Supplemental Intake of Isoflavones and Zinc-Containing Mineral Mixture Enhances Bone Components in the Femoral Tissue of Rats with Increasing Age

Zhong Jie Ma, Aki Igarashi, Masaru Inagaki, Fumio Mitsugi, and Masayoshi Yamaguchi

Laboratory of Endocrinology and Molecular Metabolism, Graduate School of Nutritional Sciences, University of Shizuoka, 52–1 Yada, Shizuoka 422–8526, Japan, Institute for Consumer Healthcare, Yamanouchi Pharmaceutical Co., Ltd., Itabashi-ku, Tokyo 174–8612, Japan, and Medical Information Group, Sanwell Co., Ltd., Taito-ku, Tokyo 111–0034, Japan

(Received July 10, 2000; Accepted July 25, 2000)

The effect of the supplemental intake of isoflavone and a zinc-containing mineral mixture on bone components in the femoral tissue of rats was investigated. Rats (5 weeks old) were orally administered either vehicle, isoflavone glycoside (5 mg/100 g body weight), aglycone isoflavone (5 mg/100 g, including 3.772 mg daidzein, 0.395 mg genistein, and 0.833 mg glycitein), zinc-containing mineral mixture (0.833 mg zinc, 50 mg calcium, 25 mg magnesium, and 0.208 μg vitamin D3 per 100 g body weight, respectively), or both aglycone isoflavone (5 mg/100 g) and zinc-containing mineral mixture for 7 d. Administrations resulted in a significant increase in femoral dry weight, calcium content, alkaline phosphatase activity, and DNA content in the femoral-diaphyseal and metaphyseal tissues. Femoral zinc content was significantly increased by the administration of zinc-containing mineral mixtures with or without aglycone isoflavone. The effect of aglycone isoflavone in elevating bone components was significantly enhanced in combination with the zinc-containing mineral mixture. Moreover, the oral administration of both aglycone isoflavones and zinc-containing mineral mixture to aged rats (50 weeks old) for 21 d resulted in a significant increase in femoral dry weight, zinc content, and bone components. This study demonstrates that supplemental intake of both aglycone isoflavone and a zinc-containing mineral mixture has an anabolic effect on bone components in aged rats, suggesting the role of nutrients in the prevention of osteoporosis with increasing age.

Key words — isoflavone, zinc, bone metabolism, bone formation, osteoporosis

INTRODUCTION

Bone loss with increasing age induces osteoporosis.1–3) This loss may be due to increased bone resorption and decreased bone formation. Osteoporosis with a decrease in bone mass is widely recognized as a major public health problem.4) Pharmacological and nutritional factors may have the potential to prevent bone loss with increasing age. Nutritional factors may be especially important in the prevention of osteoporosis, although this is poorly understood.

Isoflavone glycoside (daidzin, genistin, and glycitin) and aglycone isoflavone (daidzein, genistein, and glycitein) are present in soybeans at comparatively high concentrations. Recently, it has been demonstrated that isoflavones have an anabolic effect on bone metabolism in rats,5–9) suggesting a role in the prevention of osteoporosis. Daidzin, genistin, and glycitin are hydrolyzed to daidzein, genistein, and glycitein, respectively, by β-glucosidase in gastric juice. Daidzein and genistein have been shown to stimulate osteoblastic bone formation10,11) and to inhibit osteoclastic resorption,12,13) thereby increasing bone mass.

Zinc, an essential trace element, has been demonstrated to have a potent stimulatory effect on bone formation14,15) and an inhibitory effect on bone resorption.16) Zinc can stimulate protein synthesis in osteoblastic cells in vitro by activating aminoacyl-tRNA synthetase.16,17) The oral administration of a zinc compound can prevent bone loss in ovariectomized rats,18) an animal model of osteoporosis.

Whether the combination of nutritional factors reveals an additive or synergistic effect on bone com-
ponents has not been fully determined. This know-
ledge may be important in the prevention of bone
loss with increasing age. Recently, it has been shown
that the combination of genistein and zinc can have
a synergistic effect on bone components in femoral
tissue from elderly female rats. Furthermore, we
found that the supplemental intake of an aglycone
isoflavone-containing zinc and mineral mixture can
increase bone components in the femoral tissue of
both young and aged rats.

MATERIALS AND METHODS

Animals — Female Wistar rats (conventional)
weighing 100–110 g (5 weeks old) or 210–240 g
(50 weeks old) were obtained from Japan SLC
(Hamamatsu, Japan). The animals were fed commer-
cial laboratory chow (solid) containing 1.1% calcium
and phosphorus at a room temperature of 25°C,
with free access to distilled water.

