A novel device to prevent osteoporosis by promoting bone metabolism using a newly developed double-loading stimulation with vibration and shaking

By

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Summary: In Japan, 13 million people have osteoporosis, including approximately 9 hundred thousand people who are bedridden owing to bone fractures from falls. Preventing osteoporosis is considered to be an important and effective way of preventing fall-related fractures. Thus, we developed a novel method of locomotor stimulation and analyzed its effectiveness in mice. Specifically, we created a double-loading device that combines vibration and shaking stimulation. The device was used to continuously stimulate ovariectomy-induced decreased bone density mouse models 30 minutes daily for 10 weeks. We then collected femur samples, created undecalcified tissue slices, calculated parameters using bone histomorphomtry, and conducted comparative testing. BS/TV (bone surface/tissue volume), N.Oc/ES (osteoclast number/eroded surface), Oc.S/ES (osteoclast osteoid surface/eroded surface), Omt (osteoid maturation time), Tb.N (trabecular number), Mlt (mineralization lag time) < ($p < 0.01$), N.Ob (osteoblast number), N.Ob/TV (osteoblast number/tissue volume), sLS (single labeled suface), N.Mu.Oc/ES (multinucle osteoclast number/eroded surface), and N.Mo.Oc/ES (mononucle osteoclast number/eroded surface) ($p < 0.05$) were significantly higher in the stimulation group than in the non-stimulation group. In addition, BS/BV (bone surface/bone volume), Tb.Sp (trabecular separation), MAR (mineral apposition rate), Aj.Ar (adjusted apposition rate) ($p < 0.01$), ES (eroded surface), ES/BS (eroded surface/bone surface), and BRs.R (bone resorption rate) ($p < 0.05$) were significantly lower in the stimulation group than in the non-stimulation group. These results suggest that stimulation activated osteoblasts and osteoclasts, thereby leading to highly active bone remodeling. We anticipate that bone mineralization will subsequently occur, suggesting that this stimulation technique is effective in preventing osteoporosis by alleviating sudden bone density loss.

Introduction

Osteoporosis occurs when bone density loss leads to patients becoming more prone to bone fractures. The World Health Organization defines this condition as "a skeletal disorder characterized by compromised bone strength predisposing a person to an increased risk of fractures"[1]. Decreased mechanical load due to decreased muscle strength and lack of exercise that is associated with aging also contribute to reduced bone density. Bone resorption takes precedence over bone formation due to calcium and vitamin D deficiencies and hormonal imbalance. This process is the underlying cause of the disease in the vast majority of patients with osteoporosis[2]. Women achieve maximum bone density between their late teens and the age of 20. This level is maintained until approximately 40 years of age, after which ovarian functions begin to decrease. This, in turn, leads to a sudden decrease in the amount of estrogen they secrete, resulting in menopause and rapid loss of bone mass. The main mechanism that triggers osteoporosis is the promotion of bone resorption by estrogen deficiency related to menopause and parathormone, which cause a loss in bone mass[3, 4]. In Japan, approximately 13 million people have osteoporosis. Data show that many patients are elderly women, with 21% in their fifties, 48% in their sixties, 67% in their seventies, and 84% in their eighties, indicating an increasing trend with advancing age[1]. The third

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leading cause of becoming bedridden is bone fracture due to osteoporosis according to data from approximately 9 hundred thousand people. This becomes a significant concern as the mean age of the population continues to rise along with the number of elderly people with bone fractures. However, therapeutic procedures for osteoporosis are limited in many cases due to drug therapy. Estrogen replacement therapy increases intestinal calcium absorption and suppresses bone mass loss by activating osteoblasts. However, it has been shown to lead to adverse events that promote the onset of breast cancer and other diseases such as heart disease[1]. We believe that the prevention of osteoporosis is more important and effective than drug therapy in patients with bone fractures due to a fall. Increasing the maximum bone mass reached when an individual is young to limit the amount of bone mass loss as the individual ages is commonly considered to be the key to preventing osteoporosis[5]. It is thought that once individuals reach middle and advanced ages, they can keep bone mass loss at a minimum and prevent fractures by continuing to exercise[1]. Exercise that includes a suitable amount of load on both bones and muscles is considered effective to prevent bone density loss in post-menopausal women[6]. Research has been conducted on the effect of shaking stimulation on the alleviation of bone density loss after menopause using ovariectomy (OVX) mice, which simulate post-menopausal women[7, 8]. They took into consideration the so-called "Wolff principle," which states that "bones develop internal structures that are optimally suited to the forces that exert resistance upon them". They proposed that the application of mechanical load stimulation on bones via the tendons promote bone formation, and the resulting bone strength maintenance would alleviate decreases in bone density[9, 10]. Methods of applying a mechanical load in the form of vibration stimulation to bones has already been reported to be effective when used clinically[11, 12]. Based on the findings of these previous studies, we aimed to determine whether the combination (CB) of vibration and shaking stimulation using a novel device that we developed would increase its effectiveness. Vibration stimulation was applied in a tornado type of motion, in which fine and minute circular movements were applied at high frequency. This applied stimulation to the musculoskeletal system and bones via isometric movements without simultaneously causing joint movement. In addition, the shaking stimulation was applied by the device as it rotated a horizontal panel where the study subjects were standing while simultaneously maintaining the horizontal plane. This stimulation engaged the musculoskeletal system, particularly the lower half of the body, in a way that forced the subjects to engage in isotonic and isometric movements, which in turn directly stimulated the bones via the tendons to which they are attached[13]. We believed that this CB stimulation by compelling subjects to engage in isotonic and isometric movements mainly in the lower half of the body would be more effective in increasing muscle strength than shaking stimulation alone, and this would lead to the alleviation of bone density loss. In addition, we believe that this could be used as a preventative movement stimulation method that would increase bone density to the point where bones would be able to tolerate the load placed on them via the external force of falling. We report the use of this method to alleviate sudden bone mass loss by continuously applying stimulation movements using the vibration and shaking stimulation device that we newly developed over a fixed period of time to OVX mice that simulate post-menopausal women.

