. 7, p>0.05). At 6 weeks after LIUS, the relative variations in Cs.Th in R1 and R2 were higher in US group than CON group (Fig. 7, p>0.05), whereas no differences of Cs.Th in the R3 and R4 were observed between groups (Fig. 7, p>0.05). In the US group, the structure of tibia tends to maintain over time, and new bone formation was observed relative to in the CON group as shown in Fig. 8.
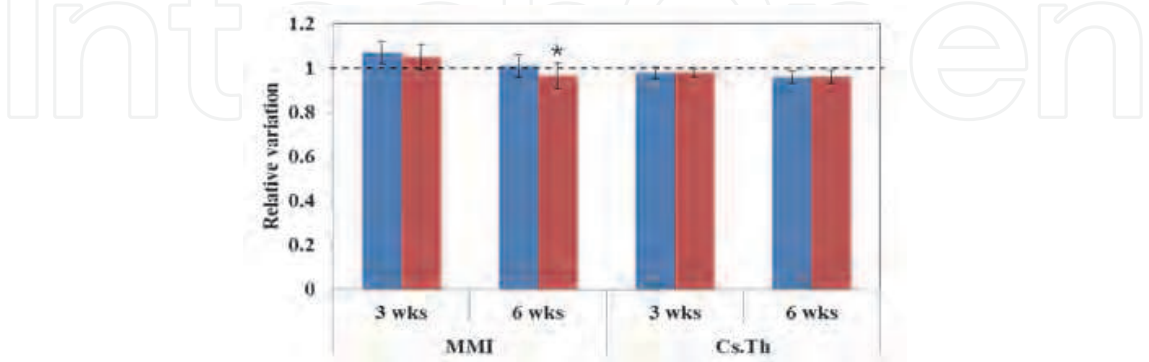

Fig. 6. Results of the structural parameter analysis for cortical bone (mean ± standard error of relative variations), *significant difference between the US and CON groups (p<0.05), blue bar: US group, red bar: CON