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Osteogenic effect of low-intensity pulsed ultrasound and whole-body vibration on peri-implant bone. An experimental in vivo study

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Abstract

Objectives: The aims of this study were (i) to compare the osteogenic impact of low-intensity pulsed ultrasound (LIPUS) and low-magnitude high-frequency (LMHF) loading achieved with whole-body vibration (WBV) on peri-implant bone healing and implant osseointegration in rat tibiae, and (ii) to examine their combined effect on these processes.

Material and methods: Titanium implants were inserted in the bilateral tibiae of 28 Wistar rats. Rats were randomly divided into four groups: LIPUS + WBV, LIPUS, WBV, and control. LIPUS was applied to the implant placement site for 20 min/day on 5 days/week (1.5 MHz and 30 mW/cm^2). WBV was applied for 15 min/day on 5 days/week (50 Hz and 0.5 g). In the LIPUS + WBV group, both stimuli were applied under the same stimulation conditions as in the LIPUS and WBV groups. After 4 weeks of treatment, peri-implant bone healing and implant osseointegration were assessed using removal torque (RT) tests, micro-CT analyses of relative gray (RG) value, and histomorphometrical analyses of bone-to-implant contact (BIC) and peri-implant bone formation (BV/TV).

Results: The LIPUS + WBV group had significantly greater BIC than the WBV and control groups. Although there were no significant intergroup differences in RT, RG value, and BV/TV, these variables tended to be greater in the LIPUS + WBV group than the other groups.

Conclusions: The combination of LIPUS and LMHF loading may promote osteogenic activity around the implant. However, further study of the stimulation conditions of LIPUS and LMHF loading is necessary to better understand the osteogenic effects and the relationship between the two stimuli.

KEYWORDS
low-intensity pulsed ultrasound (LIPUS), low-magnitude high-frequency loading, osseointegration, osteogenesis, prostheses and implants

1 | INTRODUCTION

Oral implants are a well-accepted and predictable treatment modality for the rehabilitation of partially or completely edentulous patients. However, osseointegration is not achieved in some cases. In particular, implant treatment in patients with systemic diseases such as osteoporosis and diabetes has a low success rate and an unpredictable long-term prognosis (Alsaadi et al., 2007; Ozawa et al., 2002); these comorbidities have also caused serious problems in the field of orthopedics (Russell et al., 2007; Yoneda et al., 2010).

The deterioration of bone quality due to osteoporosis is commonly treated via pharmacotherapy with bisphosphonates (BPs) (Russell et al., 2007; Yoneda et al., 2010). However, BPs suppress osteoclast activity, which suppresses bone metabolic activity. Therefore, long-term BP administration carries a risk of atypical femoral fractures and musculoskeletal pain (Black et al., 2007). In the dental field, long-term BP administration can cause serious adverse effects, such as jaw osteonecrosis and osteomyelitis (Bedogni et al., 2010; Marx, 2003; Yoneda et al., 2010). Thus, the application of implant treatment is limited in patients taking BPs (Curtis et al., 2008; Yoneda et al., 2010).

Another pharmacological treatment used for osteoporosis is parathyroid hormone (PTH), which activates the osteoblast system. However, PTH has disadvantages such as high costs, injection preparations, and the potential for osteosarcoma development with long-term use (Hodsman et al., 2005; Tashjian et al., 2002; Vahle et al., 2002). Therefore, there is a need for a non-pharmacological alternative or additional intervention.

In the treatment of bone damage, physical stimulation methods using ultrasound and mechanical vibration stimulation have recently been investigated as alternatives to drug therapy. For example, low-intensity pulsed ultrasound (LIPUS) of 0.75–3.0 MHz promotes bone fusion by applying physical stimulation via sound pressure to the fracture site. LIPUS has already been applied in the treatment of bone damage, such as fractures (Busse et al., 2002; Heckman et al., 1994; Kasturi & Adler, 2011). LIPUS for 20 min daily positively affects fracture consolidation (Busse et al., 2002; Heckman et al., 1994). In the field of dental implantation, studies have shown that LIPUS promotes the formation of peri-implant bone and shortens the time taken for osseointegration to occur (Qing et al., 2012; Tanzer et al., 1996; Ustun et al., 2008). Studies have reported positive effects on implant osseointegration after the application of LIPUS for 10 min twice daily for 21 days (Qing et al., 2012), and for 20 min daily for 4 weeks (Tanzer et al., 1996) and 6 weeks (Ustun et al., 2008).