Materials and Methods

Experimental animals

The 24 ICR mice aged 8 weeks (CLEA Japan, Inc.) with bilateral OVX were included. CB stimulation was initiated 1 week postoperatively. The animals were divided into the following two groups: OVX group (n = 12), in which the mice underwent ovariectomy, and the wild type (WT) group (n = 12), in which the mice did not undergo ovariectomy. Each of these groups was further divided into two subgroups as follows: stimulation group: plus (+) group (n = 6) and non-stimulation group: minus (−) group (n = 6). These four groups (n = 24) were then organized as follows: OVX group/stimulation group, +/+ group (n = 6); WT group/stimulation group, −/+group (n = 6); OVX group/non-stimulation group, +/−group (n = 6); and WT group/non-stimulation group, −/−group (n = 6). All animals were maintained according to protocols approved by the Institutional Animal Care and Use Committee of Fujita Health University (approval number H0702). This article does not contain any studies with humans performed by any of the authors.

Stimulation parameters

Shaking stimulation was horizontal rotation movement (movement distance: 50 mm, 150 times/min) with a variable axis of rotation that applied uniform stimulation in all directions (360 degrees). The vibration stimulation was a tornado-type of vibration (movement distance: 5 mm, 2,400 times/min). The device was made so that the lower level contained a shaking device (Nissin Scientific Corporation, Japan; NX-25D) and the upper level, a vibration device (Nissin Scientific Corporation, Japan; SK-40-D1). Stimulation was applied simultaneously to 12 mice: 6 mice each in the OVX group and the WT group. The stimulation parameters were 30 minutes of stimulation 1 times/day and 6 times /week for 10 continuous weeks.

Double-labeling of the femur

Tetracycline (TC: Merrck KGaA, Darmstadt, Germany) and calcein (CL: Dojindo Molecular Technologies, Inc. Japan) were used to double-label the femurs. Day 5

