

Effects of Aging and Dietary Antler Supplementation on the Calcium-Regulating Hormones and Bone Status in Ovariectomized SAMP8 Mice

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Abstract

This study was conducted to investigate the effects of aging and long-term dietary antler supplementation on the calcium-regulating hormones and bone status in ovariectomized (Ovx) SAMP8 mice. The female SAMP8 mice were divided into four groups (in each group $n = 6$), OvX or sham operated at the age of 2 months, and fed with 0.2% antler containing diet or control diet from the age of 2.5 months. The samples were collected at the age of 3, 6, 9, 12, and 15 months, respectively, for physicochemical analyses, biochemical analyses, and the determination of hormones by radioimmunoassay. The results showed that plasma calcium (Ca) concentrations were maintained in a narrow range in all groups throughout the whole experimental period. With aging and/or ovariectomy, plasma parathyroid hormone (PTH) and 1,25-dihydroxycholecalciferol ($1,25-(OH)_2-D_3$) levels increased, and plasma phosphorus (P) and calcitonin (CT) levels decreased, and the femoral bone densities and Ca contents increased during the earlier stage, and then decreased gradually in all groups. Plasma PTH and $1,25-(OH)_2-D_3$ levels in the OvX mice were significantly higher than those in the intact mice, and plasma P concentrations, plasma CT levels, femoral bone densities, and femoral Ca contents in the OvX mice were significantly lower than those in the intact mice. In addition, the decreases of plasma P levels, plasma CT levels, femoral bone densities, and femoral Ca contents, and the increases of plasma PTH levels were moderated by antler administration in both OvX and intact mice. However, there was no effect of the dietary antler supplementation on the plasma $1,25-(OH)_2-D_3$ levels in the female mice. It is concluded that prolonged dietary antler supplementation has important positive effects on bone loss with age and/or ovarian function deficiency.

Key Words: mice, SAM, ovariectomy, aging, antler, calcium, calcium-regulating hormones, bone

Introduction

Alterations in calcium (Ca) metabolism with aging (postmenopause) have been attributed to a

variety of highly interdependent mechanisms. Age-related bone mineral loss may be mediated by a combination of hormonal factors that impair regulation of Ca homeostasis. Although serum Ca does not

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Received: February 14, 2007; Revised: May 10, 2007; Accepted: May 28, 2007.
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change with age, some hormones that regulate Ca metabolism change markedly with age (11, 42). The levels of serum parathyroid hormone (PTH) increase (6-8, 23, 25), and the levels of plasma calcitonin (CT) decrease with age in humans (5). In rats, the alterations of serum PTH levels (11, 37, 41) are similar to those in humans, but the levels of serum CT are not decreased like those in humans and significantly higher in the older rats (11, 22, 24, 36). Recently, Chen *et al.* (1, 2) have found that the plasma PTH levels increase progressively, and the plasma CT levels decrease with advancing age in senescence accelerated mice (SAM), like those in humans.

Bone loss occurs universally with aging, and it is accelerated in women coinciding temporally with menopause, though the reduction in bone mass proceeds gradually (26). Postmenopausal osteoporosis, resulting from the loss of estrogen at menopause, is associated with a rapid reduction of bone mass, leading to porotic bones prone to fractures (9). The ovariectomized rat model and mouse model are suitable for studying problems that are relevant to postmenopausal bone loss (9, 13, 15, 16). However, the life spans of rats and mice are too long to study age-related research economically. Therefore, there is a need to explore whether other species can serve as good models of osteoporosis due to aging and ovarian hormone deficiency. Recently, the studies in our laboratory indicate that the senescence accelerated mouse-prone 8 (SAMP8) is proved to be a satisfactory animal model for longitudinal studies related to osteoporosis (2). The femoral bone densities and Ca contents in SAMP8 peak from as early as the age of 6 months and then decrease gradually, which shows that SAMP8 mice not only have the shortened life span (10 months of age) but also proceed to bone loss earlier than other mice and rats (2, 33, 34).

