〈Original Article〉

Shaking stimuli may prevent bone fracture by restraining a bone density decrease gently

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Summary When people stand for a long time, they prevent becoming tired by unconsciously shifting the center of mass of the body between the right and left. In a sense, they are performing mild whole body exercise. Standing still on a shaking plate invokes more appropriate whole body exercise than that when standing on a motionless plate. In the present study, we observed that shaking stimuli applied to a mouse model of declining bone mineral density (BMD) prevented femoral bone strength from decreasing, although the degree of prevention differed from site to site. This preventive effect on BMD decrease was obtained by morphologic, compositional, and physical analyses of the bone. We suggest that this method may be applicable to humans. Whole body exercise provoked by shaking stimuli may represent a novel physical therapy in bone fracture prevention and health promotion not only in the elderly, but also in young people.

Key words: Osteoporosis, Bone density, Ovariectomy, Shaking, Physical therapy

1. Introduction

In the body, a portion of bone is always being resorbed, while virtually the same amount is being formed at the same time by remodeling. This process proceeds continuously in normal adults, with the total bone mass remaining stable. However, in the elderly, bone formation and bone resorption are out of balance, resulting in a decreased bone mineral density (BMD), which leads to susceptibility to bone fracture. This imbalance can be corrected to maintain bone strength by appropriate exercise or stress exerted on the bone. However, the amount of exercise is dramatically reduced in the elderly, and their bone resorption is accelerated through age-related reductions of osteoblastic proliferation and function. Thus, a vicious cycle is created.

Presently, millions of Japanese people suffer from osteoporosis, which leads to femoral fractures from falls in elderly people. The incidence of osteoporosis

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increases exponentially after 70 years of age, and the number of patients is estimated to be approximately 220,000 by 2020[1]. This issue is prevalent in communities around the world in that advanced medicine increases the aged population.

Approximately 80% of osteoporosis patients are women, and women develop the disease at earlier ages. Their blood estrogen levels decline abruptly after menopause, which occurs at approximately 50 years of age. On the other hand, men may suffer from extreme reduction of BMD due to a mutation in estrogen receptor α [2]. Reduced estrogen levels are involved in acute-onset osteoporosis or in osteoporosis in elderly men[3, 4].

The amounts of Ca and P directly contribute to bone strength, and these elements are regulated by hormones, such as calcitonin. Estrogens elevate the blood concentration of calcitonin, which inhibits bone resorption[5]. Estrogen also enhances the activity of vitamin D, which accelerates intestinal Ca absorption. In human adults, approximately 500 mg of Ca cycles between the bone and the circulating blood, as Ca is released or removed by bone resorption or bone formation.

Osteocytes, osteoblasts, and osteoclasts exist in the bone and play roles in increasing the strength of bone through remodeling. Bone tissues are often considered inactive, but they are, in fact, actively metabolizing. Remodeling takes place continuously in 3-5% of the whole bone in human beings, renewing one osteon over a period of 4-5 weeks. When bone formation and resorption are out of balance and in favor of bone resorption for a long time, bone mass reduces and bone strength is weakened, which leads to the development of osteoporosis. In a molecular mechanism of osteoporosis development, estrogen acts on osteoclastic resorption by inhibiting osteoclastic responses to RANKL (receptor activator of NF- κ B ligand) through the suppression of JNK (c-Jun N-terminal kinase) and by inhibiting the binding activity of AP-1(activator protein 1)[6, 7].

The balance between bone formation and bone resorption determines bone mass. Favorable factors for bone formation are BMPs (bone morphogenetic proteins)[8], LRP5 (low density lipoprotein receptor-related protein 5)[9], Wnts (wingless ints)[10], Ocn (osteocalcin)[11], Alp (alkaline phosphatase)[12], and Osx (osterix)[13], whereas unfavorable ones are leptin[14], SOST (sclerosteosis)[15], CIZ (cas-interacting zinc finger protein)[16], and Tob (transducer of erbB2)[17]. Bone resorption is stimulated by β-catenin[18, 19] and RANK (receptor activator of NF- κ B)[20, 21], whereas it is inhibited by OPG (osteoprotegerin)[22] and calcitonin[23, 24]. Bone formation is promoted by physical stress, whereas senescence and immobilization increase bone resorption. Accelerating factors for bone resorption include the reduced production of estrogens and immobilization, whereas estrogens and calcitonin suppress bone resorption.