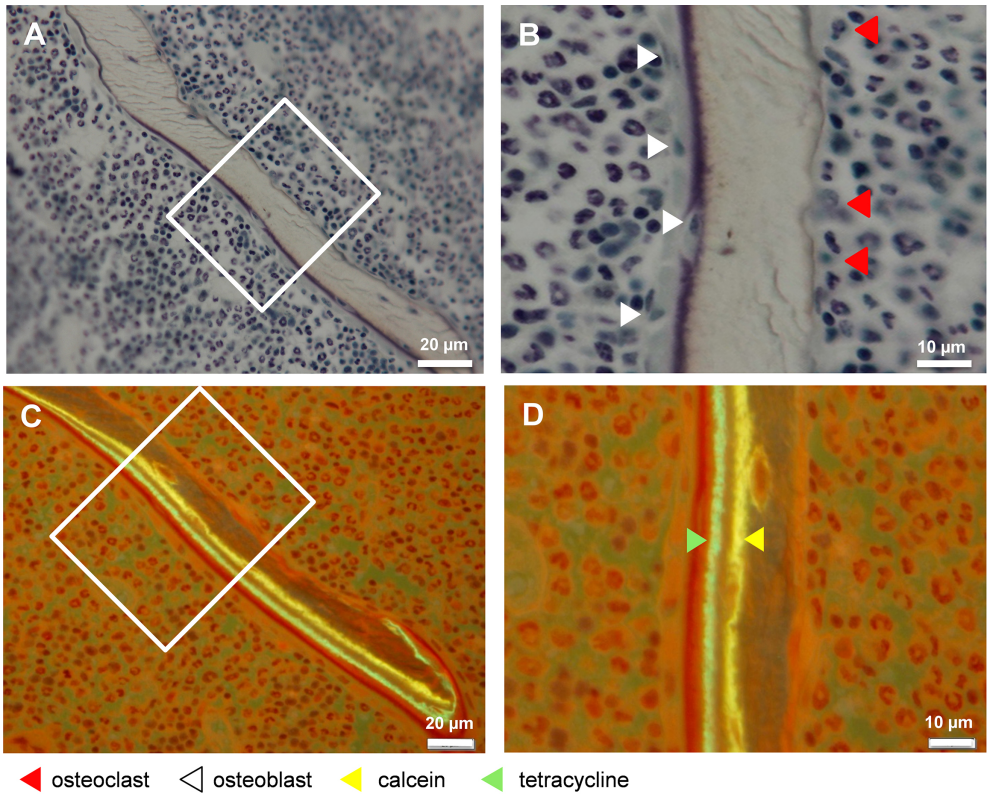

Fig. 1. The femur stained with Villanueva Bone Stain.
The osteoid surface, eroded surface, osteoblast, osteoclast, can be observed. The femur stained with Villanueva Bone Stain. The images show sagittal cross sections of femur of ICR osteoporotic model mice stained with Villanueva Bone Stain. Photo A and C show low-power fields with 20 μm scale bars. Photo B and D show high-power fields with 10 μm scale bars. Photo A and B were observed under natural light. Photo C and D were observed under fluorescent light. The symbols in the images are, red arrowhead: osteoblast, white arrowhead: osteoclast, green arrowhead: calcein, and yellow arrowhead: tetracycline.

before euthanizing the mice was designated as Day 1, on which TC was subcutaneously injected into the dorsocervical region of the mice. CL was injected in the same way 48 hours later (Day 3). Mice were killed, and their femurs resected after another 48 hours (Day 5).

Creation of non-decalcified thin-section specimens

After resecting the femurs, excess soft tissue was removed, and specimens were fixed in 70% ethanol solution in a cool, dark location. Staining was performed for four days using Villanueva Bone Stain. After dehydrating the bones, they were placed in a solution of acetone and methyl methacrylate monomer (MMA) for 24 hours.

Observation and morphological measurements of the femur tissue specimens

We observed the tissue sample under natural light and fluorescent light, and performed osteomorphometry to directly measure primary parameters (Fig. 1). The primary parameters consisted of 21 items. Observation of the bone tissue was done under natural light. Direct measurements of the area, length, and width were taken. The result of the primary parameter observations was 21 items of data. The secondary parameters were based on the primary parameters and consisted of using mathematical formulae to make calculations. These consisted of 43 items in the following 4 categories: bone mass, morphology, absorption, and calcification.

Statistical processing

Comparisons of the parameters after bone morphology measurements were taken using the Kruskal-Wallis H-test. Comparisons in cases of significant difference were conducted using the Mann-Whitney U test. The standard of significance for all statistical methods was set to less than 5%.

Results

The body weight of the mice was measured chronologically from the age of 8 to 20 weeks (Fig. 2). Body weight

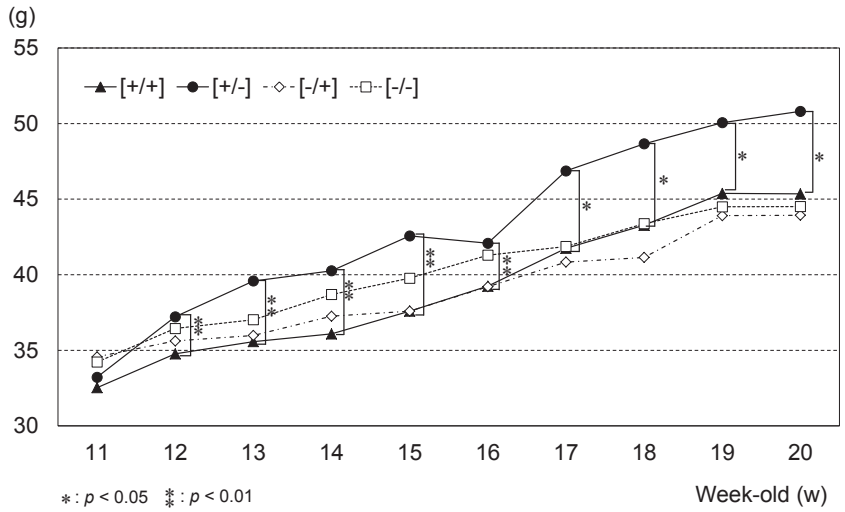

Fig. 2. Changes in body weight with age, from 11 to 20 weeks.
The body weight of the (+/−) group showed a significant increase from 12 to 16 weeks of age ($p < 0.01$), from 17 to 20 weeks of age ($p < 0.05$) as compared with the (+/+) group.

