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The Effects of a Mineral Supplement (Aquamin F®) and Its Combination with Multi-Species Lactic Acid Bacteria (LAB) on Bone Accretion in an Ovariectomized Rat Model

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Although an adequete intake of calcium (Ca) is recommended for the treatment and prevention of osteoporosis, the intake of Ca should be restricted because of its low rate of intestinal absorption. The purpose of this experiment was to identify the effect of the combined administration of Aquamin F (AQF) (a calcium agent) and lactic acid bacteria (LAB) on osteoporosis. Thirty ovariectomized (OVX) rats and six control rats were assigned to the following six groups, with six animals per group: sham Ca-deficient diet (Ca-D), OVX, LAB, AQF, and LAB-AQF. During the experiment, the body weight was measured; and after the experiment was completed, the serum biochemical analysis, the alkaline phosphatase, calcium, and inorganic phosphorus leves were measured. The tissue of the femur was stained and then scanned via CT. The body weight of the OVX group increased more significantly than that of the control group. The results of the bone mineral content (BMC), Bone mineral density (BMD), serum biochemical analysis and histological test on the femur epiphysis showed no difference between the OVX group and the LAB group, whereas the results of the AQF group were more significant than those of the OVX group. In particular, the LAB+AQF group showed more significant increases in the aforementioned results than the AQF group. This experiment showed that the combined administration of AQF and LAB in ovariectomized rats more significantly increased bone density than did a single administration of either AQF or LAB.

Key Words: Osteoporosis, Ovariectomy, Lactic acid bacteria, Aquamin F, Bone mineral density, Trabecular bone

INTRODUCTION

Osteoporosis is a systemic bone disease that increases the risk of bone fracture by increasing the fragility of the bone. Its major symptoms are a decrease in the BMD (Lee et al., 2004; Lee et al., 2005). Osteoporosis is a metabolic disease that commonly develops in 30% of menopausal women, and is a major cause of bone fractures in the elderly. Its incidence rate and prevalence are rising steeply because of the increase in the elderly population. There are about three million people with osteoporosis, including mild cases in South Korea (Byun et al., 2005). Osteoporosis is also related to low peak bone mass, which results from the insufficient intake during youth and middle age of necessary nutrients such as calcium that are related to bone formation (Lee et al., 2009).

BMD sharply decreases with a decrease in estrogen in women. Loss of trabecular bone, a major symptom of osteoporosis, is known to be sensitive to a decrease in estrogen and calcium intake; and a continuous decrease in estrogen for six to eight years accelerates bone mass loss (Whitney et al., 2002). Decrease in estrogen is related to

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Table 1. Composition of Ca-deficiency diet and typical mineral composition of Aquamin F[1)]

Ingredient	g/Kg	Mineral	Dry salt weight
Casein[2)]	200.	Calcium	31~35%
Cornstarch	397.486	Magnesium	2.5~4%
Dyetrose	132.	Moisture	<5.0%
Sucrose	100.	Iron	2,000 ppm max.
Microcrystalline Cellulose	50.	Lead	2 ppm max.
Soybean Oil	70.	Heavy Metals as Lead	10 ppm max.
t-Btylhydroquinone (TBHQ)	0.014	Arsenic	1 ppm max.
Salt Mix #310025 (without Ca)	35	Cadmium	1 ppm max.
Vitamin Mix #310025	10.	pH (1% aqueous solution)	9.5~10.5
L-Cystine	3.	Particle Size	25 μm max.
Choline Bitartrate	2.5	Bulk density	0.7~09 g/cm^3

[1)] Aquamin F is a natural calcium source produced from mineralized seaweed (*Lithothamnion sp.*). The seaweed is harvested from the seabed off the West coast of Ireland and North West coast of Iceland from clear, pollution free, mineral rich Atlantic waters.
[2)] Casein contains 300 mg/kg Calcium.

loss of ovarian function following menopause, and results in a reduction in BMD due to an imbalance between bone resorption and formation with an increase in bone resorption (Kalu et al., 1991; Balena et al., 1993).

Aquamin F (AQF) is a multi-mineral supplement derived from the red algae Lithothamnion corallioides, which is rich in calcium, magnesium, and various trace minerals including manganese, selenium, and zinc (Table 1). Its active ingredient is difficult to determine. A number of minerals in it may have anti-inflammatory and antioxidant properties, which may directly and/or indirectly influence its efficacy (Frestedt et al., 2009). While its prominent mineral component is calcium (dosage = 80% calcium RDA), its role in joint health remains unclear and warrants further study.