Another physical stimulation method expected to promote bone formation and fracture healing is low-magnitude high-frequency (LMHF) loading, which is generally defined as an acceleration of 1 g or less and a frequency of 20–90 Hz (Judex et al., 2009; Rubin et al., 2001). The mechanism by which LMHF loading affects the cellular activity of osteoblasts and osteoclasts has not yet been clarified (Coughlin & Niebur, 2012; Lau et al., 2010; Ota et al., 2016; Uzer et al., 2014 and Uzer et al., 2015). However, many studies have shown that LMHF loading by WBV promotes bone formation and bone healing (Omar et al., 2008; Sehmisch et al., 2009; Shi et al., 2010). WBV loading has been used clinically as a non-pharmacological intervention in the treatment of fracture and osteoporosis (Rubin et al., 2004; Russo et al., 2003; von Stengel et al., 2011; Verschueren et al., 2004), and has also been investigated for its potential application in implant treatment (Ogawa, Possemiers, et al., 2011 and Ogawa, Zhang, et al., 2011). To apply LMHF loading to promote osteogenesis in dental implant treatment, we have been studying the effectiveness and appropriate stimulation conditions of LHMF loading in promoting implant osseointegration. So far, we have reported that the effect of LMHF loading in healthy rat tibiae is affected in the early stages of healing by vibration stimulation parameters such as stimulation time, number of stimulations, vibration acceleration, and frequency (Ogawa, Possemiers, et al., 2011 and Ogawa et al., 2014). Furthermore, we examined the effects of LMHF loading and PTH on peri-implant bone formation in an osteoporosis model (Shibamoto et al., 2018).

The effects of physical stimulation methods such as LIPUS and LMHF loading on peri-implant osteogenesis are reportedly significantly lower than the effects of drug therapy such as BPs and PTH (Hatori et al., 2015; Shibamoto et al., 2018). Therefore, before the clinical application of physical stimulation methods in implant treatment, the optimal conditions and timing protocols that obtain osteogenesis effects must be identified. The aim of the present study was to examine the effects of LIPUS and LMHF loading (both alone and in combination) on peri-implant osteogenesis in rat tibiae. The ultimate goal is the clinical application of these two physical stimulation methods to promote osseointegration and peri-implant osteogenesis in patients with poor bone quality and bone mass.

2 | MATERIAL AND METHODS

The present study was carried out at the Institute for Animal Experimentation at Tohoku University Graduate School of Medicine under the approval of the Institutional Animal Care and Use Committee of the Tohoku University Environmental & Safety Committee (approval number 2017DnA-016). This animal care and use protocol adhered to the Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions by the Ministry of Education, Culture, Sports, Science and Technology (Notice No. 71 issued on June 1, 2006), the Standards Relating to the Care and Management of Laboratory Animals and Relief of Pain by the Ministry of the Environment (Notice No.84 issued on August 30, 2013), and the Acton Welfare and Management of Animals (the last revision on September 5, 2012) in Japan. This study conformed with the ARRIVE guidelines.

2.1 | Animals

The present experiments were conducted on 28 male Wistar rats (age 15 weeks; average weight 314.6 ± 11.1 g). To minimize the number of experimental animals, the required sample size was calculated with a level of significance of 5%, statistical power of 80%, expected difference between the test and control groups of 25%, and expected standard deviation of 15%, based on the results of the main variables (e.g. BIC and RT) from previous studies that performed LMHF loading using the similar rat tibial model (Shibamoto et al., 2018).

2.2 | Experimental design

A custom-made titanium implant (2 mm × 13 mm; cp-Titanium grade 2, machine surface) was inserted in both tibiae in each rat. The surgery was performed under anesthesia (2.5% isoflurane) (Escain; Mylan, Pittsburgh, PA, USA) in aseptic conditions. A skin incision was made on the medial side of the tibia, and both cortices were perforated with a surgical drill at a low rotational speed under constant saline cooling (Implant Motor IM-III, GC, Japan). The implant was placed approximately 10 mm distal to the knee joint (Figure 1a,b). After implant insertion, the wounds were closed with 5–0 polyglycolic acid sutures (Matsuda Ika Kogyo Co., Ltd, Japan).

28 rats were divided into four groups (n = 7 in each group): LIPUS + WBV, LIPUS, WBV, and control. The following conditions were applied from the day after surgery. LIPUS was applied via a commercially available therapeutic ultrasound device (Osteotron D^2, Ito Co., Ltd, Japan) that transmits pulsed ultrasound signals with a 1.5 MHz operation frequency consisting of a 200 μs burst of sine waves repeating at 1 kHz, which produces an average temporal and spatial intensity of 30 mW/cm^2. LIPUS was applied to the implant placement site for 20 min/day on 5 days/week. The output