was significantly more increased in the +/−group than in the +/+ group at 12–16 weeks ($p < 0.01$) and 17–20 weeks ($p < 0.05$). After primary parameters were measured (21 items), secondary parameters (43 items) were calculated using the bone histomorphometry measurements, and all groups were compared. Comparison of the OVX group/stimulation group (+/+group) and the OVX group/non-stimulation group (+/−group), which simulated post-menopausal women, showed that the +/+group was significantly more increased than the +/−group for the following items ($p < 0.01$): bone surface (tissue volume; BS/TV: μm/μm^2), trabecular number (Tb.N: N/mm), osteoclast number (eroded surface; N.Oc/ES: N/mm), osteoclast surface (eroded surface; Oc.S/ES: %), osteoid maturation time (Omt: day), and bone mineralization lag time (Mlt: day). The +/+group was significantly more increased than the +/−group for the following items ($p < 0.05$): osteoblast number (N.Ob: N), (tissue volume; N.Ob/TV: N/mm^2), single-labeled surface (sLS: μm), multinuclear Osteoclast number (eroded surface; N.Mu.Oc/ES: N/mm), and mononuclear Osteoclast number (eroded surface; N.Mo.Oc/ES: N/mm). The +/+group was significantly more decreased than the +/−group for the following items ($p < 0.01$): bone surface (bone volume; BS/BV: μm/μm^2), trabecular separation (Tb.Sp: μm), mineral apposition rate (MAR: μm/day), and adjusted apposition rate (Aj.Ar: μm/day). In addition, the +/+group was significantly more decreased than the +/−group for the following items ($p < 0.05$): eroded surface (ES: μm), (bone surface; ES/BS: %), and bone resorption rate (BRs.R: mm^2/mm^2/year).

We then compared the WT group/stimulation group (−/+group) and the WT group/non-stimulation group (−/− group), which simulated young (premenopausal) women, to examine the results of stimulation applied by the device on control populations. The −/+group was significantly more increased than the −/−group for the following measured items ($p < 0.01$; Table 1): osteoid maturation time (Omt: day) and bone surface (tissue volume; BS/TV: μm/μm^2). The −/+group was significantly more increased than the −/−group for the following items ($p < 0.05$): bone volume (BV: μm^2), labeled surface (osteoid surface; LS/OS: %), and trabecular number (Tb.N: N/mm). In addition, the −/+group was significantly more decreased than the −/−group for the following items ($p < 0.01$; Table 1): eroded surface (bone surface; ES/BS: %), mineral apposition rate (MAR: μm/day), adjusted apposition rate (Aj.Ar: μm/day), and bone surface (bone volume; BS/BV: μm/μm^2). The −/+group was significantly more decreased than the −/−group for the following items ($p < 0.05$; Table 1): eroded surface (ES: μm) and multinuclear osteoclast number (tissue volume; N.Mu.Oc/TV: N/mm^2).

In our comparisons of postmenopausal women who exercise and young (premenopausal) women who exercise, the +/+group was significantly more increased than the −/+group for the following items ($p < 0.01$; Table 2): mononuclear Osteoclast number (N.Mo.Oc: N), (bone surface; N.Mo.Oc/BS: N/mm), osteoclast number (eroded surface; N.Oc/ES: N/mm), mononuclear osteoclasts number (tissue volume; N.Mo.Oc/TV: N/mm^2), osteoclast number (tissue volume; N.Oc/TV: N/mm^2), and adjusted apposition rate (Aj.Ar: μm/day). The +/+group was significantly more increased than the −/+group or the following items ($p < 0.05$; Table 2): multinuclear osteoclast

Table 1 Bone histomorphometry (−/+ VS −/−)

	Omt (Day)	BS/ TV (μm/μm²)	BV (μm²)	LS/ OS (%)	Tb.N (N)	ES/ BS (%)
−/+	1.89 ± 0.31 **	0.008 ± 0.002 **	260704.60 ± 35929.15 *	166.78 ± 24.05 *	3.89 ± 1.22 *	22.26 ± 5.88 **
−/−	1.36 ± 0.15	0.005 ± 0.002	216560.79 ± 29182.08	137.33 ± 20.69	2.53 ± 0.84	33.32 ± 7.12

	MAR (μm/ day)	Aj.Ar (μm/ day)	BS /BV (μm/ μm²)	ES (μm)	N.Mu.Oc/ TV (N/ mm²)
−/+	1.28 ± 0.31 **	1.48 ± 0.37 **	0.026 ± 0.005 **	1310.44 ± 151.21 *	0.0007 ± 0.0003 *
−/−	2.00 ± 0.18	2.33 ± 0.36	0.051 ± 0.009	1989.74 ± 520.62	0.0011 ± 0.0003

*: $p < 0.05$ **: $p < 0.01$

Table 2 Bone histomorphometry (+/+ VS −/+)

	N.Mo.Oc (N)	N.Mo.Oc/BS (N/mm)	N.Oc/ES (N/mm)	N.Mo.Oc/TV (N/mm²)	N.Oc/TV (N)	Aj.Ar (μm/day)
+/+	9.67 ± 1.97 **	0.18 ± 0.05 **	1.47 ± 0.19 **	0.0011 ± 0.0002 **	0.002 ± 0.0001 **	1.60 ± 0.44 **
−/+	5.67 ± 1.97	0.09 ± 0.04	0.95 ± 0.27	0.0006 ± 0.0002	0.001 ± 0.0001	1.48 ± 0.37