The velvet antler is a valuable Chinese medicinal material from ancient times. It is used for a wide variety of purposes, such as strengthening, healing, and anti-aging, by practitioners of Traditional Chinese Medicine (TCM). Most studies have reported that the velvet antler has effects on anti-aging (38-40), proliferation of osteoblasts, and recovery of fracture (43). The results show positive effects which agree with the TCM practice. Therefore, the purpose of this study is conducted to investigate the effects of aging and long-term dietary antler supplementation on the plasma CT, PTH, 1,25-dihydroxycholecalciferol (1,25-(OH)₂-D₃) levels and bone status in ovariectomized SAMP8 mice.

Materials and Methods

Animals

The female SAMP8 mice were housed in a temperature-controlled room (22 ± 2°C) with 14 h of

artificial illumination daily (0600-2000). During the experimental period, they were provided with the commercial chow (0.95% Ca, 0.75% P, and 2.60 U/g vitamin D; Fwusow, Ltd., Taiwan) and water *ad libitum*.

Antler Administration

The fresh antler was sliced, dried for 36 h in the oven at 50°C, and then pulverized by the pulverizer (Model RT-34; Yeong-Shin Ltd., Taiwan). The antler powder, containing 22.3% Ca and 7.1% phosphorus (P), was mixed with the commercial chow by the Fwusow Industry Company. The antler chow contained 0.2% (2 g/kg) antler (20), 0.97% Ca, 0.76% P, and 2.67 U/g vitamin D.

Treatments

The 160 female SAMP8 mice were ovariectomized (Ovx) or sham operated (Sham) at the age of 2 months, and fed with either 0.2% antler containing diet (A) or control diet (C) from the age of 2.5 months, so that the SAMP8 mice were divided into four groups (Sham-A, Sham-C, Ovx-A, and Ovx-C; 40 mice for each group). A part of the mice (each group n = 6-8) were decapitated at the age of 3, 6, 9, 12, and 15 months, respectively. The blood samples were collected and the plasma samples were separated and stored at -20°C for biochemical analyses and the determination of hormones by radioimmunoassay (RIA). The femurs were dissected and cleaned off all soft tissue and also stored at -20°C until analysis.

Biochemical Analyses of Plasma

The plasma Ca and P concentrations and the plasma alkaline phosphatase (ALP) activities were determined by commercial kits with Kodak Ektachem DT Chemistry System (Johnson & Johnson Clinical Diagnostics, Inc., Rochester, NY, USA).

RIAs of Hormones

CT. The concentrations of plasma CT were measured by heterogenous RIA with human CT RIA kits purchased from BioSource International (Camarillo, CA, USA) as described previously (2). The sensitivity of the RIA was 4.0 pg/ml. The cold recovery rate of CT from mouse plasma pools was 95.00 ± 1.85% (mean ± SEM). The intra- and inter-assay coefficients of variation were 5.03% (n = 6) and 7.75% (n = 4), respectively.

PTH. The concentrations of plasma PTH were measured with the intact human PTH-specific RIA kits from Nichols Institute (San Juan Capistrano, CA, USA) as described previously (2). The sensitivity of the RIA was 2.2 pg/ml. The cold recovery rate of PTH from

mouse plasma pools was $95.83 \pm 1.05\%$. The intra- and inter-assay coefficients of variation were 4.25% ($n = 6$) and 6.67% ($n = 4$), respectively.

1,25-(OH) $_2$ -D $_3$. The concentrations of plasma 1,25-(OH) $_2$ -D $_3$ were measured by human 1,25-(OH) $_2$ -D $_3$ RIA kits purchased from BioSource International (Camarillo, CA, USA) as described previously (2). The sensitivity of the RIA was 5.0 pg/ml. The cold recovery rate of 1,25-(OH) $_2$ -D $_3$ from mouse plasma pools was $93.57 \pm 2.33\%$. The intra- and inter-assay coefficients of variation were 7.28% ($n = 4$) and 12.83% ($n = 4$), respectively.

Physicochemical and Chemical Analyses of Femurs

Bone density. The bone densities of the femurs were measured by Archimedes' principle (27). Each femur was placed in an unstoppered vial filled with deionized water, and agitated periodically to ensure that all trapped air diffused out of the bone. The vial was put in a desiccator connected to a vacuum for 3 h. Then the femur was blotted out tissue, weighed, and returned to the vial containing deionized water. To calculate the density, the dry weight was divided into the excluded volume.

