

<Original article>

Shaking stimuli can retard accelerated decline of bone strength of a mouse model assumed to represent a postmenopausal woman

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Summary In contrast in elderly people, if bone formation and resorption are out of balance, bone mineral density (BMD) diminishes leading to progressive development of osteoporosis, a cause of easy bone fracture. Standing still for prolonged periods leads to fatigue. Humans tend to avoid this by shifting the body weight unconsciously or consciously left and right or back and forth. Standing in an erect posture on a board that continuously shakes horizontally causes all the skeletal muscles of the body as well as the entire skeleton to act to restore balance. As a result of the application of such shaking-induced stimuli to a reduced-BMD mouse model of postmenopausal osteoporosis, rapid decline in the bone strength of the femur was suppressed. A shaking stimulus such as was used in this study could easily be applied to human beings in future once further study is conducted to validate the results.

Key words: Osteogenesis, Bone resorption, Shaking, Physical therapy

1. Introduction

A rise in average longevity along with improved treatment for organ disorders and various diseases has rendered age-associated bone mass reduction a great clinical issue. This problem needs to be solved by elucidation of its pathogenesis and establishment of appropriate therapeutic options as well as preventive methods. Postmenopausal women rapidly lose bone mass within several years of menopause due to reduced estrogen action. In human beings, bone grows in synchrony with body growth during the growth period and thereafter bone mineral density

(BMD) continues to increase for a while after cessation of linear growth. Generally, bone mass in humans reaches a peak in the late twenties to thirties, remaining in a static state for some time thereafter. With further aging, bone volume is maintained but mineral content and strength both progressively decrease. After reaching peak bone mass, women lose cortical bone by 35% and cancellous bone by 50% until the end of life. Women rapidly lose bone mineral content, especially in cancellous component-dominant bone, immediately after menopause and bone mass loss continues slowly thereafter for life¹⁾.

In humans, bone loss can be either acute or

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chronic. For example, about 1% of bone mass is lost after one week of immobilization, while 10 - 20% of bone mass is lost after several months². Bone mass is reduced by Ca, 1 - 2% over a year in postmenopausal osteoporosis. In rats, the rear legs of which were unloaded by tail suspension, their femoral bone mass was reduced within 14 days irrespective of whether the cancellous bone-rich metaphysis or the cortical bone-rich diaphysis was measured³. In the case of mice subjected to tail-suspension, cancellous bone mass was lost by up to 44% after 7 days of such treatment as compared to control. A further 7 days of such treatment did not lead to a further decrease, however⁴.

In this study, we tested exercise therapy using a physical stimulus imposed by a shaker in an attempt to prevent the accelerated reduction of BMD observed in a mouse model of postmenopausal women. The stimulus was provided as follows: A shaking board was constructed of a rotating horizontal plate, the pivot of which made circular motions. When a mouse was placed on the shaking board, the animal tried hard to keep its footing; this posture provided a kind of exercise to distort bone and accelerated reduction of BMD was thereby slowed down. However, real phenomena occurring in the living body seem to be due to indirect influences on the signal transduction pathways exerted by mechanical load-induced flows of extracellular fluid rather than by direct physical distortion of the bone.

The cells in bone that sense mechanical load are presumably osteoblasts and osteocytes. Reportedly, mechanical signals that cells recognize *in vivo* are distortion of bone tissues, changes in hydrostatic pressure, liquid current inside the bone canaliculus and electromagnetic fields generated by liquid currents⁵. Seemingly, these mechanical loads are generated by movement of skeletal muscles attached to the bones. Such movements are conducted by tendons, which passively convey stimuli to bones and these stimuli then affect bone dynamics. In this context, since we assumed that the maximum stimulus should be close to but under the threshold for falling, we determined the appropriate stimulus strength by gradually reducing the level of stimulus from the critical level of standing

firm down to the highest level of being able to keep a standing posture.

