Effects of Traditional Chinese Medicine on Bone Loss in SAMP6: A Murine Model for Senile Osteoporosis

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We evaluated the effects of the traditional Chinese medicines, Hachimi-jio-gan, Juzen-taiho-to and Unkei-to, on bone loss in murine model of senile osteoporosis (SAMP6). Two-month-old SAMP6 were divided into control and experimental groups. The control mice had the tap water available as the only drinking fluid. The experimental mice were given 0.05% aqueous solution of Hachimi-jio-gan, Juzen-taiho-to or Unkei-to for three months. The solution intake of a mouse averaged 5 ml per day. The bones were studied morphologically and histomorphometrically, together with bone mineral density (BMD), serum parathyroid hormone (PTH) and estradiol levels. In the control group, BMD and the amount of bone forming surface were low, the serum PTH level was high when compared with the normal mice SAMR1. Many osteocytes and osteoblasts showed degenerative changes and numerous mast cells were observed in the bone marrow. Compared with controls, the serum estradiol level was higher in the Unkei-to group. However, we did not find any significant changes of bones. In the Hachimi-jio-gan and Juzen-taiho-to groups, the bone mass and the amount of bone forming surface increased. Most of the osteocytes and osteoblasts appeared normal. As compared with controls, the number of mast cells in bone marrow decreased in the Hachimi-jio-gan group. The serum PTH level had declined in the Juzen-taiho-to group. The present study provides certain evidence that Hachimi-jio-gan and Juzen-taiho-to are effective in preventing bone loss in SAMP6, while Unkei-to can only improve the ovary function.

Key words Chinese medicine; senescence-accelerated mice P6 (SAMP6); senile osteoporosis; bone; histomorphometry; ultrastructure

In healthy adults, the amount of bone formation approximately balances the amount of bone resorption. In aged individuals, the balance shifts to favor bone resorption, which can result in debilitating diseases such as osteoporosis. Osteoporosis is a skeletal disorder characterized by compromised bone strength, and predisposes a person to an increased risk of fracture. Bone strength primarily reflects the integration of bone density and bone quality.1)

Osteoporosis is regarded as a heterogeneous disorder with multiple causes. It is roughly divided into two distinct types. Type I, known as postmenopausal osteoporosis, occurs in women after menopause. Type II, senile osteoporosis, occurs in both old men and women. Ovariectomy has been extensively used as an experimental model for postmenopausal osteoporosis, while senescence-accelerated mice P6 (SAMP6) can be used as a senile osteoporosis model.2) These mice exhibit a significant decrease in bone mineral density (BMD) and bone formation. It was demonstrated by using light microscopy and histomorphometric method that both osteclastogenesis and osteoblastogenesis decreased in SAMP6.3) Recently, we found that the osteoblasts showed degenerative changes and the number of osteoblasts decreased, while the mast cells in bone marrows significantly increased in SAMP6 at 2 and 5 months of age. These findings indicated that the lower bone mass in SAMP6 was mainly due to the reduction of osteoblast formation.4) However, the pathogenesis of senile osteoporosis has not fully been elucidated.

Despite numerous investigations, the treatment and prevention of osteoporosis remain unsolved problems. Several agents such as estrogens and selective estrogen receptor modulators, bisphosphonates, calcitonin (CT) and parathyroid hormone (PTH) have been used clinically in the treatment of the disease. Traditional Chinese medicine has developed its own unique system during a period of 3000 years and has recently been reevaluated in the clinical field.5) Chinese medicines are known to have low toxicity and are suitable for long-term administration compared to chemically synthesized medicines. Several Chinese medicines are claimed to be effective in treatment of osteoporosis. By measuring BMD and serum hormone levels, Sassa et al. found that Hochu-ekki-to could elevate the serum level of ovarian hormone and prevent the bone loss in ovariectomized animals.6) Berberine, an isoquinoline alkaloid, could inhibit BMD decrease in both male and female SAMP6.7) It was morphologically demonstrated that Hachimi-jio-gan, Juzen-taiho-to and Unkei-to were effective in preventing bone loss induced by ovariectomy in rats.8) However, no data are available as to the recovery of bone loss in SAMP6 by any of these Chinese medicines. In order to evaluate the effects of traditional Chinese medicine on senile osteoporosis, we examined whether Hachimi-jio-gan, Juzen-taiho-to or Unkei-to could prevent the bone loss in SAMP6.