	N.Mu.Oc (N)	N.Mu.Oc/ES (N/mm)	N.Mo.Oc/ES (N/mm)	N.Oc/BS (N/mm)	N.Mu.Oc/TV (N/mm²)	OV/BV (%)
+/+	11.33 ± 4.32 *	0.79 ± 0.18 *	0.68 ± 0.06 *	0.38 ± 0.13 *	0.0013 ± 0.0004 *	3.97 ± 0.92 *
−/+	6.67 ± 2.20	0.51 ± 0.18	0.43 ± 0.15	0.21 ± 0.08	0.0007 ± 0.0003	2.80 ± 0.77

	MS/BS (%)	QS (μm)	BV (μm²)	BV/TV (%)	LS/OS (%)	BRs.R (mm²/mm²/year)	Tb.Sp (μm)
+/+	48.20 ± 8.67 *	2823.07 ± 520.83 **	175518.44 ± 35016.62 **	19.46 ± 3.54 **	133.38 ± 12.79 **	0.15 ± 0.03 *	249.00 ± 54.93 *
−/+	38.11 ± 2.83	4604.52 ± 1008.94	260704.60 ± 35929.15	28.91 ± 4.36	166.78 ± 24.05	0.25 ± 0.10	351.68 ± 72.68

*: $p < 0.05$ **: $p < 0.01$

number (N.Mu.Oc: N), multinuclear osteoclast number (eroded surface; N.Mu.Oc/ES: N/mm), mononuclear osteoclast number (eroded surface; N.Mo.Oc/ES: N/mm), osteoclast number (bone surface; N.Oc/BS: N/mm), multinucleated osteoclast number (tissue volume; N.Mu.Oc/TV: N/mm^2), osteoid volume (bone volume; OV/BV: %), and mineralization surface (bone surface; MS/BS: %). The +/+group was significantly more decreased than the −/+group for the following items ($p < 0.01$; Table 2): quiescent surface (QS: μm), bone volume (BV: μm^2), bone volume (tissue volume; BV/TV: %), and labeled surface (osteoid surface; LS/OS: %). The +/+group was significantly more decreased than the −/+group for the following items ($p < 0.05$; Table 2): bone resorption rate (BRs.R: mm^2/mm^2/year) and trabecular separation (Tb.Sp: μm).

In our comparisons of young premenopausal women who exercise and those who do not exercise, the −/+group was significantly more increased than the −/−group for the following items ($p < 0.01$; Table 3): osteoclast number (tissue volume; N.Oc/TV: N/mm^2) and labeled surface (bone surface; LS/BS: %). The +/−group was significantly more increased than the −/−group for the following items ($p < 0.05$; Table 3): osteoclast number (bone surface; N.Oc/BS: N/mm) and mineralizing surface (bone surface; MS/BS: %). The +/−group was significantly more decreased than the −/−group for the following items ($p < 0.01$; Table 3): quiescent surface (QS: μm), bone volume (BV: μm^2), and bone volume (tissue volume; BV/TV: %). The +/−group was significantly more decreased than the −/−group in other void (Double+single) label surface area (Vd (d+s) LS: μm; $p < 0.05$; Table 3).

Discussion

The femur has a high percentage of cancellous bone in its proximal end, and structurally, it is more prone to

Table 3 Bone histomorphometry (−/+ VS −/−).

	N.Oc/TV (N/mm²)		LS/BS (%)		N.Oc/BS (N/mm)		MS/BS (%)	
−/+	0.001 ± 0.0001	**	48.22 ± 5.10	**	0.21 ± 0.08	*	38.11 ± 2.83	*
−/−	0.002 ± 0.0001		44.05 ± 3.85		0.31 ± 0.07		36.75 ± 4.96	

	QS (μm)		BV (μm²)		BV/TV (%)		Vd(d+s)LS (μm)	
−/+	4604.52 ± 1008.94	**	260704.60 ± 35929.15	**	28.91 ± 4.36	**	700.99 ± 123.05	*
−/−	4802.31 ± 1271.57		216560.79 ± 29182.08		24.29 ± 2.79		719.30 ± 195.88	

* : $p < 0.05$ ** : $p < 0.01$

the effects of decreased bone volume than the cortical bone[14, 15]. Bone mass loss associated with menopause occurs to a large degree in the cancellous bone. Thus, an effective prevention method would be to suppress bone mass decrease in the regions of bone that are abundant with cancellous bone. Exercise that applies appropriate load on bones and muscle strength can be effective in the suppressing bone density loss in postmenopausal women, and may alleviate bone mass loss in bones that bear a large portion of exercise load[16, 17]. Researchers have also suggested that exercise that applies a high degree of mechanical load, such as sudden bending load and impact load, on bones may be effective in increasing bone density[17].