Bone histomorphometry is cited as one means of assessing bone metabolism. We prepared decalcified thin sections of mouse femurs to measure cancellous bone volume, trabecular thickness, and trabecular number which are parameters of bone structure. BMD is maintained through balanced bone resorption and bone formation. Loss of this balance leads to osteoporosis. We measured osteoid surface/bone surface, osteoblast surface/bone surface, mineralizing surface/bone surface and mineral apposition rate as parameters of bone formation and eroded surface/bone surface and osteoclast number/bone perimeter as parameters of bone resorption. Increasing numbers of osteoclasts and their increased lifespan depend on animal species, bone site, and age. In rats, osteoclast formation decreases with increasing age, while in mice these parameters depend on strain. In the case of long bones, human postmenopausal bone mass reduction follows different courses between the diaphyses and metaphyses⁶. The lumbar spine rapidly loses bone mass by about 15% in 5 - 6 years after menopause and more slowly thereafter⁷. In contrast, the distal end of the forearm bone loses bone mass by as much as about 25% in a linear manner in 15 - 16 years post-menopause. Whole body bone mass decreases exponentially in the first 12 years after menopause, slowing down thereafter⁶. A lowered level of estrogen greatly affects primarily cancellous bone. The long bones of the limbs are predominantly composed of cortical bone, while their epiphyseal portions are relatively rich in cancellous bone. This is the reason for easy fracture occurring in the femoral neck or in the distal end of the radius¹. Estrogen elevates the calcitonin concentration that suppresses bone resorption. This hormone, furthermore, also stimulates vitamin D action that enhances intestinal absorption of calcium⁸.

Bone fulfils several roles in the body, acting as a store of calcium and phosphorous, providing structural support and acting as a passive locomotive organ. Maximum locomotive potential requires maintenance of bone strength as well as muscle. Bone responds to an applied mechanical load by adjusting the signals

governing remodeling. When bone cells sense a stimulus from a mechanical load, bone formation is stimulated on the surface of trabeculae, leading to an increase in bone mass. However if such stimuli are below the threshold level, bone resorption is enhanced, resulting in loss of bone mass. In adults, these processes, occur continuously so that bone mass is stable. In contrast in elderly people, if bone formation and resorption are out of balance, BMD diminishes leading to progressive development of osteoporosis, a cause of easy bone fracture.

There is a relatively new loading modality in which dynamic loads are to induce bone formation in the femur. The method of knee loading that the left knee of mice was loaded with 0.5 N force at 5, 10, or 15 Hz for 3 min/day for 3 consecutive days⁹⁾. We are interested in that evaluate the effects of loading frequencies (in Hz) on bone formation at the site away from the loading site on the knee. However, our stimulation method is the method that is almost exercise therapy by stimulation using shaker.

In this study, mice were bilaterally ovariectomized to create a mouse model (OVX mice) with suppressed secretion of estrogen. These mice were assumed to mimic postmenopausal women. We developed a method to impose a shaking stimulus which could provide indirect mechanical stress more efficiently to bone via the skeletal muscles. We analyzed the suppression of the decrease of BMD by employing bone histomorphometry in our model. Bone resorption was more suppressed in OVX mice that were stimulated compared to those that were not stimulated. In a non-ovariectomized control group, bone formation was promoted in mice that were stimulated compared to those that were not stimulated. Humans differ from mice in that they are bipedal, although mice can also stand on two feet. Such a shaking stimulus could therefore prove useful as a form of physical therapy to improve bone dynamics and could easily be applied in a clinical setting once these results are validated in the human. Moreover, such shaking-induced stimuli could provide a novel physical therapy not only for elderly people but also for health promotion in younger people with reduced BMD.

2. Materials and Method

1. Mouse Model

Ovaries were removed from 9-week-old female ICR mice under nembutal anesthesia. Estrogen secretion is decreased by ovariectomy, therefore, these mice represent an osteopenic model (OVX mouse). Animals in this study were kept in the vivarium of Fujita Health University, and studies were conducted under Animal Use Committee regulations.