group, dashed line: 1 at 0 week (Figure was modified in Journal of Orthopaedic Research, 29(2011), 116-125)
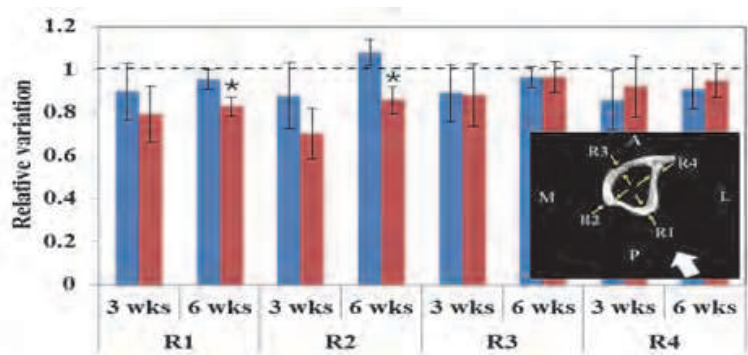
Fig. 7. Results of the Cs.Th in four regions of interest for trabecular bone (mean ± standard error of relative variations), *significant difference between the US and CON groups (p<0.05), # significant difference in each group over time (p<0.05), blue bar: US group, red bar: CON group, dashed line: 1 at 0 week, A: anterior, P: posterior, M: medial, L: lateral, white arrow: LIUS (Figure was modified in Journal of Orthopaedic Research, 29(2011), 116-125)
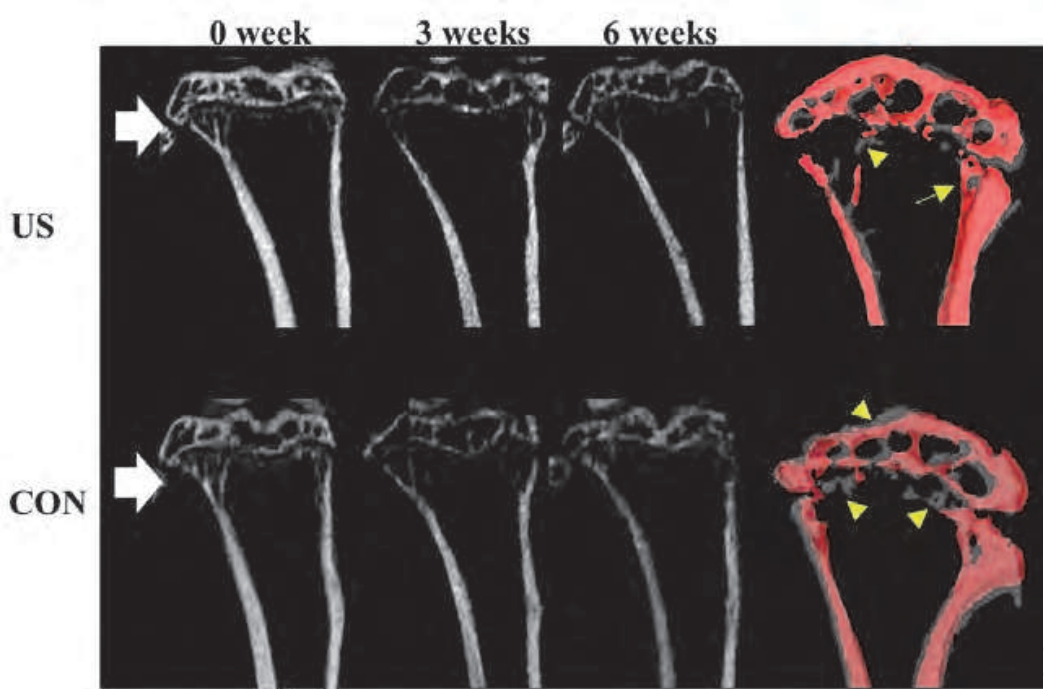