Rehabilitation after bone fractures is essential for elderly people to continue a normal life. The neglect of rehabilitation during the recovery phase after bone fracture may lead to walking difficulties, wheel chair use, muscle atrophy, pneumonia or dementia, and, finally, to a bedridden future. Appropriate stress given to the bone, as well as to the skeletal muscle during rehabilitation is considered comparable to appropriate exercise, and may help prevent bone fractures.

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 days[25]. On the other hand, our stimulation method is the method that is almost exercise therapy by stimulation using shaker. Shaking stimuli include right and left shaking, back and forward shaking, and rolling of horizontal plates on which the animals stand. When the animals were exposed to these stimuli while standing on shaking plates, they tried to balance the whole body so as not to fall. We examined how their postures of stretching the all limbs influenced the bone. 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. The results show that shaking stimuli maintained for 9 weeks affected the femoral bone

strength.

The purpose of this study was to investigate whether physical stress could activate osteoblasts to prevent a reduction of BMD, suggesting a preventive physical therapy for bone fractures in the aged. The bones of the elderly are more likely to incur fracture because of the reduced amount of fibrous components and because of declined elastic strength. Breaking energy as assessed by a three-point bending test was used be an index of elastic strength. Our results may lead to the development of new strategies for fracture prevention in humans.

2. Materials and methods

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. Animals in this study were kept in the vivarium of Fujita Health University, and studies were conducted under Animal Use Committee regulations.

2. Shaking stimuli

Twelve ovariectomized mice (OV group) and 12 non-ovariectomized mice (WT group) were further subdivided into stimulated and non-stimulated groups, respectively, to create a total of 4 groups: shaking stimuli / ovariectomy (+/+), no stimuli / ovariectomy (−/+), shaking stimuli / intact ovaries (+/−), and no stimuli / intact ovaries (−/−). Shaking stimuli were given at a rate of 150 horizontal rotations per minute for 30 minutes TAITEK NR3 (TAITEC Co., Ltd, Japan). This stimulus load was repeated 6 times a week for 10 weeks. Horizontal rotations acted as stimuli of strain to femurs from all directions. Individual mice under stimuli were separated from each other by placing them in acryl cases (W60×L140×H70 mm), the floors of which were moderately slippery. After the stimuli were completed, femurs were carefully removed without injuring them and were cleaned of adhering soft tissues. The significant difference described it in the graphs of Fig. 1B, Fig. 3 and Fig. 4. It is case-control study which the statistical analysis to compare each group with the control group assumes +/+ criteria, and compared +/−, −/+, −/− with +/+.

3. Radiographic Analysis

For radiographic imaging, a SOFRON TRS-1005 (TRS-1005 SOFRON, Co., Ltd, Japan) was used under the following conditions: X-ray tube voltage, 25 kV; tube current, 2.5 mA; and irradiation time, 90 seconds. The scanning condition of the image is the following, width 335×height 850 (284, 750 pixel), size 7×18 mm gray scale. Images were analyzed by Image J software.

4. Histological analysis

Perfusion fixation of the whole body was performed using 4% paraformaldehyde, and the femurs were removed and immersed in 70% ethanol (about 25-fold the weight of the bone tissue). The ethanol concentration was increased over a period of 7 days from 70% to 99.5% for fixation. However, for the first 3 days, the ethanol was replaced everyday and the fixation bottles were turned from time to time to mix the ethanol. We used methylmethacrylate (MMA) for embedding to prepare undecalcified thin-cut specimens. We used toluidine blue to stain the sections. Images were analyzed by Image J.