2. Shaking stimulus

Twelve ovariectomized mice (OV group) and 12 non-ovariectomized mice (i.e., wild type: WT group) were further subdivided into stimulated and non-stimulated groups, to create a total of 4 groups: Shaking stimulus/ovariectomy (+/+), no stimulus/ovariectomy (-/+), shaking stimulus/intact ovaries (+/-), and no stimulus/intact ovaries (-/-). The shaking stimulus was provided by a platform, shaking at a rate of 150 horizontal rotations per minute for 30 minutes (NR-3; TAITEC Co. Ltd Japan). This stimulus load was repeated 6 times a week for 10 weeks. Horizontal rotations acted to exert strain on the femurs from all directions. Individual mice under stimulus were separated from each other by placing them in acrylic cases (W60 × L140 × H70 mm), the floors of which were moderately slippery. After the series of stimuli were completed, mice were euthanized and femurs were carefully removed and cleaned of adhering soft tissues.

3. Administration of bone-labeling agent

For later preparation of decalcified bone specimens and subsequent bone histomorphometry, we administered calcein (Wako Pure Chemical Industries, Ltd. Japan) to animals twice at an interval of 6 days: At 8 and 2 days before bone sampling. The administration interval made it possible to measure dynamic parameters such as mineral apposition rate and bone formation rate by bone histomorphometry. Calcein was dissolved in 2% sodium bicarbonate solution at a concentration of 16 mg/kg body weight/10 ml. It was intraperitoneally administered to mice at a dose of

0.1 ml/10 g of body weight.

4. Preparation of bone specimens

After whole body perfusion fixation with 4% paraformaldehyde, the femurs were carefully removed so that the periosteum and bone were not damaged; they were then cleaned of attached muscles and other soft tissues. The excised femurs were fixed with about 25 times as large volume as sample femur of 70 - 80% ethanol. Fixation continued for 6 days with gentle agitation; fixative solutions were exchanged every other day. The femurs were embedded in glycolmethacrylate (GMA) resin with a similar hardness to hard tissue. Decalcified thin section preparations were made from the embedded femurs.

All procedures for embedding with GMA resin were performed at low temperature because the staining procedures required enzyme reactions. For staining osteoclast-specific tartrate resistant acid phosphatase (TRAP), GMA resin-embedded blocks were sectioned at a width of 4 mm and stained with the azo dye method following standard protocols. The specimens were stained with toluidine blue for morphological observation as well as histomorphometric measurements. These toluidine blue-stained specimens were used for measurement of both static and dynamic parameters.

5. Bone histomorphometry

We used bone histomorphometry as a measure of bone metabolism. Various parameters were measured in decalcified thin section specimens: Parameters related to bone structure included bone volume/total tissue volume, trabecular thickness, trabecular number, and trabecular space; Parameters related to bone formation include osteoid volume/bone volume, osteoid surface/bone surface, osteoid thickness, osteoblast surface/bone surface, mineralizing surface/bone surface, mineral apposition rate, and bone formation rate/bone surface. Since the distal ends of mouse femurs were too small to carry out histomorphometric analyses of osteoclasts, they were observed in TRAP-stained specimens. Mineral apposition rates were calculated using measurements of the interval between two fluorescence-labeled calci-

fication front lines based on the time interval between calcein administrations¹⁰.

6. Statistical analyses

We performed the statistical method to compare each group with the control group using the Tukey-Kramer method. All the statistical method of Fig. 3-6 depends on this method. Statistical analyses were conducted with JMP 8.0.1 (SAS Institute Japan Inc., Tokyo) software. For all analysis, the level of significance was set at $p < 0.05$.

3. Results

One of the causes of the postmenopausal decline of BMD in women is the reduced secretion of estrogen which plays an important role in bone formation. Therefore, we removed the ovaries of 8-week-old ICR female mice to create a mouse model of postmenopausal osteoporosis. Using these mice, we studied the effects of a shaking stimulus on the femoral bone. The stimulus was generated by horizontally rotating boards on which the experimental animals were housed.

Twenty-four model mice were assigned to 2 groups: One group consisted of 12 ovariectomized mice and the other of 12 wild type (WT) control mice. Each group was further divided into two subgroups: One was stimulated but the other was not. Four experimental groups (shaking stimulus/ovariectomy: +/+, -/+, +/-, -/-) were compared. The shaking stimulus was provided as described in Materials and Methods. For the direction of the stimulus, we chose a horizontally rotating motion with the axis moving in a circle since it would be difficult to instruct the animals to change the orientation of the body and we wanted to impose strain-stimuli on the femur from every direction. Individual mice were separated from each other while allowing them to stand on the moderately slippery floor during each stimulation period.