Ca content. The femurs were dried for 24 h at 100°C, and the dry weights were recorded. The dry femurs were then ashed in a muffle furnace at 550-600°C for 6 h, and the ash was weighed. The ash was pulverized and hydrolyzed with 6 M HCl. The hydrolysate was diluted with 0.1% lanthanum solution, and Ca content was determined by atomic absorption spectrophotometry (Hitachi, Model No. Z-6100).

ALP. The cleaned femurs were weighed and subsequently homogenized in 1.5 ml sodium bicarbonate buffer (0.15 M NaCl and 3 mM NaHCO $_3$, pH 7.2) using a polytron homogenizer (Kinematica, Model No. PT-MR 2100) for 2 min at 0°C. The ALP activities of homogenized samples were determined with commercial kits (Sigma, St. Louis, MO, USA) and colorimeter (Bio-Tek, Model No. ELx-800).

Statistical Analysis

All values are presented as the mean \pm SEM. The differences among four groups at the same age were determined by a two-way factorial analysis of variance (ANOVA) with equal replication, and determined with Duncan's multiple range test when the ANOVA indicated significant differences among means. The differences among ages within each group were determined by a one-way factorial ANOVA with

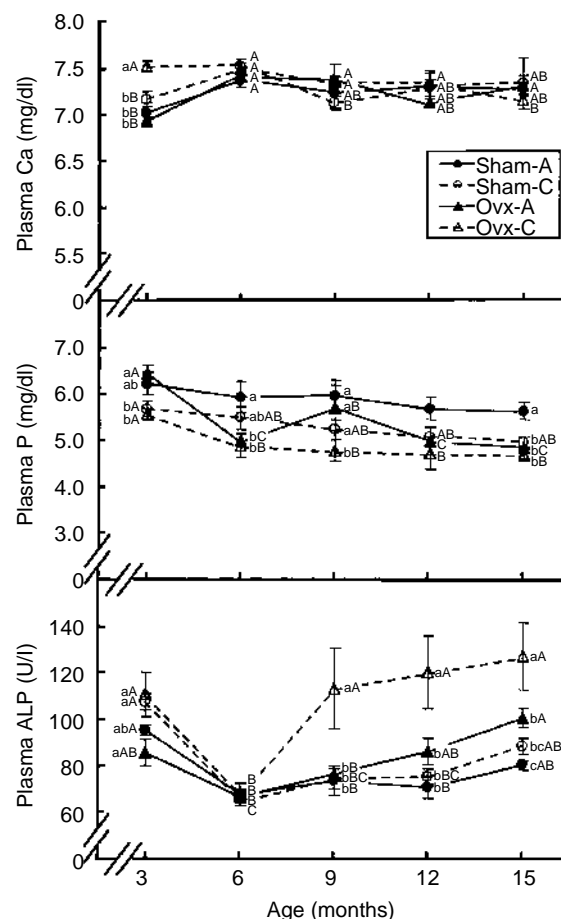


Fig. 1. Effects of dietary antler supplementation and ovariectomy on plasma calcium (top), phosphorus (central) levels, and alkaline phosphatase activities (bottom) in female SAMP8 mice. Each point represents the mean \pm SEM ($n = 6$). a, b, c: Means with same letters are not significantly different among four groups at the same age in each trial ($P > 0.05$). A, B, C: Means with same letters are not significantly different among ages within each group in each trial ($P > 0.05$).

equal replication, and determined with Duncan's multiple range test when the ANOVA indicated significant differences among means¹. A difference between means was considered significant when $P < 0.05$.

Results

Biochemistry of Plasma

Plasma Ca concentrations were maintained in a narrow range in all groups (6.9-7.5 mg/dl) throughout the whole experimental period (Fig. 1, top panel). The plasma Ca concentrations were not significantly different

¹ SAS Institute Inc. SAS/STAT Software: Changes and Enhancements, Release 8.1. Cary, NC, USA, 2000.

among four groups at the same age or among ages within each group ($P > 0.05$) except that those in Group OvX-C were significantly higher than those in the other three groups at the age of 3 months ($P < 0.05$).

With aging, plasma P concentrations decreased in all groups (Fig. 1, central panel). Plasma P concentrations in the ovariectomized mice were significantly lower than those in the intact mice ($P < 0.05$). The decrease of plasma P concentrations was moderated by antler administration in both ovariectomized and intact mice ($P < 0.05$). There were significant effects of dietary antler supplementation and ovariectomy ($P < 0.05$), but no interaction ($P > 0.05$), on the plasma P concentrations in female SAMP8 mice.