5. Measurement of BMD

We measured bone volume, as well as the BMD of cortical and cancellous bone using an XCT Research SA$^+$ equipped with Rev 6.00b software (Stratec Medizintechnik GmbH, Pforzheim, Germany). Measurements were made according to the method of peripheral quantitative computed tomography (pQCT). We measured BMD of compact bone, as well as cancellous bone of the metaphysis and diaphysis under the following conditions: at a site 1.24 mm (metaphysis) and at a site 5.5 mm (diaphysis) from the distal epiphyseal cartilage in a total of 2 slices. Slice thickness was 0.46 mm, and the cell size was 0.12 mm.

6. Bone strength test and three-point bending test

For bone dynamics testing, a femur is examined

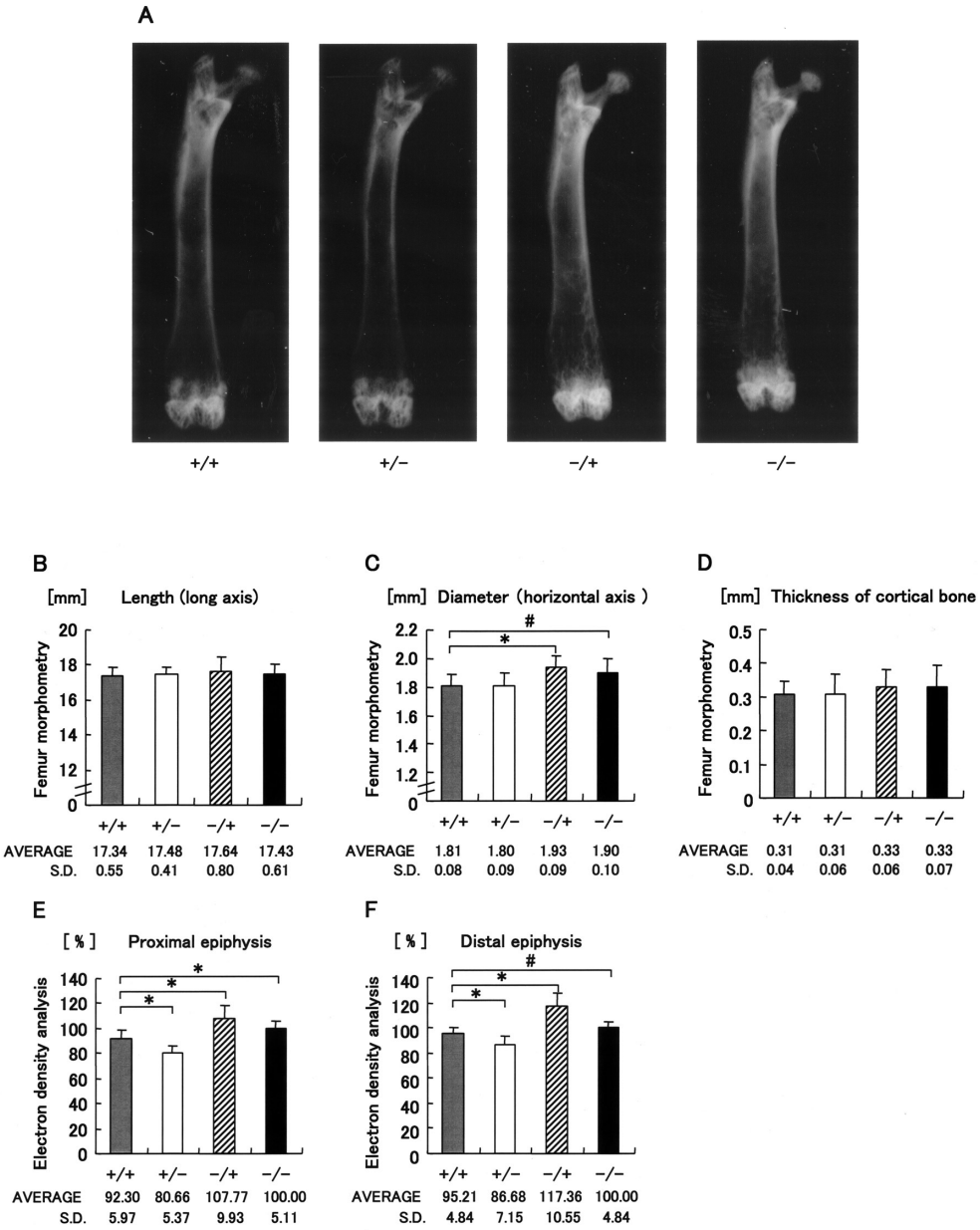

Fig. 1 Radiological examination of murine femurs.

Fig. 1A shows radiographs of murine femurs. From left to right are shown femurs from the shaking stimuli / ovariec tomy groups: +/+, -/+, +/-, and -/-. Fig. 1 B - E shows the results of morphologic measurements. Figs 1 - B, C, D, E, and F illustrate charts concerning the major axes, the thickness (or diameter) of the diaphyses, the cortical thickness of the diaphyses, results of population analyses of the proximal epiphyses, and similar results of the distal epiphyses, respectively. The gray-colored bars represent the +/+ group, the white bars the -/+ group, the shaded bars the +/- group and black bars the -/- group. Data as to the groups E and F are expressed based on data of the -/- group and data of other groups are expressed in percentage. Statistical analyses were conducted 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.

