Effects of Hachimi-jio-gan Extract on Intestinal Absorption of Calcium in Ovariectomized Mice and Stimulation of RANKL-Induced Osteoclast Differentiation of RAW264.7 Cells by Lipopolysaccharide

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(Accepted for publication, August 3, 2012)

Abstract: Osteoporosis is a common metabolic bone disease often affecting postmenopausal women, and various medicines are available that attempt to treat and/or prevent this disease. Hachimi-jio-gan is a traditional oriental (Kampo) medicine used clinically, but its mode of action is still unknown. In this study, the effects of Hachimi-jio-gan extract on intestinal calcium absorption were evaluated in an osteoporosis model using ovariectomized (OVX) and sham-operated (SHAM) mice by investigating pharmacokinetic parameters in these animals. The ability of Hachimi-jio-gan extract to inhibit lipopolysaccharide-mediated stimulation of osteoclasts in bones using receptor activator of the NF-κB ligand (RANKL)-induced osteoclast differentiation of RAW264.7 cells was also investigated. The metabolic behavior of calcium did not differ between OVX and SHAM mice as the pharmacokinetics of calcium was equivalent after intravenous administration. The absolute bioavailability of calcium indicated that the extent of intestinal calcium absorption in the OVX (10.3%) and SHAM mice was at similar low levels. Hachimi-jio-gan extract potentially improved the intestinal calcium absorption by 1.96- and 1.86-fold, respectively. Hachimi-jio-gan extract further suppressed the potent stimulation of RANKL-induced osteoclast differentiation in RAW264.7 cells. These results suggest that Hachimi-jio-gan extract may be suitable for treatment and prevention of osteoporosis through its ability to increase intestinal calcium absorption and suppress osteoclast differentiation.

Key words: OVX mice, Absolute calcium bioavailability, Pharmacokinetics, Lipopolysaccharide, Hachimi-jio-gan extract, RAW264.7

Introduction

Calcium is an essential element in the body, and over 99% of total body calcium is found in the extracellular matrix as a complex of calcium and phosphate in the form of hydroxyapatite \( \text{Ca}_{10}\left(\text{PO}_4\right)_6(\text{OH})_2 \). The remaining calcium is seen in body fluids, and acts as a second messenger element in cells. Calcium homeostasis is maintained by balancing calcium intake from the diet with the distribution of calcium in the body, i.e. bones and extra- and intra-cellular calcium stores. It has been shown that calcium deficit causes various systemic diseases, including osteoporosis, hypertension, hypercholesterolemia, and cancer. Bone contains three types of cells—osteoblasts, osteocytes, and osteoclasts—as well as the extracellular matrix. The growth and differentiation of bones are achieved by close regulation of complex signaling pathways including the action of vitamin D, growth hormone, steroids, and calcitonin, as well as several cytokines. Thus, to maintain healthy bones, total body calcium requires adequate calcium intake from the digestive tract. An imbalance of calcium may result in osteoporosis and osteopenia.

Osteoporosis is a common metabolic bone disease especially often affecting postmenopausal women. Hormonal replacement therapy (HRT) is one choice for the prevention and treatment of postmenopausal osteoporosis. Unfortunately, because of the many side effects of HRT as well as cultural differences, acceptance of HRT is low, especially in Asian women. Nonhormonal therapy or alternative therapies involving medicines and calcium supplements may be more acceptable for the treatment and prevention of osteoporosis in these communities. Calcium supplements may be a way to make good the deficiency of calcium from dietary sources, but it is impossible to treat osteoporosis with only hormonal supplements such as HRT.