Fig. 8. Representative the changes in bone structure over time in the US and CON groups, overlaid images; 0 week (white) and 6 weeks (red), white arrow: LIUS, yellow arrow head: bone resoprtion, yellow arrow: bone maintaining (Figure was modified in Journal of Orthopaedic Research, 29(2011), 116-125).

3.2 Elastic tissue modulus changes

The volumes of the higher elastic tissue modulus (3046 and 3723 MPa) in the US group were significantly increased relative to those in the CON group (Fig. 9, p<0.05). However, the volume of lower elastic tissue modulus (2369 MPa) in the US group was less than that in the CON group (Fig. 9, p<0.05). Fig. 10 shows the changes of 3D structure of tibia mapped by elastic tissue moduli. The distribution or volume of high values tends to increase in the US group over time, but it tends to decrease in the CON group.
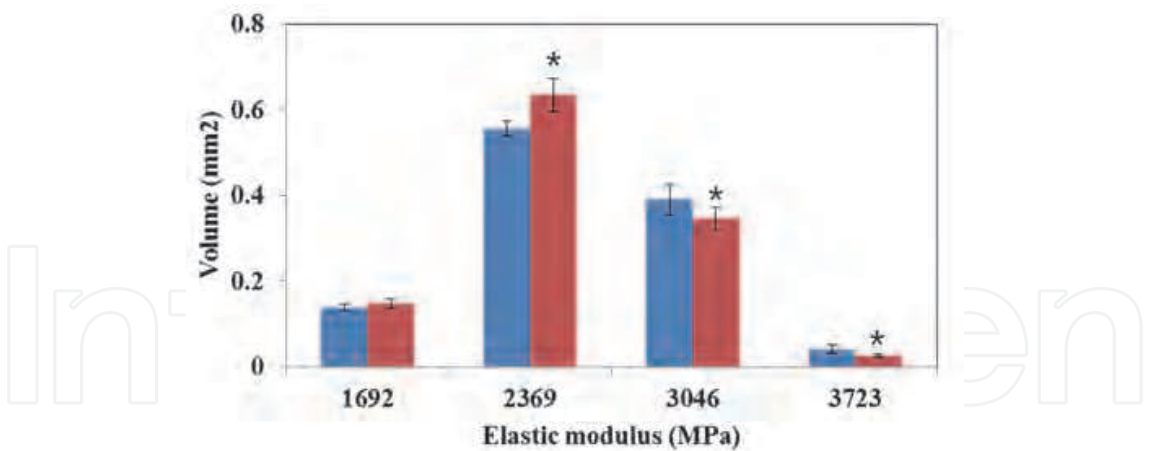
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Fig. 9. Results of the elastic tissue modulus (mean ± standard error of relative variations),
*significant difference between the US and CON groups (p<0.05), blue bar: US group, red
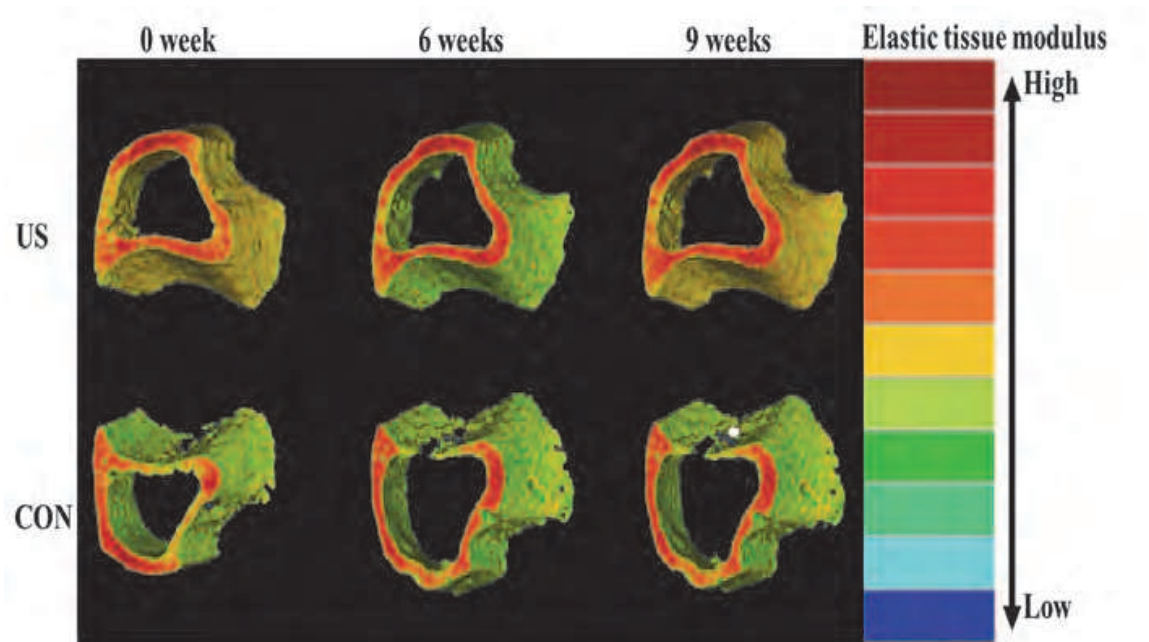bar: CON group (Figure was modified in Annals of Biomedical Engineering, 38 (2010), 2438-
2446)

Fig. 10. Representative distributions of the elastic modulus in the CON and US over time
(Figure was modified in Annals of Biomedical Engineering, 38 (2010), 2438-2446)