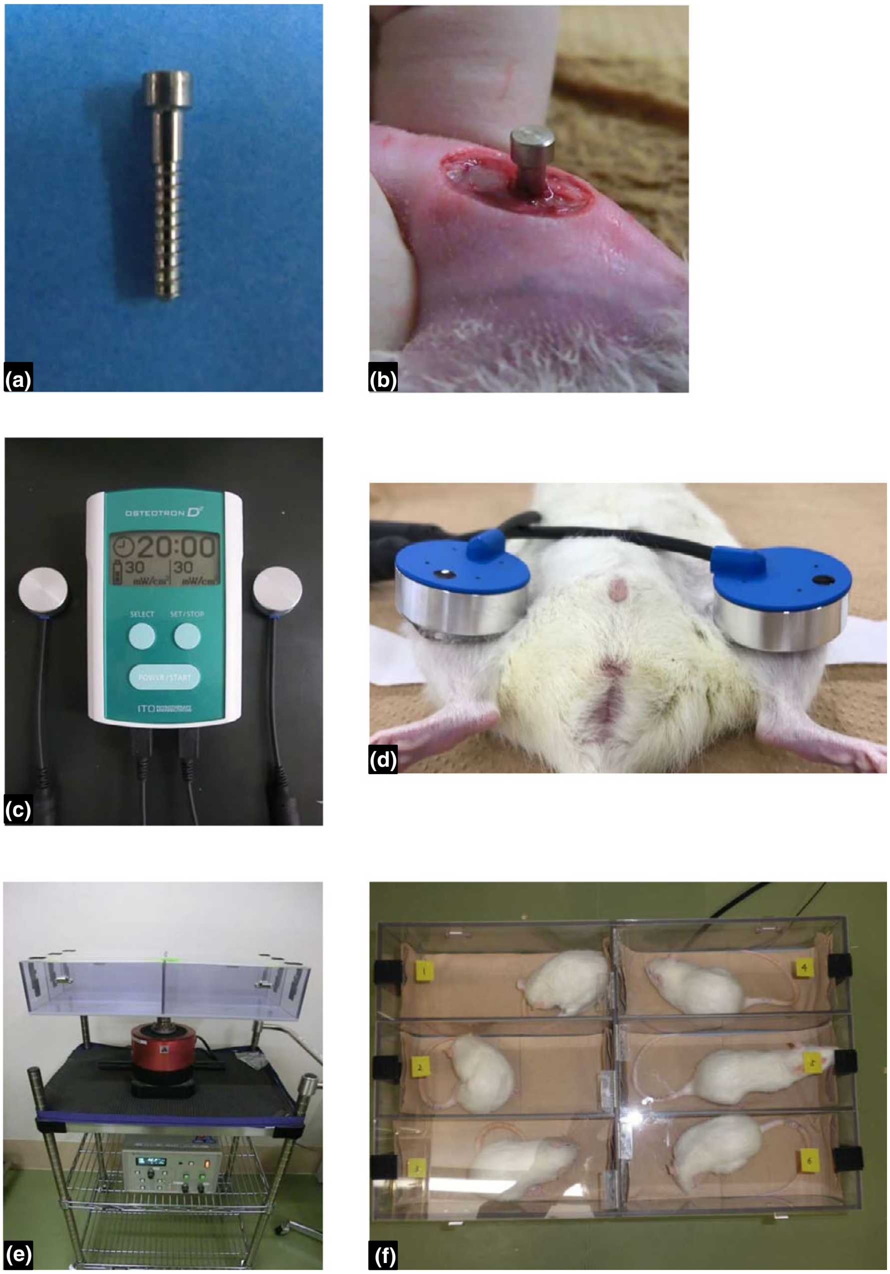


FIGURE 1 (a) The custom-made titanium implant (2 mm × 13 mm). (b) An implant was placed approximately 10 mm from the knee joint, almost perpendicular to the long axis of the tibia in each leg. (c) The low-intensity pulsed ultrasound (LIPUS) device. (d) The output surface of the ultrasound probe is almost parallel to the implant and the long axis of the tibia. (e) The whole-body vibration device. (f) Animal box set on the device. Whole-body vibration was applied to six rats simultaneously

surface of the ultrasound probe was almost parallel to the implant and the long axis of the tibia (Figure 1c,d). A Big Wave G-Master (Asahi Seisakusyo, Japan) was used to apply WBV at a frequency of 50 Hz and magnitude of 0.5 g for 15 min/day on 5 days/week (Figure 1e,f). In the LIPUS + WBV group, both stimuli were applied under the same stimulation conditions as in the LIPUS and WBV groups. The control group received no stimulation (Figure 2). The LIPUS stimulation conditions were based on the conditions used in a previous study to achieve osseointegration of rabbit tibial implants (Ustun et al., 2008). The WBV stimulation conditions were based on the conditions used in a previous study to achieve osseointegration of implants in rat tibiae in an osteoporotic model (Shibamoto et al., 2018).

The rats were euthanized 4 weeks after implantation, and the tibiae with the implants were removed. One tibia from each rat was used to evaluate the biomechanical strength of osseointegration using the RT test; the other tibia underwent micro-CT analysis to evaluate the per-implant bone density, and histological and histomorphometrical analysis to assess the biological response of the peri-implant bone.

2.3 | Removal torque test

The tibia was fixed so that the long axis of the implant was perpendicular to the fixation base. A torque gauge (ATG1.5CN/ATG12CN, Tohnichi Mfg. Co., Japan) was fitted to the implant head, and a horizontal rotational load was applied in the direction opposite to the rotational direction at the time of implant placement. The RT value was defined as the maximum rotational load until the implant was rotated horizontally.

2.4 | Micro-CT analysis

Micro-CT was performed under the conditions of 200 kV and 100 μA using a micro-CT device (ScanXmate-D225RSS270; Comscan Techno Co., Japan). After three-dimensional reconstruction, a sagittal slice along the axis of the tibia and dental implant was selected for the analysis. The relative gray (RG) value (where water = 0, implant = 100) was evaluated in a 0.4 mm^2 region of interest (ROI) set in the peri-implant cortical and cancellous bone (Figure 3a).