MATERIALS AND METHODS

Traditional Chinese Medicines Dried extract powder of the traditional Chinese medicine, Hachimi-jio-gan, Juzen-taiho-to and Unkei-to was supplied by Tsumura & Co. (Tokyo, Japan). The herbal constituents and contents are shown in Table 1.

Animals and Treatments SAMP6/Ta mice were kindly donated by the Council for SAM Research, Kyoto, Japan. Two-month-old female SAMP6 with an average body weight of 29.2 g were randomly divided into 4 groups of 15 animals each. The control group had tap water available. The other three experimental groups were given 0.05% aqueous solu-
tion of Hachimi-jio-gan, Juzen-taiho-to or Unkei-to as the only drinking fluid for 3 months. The daily solution intake of mouse averaged 5.0 ml. Fifteen SAMR1/Ta (normal mice) were used for comparison. All animals were maintained under conventional conditions and had free access to commercial diet (CE-2, CLEA Japan).

Serum Analysis and Bone Mineral Density Measurement Three months later, the blood was taken from the heart under ether anesthesia. The serum calcium (Ca) and phosphorus (P) levels were determined by standard colorimetric methods as described previously. The serum PTH, CT phosphorus (P) levels were determined by standard colorimetric methods described previously. The serum PTH level was lower in the Juzen-taiho-to group (Table 2). We did not find any significant differences between control and experimental groups with regard to the body weight (Table 2). As compared with controls, the BMD of the whole body was higher, the serum Ca and P levels were lower in the Hachimi-jio-gan and Juzen-taiho-to groups (Table 2). The serum PTH level was lower in the Juzen-taiho-to group when compared with controls. As compared with controls, the serum estradiol level was higher in the Unkei-to group when compared with controls. As compared with controls, the serum estradiol level was higher in the Unkei-to group when compared with controls. As compared with controls, the serum estradiol level was higher in the Unkei-to group when compared with controls.

RESULTS

We did not find any significant differences between the control and experimental groups with regard to the body weight (Table 2). As compared with controls, the BMD of the whole body was higher, the serum Ca and P levels were lower in the Hachimi-jio-gan and Juzen-taiho-to groups (Table 2). The serum PTH level was lower in the Juzen-taiho-to group when compared with controls. As compared with controls, the serum estradiol level was higher in the Unkei-to group (Table 2). We did not find any significant differences between control and experimental groups with regard to the serum CT level (Table 2).

The femoral dry weight, femoral Ca and P levels in the Hachimi-jio-gan and Juzen-taiho-to groups were higher than those of controls (Table 3). There was no significant difference between control and experimental groups with regard to the femoral length.

The SEM images of the vertebral body of the third lumbar vertebra showed that the amount of the trabecular bone in the Hachimi-jio-gan and Juzen-taiho-to groups was greater than that of controls and the Unkei-to group (Fig. 1, Table 3). The same results were obtained with the distal metaphysis of the femur. The trabecula of controls and the Unkei-to group were thinner and discontinuous, while those of the Hachimi-jio-gan and Juzen-taiho-to groups were thicker and continuous. In the SEM images of the endosteal surface of the femoral bone, the trabecula of controls and the Unkei-to group were thinner and discontinuous, while those of the Hachimi-jio-gan and Juzen-taiho-to groups were thicker and continuous.
Table 2. The Body Weight, BMD, Serum Ca, P, PTH, CT and Estradiol Levels