3.3 Effective structural modulus changes

During experiment, the effective moduli were significant differences over time in the US and
CON group (Fig. 11, p<0.05). The effective modulus was gradually increased in the US
group over time, whereas it was increased at 3 weeks and was decreased at 6 weeks at the
CON group. At 3 weeks after LIUS, there was no significant difference of the relative
variation in the effective structural modulus between groups (Fig. 11, p>0.05). However, the
relative variation in structural modulus in the US group was significantly bigger than that in
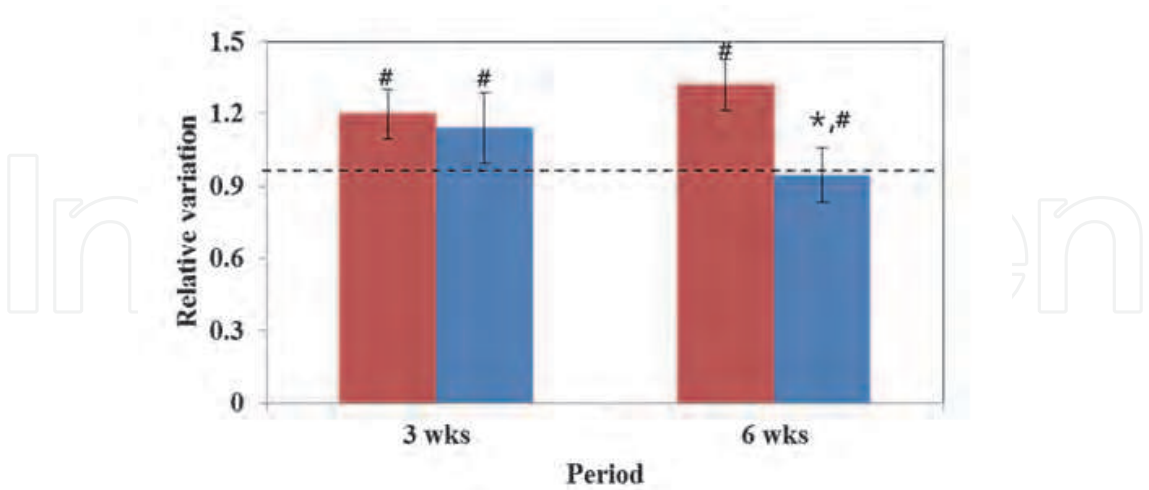the CON group (Fig. 11, p<0.05)

Fig. 11. Results of the effective structural modulus (mean ± standard error of relative variations), *significant difference between the US and CON groups (p<0.05), blue bar: US group, red bar: CON group, dashed line: 1 at 0 week (Figure was modified in Annals of Biomedical Engineering, 38 (2010), 2438-2446)

3.4 Histomorphometric analysis

More osteoid, incomplete bone mineralization, in the US group was observed relative to that the CON group (Fig. 12). Also, the trabeculae and the endosteal cortical bone, which is the near region directly treated with LIUS, in the US group were thicker than those in the CON group. These structural improvements are consistent with the results of structural parameter analysis as well as the micro-CT images, as mentioned above. Moreover, the number of osteocyte in the US group was significantly higher than that in the CON group (Fig. 13, p<0.05).
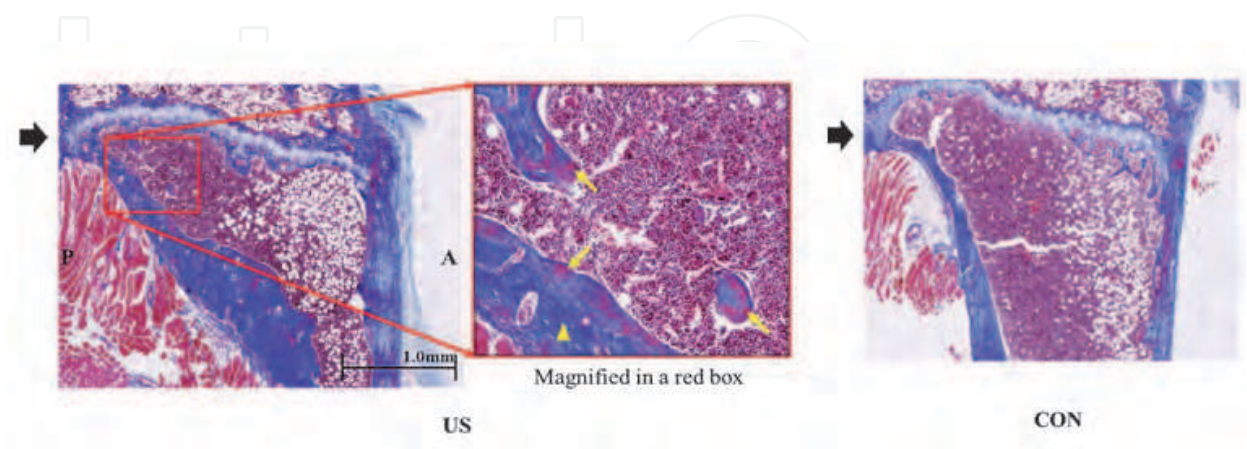