by a three-point bending test and the lumbar spine is examined by a compression test. Because mouse bone specimens are small, we tested femurs by the three-point bending test. In the bone strength test, the compressor was moved at 10 mm/min. We analyzed bone dynamics parameters acquired from the S-S curve (stress-strain diagram) of the three-point bending test. These parameters were maximum load [N], maximum extension [mm], maximum stress [N/mm^2], maximum strain [%], and energy [N· mm]. The distance between supporting points was 6 mm.

7. 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. 1B, Fig. 3 and Fig. 4 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

In order to simulate human menopause, because the BMD of women decline slowly after menopause, 9 week-old ICR female mice were ovariectomized to reduce estrogen secretion. These estrogen-deficient mice, as well as mice with intact ovaries were used to investigate the effects of shaking stimuli on bone.

Radiographs of excised femurs were obtained and subjected to population analysis using Image J software (Fig. 1A). The long axis of the femur was measured as femur length, while a length of a rectangular line drawn at the midpoint of the long axis was measured as femur thickness (diameter). Compact bone thickness was also measured at the same point as femur thickness (Fig. 1B-D). There was no significant difference in the femur length between the 4 groups, although the −/+ group tended to be slightly longer. The femur diameter was larger in the 2 non-ovariectomized groups (−/+, −/−) than in the 2 ovariectomized groups (+/−, +/+). The difference was