<table>
<thead>
<tr>
<th>Groups</th>
<th>Body weight (g)</th>
<th>BMD (mg/cm²)</th>
<th>Serum Ca (mg/100 ml)</th>
<th>Serum P (mg/100 ml)</th>
<th>Serum PTH (pg/ml)</th>
<th>Serum CT (pg/ml)</th>
<th>Serum estradiol (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAMR1</td>
<td>31.4±5.6</td>
<td>69.2±3.1</td>
<td>9.32±0.30</td>
<td>7.28±0.26</td>
<td>15.0±5.9</td>
<td>52.6±10.9</td>
<td>65.2±12.4</td>
</tr>
<tr>
<td>Control</td>
<td>34.1±6.3</td>
<td>59.8±5.5</td>
<td>9.60±0.22</td>
<td>8.24±0.36</td>
<td>38.4±9.6</td>
<td>53.2±12.1</td>
<td>35.2±5.3</td>
</tr>
<tr>
<td>Hachimi-jio-gan</td>
<td>33.9±7.9</td>
<td>61.5±2.6</td>
<td>9.55±0.36</td>
<td>8.12±0.37</td>
<td>36.3±10.2</td>
<td>51.6±12.8</td>
<td>36.7±7.1</td>
</tr>
<tr>
<td>Juzen-taiho-to</td>
<td>34.8±6.8</td>
<td>61.7±3.3</td>
<td>9.54±0.29</td>
<td>8.09±0.31</td>
<td>30.2±11.4</td>
<td>55.0±10.4</td>
<td>29.5±4.6</td>
</tr>
<tr>
<td>Unkei-to</td>
<td>32.2±7.7</td>
<td>59.6±3.7</td>
<td>9.62±0.38</td>
<td>8.25±0.41</td>
<td>37.4±10.8</td>
<td>50.8±12.7</td>
<td>52.4±8.2</td>
</tr>
</tbody>
</table>

Values are shown as mean±S.E.M.  a) p<0.05 vs. SAMR1, b) p<0.05 vs. control.

Table 3. The Femoral Weight, Length, Femoral Ca, P Levels and Trabecular Bone Volume

<table>
<thead>
<tr>
<th>Groups</th>
<th>Femoral weight (mg)</th>
<th>Femoral length (mm)</th>
<th>Femoral Ca (mg/g)</th>
<th>Femoral P (mg/g)</th>
<th>BV/TV of femur (%)</th>
<th>BV/TV of lumbar vertebra (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAMR1</td>
<td>40.5±2.4</td>
<td>16.93±0.32</td>
<td>228.3±26.5</td>
<td>119.8±17.2</td>
<td>23.5±2.6</td>
<td>13.8±2.3</td>
</tr>
<tr>
<td>Control</td>
<td>37.9±1.7</td>
<td>16.96±0.33</td>
<td>215.2±31.3</td>
<td>109.5±8.7</td>
<td>18.7±2.9</td>
<td>10.4±1.7</td>
</tr>
<tr>
<td>Hachimi-jio-gan</td>
<td>38.7±2.2</td>
<td>17.02±0.37</td>
<td>221.6±29.4</td>
<td>112.6±15.6</td>
<td>20.8±2.7</td>
<td>12.6±2.2</td>
</tr>
<tr>
<td>Juzen-taiho-to</td>
<td>38.8±2.5</td>
<td>17.00±0.30</td>
<td>222.4±28.8</td>
<td>112.9±16.1</td>
<td>20.9±3.0</td>
<td>13.1±1.7</td>
</tr>
<tr>
<td>Unkei-to</td>
<td>37.9±2.5</td>
<td>16.99±0.35</td>
<td>216.0±25.9</td>
<td>109.8±10.7</td>
<td>18.8±2.8</td>
<td>10.8±1.9</td>
</tr>
</tbody>
</table>

Values are shown as mean±S.E.M.  a) p<0.05 vs. SAMR1, b) p<0.05 vs. control.