Plasma ALP activities decreased during the early stage of the experiment in all groups, and then the activities increased in Group OvX-C, but fluctuated in the other three groups during the age of 9-15 months (Fig. 1, bottom panel). Plasma ALP activities in the ovariectomized mice were significantly higher than those in the intact mice ($P < 0.05$). The increases of plasma ALP activities were moderated by antler administration in both ovariectomized and intact mice ($P < 0.05$). There were significant effects of dietary antler supplementation and ovariectomy ($P < 0.05$), but no interaction ($P > 0.05$), on the plasma ALP activities in female SAMP8 mice.

Hormones of Plasma

With increasing of age, plasma PTH and 1,25-(OH)₂-D₃ levels increased, and plasma CT levels decreased in all groups (Fig. 2). There were significant effects of dietary antler supplementation and ovariectomy ($P < 0.05$), but no interaction ($P > 0.05$), on the plasma PTH and CT levels in female SAMP8 mice. There were significant effects of ovariectomy on the plasma 1,25-(OH)₂-D₃ levels in female SAMP8 mice ($P < 0.05$). However, there was no effect of the dietary antler supplementation on the plasma 1,25-(OH)₂-D₃ levels in the female mice ($P > 0.05$). Plasma PTH and 1,25-(OH)₂-D₃ levels in the ovariectomized mice were higher than those in the intact mice, and plasma CT levels in the ovariectomized mice were lower than those in the intact mice ($P < 0.05$). The decrease of plasma CT levels and the increase of plasma PTH levels were moderated by antler administration in both ovariectomized and intact mice ($P < 0.05$).

Physicochemistry and Chemistry of Femurs

The femoral bone densities and Ca contents increased with age during the earlier stage, and then decreased gradually in all groups (Fig. 3, top and central panels). The bone densities and Ca contents peaked at the age of 9 months in Group OvX-C, but peaked at

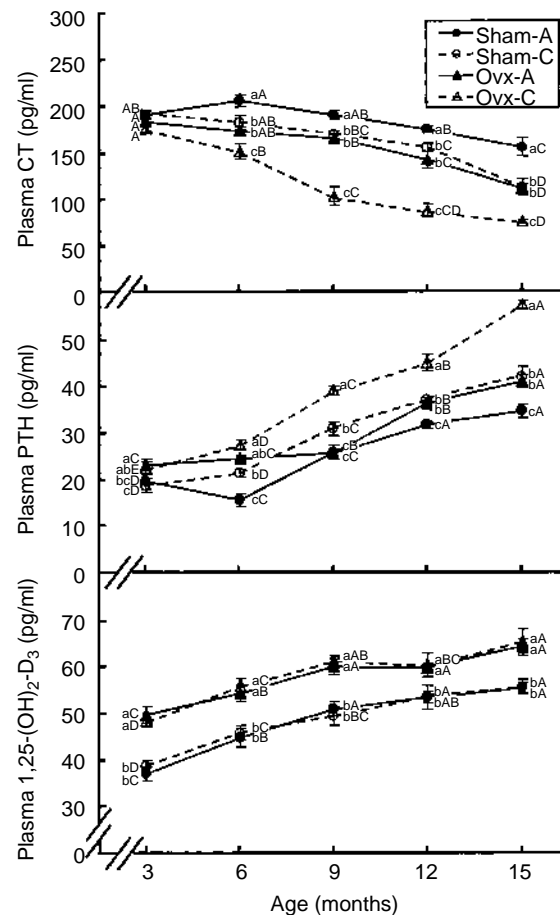


Fig. 2. Effects of dietary antler supplementation and ovariectomy on plasma calcitonin (top), parathyroid hormone (central), and 1,25-(OH)₂-D₃ (bottom) levels in female SAMP8 mice. Each point represents the mean ± SEM (n = 4). Each sample consists of a pool of plasma from two animals. a, b, c: Means with same letters are not significantly different among four groups at the same age in each trial ($P > 0.05$). A, B, C, D, E: Means with same letters are not significantly different among ages within each group in each trial ($P > 0.05$).

the age of 6 months in the other three groups. There were significant effects of dietary antler supplementation and ovariectomy ($P < 0.05$), but no interaction ($P > 0.05$), on the bone density and bone Ca content in female SAMP8 mice. The bone density and bone Ca content in the ovariectomized mice were significantly lower than those in the intact mice ($P < 0.05$). The decreases of the bone density and bone Ca content were moderated by antler administration in both ovariectomized and intact mice ($P < 0.05$).