2.5 | Histological and histomorphometrical analysis

After micro-CT analysis, the bone-implant blocks were fixed in a phosphate-buffered formalin solution and dehydrated in increasing concentrations of alcohol. After dehydration, the samples were embedded in polymethylmethacrylate. The embedded samples were cut by a diamond saw (Exakt BS-300CP; Exakt Technologies Inc., Norderstedt, Germany) along the axis of the tibia and implant. After polishing to a final sample thickness of 40 μm (Exakt MG-400CS; Exakt Technologies Inc.), the sections were stained with Villanueva Goldner stain. The histological and histomorphometrical analyses were performed using an optical microscope with a magnification of × 100 (LeicaDM3000; Leica Microsystems, Wetzlar, Germany). The samples were scanned with a high-sensitivity camera (LeicaDFC295). Image analysis software (Adobe Photoshop CS6; Adobe System Inc., USA, and ImageJ; U.S. National Institutes of Health, Bethesda, MA, USA) was used to perform the following analyses:

1. BIC (%): (summation of the lengths of contact between bone and implant (μm)/implant length extending from the most medial to the most lateral BIC point (μm)) × 100.
2. Peri-implant bone volume relative to tissue volume (BV/TV; %): (area occupied by bone (μm^2)/reference area (μm^2) × 100.

Two ROIs were set in accordance with the distance from the implant body; the BV/TV_ROI1 was the BV/TV of ROI1 located 0–100 μm from the implant body, while the BV/TV_ROI2 was the BV/TV of ROI2 located 100–500 μm from the implant body (Figure 3b).

2.6 | Statistical analysis

Data were reported as the mean ± standard deviation. Statistical analyses were conducted using SPSS version 21.0 (SPSS Inc.,

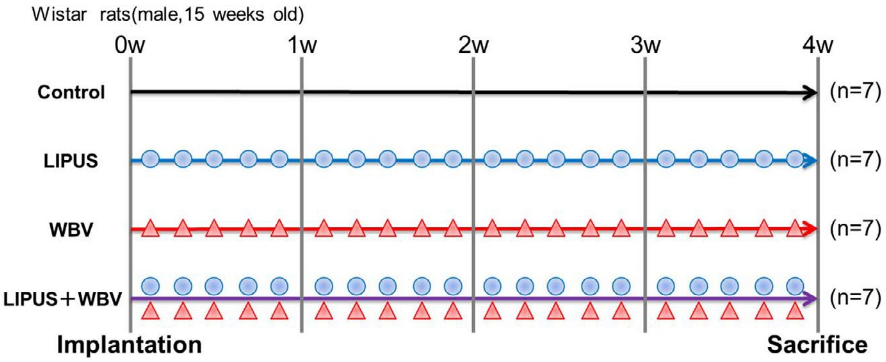


FIGURE 2 Illustration of the experimental design and grouping

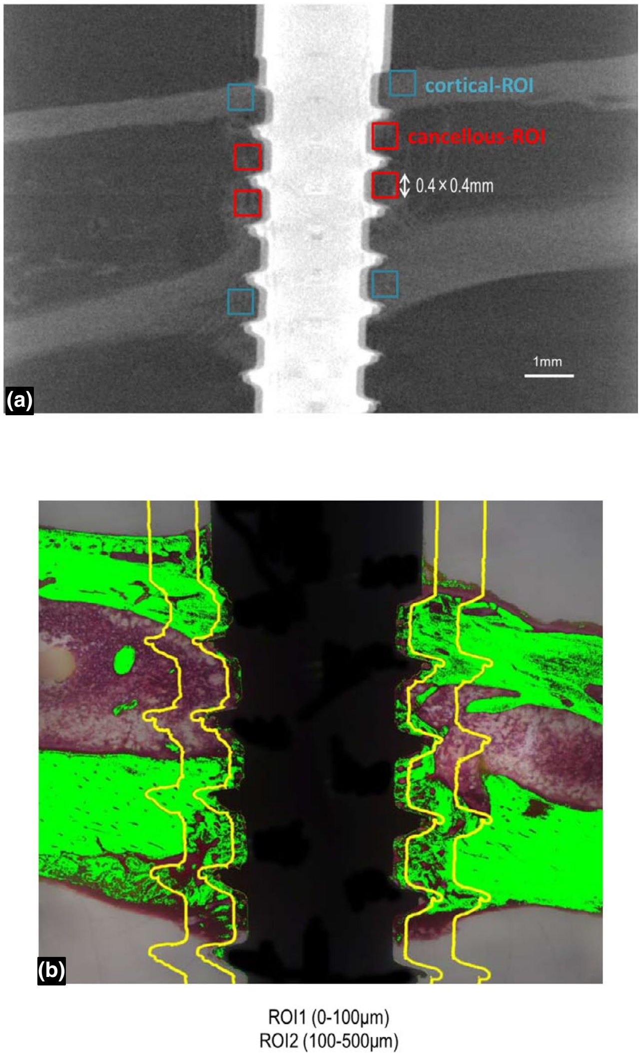


FIGURE 3 (a) The region of interest (ROI) for micro-CT analysis. The cortical-ROI and cancellous-ROI comprise 0.4 mm^2 regions in the cortical and cancellous bone adjacent to the implant surface, respectively. (b) The reference sites for the bone volume relative to tissue volume (BV/TV) evaluation. ROI1: 0–100 μm from the implant surface; ROI2: 100–500 μm from the implant surface

Chicago, IL, USA). One-way analysis of variance and Tukey's HSD test were performed to compare differences between the four groups. The significance level was set at *p* <.05.