Both probiotics and prebiotics (non-digestible oligosaccharides) are known to have independent functions in bone formation. The effect of probiotics occurs through LAB's production of metabolites or enzymes, or synthesis of vitamins. Theses functions of LAB are considered to be related to osteoporosis in that several vitamins are involved in the formation of calcium metabolites and the bone matrix (Hill, 1997; Hancock and Viola, 2001; Crittenden et al., 2003) and vitamin D, C, or K (Weber, 1999) or folate (Villa et al., 1995) is involved in bone accretion. Probiotics, because of their stimulation of Ca absorption in the intestine, have attracted attention from researchers. Probiotics are known to influence probiotic bacteria to promote calcium absorption (Cashman, 2003).

Based on these functions of LAB, Brassart and Vey (1998) reported that in a fully differentiated Caco-2 cell culture model, which is a suitable *in vitro* model for predicting calcium absorption in humans, lactobacilli stimulated the absorption of Ca by stimulating transepithelial calcium transport. Their finding has been supported by other researchers (Brassart and Vey, 1998). As such, based on LAB's function of stimulating Ca absorption, this study aimed to elucidate if the combined administration of Ca and LAB in an *in vitro* osteoporosis model could effectively alleviate the symptoms of osteoporosis through LAB's promotion of Ca absorption.

MATERIALS & METHODS

Animals and experiment design

This study was approved by the Animal Experiment Ethics Committee (CBTA-006) and complied with the Regulations on Animal Management. All the experiment protocols in this study were reviewed and approved by the Animal Care and Use Committee of Cellbiotech Co., Ltd. of Korea.

The experiment animals included 36 female Sprague Dawley rats (six weeks old). Of these, 30 were ovariectomized rats and six were sham-operated rats that were procured from SLC, Inc. (Shizuoka, Japan). For the Ca-deficient diet, a modified AIN-93G Purified Rodent Diet

Table 2. Experimental design of animals

Group	Animal	Diet	Test material	N
Sham[1)]	Sham operate	Normal diet	Vehicle	6
Ca-D[2)]	Sham operate	Ca-deficiency diet	Vehicle	6
OVX[3)]	OVX operate	Ca-deficiency diet	Vehicle	6
LAB[4)]	OVX operate	Ca-deficiency diet	Lactic acid bacteria	6
AQF[5)]	OVX operate	Ca-deficiency diet	Aqamin F	6
LAB + AQF	OVX operate	Ca-deficiency diet	Aqamin F + LAB	6

[1)] Sham: sham operation, [2)] Ca-D: Ca-deficiency diet, [3)] OVX: Ovariectomy, [4)] LAB: Lactic acid bacteria, [5)] AQF: Aquamin F.

without calcium (R&D, New Jersey, USA) (Table 1) was used, and the Aquamin F and Lactic acid bacteria (*Lactobacillus paracasei* [KCTC: 13413], *Lactobacillus rhamnosus* [KCTC: 3929] and *Streptococcus thermophillus* [KCTC: 3927]) were supplied by Marigot, Ltd. (Cork, Ireland) and Cellbiotech, Inc. (Gimpo, Korea).

The animals were acclimated for one week, and only the ones that did not show particular symptoms during this period were used in the experiment. During the acclimation, the animals grew at a temperature of 22±2℃, a relative humidity of 50±20%, and a light/dark cycle of 12 hrs / 12 hrs. A commercial rodent diet and water were allowed *ad libitum*. The animals were assigned to six groups with five rats in each group according to the experiment purpose: the sham-operation (sham group); the sham-operation + Ca-deficient diet (Ca-D group); the OVX + Ca-D + Vehicle (OVX group); the OVX + Ca-D + LAB (1×10^7 CFU/kg B.W., respectively) (LAB group); the OVX + Ca-D + AQF group (50 mg/kg B.W.) (AQF group); and the OVX + Ca-D + co-administered LAB and AQF group (LAB + AQF group) (Table 2).

The LAB and AQF, which were diluted in saline or filled with the vehicle, were orally administered. For the sham, Ca-D, and OVX groups, only the physiological saline, using the same amount and route, was administered during the experiment period

Serum and biochemical analysis

At the end of this four-month study, the rats were anesthetized with diethyl ether and their blood was collected. The serum samples were prepared via centrifugation at 1,000× g for 20 min at 4℃, and then stored at -70℃ in aliquots until they were needed.