Administration Procedures — For group 1, a
powder of isoflavone glycoside containing daidzin
(3.772 mg), genistin (0.395 mg) and glycitin
(0.833 mg) was suspended in distilled water (1 ml).
For group 2, aglycone isoflavones including daidzein
(3.772 mg), genistein (0.395 mg), and glycitein
(0.833 mg) were also suspended in distilled water
(1 ml). For group 3, a zinc-containing mineral mix-
ure consisting of zinc yeast (0.833 mg Zn), coral-
derived calcium (50 mg), magnesium (25 mg), and
vitamin D₃ (0.208 µg) was mixed and suspended in
distilled water (1 ml). For group 4, the aglycone
isoflavones of group 2 and the zinc-containing min-
eral mixture of group 3 were suspended in distilled
water (1 ml). These suspensions (1 ml/100 g body
weight) were orally administered to young rats
(5 weeks old) through a stomach tube once a day
for 7 d, and 24 h after the last administration the ani-
mals were killed by cardiac puncture under light
ether anesthesia, and the blood and femur were re-
moved immediately.

In another experiment, aged rats (50 weeks old)
were orally administered the suspension (1 ml/100 g
body weight) of aglycone isoflavones and zinc-con-
taining mineral mixture through a stomach tube once a
day for 7 or 21 d, and 24 h after the last adminis-
tration the animals were killed by cardiac puncture under
light ether anesthesia, and the blood and femur were re-
moved immediately.

Analytical Procedures — Blood samples were
centrifuged for 30 min after collection, and the se-
rum was separated and analyzed immediately. Se-
rum calcium and zinc were determined by atomic
absorption spectrophotometry. Serum inorganic
phosphorus was measured by the method of Tausky
and Shon. Serum albumin, nitrogen urea, triglyc-
eride, free cholesterol and HDL cholesterol were mea-
sured using KIT (Wako Pure Chemical Industries,
Ltd., Osaka, Japan). Serum estradiol was assayed
using a double-antibody radioimmunoassay method
using the RI KIT.

The diaphyseal and metaphyseal tissues were
washed with 16 h at 110°C and weighed. Bone tissues
were digested for 24 h at 110°C. Calcium was de-
termined by atomic absorption spectrophotometry. The
calcium content in bone tissues was expressed
as milligram per gram of dry bone.

To assay alkaline phosphatase activity, the dia-
physeal and metaphyseal tissues were immersed in
3.0 ml of ice-cold 6.5 mM barbital buffer (pH 7.4),
cut into small pieces, homogenized with a
Physcotron homogenizer, and disrupted for 60 s with
an ultrasonic device. The supernatant centrifuged at
600 × g for 5 min was used to measure enzyme ac-
tivity. Enzyme assay was carried out under opti-
mal conditions. Alkaline phosphatase activity was
determined by the method of Walter and Schutt. Enzyme activity was expressed as µmol of p-
nitrophenol liberated per minute per milligram of
protein. The protein concentration was determined
by the method of Lowry et al.

To measure bone DNA content, the diaphyseal
and metaphyseal tissues were shaken with 4.0 ml of
ice-cold 0.1 N NaOH solution for 24 h after homog-
ization of the bone tissues. After alkali extrac-
tion, the samples were centrifuged at 1000 × g for
5 min, and the supernatant was collected. DNA con-
tent in the supernatant was determined by the method of Cieriotti and expressed as the amount of DNA
(milligrams)/gram weight of bone tissue.

Statistical Analysis — The significance of the
difference between values was estimated by
Student’s t-test. p-Values of less than 0.05 were con-
sidered to indicate statistically significant differ-
ences. We also used a multiway analysis of variance
(ANOVA) and Tukey–Kramer multiple comparison
test to compare the treatment groups.
RESULTS

Effect of Isoflavones and Zinc-Containing Mineral Mixture on Bone Components in Young Rats

Rats (5 weeks old) were orally administered either vehicle, isoflavone glycoside (5 mg/100 g body weight), aglycone isoflavone (5 mg/100 g), zinc-containing mineral mixture (833 µg zinc, 50 mg calcium, 25 mg magnesium and 0.208 µg vitamin D₃ per 100 g body weight, respectively), or aglycone isoflavone (5 mg/100 g) and zinc-containing mineral mixture for 7 d, and 24 h after the last administration the animals were killed by exsanguination. Each value is the mean ± S.E.M. of six rats. * p < 0.01, compared with the control value. # p < 0.05, compared with the control value.