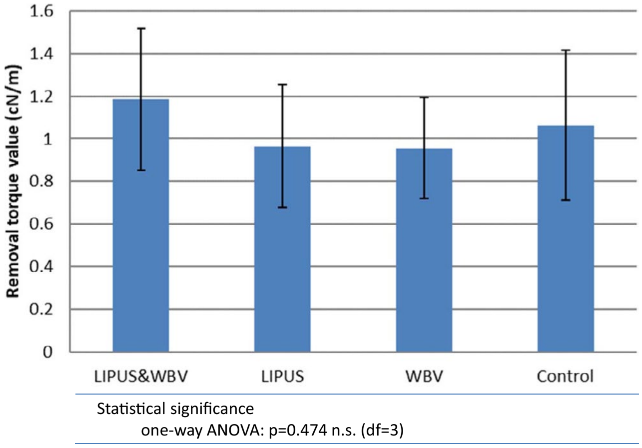


FIGURE 4 Results of the removal torque (RT) test. A torque gauge was used to measure the RT value (defined as the maximum rotational load until the implant was rotated horizontally). The graphs show the means and standard deviations of the RT value for each group. *df*: degree of freedom. n.s.: not significant

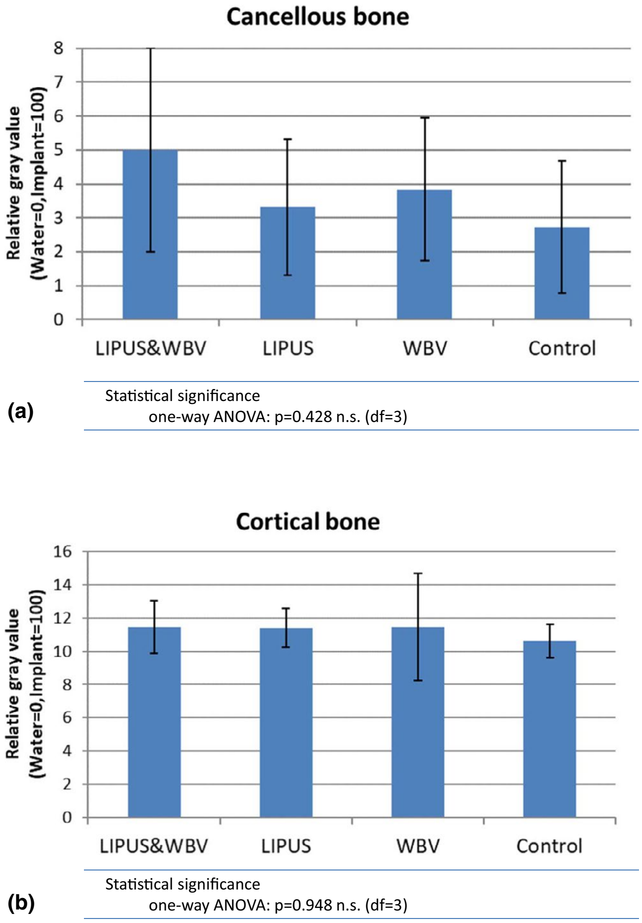


FIGURE 5 Results of micro-CT analysis in (a) cancellous and (b) cortical bone. The graphs show the means and standard deviations of the RG value (where water = 0, implant = 100) of each region of interest (ROI) for each group. *df*: degree of freedom. n.s.: not significant

3 | RESULTS

3.1 | Removal torque test and micro-CT analysis

Although there were no significant differences between the four groups in the RT and RG values, the values in the LIPUS + WBV group tended to be higher than those in the other groups (Figures 4 and 5). The average RT value was 1.19 ± 0.33 cN/m in the LIPUS + WBV group and around 1.0 cN/m in the other groups. The average RG values of cancellous bone ranged from 2.58 ± 2.04 (control group) to 4.82 ± 3.16 (LIPUS + WBV group), while the RG values of cortical bone ranged from 10.62 ± 0.99 (control group) to 11.45 ± 1.58 (LIPUS + WBV group).

3.2 | Histological and histomorphometrical analysis

Figure 6 shows representative histological sections for each group. Compared with the control group, the formation of new cancellous bone around the implant was more prominent in the stimulated groups, especially the LIPUS + WBV group. In addition, the cortical bone around the implant showed several sections of bone width increase in the groups that received LIPUS and/or WBV. The osteogenic response of peri-implant bone tended to be more visible on the apical side of the implants in most cases.

The BIC was higher in the LIPUS + WBV group than in the other groups, and the BIC was significantly higher in the LIPUS + WBV group than the WBV group and the control group ($p < .05$) (Figure 7a). The BV/TV did not significantly differ between the groups; however, the value tended to be higher in the LIPUS + WBV group than in the other groups (Figure 7b,c).