The level of calcium, inorganic phosphorus (IP), and alkaline phosphatase (ALP) activity in the serum was measured using a Fuji DRI-CHEM 4000i (Fuji Photo Film Co., Ltd., Japan).

Bone histopathology and densitometry

The femurs were removed and fixed in a 10% NBF (neutral buffered formalin) solution. A bone histopathological study was carried out as follows: After fixing specimen, a 30 mm cross-section of the femur was prepared and decalcified in 10% nitric acid for six hrs. Then the specimen was treated using the general tissue treatment process, embedded in paraffin, and cut into 4 μm-thick sections, which were subsequently stained with hematoxylin and eosin (H&E). The bone morphology was then examined under an optical microscope (Olympus, Japan).

A representative femur from each group was selected for the evaluation of the trabecular microarchitecture of the femoral metaphysis using the Explore Locus SP Pre-clinical Specimen Micro-CT (GE Healthcare, USA).

For the measurements of BMC and BMD, the tibias were fixed in 10% NBF, processed, and scanned with XCT Research SA+ (Stratec, Germany) at a voxel size of $0.1 \times 0.1 \times 1$ mm^3. Four consecutive slices that were separated by 0.2 mm were scanned for each tibia, beginning at 2 mm distal to the growth plate.

Statistical analysis

The results are expressed as means and their standard errors. The statistical differences between the groups were evaluated via the Student's *t* test using Prism 5 (GraphPad Software, USA). $P<0.05$ was considered significant.

RESULTS

Body weight

The body weights of the experiment animals were measured once a week at regular times after the start of the administration of the test substance. The body weights of the sham-operated group and the ovariectomized group differed considerably (sham group: 270±19 g; OVX group: 347±20 g). In contrast, among the ovariectomized groups, only the LAB group (323±14 g) showed a more significant change in body weight than the OVX group, and the other groups (AQF group: 363±11 g and LAB + AQF group: 356±31 g) did not show more significant changes in body weight than the OVX group (Fig. 1).

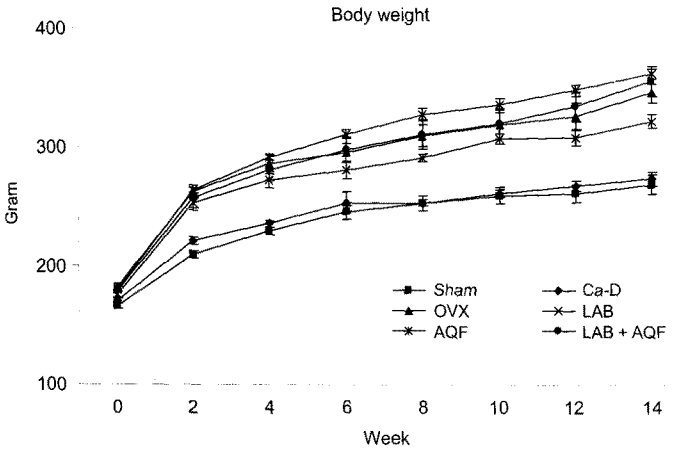

Fig. 1. Changes in body weight of ovariectomized rats. Changes in body weight of ovariectomized rats treated with test meterial for 14 weeks. Data are expressed as mean ± SEM (all group n=5). **Sham:** sham operation, **Ca-D:** sham operation and Ca-D diet, **OVX:** ovariectomized and Ca-D diet, **LAB:** ovariectomized and treated with lactic acid bacteria (1×10^7 CFU/kg B.W., respectively), **AQF:** ovariectomized and treated with Aquamin F 50 mg/kg BW, **LAB + AQF:** ovariectomized and treated with lactic acid bacteria (1×10^7 CFU/kg B.W., respectively) and Aquamin F 50 mg/kg BW treatment.