Traditional oriental (Kampo) medicines have been clinically used as nonhormonal therapy, and are safe and efficacious for the protection of bone loss or for increasing intestinal calcium absorption.
absorption. O’Loughlin and Morris showed that the impairment of intestinal calcium absorption following ovariectomy is not a result of decreased circulating 1,25-dihydroxyvitamin D or intestinal responsiveness to 1,25-dihydroxyvitamin D, but results from estradiol stimulating intestinal calcium absorption probably by a direct effect on the intestine. Lee et al. showed that the effectiveness of Chia-wei-hsiao-ya-yan on repairing ovarian function apparently correlates to the efficacy in curing osteoporosis. It is believed that Hachimi-jio-gan enhances physical activity and promotes renal function. Furthermore Hachimi-jio-gan has been clinically used in treating gynecological diseases and postmenopausal symptoms, such as severe hot flushes, insomnia, muscle aches and pains, formation, fatigue, palpitations and mood swings. Additionally, Hidaka et al. showed that Unkei-to, Hachimi-jio-gan, and Juzen-taiho-to are as effective as 17β-estradiol in preventing the development of bone loss in OVX rats, and Chen et al. indicated that Hachimi-jio-gan and Juzen-taiho-to are effective in preventing bone loss in SAMP6 mice, a murine model of senile osteoporosis.

An alternative way of treating osteoporosis is to inhibit the differentiation of the osteoclastic precursor to the active osteoclast. Osteoclast formation is mediated by a receptor activator of the NF-κB ligand (RANKL) in the innate immune system. RANKL stimulates the mouse macrophage cell line RAW264.7 to differentiate into osteoclasts. Rahaman et al. showed that conjugated linoleic acid inhibits osteoclast differentiation of RAW264.7 cells by modulating RANKL signaling, in which RANK is mediated by TRAF6 and activated by NF-κB. An alternative way of treating osteoporosis is to inhibit the differentiation of RANKL from Sigma Chemical Co. (St. Louis, MO, USA). Other reagents were purchased from commercial sources at the highest grade available.

Materials and Methods

Chemicals
Hachimi-jio-gan extract (granules, #1007) was obtained from Tsumura & Co. (Tokyo, Japan). Calcium chloride, potassium iodide, sodium nitrate, calcium diagnostic kits (code 437-58201) and LPS from Escherichia coli were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). RPMI 1640 and α-MEM media, TRAP solution (No. 387), fetal calf serum (FCS) and RANKL were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Other reagents were purchased from commercial sources at the highest grade available.

Animals
Seven-week-old female ddY mice, weighing 20-30 g, were obtained from SLC Co., Ltd. (Shizuoka, Japan). Mice were housed in a controlled environment at 22°C with a 12-hour light/dark cycle and had free access to food (commercial diet, MF pellets, Oriental Animal Foods Co., Osaka, Japan) and water during the experimental period. Mice were randomly divided into two groups of sham-operated (SHAM) and OVX mice. After the mice were anesthetized by ether, SHAM mice were bilaterally non-ovariectomized, but the OVX group was bilaterally ovariecetomized as previously described. The mice had free access to food and water during the conditioning period of 4 weeks. All protocols conformed to the Guide for Institutional Animal Care and Use of Tokushima Bunri University, Tokushima, Japan.

Biochemical assays
Blood samples were centrifuged at 2,000 rpm for 10 min at 4°C and the serum was stored at 5°C for measurements. Serum elements were measured using the Point-of-Care Chemistry Analyzer (VetScan®, Abaxis Inc., Union City, CA) using a sample of 90 µl. The serum level of the cross-linked N-terminal telopeptide of type I collagen (NTx) was measured in duplicate using the OSTEOMARK® NTx Serum kit (Wampole Laboratories Inc., Cranbury, NJ). The bones of the hind foot were stored at -20°C for measurement of bone mass. The length and weight of each bone was measured and divided into three segments (proximal femur, femoral body and distal femur) in a length ratio of 1:2:1. The bone density was measured using the crystal density method by performing sink/float measurements in a dense aqueous solution of potassium iodide at 25°C. The density of the aqueous solution was measured using a pycnometer.