4 | DISCUSSION

The present study evaluated the osteogenesis-promoting effects of LIPUS and LMHF loading. The aim of the present study was to assess the effects of LIPUS and LMHF loading (alone and in combination) on peri-implant bone formation in rat tibiae to determine whether these methods should be applied in dental implant treatment. The results of this study suggest that the osteogenic effects of LIPUS and LMHF loading on peri-implant bone healing may be enhanced when both stimuli are used in combination. The mechanism by which cells sense mechanical stimuli and convert them into biochemical signals is called mechanotransduction. In the present study, osteocytes may have promoted osteoblast differentiation by sensing the mechanical stimuli of LIPUS and WBV, which leads to the activation of bone remodeling and promotes bone formation (Baron et al., 2020; Wu et al., 2016).

LIPUS reportedly affects bone healing and bone metabolic activity during the healing process. Although the mechanism of action at the cellular level is still unknown, LIPUS is considered to cause mechanical stress that increases cellular activity (Kasturi & Adler, 2011). LIPUS increases the number of osteoblasts by

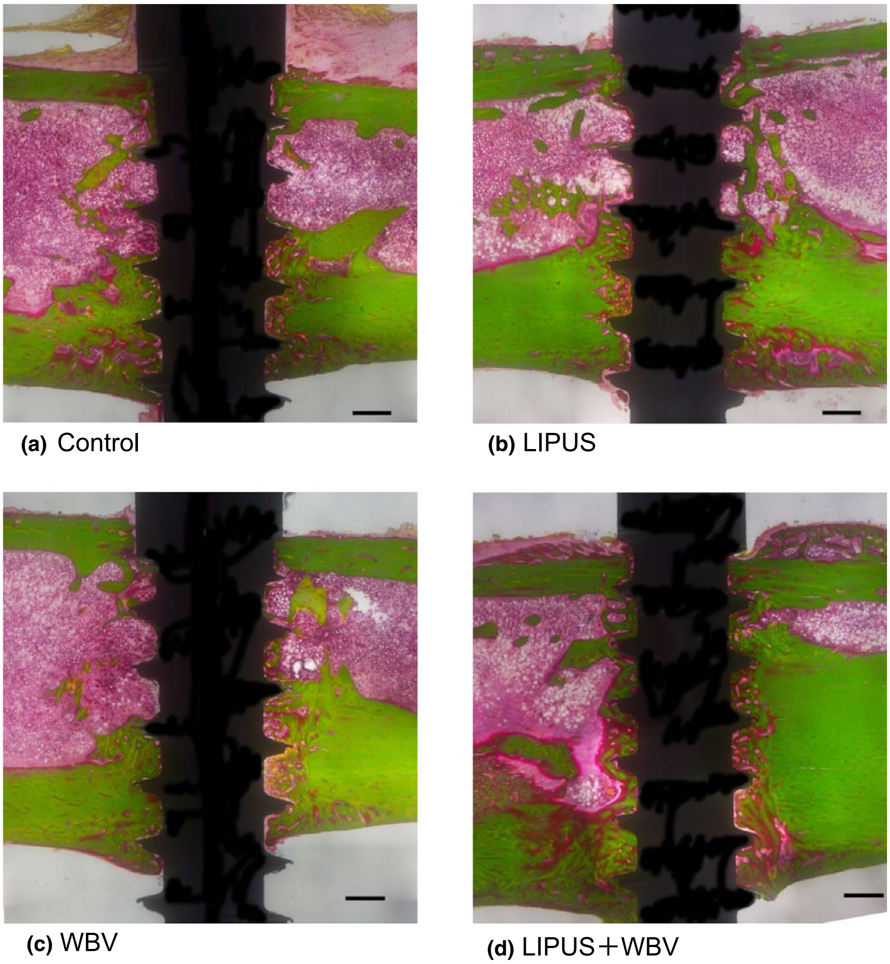
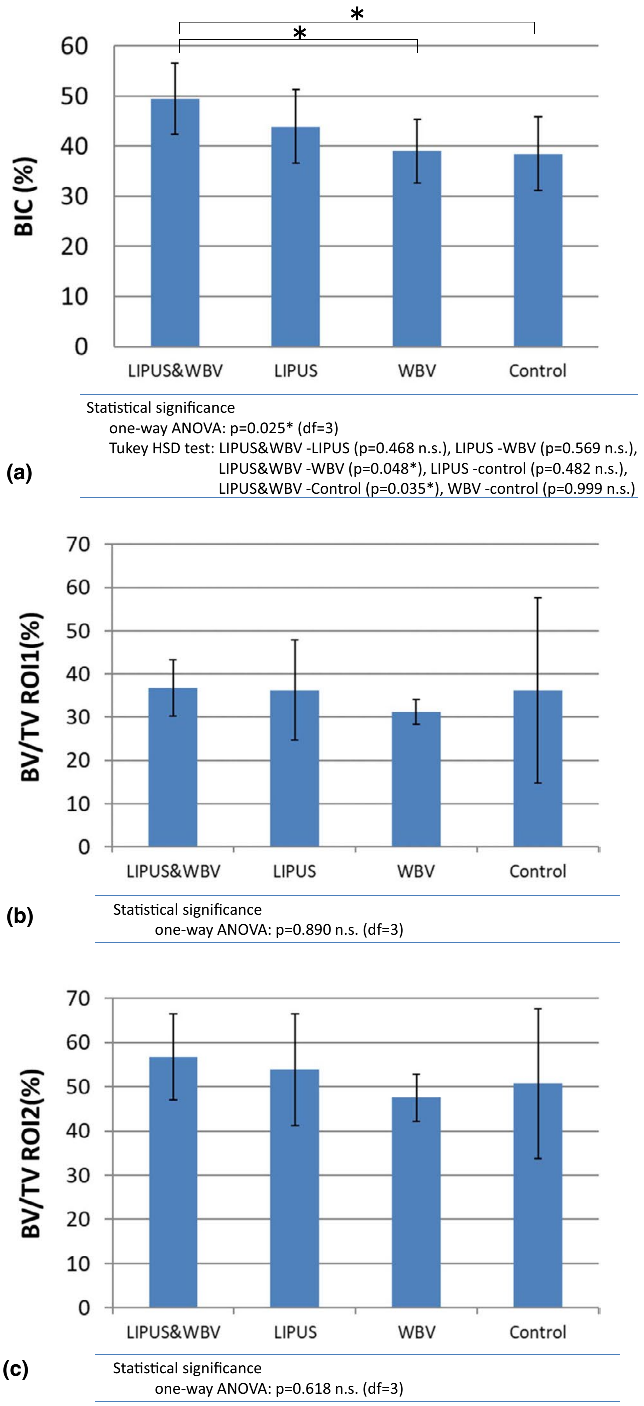