To assess bone metabolism, we employed bone histomorphometry, preparing toluidine blue-stained specimens that were suitable for histomorphometric observation and measurement (Fig. 1). However the distal ends of the femurs were too small for histo-

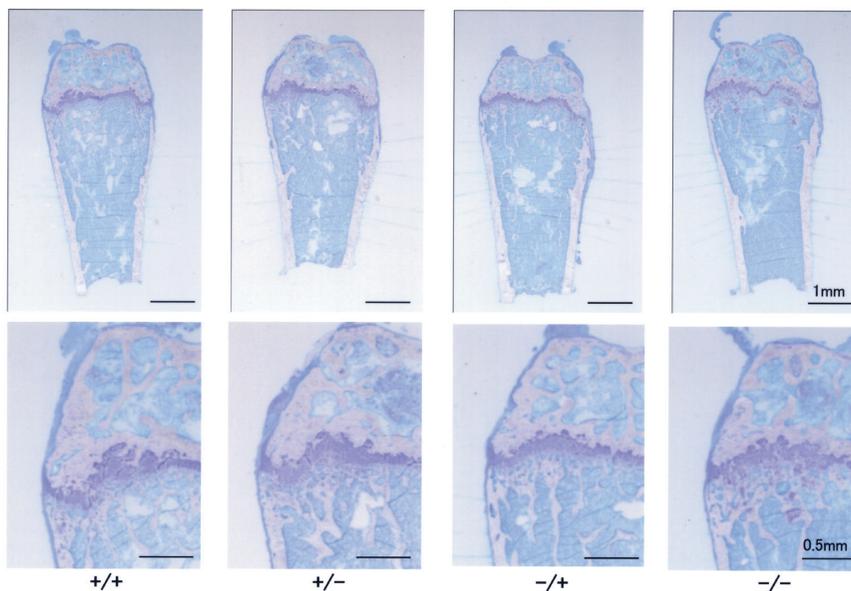


Fig. 1 Histological analysis using toluidine blue staining of the murine femur. Photographs of toluidine blue-stained undecalcified thin preparations of the murine femur (from the diaphysis to the distal epiphysis). Upper panel shows low-power views and the lower panel shows high-power views of the epiphyses. Images of the $+/+$, $-/+$, $+/-$, and $-/-$ groups are from left to right. The scale bar denotes 1 mm in the low-power view and 0.5 mm in the high-power view.

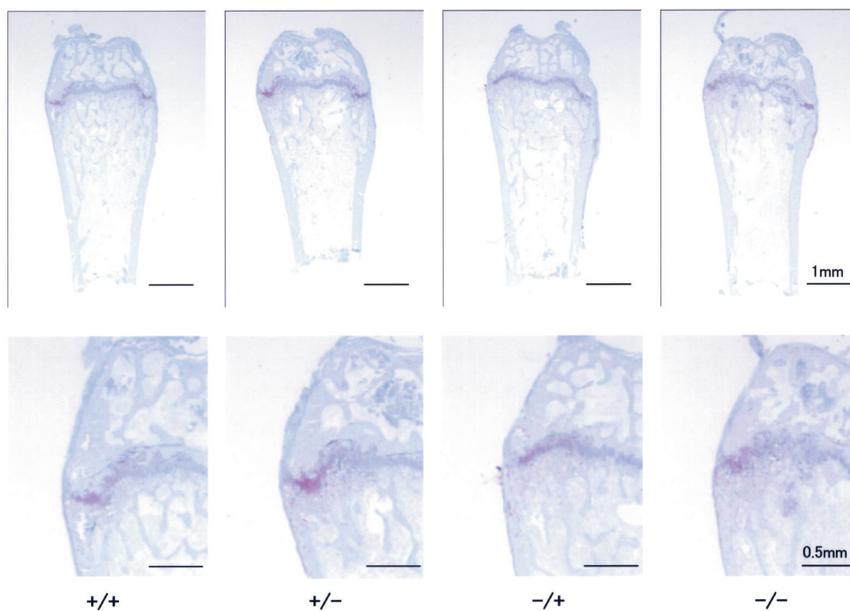


Fig. 2 Histological analysis using TRAP (Tartrate Resistance Acid Phosphatase) staining of the murine femur. Photographs of TRAP-stained undecalcified thin preparations of the murine femur (from the diaphysis to the distal epiphysis). Upper panel shows low-power views and the lower panel shows high-power views of the epiphyses. Images of the $+/+$, $-/+$, $+/-$, and $-/-$ groups are from left to right. The scale bar denotes 1 mm in the low-power view and 0.5 mm in the high-power view.