Table 4. Bone Surface Types on Endosteal Surface of the Femoral Diaphysis

<table>
<thead>
<tr>
<th>Groups</th>
<th>Forming (%)</th>
<th>Resting (%)</th>
<th>Resorbing (%)</th>
<th>Forming/Resorbing</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAMR1</td>
<td>12.3±2.5</td>
<td>66.5±12.7</td>
<td>21.2±6.9</td>
<td>0.58±0.07</td>
</tr>
<tr>
<td>Control</td>
<td>5.7±2.4</td>
<td>79.1±24.3</td>
<td>15.2±7.4</td>
<td>0.38±0.06</td>
</tr>
<tr>
<td>Hachimi-jio-gan</td>
<td>9.6±3.1</td>
<td>71.3±19.6</td>
<td>19.1±5.8</td>
<td>0.50±0.08</td>
</tr>
<tr>
<td>Juzen-taiho-to</td>
<td>9.8±3.4</td>
<td>70.6±18.4</td>
<td>19.6±4.7</td>
<td>0.50±0.08</td>
</tr>
<tr>
<td>Unkei-to</td>
<td>6.0±2.9</td>
<td>77.2±22.6</td>
<td>16.8±3.9</td>
<td>0.36±0.07</td>
</tr>
</tbody>
</table>

Values are shown in mean±S.E.M.  a) p<0.05 vs. SAMR1, b) p<0.05 vs. control.

diaphysis, we found that the amount of the forming surface and the ratio of forming/resorbing surfaces in the Hachimi-jio-gan and Juzen-taiho-to groups were much higher than those of controls (Table 4).

The trabecular bone of the proximal tibia was observed with LM and TEM. Osteoclasts, osteoblast and osteoclasts of the tibia are easily identified in Azur II-stained section. The osteoblast number of the trabecular bone in the Hachimi-jio-gan and Juzen-taiho-to groups was higher than that of controls (Table 5).

Ultrastructurally, we found that the osteoblasts had abundant cisternae of granular endoplasmic reticulum and prominent well-developed Golgi complexes. Mitochondria demonstrated pronounced vacuolization and reticulum and prominent well-developed Golgi complexes. The amount of the trabecular bone in the Hachimi-jio-gan and Juzen-taiho-to mice is greater than that of the control and Unkei-to mice. Scale bar: 0.5 mm.

Fig. 1. Scanning Electron Micrographs of the Vertebral Body in the Third Lumbar Vertebra of the Mouse

A: control group; B: Hachimi-jio-gan group; C: Juzen-taiho-to group; D: Unkei-to group. The amount of trabecular bone in the Hachimi-jio-gan and Juzen-taiho-to mice is greater than that of the control and Unkei-to mice. Scale bar: 0.5 mm.

Table 5. The Number of Trabecular Osteoblasts, Osteoclasts and Bone Marrow Mast Cells

<table>
<thead>
<tr>
<th>Groups</th>
<th>Number of osteoblasts (per mm²)</th>
<th>Number of osteoclasts (per mm²)</th>
<th>Number of mast cells (per mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAMR1</td>
<td>45.1±16.3</td>
<td>1.32±0.58</td>
<td>2.6±0.3</td>
</tr>
<tr>
<td>Control</td>
<td>27.7±8.9</td>
<td>1.19±0.49</td>
<td>13.1±2.8</td>
</tr>
<tr>
<td>Hachimi-jio-gan</td>
<td>35.3±9.8</td>
<td>1.36±0.63</td>
<td>3.5±0.4</td>
</tr>
<tr>
<td>Juzen-taiho-to</td>
<td>36.0±7.9</td>
<td>1.24±0.58</td>
<td>11.9±2.5</td>
</tr>
<tr>
<td>Unkei-to</td>
<td>28.4±6.7</td>
<td>1.30±0.70</td>
<td>15.0±3.1</td>
</tr>
</tbody>
</table>

Values are shown in mean±S.E.M.  a) p<0.05 vs. SAMR1, b) p<0.05 vs. control.
to the reduction in the number and function of osteoblasts. SAMP6 mainly arise from the decreased bone formation due to osteoclasts in SAMP6. We consider that the bone loss in any significant changes in the number and morphology of osteoblasts and osteocytes was similar among all four groups.

