Nutrition for Enhancing Bone Volume in Mice

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1. Introduction

It is well known that postmenopausal osteoporosis is related to severe decreases in the serum estrogen levels. Estrogen deficiency produces an imbalance in the bone remodeling balance which is associated with a reduced bone volume and higher fracture risk. Androgen deficiency also produces osteoporosis by directly affecting bone cells. In addition, androgen was demonstrated to be present during the fetal period, and it is considered to play an essential role in the sexual differentiation of males. Therefore, both sex hormones, androgen and estrogen, are great important for bone homeostasis not only in females but also in males. This is further supported by the fact that both males and females express androgen receptor (AR), estrogen receptor-alpha ($ER\alpha$) and estrogen receptor-beta ($ER\beta$). Androgen and estrogen react with the AR and $ER\alpha$ and $ER\beta$, respectively. It is thus speculated that they mutually regulate themselves during bone development and remodeling.

Up to present, various studies have examined the roles of estrogen and its receptors to explore potential methods for preventing or treating postmenopausal osteoporosis (Judd et al., 1983; Kousteni et al., 2002; Martin-Milan et al., 2010; Pietschmann et al., 2008). Recent clinical studies have reported that the prevention of hip bone fractures and vertebral deformities is highly pertinent to improving the quality of life for older people (Chang et al., 2004; O'Neill et al., 2009). To avoid these incidents, we have to try to achieve adequate peak bone mass during adolescent growth, it is also important to understand the bone growth, because this is currently an under-investigated area. In adult animal experiments, orchiectomy and ovariectomy reduced both the bone volume and density. Furthermore, the bone volume loss was induced not only in the long bones but also in the mandibular condyles (Fujita et al., 2001). In addition, we have reported that these phenomena were also shown in immature mice (Fujita et al., 2006). Therefore, the influence of sex hormones on bone remodeling has been demonstrated in the craniofacial region. In orthodontic and orthopedic treatment, it is especially, difficult to predict bone growth including craniofacial and mandibular growth during adolescence. The full nature of bone growth, in association with sex hormones remains to be fully elucidated.

The ratio of osteoporosis patient in Japan based on the diagnosed by Japanese society for bone and mineral research is reported 24% of over fifty-year-old people and it is indicated the number of patients is higher than those in USA and European countries (Orimo, 2000).

Therefore, we all recognize osteoporosis is one of serious social problems in Japan. This fact indicates that the influences of sex hormones on bone metabolism are very complicated, and the mechanism is very difficult to be understood.

Peak bone mass, in general, is acquired from childhood to adolescence, but 35% of cortical bone and 50% of trabecular bone are lost gradually thereafter. The importance of preventive medicine thus has been gradually recognized in the field of orthopaedic surgery with a concept that peak bone mass is to be increased and saved during childhood as much as possible (Lapauw et al., 2009; Kaufman, 2009). Such an idea has been attracting a special attention in the field of clinical medicine for the prevention of osteoporosis. The importance of normal development and growth of bone until adolescence has also been reported (Karlsson et al., 2008; Wang et al., 2008). This fact suggested that if we are able to increase the peak bone mass during adolescence as possible by providing some effective nutrition for increasing bone volume, it may lead to prevent the risk and to delay the appearance of osteoporosis.

According to the prevention and treatment of guideline in osteoporosis, it was recommended to intake daily dose of calcium is higher (Rosen & Gallagher, 2011). However, the amount of calcium intake is approximately 500mg per one day in practically, and even added the consumption applied by supplement, could not reach daily objective intake.

A newly developed snack used in our experiments contains appropriate amounts of minerals (calcium and magnesium) and casein phosphopeptide (CPP) as well as soybean isoflavone which has a sex hormone-like action, which is recognized as a specified food for health, is also included. The objective of this chapter was to introduce the effects of bone remodeling including bone volume, density and strength in osteoporosis and healthy growing mice fed a special composition nutrition.