morphometric observation of osteoclasts. Therefore, we observed osteoclasts in the bone using TRAP-stained specimens (Fig. 2), which were analyzed for bone structure parameters including bone volume/total tissue volume, trabecular thickness, trabecular number, and trabecular spacing (Fig. 3).

The bone volume/total tissue volume and trabecular number were increased in the OVX-stimulation group (+/+) compared to the OVX-non-stimulation group (+/-) ($p < 0.05$). Also in comparison between the non-OVX groups (-/+ and -/-), these parameters were increased in the stimulation group (-/+ ($p < 0.05$). The trabecular spacing was greatest in the OVX-non-stimulation group (+/-), which showed a remarkable reduction of cancellous bone mass. There was a trend towards increased trabecular thickness with stimulation.

We next analyzed bone formation parameters including osteoid volume/bone volume, osteoid surface/bone surface, osteoid thickness, osteoblast surface/bone surface, mineralizing surface/bone surface, mineral apposition rate, and bone formation rate/bone surface (Fig. 4, 5). Analysis of bone formation (osteoblastic function) was made using specimens from animals whose bones were labeled with calcein before sampling. Osteoblasts form bone via two steps, i.e., bone matrix protein production and mineral apposition to osteoid. Since the bone-labeling agent chelates Ca, observable fluorescent lines represent the mineralizing front of the mouse concerned at the moment of administration of calcein. Bone was labeled with calcein administered twice at an interval of 6 days.

We calculated the mineral apposition rate, which represented bone mass produced during the interval, by measuring the distance between the two fluorescence-labeled mineralization front lines under a fluorescent microscope. The results showed that the osteoblast surface/bone surface, the mineral apposition rate, and the bone formation rate/bone surface were increased in the non-OVX/stimulation group (-/+) compared to the non-OVX/non-stimulation group (-/-) ($p < 0.01$). These parameters were also slightly increased in the OVX/stimulation group (+/+) compared to the OVX/non-stimulation group (+/-) to

the same degree as seen between the (-/+) and (-/-) groups. The osteoid thickness varied greatly between individual mice but a trend towards stimulation was observed (Fig. 3).

In addition, we analyzed bone resorption parameters including eroded surface/bone surface, osteoclast number/bone perimeter, and osteoclast surface/bone surface (Fig. 6). Although the osteoclast number/bone perimeter and osteoclast surface/bone surface showed great individual differences, these parameters were larger in both the OVX groups (+/+, +/-) than in the non-OVX groups (-/+, -/-). The bone resorption parameters in stimulation and non-stimulation groups were compared but individual differences were great and there was no significant difference between the two groups. The mean values and standard deviations of the bone histomorphometric data are shown in Figure 3-6.

4. Discussion

It is clear that mechanical load influences bone. Reduction of mechanical load leads to a decline in bone formation and acceleration of bone resorption. In cortical bone of the tibia of 2-month-old male rats, bone formation was halted in both the periosteum and the endosteum after a 19.5-day space flight. Reduced bone formation is said to recover by 26 days after the return to Earth¹¹. Reduction of mechanical load results in a decline in bone formation not only in cortical bone but also in cancellous bone. The number of osteoclasts was found to be increased twofold in the metaphysis of the tibia of a 3-month-old rat that was freed of load by a 5-day space flight¹².