Fig. 12. Representative histology in the US and CON group, yellow arrow head: osteocyte, yellow arrow: osteoid, black arrow: LIUS (Figure was modified in Journal of Orthopaedic Research, 29(2011), 116-125).
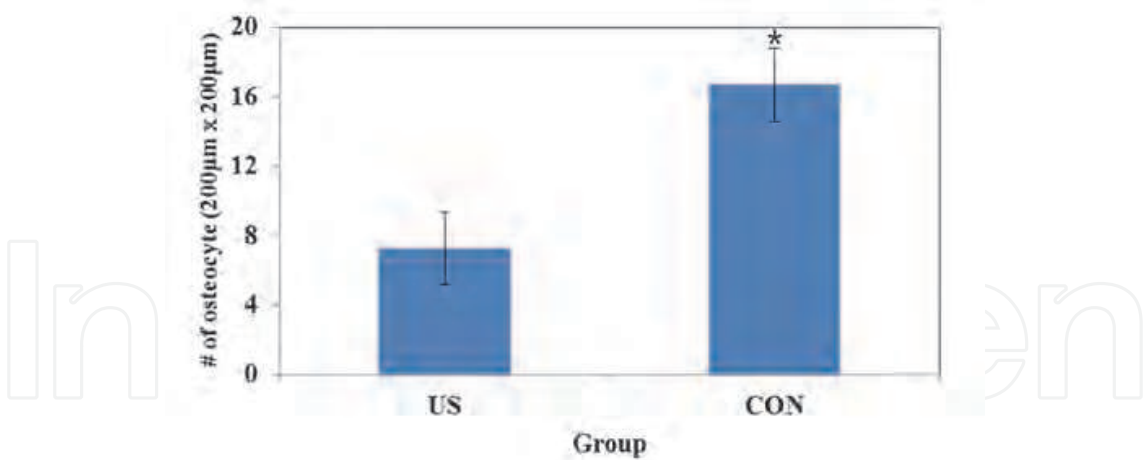
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Fig. 13. Comparison of the number of osteocyte, *significant difference between the US and CON groups (p<0.05)

4. Discussion

We evaluated the feasibility of LIUS for treatment or prevention of osteoporosis induced by estrogen deficiency through an analysis of biomechanical characteristics, bone structural characteristics and mechanical characteristics. Bone quality and quantity are regulated by external mechanical loading, because it is a sensitive tissue responding to external mechanical loading. The relationship between bone and mechanical loading is supported by mechanosensory mechanisms, including mechanoreception and mechanotransduction (Chen et al., 2003;Cowin, 2000). These factors suggested that LIUS can enhance bone healing and regeneration. However, the effects of LIUS for treatment or prevention of osteoporosis are controversial (Carvalho,Cliquet Jr, 2004;Perry et al., 2009;Warden et al., 2001). These controversial results may be contributed to the limitations of conventional experimental and analytic methods, lack of longitudinal study and ignoring individual differences, bone heterogeneity and partial effects of LIUS considering irradiation location and direction. It is widely known that in-vivo micro-CT can detect and track longitudinal changes in 3D bone structure of identical small animals without sacrificing them. Moreover, micro FEA is also widely used to evaluate longitudinal changes in bone mechanical strength and predict bone fracture risks. Therefore, we attempted to overcome these limitations using in-vivo micro-CT and micro FEA.

After 6 weeks of LIUS treatment, the relative variations of BV/TV and Tb.N were increased and the relative variation of Tb.Pf was decreased for trabecular bone compared to the non-LIUS treatment group. Moreover, the relative variation in MMI for the cortical bone was increased. These results indicated that 6 weeks of LIUS treatment might prevent bone loss and disconnection, suggesting suppression of the continuous progress of osteoporosis-associated bone loss in both trabecula and cortical bones.

To determine whether irradiation direction/location of LIUS affects bone adaptation site-specifically, BV/TV in five ROIs for trabecular bone and Cs.Th in four ROIs for cortical bone were measured and calculated. In the site of direct LIUS irradiation (R1), the relative variation of BV/TV and Cs.Th were significantly increased in the US group compared to the non-US group. However, in the furthest site of direct LIUS irradiation (R3 and R4), the relative variations of BV/TV and Cs.Th were different between groups. These data indicated

that irradiation location/direction of LIUS application might induce site-specific bone adaptation and thereby cause treatment outcome. These results are consistent with previous studies that Responses in bone cell corresponding to the local stimuli, such as strain-energy density and deformation of the bone matrix, may be temporal and positional (Henderson,Carter, 2002;Huiskes et al., 2000;Waarsing et al., 2004;Wolpert, 1989).