The bone ALP activities tended to decrease with age, although the activities elevated slightly at the age of 15 months in all groups (Fig. 3, bottom panel). There were significant effects of dietary antler supplementation and ovariectomy ($P < 0.05$), but no interaction ($P > 0.05$),

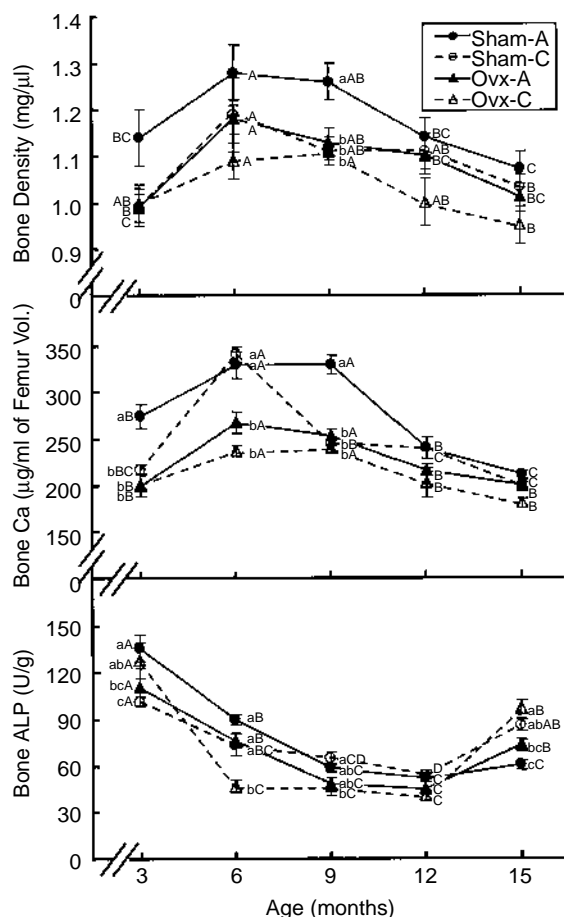


Fig. 3. Effects of dietary antler supplementation and ovariectomy on femoral bone densities (top), femoral calcium contents (central), and femoral alkaline phosphatase activities (bottom) in female SAMP8 mice. Each point represents the mean \pm SEM ($n = 6$). a, b, c: Means with same letters are not significantly different among four groups at the same age in each trial ($P > 0.05$). A, B, C, D: Means with same letters are not significantly different among ages within each group in each trial ($P > 0.05$).

on the bone ALP activities in female SAMP8 mice.

Discussion

Due to aging or ovariectomy, the plasma P and CT levels, the femoral bone densities, and the femoral Ca contents decrease, and the plasma PTH and $1,25-(\text{OH})_2\text{-D}_3$ levels increase in SAMP8 mice. These results indicate that the female SAMP8 develops osteoporotic signs earlier by aging and/or ovariectomy.

In this study, the plasma Ca concentrations are maintained within 6.9–7.5 mg/dl throughout the experiment without ovariectomized variation in all groups of SAMP8 mice. The absence of variation of plasma Ca concentrations with ovariectomy have also

been found in rats (9, 29–31). In women, plasma Ca concentrations are unchanged (19, 28, 35) or decrease slightly (25) with postmenopause. On the other hand, the plasma P concentrations decrease with age and/or ovariectomy in all groups of SAMP8 mice, and the similar tendency is also found in postmenopausal women (19, 25, 28, 35). It is suggested that much more P than Ca is lost through the urine, and mobilization of P from bone might not keep pace with the renal loss; hence, senescent and/or ovariectomized animals become slightly but significantly hypophosphatemic (14, 17, 18). In this study, the hypophosphatemia is moderated by antler administration in SAMP8 mice, especially during senescence (Fig. 1, central panel). Furthermore, the effects of antler supplementation are not mediated by gonad because they also occur in the ovariectomized groups.