The number of osteoclasts and the contact area between osteoclasts and bone both increase within 1 week of load deprivation, while the number and the area decrease again and return to control levels when loaded over 2 weeks after 2-week load deprivation¹³. As described above, the earth exerts gravity as a load on any living body, even when not exercising consciously or unaware of load. Therefore organisms living on the earth are unconsciously influenced by mechanical load. Conscious mechanical load in this experiment was the shaking stimulus, which was

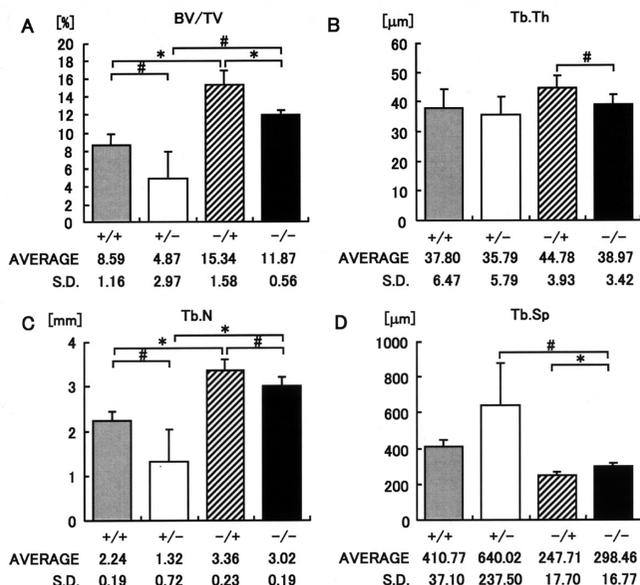


Fig. 3 Parameters of bone structure are shown. A: bone volume/total tissue volume (BV/TV); This parameter represents a volume ratio of the total area of trabeculae to that of the whole bone tissues as a percentage (%). B: trabecular thickness (Tb. Th); (mm), C: trabecular number (Tb. N)/(mm). D: trabecular separation (Tb.Sp); (mm). Statistical analyses were performed between individual groups with reference to the +/+ group. *denotes $p < 0.01$ and # denotes $p < 0.05$. The number of animals in each group was 6.

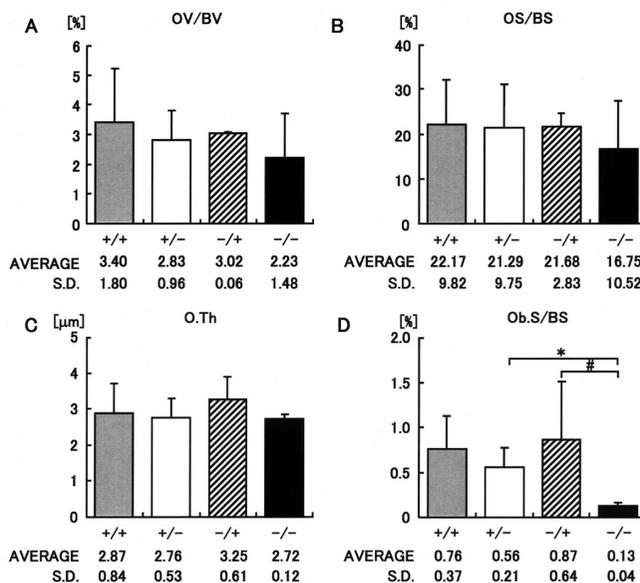


Fig. 4 Parameters of bone formation are shown. A: osteoid volume/bone volume (OV/BV); (%). B: osteoid surface/bone surface (OS/BS); (%); This parameter represents a ratio of the length of the osteoid surface to the whole trabecular surface as a percentage. BS= OS+ ES+ QS [mm] OS; osteoid surface, ES; eroded surface, QS; quiescent surface, C: osteoid thickness (O. Th); (mm). D: osteoblast surface/bone surface (Ob.S/BS). Osteoblast surface; this parameter represents a ratio of the length of the surface covered by osteoblasts to the whole trabecular surface (%). Statistical analyses were performed between individual groups with reference to the +/+ group. *denotes $p < 0.01$ and # denotes $p < 0.05$. The number of animals in each group was 6.