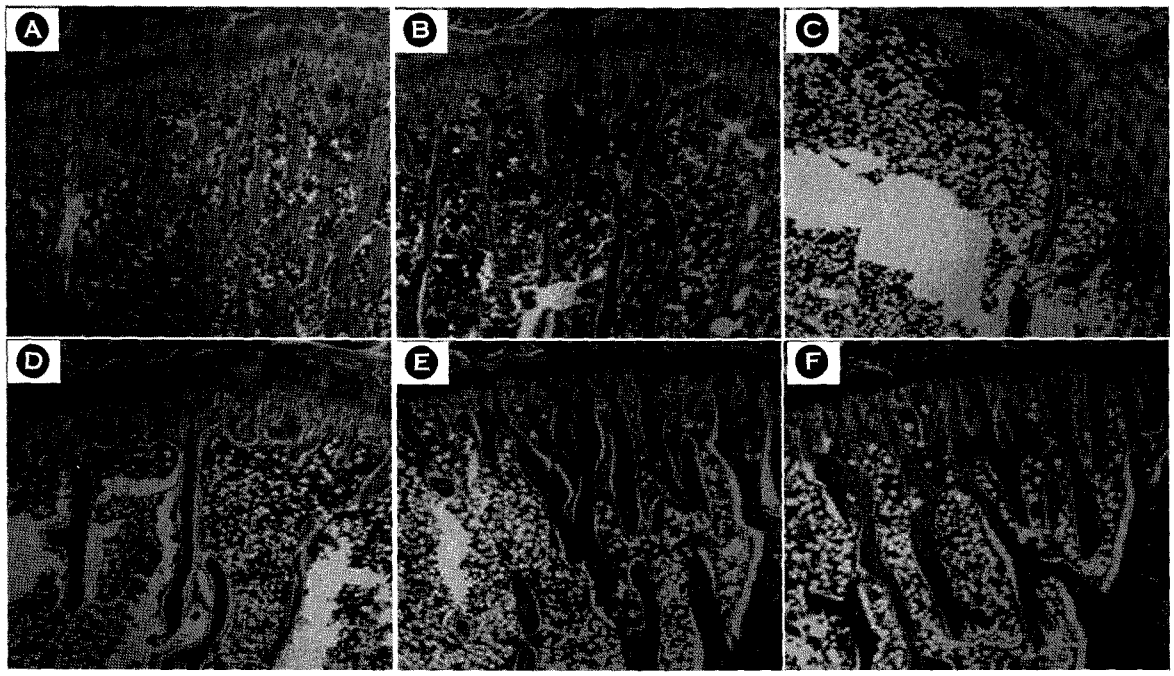

Fig. 2. Photographs of longitudinal sections of the distal end of femurs. Femurs were removed and fixed in 4% formalin after 14 weeks of oral treatment with AQF and or LAB from 1 months after ovariectomy. Fixed femurs were stained hematoxylin and eosin. Trabecular bone in the distal femur metaphysis was observed under a microscope (×40). **(A)** sham operation, **(B)** sham operation and Ca-D diet, **(C)** ovariectomized and Ca-D diet control, **(D)** ovariectomized and treated with lactic acid bacteria (1×10^7 CFU/kg B.W., respectively), **(E)** ovariectomized and treated with Aquamin F 50 mg/kg BW, **(F)** ovariectomized and treated with lactic acid bacteria (1×10^7 CFU/kg B.W., respectively) and Aquamin F 50 mg/kg BW treatment.

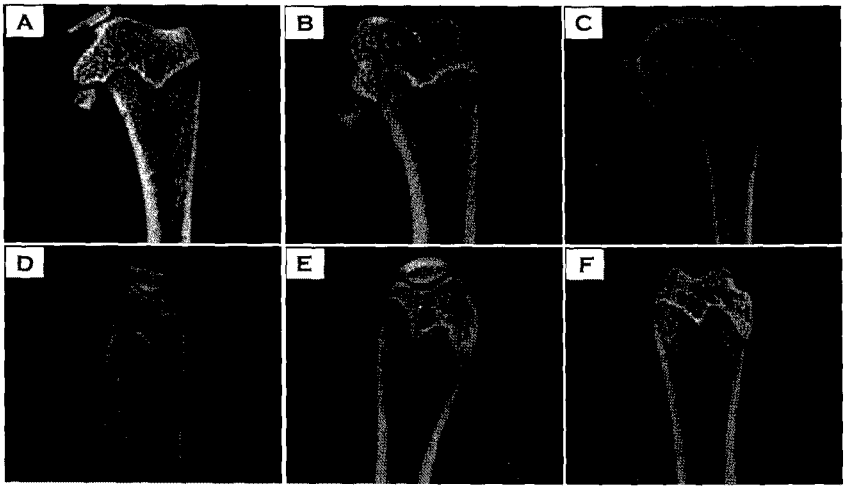

Fig. 3. Micro-focus CT images of distal portion of femurs. Femurs were removed after 14 weeks of oral treatment with Aquamin F and or LAB from 1 months after ovariectomy. **(A)** sham operation, **(B)** sham operation and Ca-D diet, **(C)** ovariectomized and Ca-D diet control, **(D)** ovariectomized and treated with lactic acid bacteria (1×10^7 CFU/kg B.W., respectively), **(E)** ovariectomized and treated with Aquamin F 50 mg/kg BW, **(F)** ovariectomized and treated with lactic acid bacteria (1×10^7 CFU/kg B.W., respectively) and Aquamin F 50 mg/kg BW treatment.