In our present study, we applied stimulation using a new double-loading device that combined vibration and shaking to a postmenopausal women mice model and analyzed femur tissue specimens using bone histomorphometry measurements. The results indicated that the +/+group, consisting of OVX mice to which stimulation was applied, had significantly greater osteoblast number (N.Ob) and osteoblast number (tissue volume; N.Ob/TV), which are indicators of bone formation, than the +/− group, to which stimulation was not applied (Fig. 3A, B). During bone remodeling, osteoblasts are the cells that perform bone formation because they have an affinity for calcium and regulate osteoid mineralization[18]. Increases in osteoblasts are thought to simply be an indication that they assist in osteoid mineralization. Bone surface (tissue volume; BS/TV) and single-labeled surface (sLS), which are indicators of bone structure, were significantly higher in the +/+group than in the +/−group. We believe this indicates that bone volume increased (Fig. 3C, D). In addition, we believe that MSC bone formation induced by the mechanical stimuli was activated by the major pathway for bone formation (Wnt-β-catenine pathway)[19, 20]. The dynamic index of the trabecular bone number (Tb.N), which is an indicator of bone structure, was significantly higher in the +/+group than in the +/−group (Fig. 3E). The direction of distribution of trabecular bone is consistent with the line of force of load. This is closely related to the mechanical strength against the load[14, 15]. Increases in trabecular bone due to stimulation suggest increased bone strength. An equal amount of bone resorption and bone formation is needed in all areas where bone remodeling occurs throughout the body to maintain decreasing bone density[21]. Trabecular separation (Tb.Sp), which are an indicator of bone structure, were significantly lower in the +/+group than in the non-stimulation group (+/− group; Fig. 3F). We believe this indicates that trabecular bone spaces became smaller, the cylindrical structures within the bone structure underwent relative declines, connectivity increased, and structural complexity increased[22]. Bone surface (bone volume; BS/BV), which is an indicator of bone structure, was significantly lower in the +/+group than in the +/−group (Fig. 3G). However, bone surface (tissue volume; BS/TV) was significantly higher in the +/+group (stimulation group). In addition, bone volume (BS/TV) increased significantly more in the +/+group than in the +/−group. Eroded surface (ES, bone surface; ES/BS), annual bone resorption rate (BRs.R), and trabecular separation (Tb.Sp), which are indicators of bone resorption, had low values, indicating that bone resorption was alleviated in the +/+group. This suggests the possibility that the groups that underwent stimulation had abundant bone mass. Osteoclast surface (eroded surface; Oc.S/ES); multinuclear osteoblast number (eroded surface; N.Mu.Oc/ES), mononuclear osteoclast number (eroded surface; N.Mo.Oc/ES), osteoclast number (eroded surface; N.Oc/ES), which is an indicator of bone resorption, was significantly higher in the +/+group than in the +/−group (Fig. 3H, I, J, K). Although possible causes include sudden hormonal imbalance following OVX, it is thought that continuous stimulation induces active remodeling. During bone remodeling, osteoclasts, which are large multinucleated cells, play a role in absorbing bone tissue. Eroded surface (ES, bone surface; ES/BS) and bone resorption rate (BRs.R), which are linear indicators associated with bone resorption, were significantly lower in the +/+group than in the +/−group (Fig. 3L, M,