FIGURE 6 Representative histological sections from each group. Scale bars: 500 μm



(a)

Statistical significance
one-way ANOVA: p=0.025* (df=3)
Tukey HSD test: LIPUS&WBV -LIPUS (p=0.468 n.s.), LIPUS -WBV (p=0.569 n.s.),
LIPUS&WBV -WBV (p=0.048*), LIPUS -control (p=0.482 n.s.),
LIPUS&WBV -Control (p=0.035*), WBV -control (p=0.999 n.s.)

(b)

Statistical significance
one-way ANOVA: p=0.890 n.s. (df=3)

(c)

Statistical significance
one-way ANOVA: p=0.618 n.s. (df=3)

FIGURE 7 Histomorphometrical results for the bone-to-implant contact (BIC). (a) The means and standard deviations of the BIC for each group (*$p < .05$; one-way analysis of variance followed by Tukey's HSD test). Histomorphometrical results for the bone volume relative to tissue volume (BV/TV). The graphs show the means and standard deviations of the BV/TV at (b) BV/TV_ROI1 (0–100 μm from the implant surface) and (c) BV/TV_ROI2 (100–500 μm from the implant surface). *df*: degree of freedom. n.s.: not significant

increasing the expression of growth factor cytokines such as bone morphogenetic protein, which promotes cell differentiation and promotes bone resorption and angiogenesis. Thus, LIPUS may promote bone formation by activating bone remodeling (Busse et al., 2002; Heckman et al., 1994; Kasturi & Adler, 2011). Several studies have reported that LIPUS promotes osteoblast differentiation in peri-implant bone and promotes osteogenesis, which shortens the treatment period and maintains long-term osseointegration (Qing et al., 2012; Tanzer et al., 1996; Ustun et al., 2008). Zhou et al., (2016) reported that osteoporotic rats that received LIPUS attained a higher RT value, BIC, and BV/TV compared with osteoporotic rats without stimulation, and that LIPUS effectively improved peri-implant bone healing. However, the present study found no significant differences between the LIPUS group and the control group in the RT value, RG value, and histomorphometrical analysis. This is presumably because the rats used in the present study were healthy, while the osteoporotic rats used by Zhou et al., (2016) had lesser bone quality and quantity than normal rats, which may have made the effect of LIPUS more prominent. Therefore, it is necessary to study the conditions of LIPUS that promote the osteogenic activity of peri-implant bone, even in healthy models.

Previous studies have shown that LMHF loading is effective in fracture healing and dental implant treatment (Ogawa, Possemiers, et al., 2011; Ogawa, Zhang, et al., 2011; Rubin et al., 2004; Russo et al., 2003; von Stengel et al., 2011; Verschueren et al., 2004). Regarding the stimulation conditions of LMHF loading, Ogawa, Possemiers, et al., (2011) and Ogawa et al., 2014) reported that the effectiveness of LMHF loading increased in tandem with the stimulation time and number of stimulations, and with the acceleration and frequency. However, the acceleration, frequency, and amplitude conditions were related to each other. Thus, it was considered inappropriate to specify only one of the parameters and examine the effect of the other parameters. Furthermore, Shibamoto et al., (2018) investigated the effects of LMHF loading and PTH on the peri-implant bone in a rat model of osteoporosis, and found that LMHF loading and PTH (alone and in combination) resulted in higher RT values, RG values in cortical bone, and BIC than controls, indicating that these methods effectively promoted peri-implant bone healing and implant osseointegration. However, in the present study, there was no significant difference between the WBV group and the control group in the RT value, RG value, and histomorphometrical analysis. Similarly to the reasons for the lack of significant effects of LIPUS, this is probably because the rats used in the present study were healthy. Therefore, it is necessary to study the conditions of LMHF loading that promote the osteogenic activity of peri-implant bone in a healthy model.