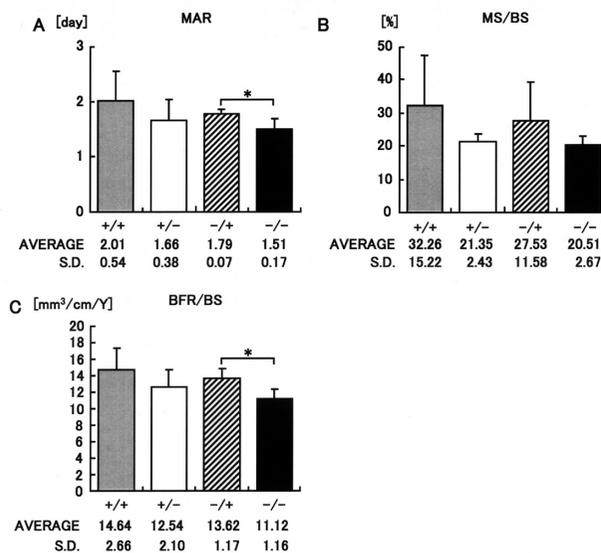


Fig. 5 Parameters of bone formation. A: eroded surface/bone surface (ES/BS); This parameter represents the ratio of uneven surface due to erosion to the whole trabecular surface (%). B: osteoclast number/bone perimeter (N.Oc/B.Pm); This parameter represents the number of osteoclasts per unit perimeter of trabecular bone (number/100mm). C: osteoclast surface/bone surface (Oc.S/BS); This parameter represents the ratio of the length of the surface covered by osteoclasts to the whole trabecular surface as a percentage (%). Statistical analyses were performed between individual groups with reference to the +/+ group. *denotes $p < 0.01$. The number of animals in each group was 6.

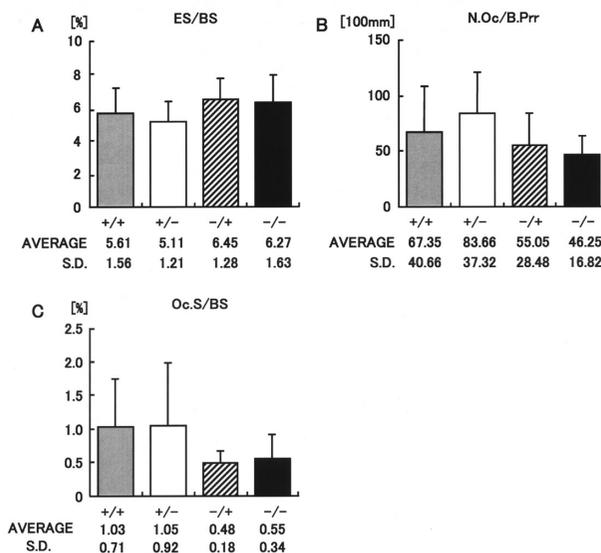


Fig. 6 Parameters of bone resorption are shown. A: mineral apposition rate (MAR); This parameter represents a value that is obtained by dividing the distance between two calcein-labeled mineralization surfaces by the labeling interval in days (mm/day) B: mineralizing surface/bone surface (MS/BS); This parameter represents the ratio of the mineralization surface to the total of all trabecular surfaces as a percentage (%). C: bone formation rate/bone surface (BFR/BS); This parameter represents the bone formation rate per year per unit of trabecular surface. ($\text{mm}^3/\text{cm}^2/\text{year}$) $\text{BFR} = \text{MAR} \times (\text{MS/BS})$, Statistical analyses were made between individual groups with reference to the +/+ group. The number of animals in each group was 6.

given for one 30 minute session daily. This session of stimulus was imposed on the animals once a day for 6 successive days, followed by a 1-day break. This 1-week course was repeated 10 times. Mechanisms of promotion of bone formation and suppression of bone resorption were not clarified but the shaking stimulus was considered to have significantly accelerated bone formation, thus slowing down the decrease in BMD of the femur seen in the mouse osteopenic model.

Bone tissues are a target of estrogen, so if rats are ovariectomized, estrogen receptor alpha (ER α) and estrogen receptor beta (ER β) in cancellous bone are down regulated¹⁴. It is not clear which subtype mediates estrogenic actions; however ER α expression in human osteocytes is augmented by estrogen¹⁵. The ovariectomy-induced decrease in bone mass in the rat is blocked by administration of nitrogen monoxide (NO)¹⁶. Estrogen increases the expression of endothelial nitric oxide synthase (eNOS), an enzyme that produces NO, in human osteoblasts¹⁶. This hormone induces apoptosis in osteoclasts¹⁷, whereas estrogen depletion stimulates apoptosis in osteocytes¹⁸.