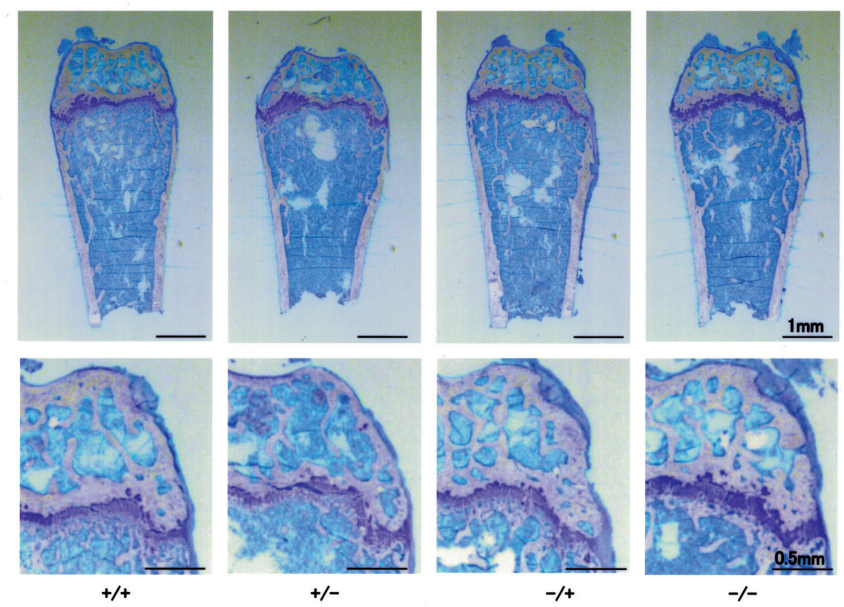

Fig. 2 Histological analysis using toluidine blue staining of the murine femur.
Photographs of toluidine blue-stained undecalcified thin preparations of the murine femur (from the diaphysis to the distal ephiphysis). Upper panel shows low-power views and the lower panel shows high-power views of the 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.

significant between the +/- and -/+ groups ($p < 0.01$).

The thickness of the diaphysis cortex showed no significant difference between the 4 groups. Since the border between compact and cancellous bone at the epiphysis in radiographs was ambiguous, we performed population analysis on 2 areas of the proximal and distal epiphyses exclusive of the femoral head and neck (Fig. 1 E-F). The results from both proximal and distal epiphyses were lowest in the +/− groups, followed by the +/+ group, and the −/+ group showed the highest value ($p < 0.01$). These results suggest that the shaking stimuli affected the BMD of cancellous bone at the epiphysis.

We stained sections of the femoral distal epiphyses and diaphyses with toluidine blue and measured the thickness of compact bone of the diaphysis. Compact bone thickness was greatest in the −/+ group, followed by the −/−, +/+, and +/− groups in that order (data not shown). In the epiphyses of the +/− group, cancellous bone were observed to have sparse trabeculae with a dominant marrow space (Fig. 2). Results were similar to those of the radiographic imaging analyses, suggesting an increase of bone formation.

We measured the BMD of compact bone, as well as cancellous bone at both the epiphyses and diaphyses using the XCT Research SA$^+$ and pQCT methods (Fig. 3 A-D). The +/− group showed a slightly higher BMD, suggesting that the stimuli prevented its deterioration. The results also showed that the BMD for compact and cancellous bone was lowest at the epiphyses in the +/− group. The results of this BMD study agreed with those obtained by the imaging analyses of radiographs and tissue preparations, suggesting a relationship between bone formation and BMD. This relationship was especially clear in