From the results of histological analysis, on near the region stimulated directly by LIUS, new bone formation in the endosteal cortical bone was observed in the US group. Moreover, thicker cortical bone and new bone formation in trabecular bone were also observed in the US group. These results show that LIUS stimulation for 6 weeks might enhance new bone formation or suppress bone resorption, consistent with previous studies (Carvalho,Cliquet Jr, 2004;Perry et al., 2009;Pilla et al., 1990). Furthermore, the number of osteocytes in the US group were higher than that in the CON group. Osteocyte regulates activity of bone forming cell (osteoblast) and bone resoprtion cell (osteoclast) and play an important role in mechanotransduction responding to mechanical loading (Skerry, 2008). Moreover, the estrogen deficiency-induced osteocyte apoptosis increased bone fragility and thereby fracture risks (Tatsumi et al., 2007). These results showed that LIUS may prevent estrogen deficiency-induced osteocyte apoptosis and bone fragility, suggesting increase in bone strength and decrease in bone fracture risks. Moreover, LIUS might affect mechanotransduction.

At 6 weeks after LIUS, the volume of higher tissue elastic moduli were increased compared with non-LIUS treatment group. This result showed that LIUS might not only increase bone volume enhance but also tissue material properties. Thus, LIUS might improve bone quantity, which was consistent with the results of structural parameters and histological analysis mentioned above, as well as bone quality, leading to increase in bone strength and thereby decrease in osteoporotic bone fracture risks.

The results of structural, histological and material analysis suggested that LIUS could reduce bone fracture risk. We verified the improvement of bone mechanical characteristics via performing micro FEA. At 6 weeks after LIUS, the effective structural modulus was significantly increased compared with non-LIUS treatment group. This data showed that LIUS improve bone structural strength. Therefore, LIUS could suppress the progressive bone weakening induced by estrogen deficiency, thereby would be effective in preventing or decreasing osteoporotic bone fracture risk.

However, the magnitude of the effects of LIUS for preventing/treating osteoporotic bone loss and weakening seemed to be small. This contributed to an inability of the US to reach osteoporotic bone due to absorption, scattering and reflection during transmitting soft tissues (Malizos et al., 2006;Warden, 2001). Moreover, local site-specific stimuli in partial bone can be contributed to systemic bone adaptation in others bone by neuronal regulation (Sample et al., 2008). Therefore, bones in the CON group have adapted via neuronal regulation despite indirect LIUS stimulation to them. This hypothesis might be supported by temporary increase in the effective structural modulus in the CON group at 3 weeks after LIUS.

In summary, our data show that LIUS may improve the osteoporotic bone microarchitectural characteristics, and bone material properties by regulation of bone homeostasis, enhancing bone formation and suppressing bone resorption, and mecahnostraduction, preventing osteocyte apoptosis caused by estrogen deficiency. Also, LIUS may prevent bone weakening and thereby decrease bone fracture risks. Therefore, the QOL of patients with osteoporosis can improve.

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5. Conclusion

LIUS may improve the microarchitectural characteristics, material properties and mechanical strength in the osteoporotic bone, leading to decrease in bone fracture risks. Thus, LIUS may be effective to prevent and treat osteoporosis and thereby contribute to improve the QOL of patients with osteoporosis.

6. Acknowledgment

7. References

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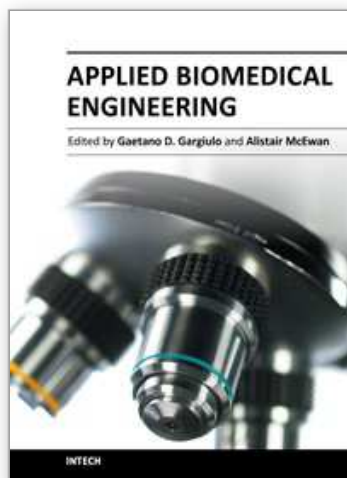
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