The bone marrow of the proximal tibia was observed with LM and TEM. Many mast cells were found in the bone marrow of controls, Juzen-taiho-to and Unkei-to groups. Some mast cells were in close proximity to the bone surface and degranulating. The mast cells of the bone marrow exhibited a striking decrease in the Hachimi-jio-gan group (Table 5). A few mast cells were found near the bone surface in the Hachimi-jio-gan group.

DISCUSSION

SAMP6 was established to be the murine model of senile osteoporosis. These mice exhibited a decrease in bone volume and bone formation early in life when compared with their normal controls, SAMR1. The number of osteoblast per unit trabecular bone in SAMP6 significantly decreased, and this decline was accompanied by a reduction in the area of bone forming surface and trabecular bone volume, and ultrastructural changes of osteoblasts. However, we did not find any significant changes in the number and morphology of osteoclasts in SAMP6. We consider that the bone loss in SAMP6 mainly arise from the decreased bone formation due to the reduction in the number and function of osteoblasts.

Hachimi-jio-gan, the representative for compensating the function of kidney, was first described in ancient Chinese medicine book about 1800 years ago. Kidney in Chinese medicine refers to the urinary and reproductive organs, a part of endocrine and nervous systems in modern medicine. Kidney also has the ability to govern the function of bones. Previous study has shown that Hachimi-jio-gan is effective against auto-antibody production and nephritis in senescence accelerated mice (SAM-P1). Theoretically, Hachimi-jio-gan can be used for preventing and treating bone diseases. It was reported that the administration of Hachimi-jio-gan to ovariectomized rats for seven weeks was as effective as 17β-estradiol in preventing osteoporosis.

Juzen-taiho-to was formulated in Chinese Song Dynasty in AD 1200. It is prepared by extracting a mixture of ten medical herbs that tone the blood and vital energy, and strengthen health and immunity. This potent and popular prescription has traditionally been used against anemia, anorexia, extreme exhaustion, fatigue, kidney and spleen insufficiency and general weakness, particularly after illness. Juzen-taiho-to can restore immunity in cancer patients, potentiate the therapeutic activity in chemotherapy and radiotherapy, inhibit the recurrence of malignancies, prolong survival, as well as ameliorate and/or prevent adverse toxicities of many anticancer drugs.

Unkei-to is manufactured from 12 medicinal herbs. Unkei-to is generally used to treat women who are suffering from irregular menstruation, sterility, and climacteric disturbances. Animal studies showed that Unkei-to acts on the endocrine system such as the hypothalamus-pituitary-ovarian axis. Unkei-to has direct stimulatory effects on human granulosa cells to stimulate the steroidogenesis and secretion of cytokines. The various beneficial actions of Unkei-to on the ovary may result from a combination of different ingredient herbs with different stimulatory effects on both steroidogenesis and the ovolatory process within the ovary, as well as stimulatory effect on the hypothalamus-pituitary axis.

The present study demonstrated that long-term treatment with Hachimi-jio-gan or Juzen-taiho-to to SAMP6 for 3 months stimulated the bone formation and restored the amount of bone volume. The functional morphology of the osteoblasts and osteocytes was improved. We consider that Hachimi-jio-gan and Juzen-taiho-to are effective in preventing the development of bone loss in murine model of senile osteoporosis.

One of the main components for Hachimi-jio-gan and Juzen-taiho-to is rehmanniae radix. Several studies were undertaken concerning the pharmacological function of rehmanniae radix. It was reported that rehmanniae radix could prevent an inducement of impediment in the peripheral microcirculation of various chronic diseases through the improvement of hemorheology. Rehmanniae radix could inhibit systemic allergic reaction and had beneficial effects on the regulation of immediate type allergic reaction. It also had an anti-inflammatory activity in the central nervous system. Hoelen, another component of Hachimi-jio-gan and Juzen-taiho-to, had anti-aging impact, prolonged the life span of SAM, prevented body weight decrease with aging and tended to improve the senile syndrome. However, there
have been no reports on the treatment or prevention of senile osteoporosis by these herbal medicines.