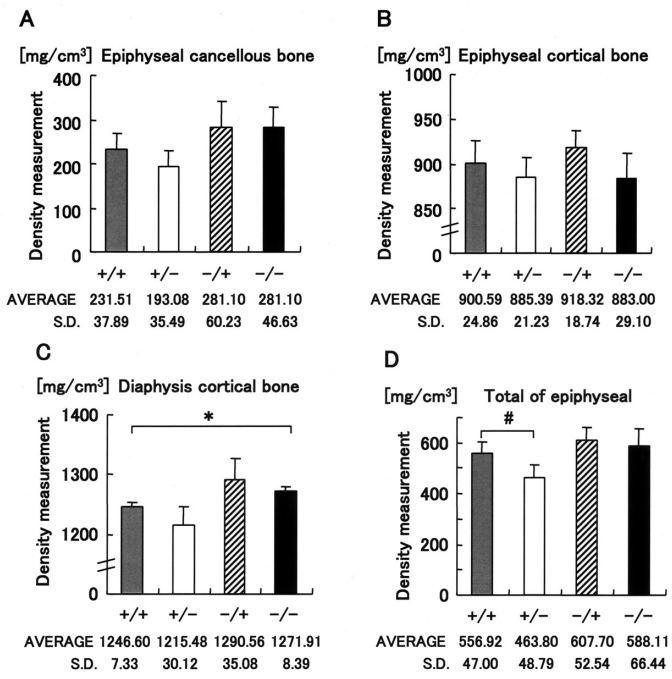

Fig. 3 The bone density measurement in each femur region. Charts of bone mineral density of the murine femurs. Charts A, B, C, and D show data of the cancellous bone of the epiphyses, the cortex of the epiphyses, the cortex of the diaphyses, and the whole femurs, respectively. The gray-colored bars represent the +/+ group, the white ones the -/+ group, the shaded ones the +/- group, and the black one the -/- group. Statistical analyses were made 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.

the whole bone ($p < 0.05$).

Morphological and component improvement in the bone indicates an acceleration of bone formation. We examined bone strength using the three-point bending test and acquired bone dynamics parameters from the S-S curve (stress-strain diagram) (Fig. 4 A, B). We obtained results of maximum load that were similar to above morphological and component analyses ($p < 0.01$). Break power results were similar, although SD was large, and bone strength was found to be correlated with bone formation or BMD. Furthermore, the bone strength-related components of bone Ca and P were determined (Fig. 3 C, D). There was no significant difference in Ca content between the +/+ and +/− groups, but the +/+ group tended to show a slightly higher levels of P than the −/+ group ($p < 0.05$).

4. Discussion

Bone is important for body structure. Bone strength is critical for locomotive function. The bone orients itself to physical stress following specific rules. Daily life physical stress influences remodeling of bone, and stimuli exceeding a certain threshold promotes bone formation on the surface of trabeculae and increase bone mass. In contrast, stimuli under a specific threshold alters the balance between bone formation and resorption toward bone mass reduction. For example, longterm recumbency, paralysis due to a spinal cord injury, or immobilization with a cast reduces physical activity, and bone mass begins to be lost.

On the other hand, aging is correlated with a decrease in bone cell activities, as well as of fibrous components, resulting in a reduction of elasticity

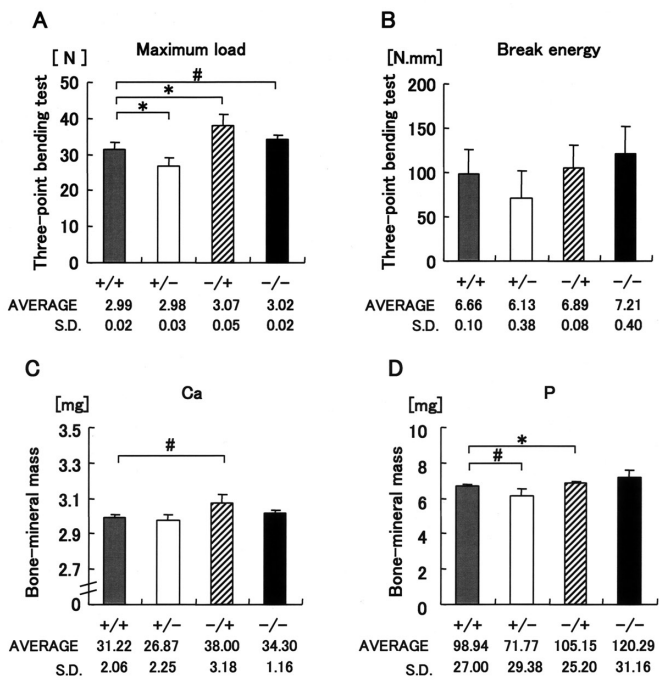

Fig. 4 The bone intensity study and the measurement of the inorganic mass. Data of the bone strength of the murine femurs. Charts A and B illustrate maximum load and break power, respectively, acquired using the three-point bending test. Charts C and D show bone Ca and P contents, respectively. The gray-colored bars represent the +/+ group, the white ones the -/+ group, the shaded ones the +/- group, and the black one the -/- group. Statistical analyses were made 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.

leading to bone fracture caused by a minimal physical load. Bone mass is reduced by approximately 1% after 1-week recumbency and by approximately 10-20% after several months of recumbency, whereas BMD declines by approximately 2-4% in 1 year following menopause[26]. The mechanisms that bone uses to sense physical stress are unclear, but osteocytes and osteoblasts may sense it.