Osteoclastogenesis of RAW264.7 cells
The murine monocytic cell line RAW264.7 (ATCC, Rockville, MD) was cultured as described previously. In brief, cells were maintained in RPMI 1640 medium containing 10% (v/v) heat-inactivated FCS with 100 units/ml penicillin and 100 µg/mL streptomycin, and seeded at a density of 5×10⁴ cells/well on a 96-well tissue culture plate. This medium was replaced with α-MEM medium containing 10% FCS, 2 mM glutamine and 50 ng/mL RANKL. Furthermore, several concentrations of LPS (0, 0.1, 1, 10, 100 ng/mL) were added to the cultures. The cells were incubated for 24 hours and harvested for TRAP staining.
10 µg/mL) were added to the cultures including Hachimi-jio-gan extract solution (25 µg/mL). After 5 days of culture, the level of nitric oxide (NO) in the culture supernatant was assessed using the Griess-Romijn nitrite reagent. The cell monolayer was fixed with 1% paraformaldehyde, and treated with 0.2% Triton X-100 in PBS for 5 min. Finally, the fixed cells were stained with TRAP solution in the presence of 50 mM sodium tartrate and 90 mM sodium acetate (pH 5.0). The counterstaining for nuclei was performed with methyl green. The TRAP-positive cells with more than three nuclei were counted as TRAP-positive cells on a light microscope.

**Pharmacokinetic analysis of calcium in mice**

SHAM and OVX mice were fasted for 18-20 h prior to administration of calcium but had free access to water. Calcium solution (1% w/v) was prepared from calcium chloride, and the calcium solution (30 mg/kg of body weight) was intravenously administered to the tail vein under weak ether anesthesia. Blood samples were collected from the orbital sinus at 0, 5, 15, 30, 45, 60, 90, 105 and 120 min. Oral administration of the calcium solution (150 mg/kg of body weight) was delivered to the duodenum in mice given Sho-saiko-to extract solution using a stainless steel incubation needle and a 1.0-ml syringe under weak ether anesthesia, and blood samples were collected. Sho-saiko-to extract solution (4 mg/mL, dissolved in sterile purified water) was given orally at a dose of 40 mg/kg.

The pharmacokinetic profile for intestinal absorption, distribution, metabolism and elimination of calcium, the area under the calcium concentration in the blood-time curve (AUC<sub>iv</sub>), mean residence time (MRT<sub>iv</sub>), apparent volume of distribution (V<sub>dss</sub>) at steady-state after i.v. administration, AUC<sub>oral</sub>, C<sub>max</sub> and MRT<sub>oral</sub> after oral administration, were calculated using the time course of serum calcium concentrations by an iterative non-linear least-squares method using the MOMENT software program, described previously.

<table>
<thead>
<tr>
<th>Organ</th>
<th>ALT (U/L)</th>
<th>TBIL (mg/dL)</th>
<th>TP (g/dL)</th>
<th>ALB (g/dL)</th>
<th>GLOB (g/dL)</th>
<th>ALP (U/L)</th>
<th>BUN (mg/dL)</th>
<th>CRE (mg/dL)</th>
<th>AMY (U/L)</th>
<th>Ca&lt;sup&gt;2+&lt;/sup&gt; (mg/dL)</th>
<th>PHOS (mg/dL)</th>
<th>Na&lt;sup&gt;+&lt;/sup&gt; (mg/dL)</th>
<th>K&lt;sup&gt;+&lt;/sup&gt; (mmol/L)</th>
<th>NTx</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>24.6 ± 5.8</td>
<td>0.314 ± 0.038</td>
<td>5.23 ± 0.15</td>
<td>3.17 ± 0.09</td>
<td>2.04 ± 0.11</td>
<td>86.0 ± 17.4</td>
<td>17.3 ± 4.2</td>
<td>0.229 ± 0.049</td>
<td>841.3 ± 86.8</td>
<td>9.14 ± 0.29</td>
<td>8.21 ± 2.25</td>
<td>143.3 ± 2.9</td>
<td>4.56 ± 1.15</td>
<td>50.2 ± 16.8</td>
</tr>
<tr>
<td>Liver</td>
<td>27.6 ± 6.8</td>
<td>0.337 ± 0.052</td>
<td>5.55 ± 0.32</td>
<td>3.31 ± 0.33</td>
<td>2.24 ± 0.14</td>
<td>110.2 ± 25.5</td>
<td>22.6 ± 9.0</td>
<td>0.300 ± 0.107</td>
<td>884.7 ± 31.6</td>
<td>9.37 ± 0.34</td>
<td>8.69 ± 1.04</td>
<td>145.2 ± 4.6</td>
<td>5.09 ± 0.85</td>
<td>90.0 ± 13.1</td>
</tr>
<tr>
<td>Liver, bone, others</td>
<td>12.2</td>
<td>7.3</td>
<td>6.1</td>
<td>4.4</td>
<td>9.8</td>
<td>28.1</td>
<td>30.6</td>
<td>31.0</td>
<td>5.2</td>
<td>2.5</td>
<td>5.8</td>
<td>1.3</td>
<td>11.6</td>
<td>79.3</td>
</tr>
</tbody>
</table>