2. Nutrition

2.1 Function of nutrition

Recently, it was focused on the prevention of osteoporosis and life style related disease like a diabetic and heart disease. Under such situation, people are well considered the food function. It is well known that the function of foodstuff has mainly for nutrition and taste. Additionally, it has also the important function as a decreasing of risk in some disease. The development of foodstuff which has the specific function for living body adjustment is going forward actively in nowadays, therefore the daily food which we intake were diversify. Now it was reported that there are detected and demonstrated in some nutrition that the function of anti-thrombosis, anti-oxidation, encouragement to assimilate calcium, controlling of blood pressure and improvement of cholesterol readings as a food for special health uses and Japanese government had approved it (Yamada et al., 2008). For example, beta-carotene was known as a great nutrition because of its essential function, foodstuff factor as a provitamin, taste factor by its color in vegetable and fruit, and the role of anti-oxidation (Kim et al., 2010).

2.2 Casein phosphopeptide

CPP is also well known as a factor of encouragement to assimilate calcium. Milk and dairy products contains CPP together with functional protein like a milky basic protein and lactoferrin. Casein as a milk protein is a protein containing phosphoric acid, and is decomposed into oligopeptide of various sizes by the digestive enzyme. It became

clarified that special protein containing many phosphoric acid called CPP. Generally, calcium is the fifth abundant element by mass in the human body, where it is a common cellular ionic messenger with many functions, and serves also as a structural element in bone. And it has four stable isotopes (^{40}Ca , ^{42}Ca , ^{43}Ca and ^{44}Ca) plus two more isotopes (^{46}Ca and ^{48}Ca). 97% of naturally occurring calcium is in the form of ^{40}Ca which is a one of daughter products of ^{40}K decay, along with ^{40}Ar (Mueller & Boehm, 2011; Skulan & DePaolo, 1999). Calcium is hard to be absorbed in human body since it combines with negative ion such as a phosphoric acid in a small intestine. However, CPP prevents own insoluble and promote its efficiency of absorption in a small intestine due to a part of serine residue is phosphorylated and the ionic bond of CPP is carried out to calcium with the negative ion of phosphate group.

Moreover, the function in the promotion of absorption efficacy of CPP, it is positively applied in the form of casein phosphopeptide-amorphous calcium phosphate (CPP-ACP), and is widely well-known as Recaldent. CPP from the major protein of milk have the remarkable ability to stabilize calcium, phosphate and fluoride ions as water soluble amorphous complexes that provide bioavailable calcium, phosphate and fluoride ions to the tooth. Animal and short-term human clinical trials to repair early staged of tooth decay by replacing the calcium and phosphate ions lost due to decay (Reynolds, 2009; Llena et al., 2009). Long-term controlled clinical trials also have demonstrated the efficacy of CPP-ACP is slowing the progression of dental caries and in regressing early stages of tooth decay (Andersson et al., 2007; Morgan et al., 2008). Moreover, it was reported that it is effective rather than the 500ppm sodium fluoride paste which the dentists have used conventionally for the purpose of prevention of tooth decay (Zhang et al., 2011; Altenburger et al., 2010).

On the other hand, it was reported that the calcium in food exists 50~60% in milk, approximately 20% in small fish and approximately 30% in vegetable. But, absorption efficacy of calcium in human body is not much excellent. Therefore, CPP is further developed as "food suitable for the people of calcium shortage" such as sugar-free gum, tooth cream, dairy milk and another drink and Japanese tofu. However, with such professional tool, food and drink form, we speculated that it will be probably difficult to take freely and daily for the purpose of increasing the absorption efficacy of calcium.

3. Animal study

3.1 Materials and methods

Fifty male and fifty female C57BL/6J mice of twelve-week-old and thirty male and thirty female C57BL/6J mice of five-day-old (Jackson Laboratory, Bar Harbor, ME, USA) were used in this experiment and divided respectively into eight groups with ten in each sex. In the experimental groups, orchietomy (ORX) and ovariectomy (OVX) were performed for ten mice of twelve-week-old in each gender at the beginning of experiment to create the situation of osteoporosis model. ORX and OVX were performed by means of a stereoscopic microscope (SZX9, Olympus Optical Co., Tokyo, Japan) under general anesthesia with sodium pentobarbital, whereas the control mice underwent sham operation. The detail of time schedules in adult and growing mice during a series of experiment are shown in Table 1 and 2, respectively. These animals were treated under the ethical regulations defined by the Ethics Committee, Hiroshima University Faculty of Dentistry.