Femoral dry weight was significantly enhanced by the combination of aglycone isoflavone and zinc-containing mineral mixture.

Zinc content in the femoral-diaphyseal and metaphyseal tissues was significantly increased by the administration of the zinc-containing mineral mixture with or without aglycone isoflavone as compared with that of control rats (Fig. 2). This increase was not seen with the administration of isoflavone glycoside or aglycone isoflavone.

Calcium content, alkaline phosphatase activity, and DNA content in the femoral-diaphyseal and metaphyseal tissues were significantly elevated by the administration of isoflavone glycoside or aglycone isoflavone, zinc-containing mineral mixture, or both aglycone isoflavone and zinc-containing mineral mixture (Figs. 3, 4, and 5). These increases in the femoral-metaphyseal tissues were significantly enhanced by the combination of both aglycone isoflavone and zinc-containing mineral mixture compared with that of isoflavone glycoside, aglycone isoflavone or zinc-containing mineral mixture alone (Figs. 3, 4, and 5).

Effect of Supplemental Intake of Aglycone Isoflavone and Zinc-Containing Mineral Mixture on Bone Components in Aged Rats

Rats (50 weeks old) were orally administered the suspension (1 ml/100 g body weight) of supplemental tablets containing aglycone isoflavone (5 mg/ml,
Fig. 3. Changes in Calcium Content in the Femoral-Diaphyseal and Metaphyseal Tissues of Young Rats Orally Administered Isoflavones and Zinc-Containing Mineral Mixture

Rats were administered isoflavones as described in the legend to Fig. 1. Each value is the mean ± S.E.M. of six animals. * \( p < 0.01 \), compared with the control value. \# \( p < 0.01 \), compared with the value for isoflavone glycoside or aglycone isoflavone administration. □ Control; ■ isoflavone glycoside; □ aglycone isoflavone; □, zinc-containing mineral mixture; ▲ aglycone isoflavone and zinc-containing mineral mixture.

Table 1. Effect of Supplemental Intake of Aglycone Isoflavone and Zinc-Containing Mineral Mixture on Serum Components in Aged Rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Calcium group (mg/dl)</th>
<th>Inorganic phosphorus (µg/dl)</th>
<th>Zinc group (µg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 weeks old</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>10.82 ± 0.38</td>
<td>7.91 ± 0.22</td>
<td>152.5 ± 15.8</td>
</tr>
<tr>
<td>50 weeks old</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>9.25 ± 0.12</td>
<td>4.55 ± 0.19</td>
<td>127.1 ± 6.6</td>
</tr>
<tr>
<td>7 d</td>
<td>9.28 ± 0.16</td>
<td>4.57 ± 0.18</td>
<td>151.7 ± 4.6*</td>
</tr>
<tr>
<td>21 d</td>
<td>9.27 ± 0.15</td>
<td>4.58 ± 0.22</td>
<td>167.0 ± 9.2**</td>
</tr>
</tbody>
</table>

Rats (50 weeks old) were orally administered the suspension (1 ml/100 g body weight) of supplement containing aglycone isoflavone (5 mg/ml), zinc (833 µg/ml), calcium (50 mg/ml), magnesium (25 mg/ml), and vitamin D3 (0.208 µg/ml) for 7 or 21 d. Each value is the mean ± S.E.M. of six animals. * \( p < 0.05 \), compared with the control value obtained from 5-week-old rats. * \( p < 0.025 \) and ** \( p < 0.01 \), compared with the control value of aged rats.

Fig. 4. Changes in Alkaline Phosphatase Activity in the Femoral-Diaphyseal and Metaphyseal Tissues of Young Rats Orally Administered Isoflavones and Zinc-Containing Mineral Mixture

Rats were administered isoflavones as described in the legend to Fig. 1. Each value is the mean ± S.E.M. of six animals. * \( p < 0.05 \) and ** \( p < 0.01 \), compared with the control value. \# \( p < 0.01 \), compared with the value for isoflavone glycoside or aglycone isoflavone administration. □ Control; ■ isoflavone glycoside; □ aglycone isoflavone; □, zinc-containing mineral mixture; ▲ aglycone isoflavone and zinc-containing mineral mixture.