Ovariectomy increases the plasma PTH and $1,25-(\text{OH})_2\text{-D}_3$ levels, and the increase of plasma PTH is prevented by estrogen therapy, but there is no significant effect on the plasma $1,25-(\text{OH})_2\text{-D}_3$ levels in C57BL and C3H mice (13). On the other hand, ovariectomy decreases the plasma CT levels (10, 36), and the increase of the plasma CT levels is enhanced after progesterone treatment in rats (21). Also, there are significant effects of long-term dietary antler supplementation on the plasma PTH and CT levels in female SAMP8 mice. The plasma PTH levels are lower in the antler diet groups than those in the control diet groups; the plasma CT levels are higher in the antler diet groups than those in the control diet groups. However, there is no effect of the dietary antler supplementation on the plasma $1,25-(\text{OH})_2\text{-D}_3$ levels in the SAMP8 mice. These results indicate that the increase of plasma PTH levels and the decrease of plasma CT levels are moderated by antler administration. Furthermore, the effects of antler supplementation require a period of time to be significant (Fig. 2, top and central panels), which are not mediated by gonad because they also occur in the ovariectomized groups.

Ovariectomy decreases the femoral bone densities and Ca contents, and the decreases are moderated by antler administration in SAMP8 mice. The bone densities and Ca contents are higher in the antler diet groups than those in the control diet groups. The results show that the increases of the bone densities and Ca contents are elevated by antler administration which is not mediated by gonad because the effects also occur in the ovariectomized groups. In rats and other strain mice, the bone densities and Ca contents are decreased by ovariectomy, and they are enhanced after high dietary Ca supplementation, phytoestrogen (soybean isoflavones) treatment or estrogen therapy (9, 12, 17). The above results suggest that the antler administration, the same as high dietary Ca supplementation, phytoestrogen treatment or estrogen therapy, has positive effects on

bone status.

The activities of the plasma and femoral ALP are slightly elevated in the ovariectomized groups during senescence. The elevated ALP activity indicates that the bone formation and bone turnover are also stimulated. Much evidence has shown that bone formation and resorption are enhanced by ovariectomy, with resorption exceeding formation, and the exceeding resorption leads to the decreases of bone density and bone Ca content and the increase of bone loss (4, 12, 17, 18, 32). In SAMP8 mice, the plasma and femoral ALP activities are lower in the antler diet groups than those in the control diet groups; it indicates that the bone formation and bone turnover are moderated by antler administration. The moderated bone turnover rate by long-term antler administration contributes to the restraint of the decreases of bone density and bone Ca content as well as the increase of bone loss. The activities of the plasma ALP are higher in ovariectomized rats than those in intact female rats, and the increase of the plasma ALP activity is prevented by estrogen therapy (9, 32). The results suggest that the antler administration, the same as estrogen therapy, is able to inhibit the increases of the plasma and femoral ALP activities.

In aged and/or ovariectomized animals, the plasma Ca concentrations are always maintained at homeostasis, and the bone densities and Ca contents decrease owing to the decreases of the intestinal Ca absorption and the renal Ca reabsorption. Aging and/or ovariectomy rise the plasma PTH and 1,25-(OH)₂-D₃ levels, and decrease the intestinal absorption and the renal reabsorption of Ca and P (3) in SAMP8 mice; it seems that there is a tendency to reduce the responsiveness of intestinal 1,25-(OH)₂-D₃ and renal PTH action with aging and/or ovarian function deficiency. PTH and CT are the resorption-stimulating hormone and the resorption-inhibiting hormone respectively. Thus, to prevent hypocalcemia from Ca excretion, plasma PTH secretion increases and plasma CT secretion decreases, stimulating bone resorption. In this study, the increase of plasma PTH levels, and the decrease of plasma CT levels are prevented by antler supplementation in SAMP8 mice. The above results show that the long-term antler administration mainly inhibits bone resorption.

In conclusion, aging and/or ovariectomy accelerate bone loss by the decreases of plasma P levels, plasma CT levels, femoral bone densities, and femoral Ca contents, and the increases of plasma PTH and 1,25-(OH)₂-D₃ levels in SAMP8 mice. In addition, the prolonged dietary antler supplementation has important positive effects on bone loss with age and/or ovarian function deficiency.

Acknowledgments

This study was conducted at the Animal Science

and Biotechnology Department, Tunghai University, Taichung, Taiwan, R.O.C.

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