Table 1. Time schedule during experiments in adult mice

Table 2. Time schedule during experiments in growing mice

We prepared three types of powder diet *e.g.*: normal calcium diet (NCD, Ca : 0.9%, Clea Japan Co., Tokyo, Japan), low calcium diet (LCD, Ca : 0.63%, Clea Japan Co.,) and special diet (SCD, Ca : 0.9%) developed as a new bean snack. NCD with 0.9% calcium was made on basis of objective consumption of calcium intake in humans defined by the Ministry of Health, Labour and Welfare (MHLW) in Japan. LCD including 0.63% calcium was made on the basis of actual calcium consumption in Japanese. The SCD containing LCD and the newly developmental snack, was supplied as a diet including calcium content same as NCD. The newly developmental snack was composed of calcium, magnesium, CPP and black soybean. As the mainly composition, 1.3% of calcium, 0.6% of magnesium, 0.15% of CPP and 12.8% of black soybean were included in this snack. NCD was given up to six weeks after sham operation in the group 1 and 6. Other six groups were given LCD until six weeks after gonadectomy. Six weeks after surgery, group 3, 5 and 8 in each sex were given SCD including the newly developed bean snack.

Eighteen weeks after surgery, all the animals were sacrificed under general anesthesia and the femur was fixed with 4% formaldehyde and prepared for histomorphometric analysis. Peripheral Quantitative Computed Tomography (XCT Research SA, Stratec Medizintechnik GmbH, Pforzheim, Germany) was used to quantify bone density and bone mineral content. Femur was measured at a point 1.4mm distal area from chondrocyte growth plate. The cortical bone area was defined as over 690mg/cm³ threshold and the trabecular bone area was defined as under 395mg/cm³ threshold. Moreover, bone strength of femur performed by under three point flexural test was also executed in growing mice.

We performed pairwise comparisons (Fisher) to examine the difference in measured values between the groups with a confidence level greater than 95%. All the data are presented as means±standard deviations.

3.2 In ORX and OVX mice

Figure 1 shows the photographs of femur section examined by pQCT at the end of experiment. Irrespective of the sex differences, the trabecular bone volume surrounded by the thick cortical bone was maintained at high level in the control groups, in sham operated mice fed NCD in particular (group 1). Meanwhile, trabecular bone volume was decreased moderately in the experimental group 2 with LCD as compared with control mice. Furthermore, the group 4, gonadectomized mice fed LCD exhibited an excessive decrease in bone volume as compared with the groups 1 and 2. On the other hand, the femur section in the group 5, osteoporosis model mice given SCD, presented a prominent recovery of trabecular bone volume.

Fig. 1. Photographs of femoral section in adult mice.

As a result of pQCT analysis, similar phenomena were observed as mentioned above for the femur structures. The trabecular bone density of gonadectomized mice fed SCD was significantly increased as compared with sham operated mice given LCD in both genders (Fig. 2). Moreover, the bone density was also significantly increased only in the female mice of group 5 as compared with sham operated mice fed NCD.

For the cortical bone density, different findings from above mentioned results were obtained. Improvement effect was especially revealed in the gonadectomized mice given SCD (group 5) (Fig. 3). Additionally, it was also shown in the sham operated mice fed SCD (group 3) compared with the sham operated mice fed LCD (group 2). These tendencies were observed in both sexes.

Fig. 2. Trabecular bone density in adult mice

Fig. 3. Cortical bone density in adult mice

Subsequently, the total bone density was apparently increased as compared with other sham operated mice. The reason may be due to SCD supplied to the gonadectomized mice, especially in the female mice (Fig. 4).

For bone mineral content, the groups 3 and 5 mice given SCD exhibited a significant increase as compared with the groups 1 and 2, especially in female mice. It is demonstrated from these findings that the newly development snack is very effective for the improvement of reduced bone quality and controlling of osteoporosis (Fig. 5).

Fig. 4. Total bone density in adult mice

Fig. 5. Bone mineral density in adult mice

3.3 In young mice

The results of trabecular bone density were shown in Figure 6. The mice fed NCD revealed around 40 to 50 mg/cm³ in density, but by the cause of changing food to LCD, it lead to decrease in both sexes. Under such situation, in case of group 8, mice fed SCD from after six weeks to the end of experiment, trabecular bone density showed significantly increased

compared with mice fed LCD, therefore, there is no significant differences between control mice of group 6 and mice fed food which is changing to SCD after feeding LCD of group 8. In the results of cortical bone density, similar phenomena were observed with trabecular bone density, the cortical bone density of mice fed LCD revealed significantly decreased compared with mice given NCD (Fig. 7). However, by the feed changing to SCD, cortical bone density wasn't getting better as contrasted with the results of trabecular bone density and the results of cortical bone density in adult mice. These tendencies were observed in both sexes. As a result, only in female mice, total bone density also showed the sufficient effect of improvement (Fig. 8). There is no significant difference between group 6 and 8 in male mice.