Serum biochemical analysis

In the serum biochemical analysis, the OVX group showed a two fold higher level of ALP than the sham group (sham group: 593±19.9 IU/L and OVX group: 1159 ±103.6 IU/L), and such high level of ALP was significant in the LAB group (927±63.4 IU/L) and the AQF group (880±89.3 IU/L). Particularly, the LAB + AQF group (683 ±46.7 IU/L) showed the greatest decrease in ALP. In the measurement of CA and IP, there was no significant difference between the sham group and the OVX group, and between the OVX group and the LAB + AQF group.

Microarchitecture of the trabecular bone

Removal of an ovary is known to accelerate bone loss. As mentioned, the level of ALP increased in the ovariectomized rats, which indicates the mal-metabolism of the bone. To examine if AQF and/or LAB can affect the microarchitecture of the bone tissue, the morphological changes in the trabecular bone (TbB) in the distal femoral metaphysis were examined. Fig. 2 shows that TbB stained with H&E developed more poorly in the OVX group [Fig. 2 (C)] than in the sham group, whereas its area increased after treatment with AQF and the LAB + AQF mixture [Fig. 2 (E) and (F)]. These indicate that LAB + AQF are effective in recovering bone loss due to ovariectomy in rats.

The recuperation of the bone microarchitecture with AQF and the LAB and AQF mixture was further confirmed by examining TbB in the distal femoral metaphysis with micro-CT. More severe resorption of the TbB was observed in the OVX group [Fig. 3 (C)] than in the sham group, and treatment with LAB alone did not significantly affect the bone tissue of the rats [Fig. 3 (D)]. In contrast, the TbBs were better developed in both the AQF and LAB + AQF groups than in the sham group [Fig. 3 (E) and (F)].

Effect on bone mineralization

BMC and BMD are commonly used to assess bone mineralization. To assess the effect of AQF or/and LAB on bone mineralization, the BMC and BMD in the tibia were measured using XCT Research SA+. As shown in Fig. 4, the BMC of the OVX group (5.22±0.53 mg/mm) decreased more significantly than that of the sham group (11.79± 0.47 mg/mm). Although the LAB group (5.00±0.36 mg/mm) did not show a significant difference, the BMCs of the AQF (7.87±0.43 mg/mm) and LAB + AQF (9.43± 0.81 mg/mm) groups increased more significantly than did that of the OVX group ($P<0.0001$, respectively). When the BMCs of the AQF and the LAB + AQF groups were compared, the LAB + AQF group showed an approximately 30% higher BMC than the AQF group (Fig. 4A; $P<0.0001$).

On the other hand, ovariectomy led to a more significant decrease in the BMD of the OVX group (288.03±29.66 mg/mm^2) than in that of the sham group (709.53±6.47 mg/mm^2) (Fig. 4B). The effect of treatment with LAB alone on BMD was little; but the AQF group (498.13± 79.62 mg/mm^2) and the LAB + AQF group (603.38±50.41 mg/mm^2) had more significant BMD increases than the