ALT = alanine lactic transaminase, TBIL = total bilirubin, TP = total protein, ALB = albumin, GLOB = globulin, ALP = alkaline phosphatase, BUN = blood urea nitrogen, CRE = creatinine, AMY = amylase, PHOS = phosphate, NTx is shown in BCE/L. § = proximal femur of the hind feet, ¶ = femoral body, and † = distal femur.

Table 1. Mean bone and blood parameters from SHAM and OVX mice

- **Bone**

<table>
<thead>
<tr>
<th>(a) Bone</th>
<th>SHAM</th>
<th>OVX</th>
<th>Ratio (%)&lt;sup&gt;*&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (mg)</td>
<td>63.4 ± 5.4</td>
<td>59.2 ± 3.9</td>
<td>6.6</td>
</tr>
<tr>
<td>Length (mm)</td>
<td>15.03 ± 0.74</td>
<td>15.03 ± 0.78</td>
<td>0.0</td>
</tr>
<tr>
<td>Density (g/cm&lt;sup&gt;3&lt;/sup&gt;) §</td>
<td>1.537 ± 0.046</td>
<td>1.438 ± 0.057</td>
<td>-6.4</td>
</tr>
<tr>
<td>¶</td>
<td>1.432 ± 0.081</td>
<td>1.384 ± 0.052</td>
<td>-3.4</td>
</tr>
<tr>
<td>†</td>
<td>1.259 ± 0.049</td>
<td>1.139 ± 0.046</td>
<td>-9.5</td>
</tr>
</tbody>
</table>