Fig. 6. Trabecular bone density in growing mice

Fig. 7. Cortical bone density in growing mice

Fig. 8. Total bone density in growing mice

In the results of bone mineral content, Male mice after fed SCD showed no significant differences as compared with the control mice (Fig. 9). On the other hand, female mice of group 7 and 8 showed significantly reduced bone mineral content as compared with mice of group 6.

According to the results of total bone density and bone mineral content, the results of three point flexural test also showed same tendency in male mice (Fig. 10). Meanwhile, although the results of group 8 showed significant lower than the one of group 6 in total bone density and bone mineral content, group 7 and 8 demonstrated same level of group 6.

Fig. 9. Bone mineral content in growing mice

Fig. 10. Three point flexural test in growing mice

4. Discussion

Osteoporosis is generally thought of as a disease that affects females, because the prevalence of osteoporosis and the rate of fracture are much higher in postmenopausal females than in older males. However, the absolute number of male patients affected by osteoporosis and fractures has been reported to be large. Older Asian males with low serum estradiol levels also display elevated bone loss and increased risk of fractures similar to the findings in Caucasians (Woo et al., 2011; Moayyeri et al., 2009). The main concern of treatment in osteoporosis research is to prevent bone volume loss by decreasing the progression of bone resorption. Nevertheless, the current knowledge is not sufficient to identify the precise causes of osteoporosis and all of the subjects at risk. As a result, recent studies have indicated that weight-bearing activity and possibly calcium supplements are beneficial if they are begun during childhood, preferably before puberty. The achievement of optimal peak bone mass is important to prevent the risk of bone fracture due to osteoporosis in the future.

The sex hormones are known to be important for the regulation of reproductive functions. They induce sexual differentiation before birth, and sexual maturation during puberty in both genders. They also exert influences on the nervous and cardiovascular systems and are important in the development of the skeletal structure. There are two types of sex hormones, androgens and estrogens. These hormones are secreted mainly from the testis and ovaries. Testosterone can activate the androgen receptor either directly or indirectly after conversion to DHT by 5 α -reductase. Moreover, it is well known that testosterone is also converted into 17 β -estradiol by P450 aromatase, which activates the estrogen receptors (alpha and beta). Based on these facts, Ovariectomized and Orchiectomized animals provide an excellent model to study osteoporosis due to estrogen and androgen deficiency in both genders. Both OVX and ORX mice exhibit marked bone loss with increased bone resorption. It was also reported that a loss in bone mineral density in ORX mice was evident between 1 to 4 months post-surgery, and histomorphometric evaluations revealed that this occurred more rapidly and with greater sensitivity in a rat model.

This snack we had developed was composed of calcium, magnesium, CPP and black soybean. We have examined into body weight and height of all mice during experimental period. However, all mice of supplied food in these measurements were no significant difference compared with the control groups in both sexes (data were not shown). In addition, the amount of all nutrition including supplied food was restrained according the instructions of MHLW in Japan. Therefore, we assumed that supplied special diet food has no occurrence of side effect following snack administration.

It is well known that calcium intake decreases the risk of bone fracture. It was also reported that the calcium supplementation suppress bone formation when magnesium is deficient (Mora & Gilsanz, 2003). Based on these reports, we designed to mix calcium and magnesium at an appropriate proportion of 2 to 1. These contents in this snack were compounded to fulfill ninety percentage of the daily-required nutrition defined by MHLW in Japan.

It was also reported that addition of CPP will have a beneficial effect on the absorption of calcium (Dontas & Yiannakopoulos, 2007). In Japan, CPP was accredited as a food for qualified health uses by MHLW. Accordingly, we add a certain amount of CPP to absorb mineral content easily.