It was reported that Unkei-to could prevent the development of osteoporosis induced by ovariectomy in rats. In the present study, we did not find any significant changes of blood parameters, though the serum estradiol level was higher after administration with Unkei-to. Ovariectomy causes significant decrease of serum estradiol levels, leading to osteoporotic bone loss by stimulating the bone resorption. On the contrary, SAMP6 showed a significant reduction of osteoblast formation. So the changes of the bone metabolism in ovariectomized rat and SAMP6 are quite different. However, it is not clear why Unkei-to has no effect on bone morphology in SAMP6.

Hormones such as PTH and CT are important in regulating Ca metabolism and bone remodeling. Whether the Chinese medicines affect these hormones is not clear. CT is a known inhibitor of the bone resorption. CT deficiency has been implicated in the pathogenesis of accelerated bone loss. Multiple studies have confirmed that CT has hypocalcemic effect, which is mediated by inhibiting the bone resorption. In the present study, we did not find any significant changes of the serum CT levels in SAMP6 control and experimental animals. We consider that the functional activity of the thyroid C-cells may not be affected by Hachimi-jio-gan, Juzen-taiho-to and Unkei-to.

PTH is an 84 amino acid polypeptide hormone. The overall effect of PTH is to raise plasma Ca level, partly through the bone resorption. Senile osteoporosis is thought to be caused by age-related factors, such as decrease in osteoblast function and Ca absorption, and a secondary increase in the circulating level of PTH owing to decreased Ca absorption. The increased PTH, by binding to its receptor on bones, stimulates the osteclast formation and the bone resorption, inducing to osteoporosis. Recently we found that the serum PTH level in SAMP6 was more than twice as high as in SAMR1 at 2 and 5 months of age. The serum Ca and P levels were higher, the femoral Ca and P levels were lower in SAMP6. Accordingly, we consider that the bone loss in SAMP6 is partially due to stimulated activity of the parathyroid gland. In the present study, we found that the serum Ca, P and PTH levels were lower, the femoral Ca and P levels were higher in the Juzen-taiho-to group than those of controls. We speculate that Juzen-taiho-to have an effect on the parathyroid function.

A number of factors produced in the bone marrow microenvironment are capable of influencing the development, differentiation and function of osteoblasts and osteoclasts. Alternations of cytokines in bone marrow might cause the disturbances in the differentiation of bone marrow stromal cells to osteoblasts, resulting in bone loss. There is evidence that mast cells of the bone marrow release biological mediators, such as heparin, histamine, IL-1, IL-3, IL-6 and TNF, potentially involved in bone metabolism. An increase in the number of mast cells has been described in the bone marrow of elderly women with osteoporosis. It has been suggested that mast cells in the bone marrow may be involved in the events leading to bone loss by changing bone remodeling activity. Recently, we found that the number of mast cells in the bone marrow of SAMP6 at 2 and 5 months of age dramatically increased and that some of them were degranulating, i.e., they were in an activated state. Moreover, some mast cells were in close proximity to the bone surface. We consider that mast cells in the bone marrow provide a paracrine mechanism which regulates the recruitment and function of osteoblast progenitors. In the present study, we found that the number of mast cells in the bone marrow dramatically decreased after treatment with Hachimi-jio-gan. Accordingly, it is suggested that Hachimi-jio-gan affect the metabolism of mast cells in the bone marrow.

In conclusion, this initial study provides certain evidence that the traditional Chinese medicine, Hachimi-jio-gan and Juzen-taiho-to are effective in preventing the development of bone loss in SAMP6, while Unkei-to can only improve ovary function.

REFERENCES