In the present study, the LIPUS + WBV group had a significantly higher BIC than the control group. Furthermore, although no significant differences were observed, the other parameters tended to be highest in the LIPUS + WBV group compared with the other groups. These results suggest that the combination of LIPUS and WBV may be effective in facilitating osseointegration. Perry et al., (2009) studied the effects of LIPUS and mechanical loading on the calcification rate and bone formation rate in rat ulnae, and found that the combined use of LIPUS and mechanical loading was more effective than LIPUS only, but achieved similar results to mechanical loading only.

Although this is slightly different from the present results, it suggests that concomitant LIPUS and mechanical loading may achieve combined effects. Perry et al., (2009) thought that LIPUS would not further promote bone formation if the mechanical loading had already maximized the osteogenic response. In the present study, the LIPUS group and the WBV group were not significantly different from the control group, and the relationship between the two stimulation methods could not be considered. In the future application of such stimulation methods in implant treatment, it is necessary to understand the characteristics of both stimulation methods in more detail, rather than just using both simultaneously. This will make it possible to apply each physical stimulus under appropriate conditions and also to use an appropriate combination method, which will lead to safer and more effective implant treatment.

This study has some limitations. The study was planned as a basic study to examine the fundamental phenomenon, before progressing to an oral implant model using higher level experimental animals. As long bones such as the tibia are different from craniofacial bones, further studies using jaw bone models are necessary to optimize the performance of vibration devices for local application and explore the optimal stimulation conditions for peri-implant bone healing and implant osseointegration. However, the rat tibia, which has been used successfully in previous experiments (Ogawa, Zhang, et al., 2011 and Ogawa et al., 2014), is considered a suitable and reliable location for implant surgery and can be maintained without unpredictable loading. Therefore, the findings of the current study will increase our understanding of the peri-implant bone response to LIPUS and LMHF loading, prior to subsequent studies using an oral implant model.

The present study focused on the morphological results of the osteogenic effects of LIPUS and LMHF loading and did not assess the properties of cells or proteins exhibiting bone remodeling activity. Wnt is a secreted glycoprotein that is conserved across species and controls body axis determination and organogenesis during early development. When Wnt binds to the receptor complex of Frizzled and low-density lipoprotein receptor-related protein 5/6, β-catenin degradation is suppressed and β-catenin is stabilized. As a result, β-catenin translocates into the nucleus, promotes osteoblast differentiation, and promotes bone formation (Behrens et al., 1996; Eastman et al., 1999; Yost et al., 1996). Sclerostin inhibits the Wnt/β-catenin pathway and suppresses bone formation, whereas LIPUS and WBV are thought to promote bone formation by suppressing the expression of sclerostin (Jing et al. 2016; Kumagai et al., 2017). To investigate the osteogenic effects of LIPUS and WBV in more detail, future studies are warranted to assess the properties of cells and proteins exhibiting bone remodeling activity.

In the present study, the healing period was only 4 weeks. Although osseointegration is established approximately 3 months after implant placement in the human mandible, osseointegration is observed in about 4 weeks in the rat tibia (Ogawa, Possemiers, et al., 2011 and Ogawa, Zhang, et al., 2011). Furthermore, our preliminary experiment showed that no major peri-implant bone remodeling occurred after 4 weeks, and we previously reported that the WBV parameters affect osseointegration in healthy rat tibiae during 4 weeks of healing (Ogawa, Possemiers, et al., 2011 and Ogawa et al., 2014). Therefore, in the present study, to reduce the number of experimental animals, the healing was assessed 4 weeks after implantation, as this was assumed to be the time at which osseointegration was acquired. However, in the future, a short experimental period such as 1 or 2 weeks after implantation is required to evaluate the effect of these stimulations on peri-implant bone remodeling before osseointegration is obtained.

5 | CONCLUSIONS

The present results suggest that the combined use of LIPUS and LMHF loading promotes osteogenic activity in peri-implant bone. However, to obtain more information about the effects of these stimuli on osteogenic activity and to better understand the relationship between the two stimuli, further study of the stimulation conditions of LIPUS and LMHF loading is necessary.

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CONFLICT OF INTEREST

The authors have stated explicitly that there are no conflicts of interest in connection with this article.

AUTHOR CONTRIBUTION

Kenta Shobara, Conceptualization-Equal, Data curation-Lead, Formal analysis-Equal, Investigation-Lead, Project administration-Equal, Writing-original draft-Lead, Writing-review and editing-Supporting. Toru Ogawa, Conceptualization-Lead, Data curation-Supporting, Formal analysis-Equal, Investigation-Equal, Methodology-Equal, Project administration-Lead, Supervision-Lead, Validation-Equal, Writing-review & editing-Lead. Aya Shibamoto, Conceptualization-Supporting, Data curation-Supporting, Investigation-Supporting, Methodology-Lead, Project administration-Supporting, Software-Supporting, Supervision-Supporting. Makiko Miyashita, Data curation-Supporting, Investigation-Supporting, Methodology-Equal, Supervision-Supporting. Akiyo Ito, Data curation-Supporting, Methodology-Supporting. Ratri M. Sitalaksmi, Writing-original draft-Equal, Writing-review and editing-Equal.

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