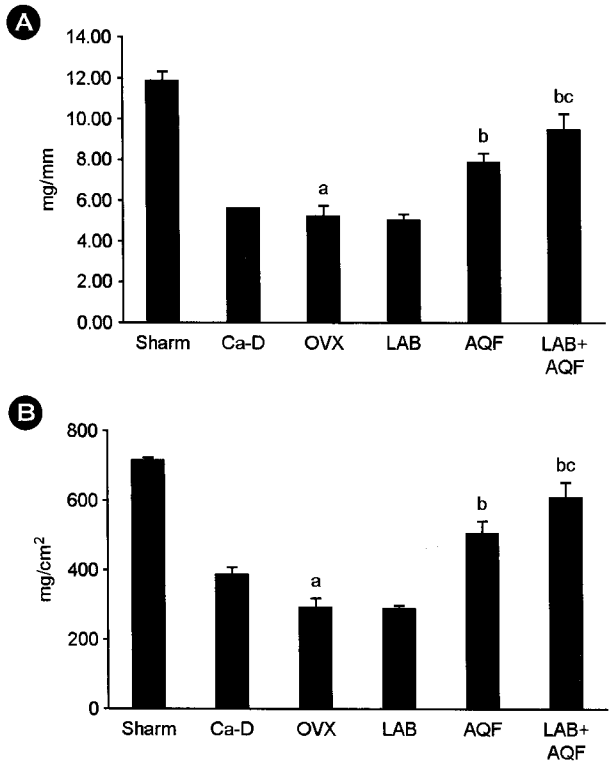

Fig. 4. BMC (bone mineral content, mg/mm) and BMD (bone mineral dencity, mg/mm^2) of ovariectomized rats. BMC (bone mineral content, mg/mm) and BMD (bone mineral density, mg/mm^2) of ovariectomized rats treated with test meterial for 14 weeks. **Normal:** normal, **Sham:** sham control, **OVX:** ovariectomized control, **LAB:** ovariectomized and treated with lactic acid bacteria (1×10^7 CFU/kg B.W., respectively), **AQF:** ovariectomized and treated with Aquamin F 50 mg/kg B.W, **LAB + AQF:** ovariectomized and treated with lactic acid bacteria (1×10^7 CFU/kg B.W., respectively) and Aquamin F 50 mg/kg BW treatment. Values are means ± SEM, n=5 per group, a=P<0.05 vs. Sham group, b=P<0.05 vs. OVX group, c=P<0.05 vs. AQF group by Unpaired t-test.

OVX group (Fig. 4B; P<0.0001, respectively). Moreover, the BMD of the LAB + AQF group was 18% higher than that of the AQF group, but the difference was only borderline significant (Fig. 4B; P=0.0764).

DISCUSSION

Osteoporosis is a skeletal disease in which reduced bone strength increases the risk of bone fracture. Bone mass, a major factor of bone strength, reaches its peak level at the ages of 20~30 years. If peak bone mass is not fully achieved due to either genetic or environmental reasons, the risk of developing osteoporosis increases (Lee et al., 2009). After peak bone mass is achieved, bone loss begins with age. In women, bone loss occurs faster than in men because of the increased number and activity of osteoclasts due to post-menopausal estrogen deficiency. An imbalance between such bone loss and the bone formation leads to a decrease in bone mass (Kalu et al., 1991; Balena et al., 1993).

Many studies highlighted the importance of calcium and vitamins in the prevention and alleviation of osteoporosis. Studies showed that such environmental factors as calcium and vitamin D play a critical role in the formation of peak bone mass (Eriksen, 1986; Ettinger, 2003) and that administration of calcium and vitamin D was effective in alleviating the symptoms of osteoporosis caused by estrogen deficiency due to diminished ovarian function (Lee et al., 2009).

Probiotics, because of their stimulation of Ca absorption in the intestine, have attracted the attention of researchers. Probiotics are known to influence probiotic bacteria to promote calcium absorption (Cashman, 2003). Thus, this study aimed to ascertain the effect of the combined administration of AQF (a calcium- and magnesium-rich seaweed-derived multimineral supplement) and three LAB (*Lactobacillus caseii*, *Lactobacillus rhamnosus*, and *Streptococcus thermophillus*) on an *in vivo* osteoporosis model the condition of which was induced by ovariectomy-caused estrogen deficiency and a calcium-deficient diet.

The body weight of the sham-operated group and the ovariectomized group differed considerably (sham group: 270±19 g and OVX group: 347±20 g). In contrast, among the ovariectomized groups, only the LAB group (323±14 g) showed a more significant change in body weight than the OVX group, and the other groups (AQF group: 363±11 g and LAB + AQF group: 356±31 g) did not show a more significant change in body weight than the OVX group (Fig. 1).