The black soybean contains isoflavone which is widely accepted to have a weak estrogen activity, and to be able to bind estrogen receptor. Actually, Brandi and Miyauchi et al., reported that isoflavone had either effect for the suppression of bone resorption or enhancement of bone formation, affecting directly both osteoclasts and osteoblasts (Brandi, 2003; Miyauchi et al., 1996). In addition, isoflavone was revealed to affect bone metabolism similarly to the sex hormone-like effect in male (Chavarro et al., 2008). Recently, it was also reported that isoflavones have revealed inhibition of bone loss in castrated male mice and growing male mice respectively (Fujioka et al., 2007; Ishimi et al., 2002; Khalil et al., 2005). Under such background, a new snack was developed by use of black soybean. The amount of isoflavone in this snack was determined with a special reference to the amount of safety surplus nutrition per day defined by MHLW.

Decrease in the trabecular and cortical bone volumes after gonadectomy has already been demonstrated by our previously reports (Fujita et al., 2001, 2004). We also reported that bone growth was significantly suppressed in the gonadectomized mice immediately after birth (Fujita et al., 2006). Moreover, we clarified that decrease in bone volume was occurred four weeks after gonadectomy. According to these results, we examined bone density of the femur six weeks after ovariectomy and orchiectomy.

The density of trabecular and cortical bones in the gonadectomized mice given LCD was significantly lower than in the sham operated mice given NCD and LCD, respectively. It was revealed that the deficiency of calcium intake caused decrease in bone density, under sex hormone disturbances in particular. Therefore, it is speculated that the bone density is below the optimal level in Japanese and fracture risk may become higher for aged people. These findings also support that the incidence of primary osteoporosis is higher in Japan than in American and European countries.

The bone metabolism is classified into two types, high turnover type to accelerate both bone formation and resorption and low turnover type caused by degradation of bone formation. In this study, twelve-week-old mice with sham operation fed LCD are regarded as young growing humans with low turnover type, whereas gonadectomy mice to simulate sex hormone disturbance in the experimental groups are assumed to be under the condition of

postmenopausal osteoporosis as a high turnover type. Irrespective of the turnover types, however, the bone density in the group 5 given appropriate amount of calcium by supplying SCD exhibited a remarkable increase. It is thus suggested that sufficient calcium quantity through nutrition of newly developed bean snack enhanced bone formation irrespective of age.

It is reported that postmenopausal women with daily calcium intake of less than 400mg experience significant bone loss and that calcium intake of 800mg per day is effective for improving postmenopausal bone loss (Dawson-Hughes et al., 1990; Reid et al., 1993). On the other hand, it is well known that improvement effect against bone volume loss by calcium intake is available only at the initial stage of treatment. In addition, Riggs et al., clarified that the effect on bone loss is weaker than those reported for estrogen and bisphosphonates therapy, indicating that calcium supplements alone can't substitute treatment for osteoporosis (Riggs et al., 1998). In this study, longitudinal effect of newly developmental snack intake was not examined. However, It is hopefully anticipated that new bean snack could contributed to the enrichment of QOL as a nutrition function food, because of it was contain of several ingredients to promote assimilation efficiency of calcium.

5. Conclusions

The new snack we developed included proper amount of calcium, magnesium, CPP and black soybean. When this product was given to the osteoporosis model mice, bone density of the femur was significantly increased. From these results, it is suggested that this product supplement promote bone formation irrespective of gender and age. We demonstrated that newly developmental snack supplements may be a useful preventive measure for the people whose bone mineral density values are less than the ideal condition.

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7. References

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Osteoporosis is a public health issue worldwide. During the last few years, progress has been made concerning the knowledge of the pathophysiological mechanism of the disease. Sophisticated technologies have added important information in bone mineral density measurements and, additionally, geometrical and mechanical properties of bone. New bone indices have been developed from biochemical and hormonal measurements in order to investigate bone metabolism. Although it is clear that drugs are an essential element of the therapy, beyond medication there are other interventions in the management of the disease. Prevention of osteoporosis starts in young ages and continues during aging in order to prevent fractures associated with impaired quality of life, physical decline, mortality, and high cost for the health system. A number of different specialties are holding the scientific knowledge in osteoporosis. For this reason, we have collected papers from scientific departments all over the world for this book. The book includes up-to-date information about basics of bones, epidemiological data, diagnosis and assessment of osteoporosis, secondary osteoporosis, pediatric issues, prevention and treatment strategies, and research papers from osteoporotic fields.

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