It is absolutely certain that estrogen influences bone tissues. In our study, the shaking stimulus affected bone in a positive way, i.e. in the direction of promoting bone formation, both in ovariectomized, that is, estrogen deficient and in normal mice. Although the mechanisms underlying this result are unclear, the shaking stimulus promoted bone formation especially in the cancellous bone, known to be greatly influenced by estrogen. We may therefore safely say that a shaking stimulus is an effective method of imposing mechanical loading.

Mechanical load, if imposed on osteocytes *in vitro*, stimulates the flow of extracellular calcium ions (Ca²⁺) into these cells through positive ion channels via indirect factors such as shear stress, liquid current, and hydrostatic pressure. As a result, the intracellular Ca²⁺ concentration is elevated. This Ca²⁺ flow activates protein kinase A (PKA), followed by cAMP elevation, which triggers intracellular signal transduction as well as extracellular autocrine /paracrine signal transduction, ultimately affecting gene expression¹⁹.

At 30-60 minutes after the beginning of myotaxis,

c-fos is transiently increased and cyclooxygenase-2 (COX-2) is upregulated in osteocytes. The COX-2 activity reaches a peak at about 8 hours²⁰⁻²². At 24 hours, genes for insulin-like growth factor (IGF-1) as well as bone matrix proteins such as osteocalcin, are increasingly expressed on the trabecular surface²⁰. Osteoblastic proliferation and differentiation follows, leading to bone formation. Bone is a passive-type locomotive organ, so shaking stimuli must affect bone via these molecular mechanisms mediated by indirect factors induced by skeletal muscle contraction/relaxation.

There is the report that the osteogenic potential of short durations of low-level mechanical stimuli was examined in children with disabling conditions. This study of the pilot RCT have shown for the first time that low-magnitude, high-frequency mechanical stimuli are anabolic to trabecular bone in children, possibly by providing a surrogate for suppressed muscular activity in the disabled²³. On the other hand, there is the report that 1-year prospective, randomized, double-blind, and placebo-controlled trial of postmenopausal women demonstrated that brief periods of a low-level vibration applied during quiet standing can effectively inhibit bone loss in the spine and femur, with efficacy increasing significantly with greater compliance, particularly in those subjects with lower body mass²⁴. However, our stimulation method is totally different from these conventional stimulation methods. Shaking stimuli were given at a rate of 150 horizontal rotations per minute for 30 minutes. This stimulus load was repeated 6 times a week for 9 weeks. Originality of this stimulation method is that horizontal rotations acted as stimuli of strain to femurs from all directions. Our condition of shaking stimuli give at a rate of 150 horizontal rotations per minute for 30 minutes. This stimulus load was repeated 6 times a week for 9 weeks. Originality of this stimulation method is that horizontal rotations acted as stimuli of strain to femurs from all directions.

Skeletal muscles are attached around bones, which may be explained by the concept that bones are passive-type locomotive organs. When bipeds or quadrupeds are in danger of falling, they brace their

legs, especially the lower legs, and try to maintain the balance of the whole body so as to prevent falling due to a stimulus. Skeletal muscles play an important role in this process. Skeletal muscles are connected to bones with tendons at the end. The present method of stimulation imposed greater load on the epiphyses, which have a higher concentration of attachment point, so that bone resorption was suppressed and bone formation was stimulated there in cancellous bones. Although none of these effects were dramatic, this method is likely to play an important role in prevention of fall fracture, taking into account that this method contributes to strengthening of the skeletal muscles.

Shaking stimuli are expected to be a useful physical therapy for postmenopausal women, acting gently on bone to retard the BMD decline occurring over a very short period. Practical application of this shaking stimulus method is expected to promote bone formation and health in younger people as well, who may have decreased BMD due to lack of exercise or an unjustifiable diet.

Conflicts of interest

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