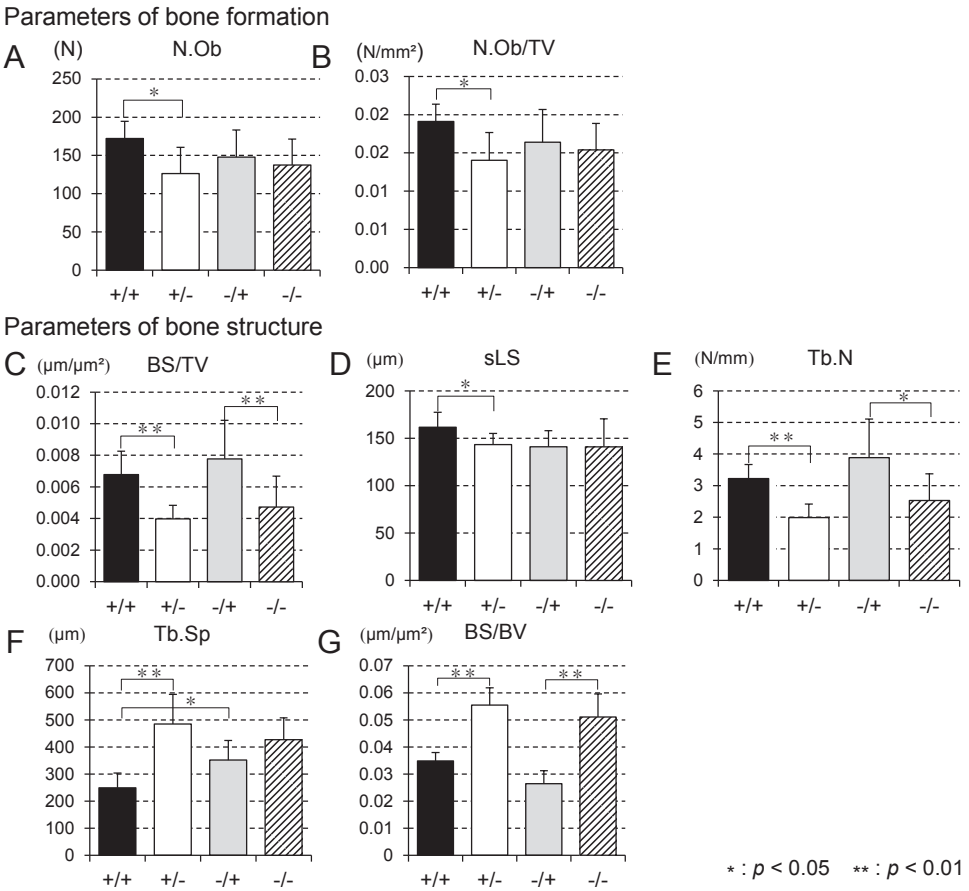

Fig. 3. Analysis of calculated parameters using bone histomorphomtry.

Parameters of bone formation show to graph A and B. Graph of A: osteoblast number (N.Ob; N), Graph of B: osteoblast number (N.Ob/TV; N/mm^2). Parameters of bone structure show to graph from C to G. Graph of C: bone surface (BS/TV; μm/μm^2), Graph of D: single labeled suface (sLS; μm), Graph of E: trabecular number (TB.N; N/mm), Graph of F: trabecular separation (Tb.Sp; μm), Graph of G: bone surface (BS/BV; μm/μm^2). Parameters of bone resorption show to graph from H to N. Graph of H: osteoclast surface (Oc.S/ES; %), Graph of I: multi-nuclear Osteoclast number (N.Mu.Oc/ES; N/mm), Graph of J: mononuclear Osteoclast number (N.Mo.Oc/ES; N/mm), Graph of K: osteoclast number (N.Oc/ES; N/mm), Graph of L: eroded surface (ES; μm), Graph of M: single labeled suface (ES/BS; %), Graph of N: bone resorption rate (BRs.R; mm^2/mm^2/year). Parameters of bone mineralization show to graph from O to R. Graph of O: osteoid maturation time (Omt; day), Graph of P: mineralization lag time (Mlt; day), Graph of Q: adjusted apposition rate (Aj.Ar; μm/day), Graph of R: mineral apposition rate (MAR; μm/day). Statistical analyses were performed to assess the significance of differences between individual group with reference to the (+/+). The closed bars represent the OVX/stimulation (+/+), the open bars represent the OVX/non-stimulation (+/−), the shaded bars represent WT/stimulations (−/+), and the hatched bar represent the WT /non-stimulation (−/−). *: $p < 0.05$ and **: $p < 0.01$.

N). This indicates that osteoclast number (N.Mu.Oc/N.Mo.Oc) based on eroded surface area was promoted, but the linear indicator that shows a more comprehensive picture of bone resorption was lower. This result seems to indicate that interleukin-6, which stimulates osteoclasts and osteoblasts, was produced[23]. Osteoid maturation time (Omt) and bone mineralization lag time (Mlt), which are indicators associated with mineralization, was significantly higher in the +/+group than in the +/−group (Fig. 3O, P). In humans, the shift from bone resorption to bone formation during the bone remodeling process is as follows in humans: Bone resorption at the site of remodeling is complete after several weeks, and bone formation occurs over a period of several months[24]. Bone formation takes longer than bone resorption, and bone cells are absorbed by osteoclasts before they are fully mature. We assume that the +/+group, were in the middle of the remodeling process and, as a result, absorption and formation were in an activate state. We believe that this is what caused revisions in the osteoid maturation and delays in mineralization. The +/+group had significantly lower values for Adjusted mineralization rate (Aj.AR) and mineral apposition rate (MAR), which are related to bone mineralization, were significantly lower in the +/+group