Physical stresses as sensing factors include strain to bone tissue caused by external forces, hydrostatic pressure, liquid current in bone canaliculi, and electromagnetic fields caused by metabolic current[27]. In this study, we focused on strain to bone tissue caused by external forces to develop a method to provide stimuli that would stimulate osteocytes, as well as osteoblasts and accelerate bone formation. The aim of this study was to slow the chronic decline of BMD in postmenopausal women, which would help women prevent bone fracture by continuing maintenance of bone strength.

Intracellular signaling systems are involved in the mechanisms of bone formation through stimulation by physical stress[28]. Physical stress induces the flow of extracellular Ca ions into cells, which increases intracellular Ca ion concentrations. This increase of intracellular Ca ions activates PKA (protein kinase A), followed by an increase of cAMP (adenosine 3', 5'-cyclic monophosphate AMP), and second messengers or PG (prostaglandin) and NO (nitric oxide) are generated to act in an autocrine or paracrine fashion[29]. At 30-60 minutes after stimuli, c-fos, an early gene in bone cells, increases transiently[30]. This is followed by an upregulation of COX-2 (cyclooxygenase-2)[31, 32], and, 24 hrs later, IGF-1 (insulin-like growth factor-1) and matrix protein like osteocalcin are expressed[30]. These factors promote proliferation, as well as differentiation of osteoblasts and bone formation.

In this study, we attempted to search for proper stimuli that might promote efficient bone formation, the mechanisms for which were being clarified. We placed mice on shaking plates that rotated horizontally. The animals tried to balance themselves to prevent falls by stretching their four limbs. This stretch was physical stress or a load that placed strain on bone and stimulated bone formation.

Osteoblasts respond to strain *in vitro*, the intensity of which needs to be as high as about 119MPa[17, 33, 34, 35]. However, in the living body, the intensity of strain cannot exceed about 24 MPa[36]. We assume that signal transduction is affected by mechanical load rather than by physical strain. In this experiment, the bone was considered to be affected by stimuli that were passively conveyed to the bone by muscle motion. Therefore, we determined appropriate intensity of stimuli by decreasing the shaking intensity from the maximum that the animals could manage to stand by stretching the limbs.

The present animal groups represented four combinations of shaking stimuli and ovariectomy. The −/+ group, which had no shaking stimuli and had an ovariectomy, represented postmenopausal women, while the +/+ group represented postmenopausal women who did appropriate exercise everyday. Adult women were simulated by the +/− group representing those that participate in exercise or by the −/− group representing those that do not participate in exercise. Importantly, there was a difference in results between the two ovariectomized animal groups (+/− and +/+) and the two non-ovariectomized groups (−/+ and −/−). Furthermore, it was noteworthy that there was a difference in results between the +/− and +/+ groups.

We assumed that the shaking stimuli provided by rotary motions of horizontal plates was sufficient, although indirectly, to apply strain to bone. However, the results were not statistically significant in the stimulation of bone formation, but bone resorption may have been reduced. The slope of bone resorption progressing with time was less steep. However, Ca, P, and other factors, such as activated vitamin D, should have been supplemented as basic elements for bone formation.

However, our results showed that shaking stimuli reduced bone resorption. More efficient programs to prevent BMD reduction may be developed when other factors are taken into account. In addition, further studies on shaking stimuli will contribute to the development of preventive measures for bone fractures due to falls. Currently, more attention is being focused on preventive medicine that avoids new disease,

instead of therapies that aid in recovering from disease. Shaking stimuli using horizontally rotating plates may play a role in preventive medicine as part of efficient programs for suppressing BMD reduction in humans.

Conflicts of interest

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