including 3.772 mg daidzein, 0.395 mg genistein, and 0.833 mg glycitein), zinc (833 µg/ml), calcium (50 mg/ml), magnesium (25 mg/ml), and vitamin D3 (0.208 µg/ml) for 7 or 21 d. Serum calcium and inorganic phosphorus concentrations in aged rats were significantly reduced as compared with those in young rats (5 weeks old) (Table 1). They were not significantly altered by the administration of aglycone isoflavone and zinc-containing mineral mixture. The serum zinc concentration decreased slightly with increasing age. This level was significantly elevated with the administration of aglycone isoflavone and zinc-containing mineral mixture (Table 1). The serum concentration of albumin, nitrogen urea, triglyceride, free cholesterol, HDL cholesterol, and 17 β-estradiol in aged rats was not significantly altered by the administration of agly-
cone isoflavone and zinc-containing mineral mixture for 7 or 21 d (data not shown). Body weight did not change in rats administered the supplements (data not shown). These results indicate that the supplemental intake had no toxic effect.

The femoral dry weight of aged rats was significantly increased with the administration of aglycone isoflavone and zinc-containing mineral mixture for 21 d (Fig. 6).

The zinc content in the femoral-diaphyseal and metaphyseal tissues of aged rats was significantly increased with the administration of aglycone isoflavone and zinc-containing mineral mixture for 7 or 21 d (Fig. 7). Calcium content, alkaline phosphatase activity, and DNA content in the femoral-diaphyseal and metaphyseal tissues of aged rats were significantly elevated with the administration of aglycone isoflavone and zinc-containing mineral mixture (Figs. 8, 9, and 10).

**DISCUSSION**

Osteoporosis is widely recognized as a major public health problem. Nutritional factors may be important in preventing bone loss with increasing age. Recently, it has been demonstrated that aglycon isoflavone (daidzein and ginistene), which is present in soybeans, has an anabolic effect on bone metabolism.5–9 Zinc, an essential trace element, has been shown to have a potent stimulatory effect on bone formation and an inhibitory effect on bone resorption,14–18 increasing bone mass. Dietary supplementation with a combination of isoflavone and zinc may be important in the prevention of bone loss with
increasing age. The present study demonstrates that the supplemental intake of isoflavone and a zinc-containing mineral mixture has a stimulatory effect on bone components in the femoral tissues of young and aged rats.

We administered isoflavone glycoside (including daidzin, genistin, and glycitin) and aglycone isoflavone (including daidzein, genistein, and glycitein) to rats. Isoflavone glycoside is hydrolyzed to aglycone isoflavone by β-glucosidase in gastric juice. The oral administration of isoflavone glycoside or aglycone isoflavone to young rats for 7 d caused a significant increase in femoral dry weight, calcium content, alkaline phosphatase activity and DNA content in the femoral-diaphyseal and metaphyseal tissues of young rats. The effect of isoflavone glycoside or aglycone isoflavone in elevating bone components was equivalent, suggesting that isoflavone glycoside is partly converted to aglycone isoflavone. Alkaline phosphatase is a marker enzyme of osteoblasts, and the enzyme participates in bone mineralization. DNA content is an index of bone growth and of a number of bone cells including osteocytes, osteoblasts, and osteoclasts in bone tissues. Presumably, aglycone isoflavone has a stimulatory effect on osteoblastic bone formation and mineralization in rats.

Zinc has been demonstrated to have a potent stimulatory effect on bone formation. Our zinc-containing mineral mixture was composed of zinc yeast, coral-derived calcium and magnesium, and vitamin D₃ which has been reported to have a stimulatory effect on intestinal calcium absorption. The administration of the zinc-containing mineral mixture had an anabolic effect on bone components in young rats, confirming its stimulatory effect on bone formation and mineralization.

The combination of aglycone isoflavone and zinc-containing mineral mixture had an additive enhancing effect on bone components in young rats. This enhanced effect may have resulted from the action of daidzein, genistein, and zinc on osteoblastic cells in the femoral tissues, since these factors are known to stimulate osteoblastic cell function. Moreover, the supplemental tablets, including aglycone isoflavone and zinc-containing mineral mixture, for 21 d caused a significant elevation of femoral dry weight, calcium content, DNA content, alkaline phosphatase activity, and DNA content in the femoral-diaphyseal and metaphyseal tissues of aged rats. Bone metabolism deteriorates with increasing age. This finding suggests that the supplemental intake of aglycone isoflavone and zinc-containing mineral mixture can prevent bone loss with increasing age. The combination of nutritional factors presumably plays a beneficial role in the prevention of osteoporosis.

In conclusion, it has been shown that the supplemental intake of isoflavone and zinc-containing mineral mixture has an anabolic effect on bone components in the femoral tissues of young and aged rats.
REFFERENCES