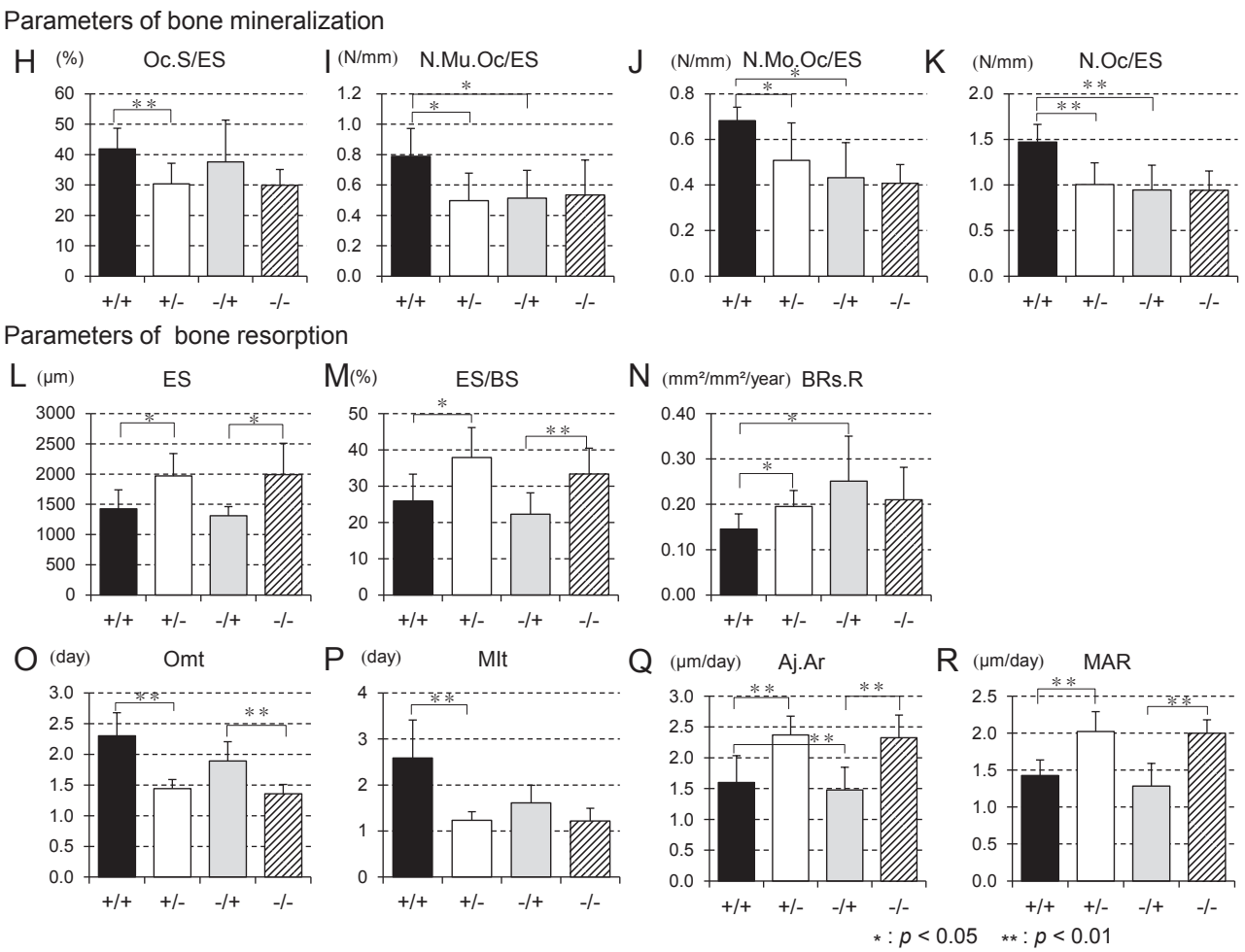

Fig. 3. Continued.

than in the +/−group (Fig. 3Q, R). Mice that underwent stimulation had delayed mineralization rates, and thus, it is possible that mineralization was insufficient. This is because the promotion of bone remodeling causes the lifespan of the bone matrix to be reduced, which prevents the development of full secondary mineralization. This, in turn, causes reduced mineralization in terms of wall thickness[25, 27]. These then are the factors that lead to reduced mineralization as a result of the promotion of bone remodeling. Although CB stimulation lowered the degree of mineralization in terms of wall thickness, this suggests that non-uniformity of intra-bone mineralization was maintained. This study was an analysis of 20-week-old mice. A mouse's lifespan is 100 weeks, and their bone turnover rate is 30 times that of humans[27]. In addition, 20-week-old mice are at an age equivalent to middle-aged humans and display the same physiological functions at that stage, including continuous decline in bone metabolism. The non-stimulation groups showed decreased metabolic turnover and had an accumulation of bone due to the lack of bone resorption, which increased the degree of mineralization. This, in turn, promoted bone rigidity. Sites with older bone have a high degree of mineralization with a simultaneous increase in consistency in the intra-bone degree of mineralization which leads to decreased bone hardness. This also promotes bone rigidity. It is thought that neighboring sites of highly mineralized bone and less mineralized bone that are intermixed are important aspects associated with bone micro damage prevention. Thus, it is important to ensure increased mineralization throughout bone tissue and non-uniform degree of mineralization[28]. These results demonstrated a significant difference between the groups that underwent vibration and shaking CB stimulation and may indicate that stimulation had an effect on the process of bone mineralization. The promotion of bone metabolic turnover slows the mineralization rate, and although the degree of mineralization is lower, the distribution of that degree is more uniform. We believe that may have the effect of preserving bone rigidity.

Conclusions

The results of our study indicate that CB stimulation of mice with vibration and shaking had an effect on osteoblasts and osteoclasts, which suppressed bone resorption and promoted bone formation. In addition, this type of stimulation promoted bone remodeling, which suggests that it had an effect on bone mineralization. Although promoting bone metabolic turnover causes lower mineralization, the distribution of the surface area that undergoes mineralization increases, which we believe may preserve bone rigidity. Further studies are needed on the clinical application of this type of CB stimulation with vibration and shaking in humans. In addition, studies are warranted to confirm that it is effective as a novel preventative measure against osteoporosis that leads to decreased risk of bone fractures from the perimenopausal stage to the postmenopausal stages in elderly women.

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