It is believed that these results were due to the non-suppression of body fat accumulation because of the reduced lipoprotein lipase activity and the decreased hormone-sensitive lipase caused by ovariectomy-induced estrogen deficiency (Ross, 1989; Women's Health Initiative Steering Committee, 2004). Considering reports that osteoblasts and adipocytes differ from blastocytes and that

Table 3. Effects on body weight and serum parameters

Group	ALP (IU/L)	CA (mg/dL)	IP (mg/dL)
Sham	593±19.9	11.1±0.7	5.2±0.7
Ca-D	666±80.3[a]	10.5±0.3	4.4±0.1
OVX	1159±103.6	9.8±0.2	5.8±0.5
LAB	927±63.4[b]	10.1±0.3	4.7±0.2
AQF	880±89.3[b]	10.4±0.2	3.9±0.3
LAB + AQF	683±46.7[b,c]	10.8±0.3	3.7±0.5

Meansurement of serum ALP (Alkaline phosphatase), CA (Calcium) and IP (Ignore phosphorus) of ovariectomized rats treated with test material for 14 weeks. Normal: normal, Sham: sham control, OVX: ovariectomized control, LAB: ovariectomized and treated with Lactic acid bacteria (1×10^7 CFU/kg B.W., respectively), AQF: ovariectomized and treated with Aquamin F 50 mg/kg B.W, LAB + AQF: ovariectomized and treated with Lactic acid bacteria (1×10^7 CFU/kg B.W., respectively) and Aquamin F 50 mg/kg BW treatment. Values are means ± SEM, n=5 per group, a=P<0.05 vs. Sham group, b=P<0.05 vs. OVX group, c=P<0.05 vs. AQF group by Unpaired t-test.

estrogen promotes differentiation of blastocytes into osteoblasts and suppresses differentiation of blastocytes into adipocytes, it is believed that the increase in body weight was due to the body fat accumulation caused by the ovariectomy-induced estrogen deficiency, and is a characteristic of ovariectomized rats (Okasaki et al., 2002).

ALP belongs to the group of target enzymes for the parathyroid hormone, is an osteoblast-secreted glycoprotein, and is the most widely used marker for bone formation. It was reported that the serum ALP level increased when osteoporosis, fracture, and calcium deficiency existed during the growth period, throughout which bone formation is active (Price et al., 1980). There was also a report that an increased level of ALP was observed in a post-menopausal osteoporosis patient (Sarioglu, 2006).

In this study, a serum biochemical analysis showed that the ALP level of the OVX group was almost double that of the sham group (sham group: 593±19.9 IU/L and OVX group: 1,159±103.6 IU/L), and was significantly low in the LAB group (927±63.4 IU/L) and the AQF group (880 ±89.3 IU/L). In particular, the ALP level decreased most in the LAB + AQF group (683±46.7 IU/L). In the measured CA and IP, there was no significant difference between the sham group and the OVX group, and between the OVX group and the LAB + AQF group (Table 3). These results indicate that combined administration of LAB and AQF can be more effective than single administration of either LAB or AQF for osteoporosis, although such mono administration can also be effective.

Histopathological observation of the TbB of the tibia or femur can be useful for evaluating the status of the bone remodeling, because the TbB has a bone resorption rate of about 25% per year when the bone meatabolism is abnormal, whereas the cortical bone shows a bone resorption rate of only 3%, and thus allows a more accurate comparison (Pacifici, 1996).

Ovariectomy results in decreased BMD in rats and thus, has been used as a study model for postmenopausal bone loss (Wronski et al., 1988). Turner et al. reported that the trabeculae volume in the tibial trabecular bone of ovariectomized rats was reduced by more than 60% 28 days after ovariectomy (Turner, 1987). Takano-Yamamoto and Rodan reported that ovariectomy caused a 50% loss in the trabeculae volume in the cancellous bone of a rat femur after 22 days, doubling of the number of osteoclasts, tripling of the number of osteoblasts, and an eight-fold increase in the relative osteoid surface area (Takano-Yamamoto and Rodam GA, 1990).

In this study, morphological observation of the head of a femur is using H&E staining and micro-CT scanning revealed considerable resorption of the TbB in the OVX group. Although such resorption of the TbB did not improve in the LAB group, it was as low in the AQF group and the LAB + AQF group as in the sham group. Besides, the BMD and BMC results were consistent with the results of the morphological analysis.

These results indicate that the combined treatment could be more effective in improving the bone density than treatment with only AQF.

CONCLUSION

Combined treatment of ovariectomized rats with LAB and AQF effectively inhibited bone loss and significantly reduced the serum ALP level, a biomarker of bone formation. This study suggests that treatment with LAB in combination with AQF would better prevent and cure bone loss caused by post-menopause than only AQF treatment.

The effects in human need to be further addressed.

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