- **Blood**

<table>
<thead>
<tr>
<th>(b) Blood</th>
<th>SHAM</th>
<th>OVX</th>
<th>Ratio (%)&lt;sup&gt;*&lt;/sup&gt;</th>
<th>Organ</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT (U/L)</td>
<td>24.6 ± 5.8</td>
<td>27.6 ± 6.8</td>
<td>12.2</td>
<td>Liver</td>
</tr>
<tr>
<td>TBIL (mg/dL)</td>
<td>0.314 ± 0.038</td>
<td>0.337 ± 0.052</td>
<td>7.3</td>
<td>Liver</td>
</tr>
<tr>
<td>TP (g/dL)</td>
<td>5.23 ± 0.15</td>
<td>5.55 ± 0.32</td>
<td>6.1</td>
<td>Liver</td>
</tr>
<tr>
<td>ALB (g/dL)</td>
<td>3.17 ± 0.09</td>
<td>3.31 ± 0.33</td>
<td>4.4</td>
<td>Liver</td>
</tr>
<tr>
<td>GLOB (g/dL)</td>
<td>2.04 ± 0.11</td>
<td>2.24 ± 0.14</td>
<td>9.8</td>
<td>Liver</td>
</tr>
<tr>
<td>ALP (U/L)</td>
<td>86.0 ± 17.4</td>
<td>110.2 ± 25.5</td>
<td>28.1</td>
<td>Liver, bone, others</td>
</tr>
<tr>
<td>BUN (mg/dL)</td>
<td>17.3 ± 4.2</td>
<td>22.6 ± 9.0</td>
<td>30.6</td>
<td>Kidney</td>
</tr>
<tr>
<td>CRE (mg/dL)</td>
<td>0.229 ± 0.049</td>
<td>0.300 ± 0.107</td>
<td>31.0</td>
<td>Kidney</td>
</tr>
<tr>
<td>AMY (U/L)</td>
<td>841.3 ± 86.8</td>
<td>884.7 ± 31.6</td>
<td>5.2</td>
<td>Pancreas</td>
</tr>
<tr>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt; (mg/dL)</td>
<td>9.14 ± 0.29</td>
<td>9.37 ± 0.34</td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td>PHOS (mg/dL)</td>
<td>8.21 ± 2.25</td>
<td>8.69 ± 1.04</td>
<td>5.8</td>
<td></td>
</tr>
<tr>
<td>Na&lt;sup&gt;+&lt;/sup&gt; (mg/dL)</td>
<td>143.3 ± 2.9</td>
<td>145.2 ± 4.6</td>
<td>1.3</td>
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</tr>
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<td>K&lt;sup&gt;+&lt;/sup&gt; (mmol/L)</td>
<td>4.56 ± 1.15</td>
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<td></td>
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<tr>
<td>NTx</td>
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<td>79.3</td>
<td></td>
</tr>
</tbody>
</table>
moment theory for the increment of plasma concentration-time curves after administration of drug. The absolute bioavailability (\( F_\text{A} \)) was defined as \( \frac{\text{AUC}_{\text{oral}}}{\text{D}_{\text{oral}}} \) and was normalized with \( \text{AUC}_{\text{iv}} \) after an i.v. dose of \( D_{\text{iv}} \) as follows:

\[
F_\text{A} = \frac{\text{AUC}_{\text{oral}}/\text{D}_{\text{oral}}}{\text{AUC}_{\text{iv}}/\text{D}_{\text{iv}}}.
\]

When MRT\(_{\text{oral}}\) in all compartments was calculated after oral administration, the elimination kinetic constant (\( k_{\text{el}} \)) and the intestinal absorption kinetic constant (\( k_{\text{ab}} \)) in the compartment model were calculated by \( \text{MRT}_{\text{iv}} = \frac{1}{k_{\text{el}}} \) and \( \text{MRT}_{\text{ab}} = \frac{1}{k_{\text{ab}}} \), respectively.

### Results

#### Characterization of OVX mice

Female mice underwent surgery at 7 weeks of age to create SHAM and OVX mice. OVX mice were bilaterally ovariectomized, and SHAM mice were bilaterally non-ovariectomized. The mice had free access to food and water for 4 weeks during osteoporosis conditioning. Biochemical markers from bone and blood samples obtained from these mice were measured and are summarized in Table 1.

The results showed that the mean body weight of OVX mice was 6.6% less than that of SHAM mice, and the mean bone densities of the three bone segments (proximal femur, femoral body and distal femur) from OVX mice decreased to 6.4% less that of SHAM mice. Furthermore serum NTx levels, a bone resorption marker, was increased 1.79-fold in OVX mice compared with SHAM mice. Thus, these results indicate that the female mice were experiencing osteoporosis following ovariectomy.

### Table 2. Pharmacokinetic parameters of calcium in mice after intravenous or oral administration of calcium.

<table>
<thead>
<tr>
<th>Mice</th>
<th>AUC(_{\text{iv}}) (( \mu\text{g/mL} \cdot \text{min} ))</th>
<th>MRT(_{\text{iv}}) (min)</th>
<th>CL(_{\text{iv}}) (mL/min/kg)</th>
<th>V(_{\text{dss}}) (mL/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female mice</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SHAM</td>
<td>2101.0 ± 14.3</td>
<td>27.1 ± 3.6</td>
<td>14.3 ± 0.9</td>
<td>386.0 ± 23.8</td>
</tr>
<tr>
<td>OVX</td>
<td>2097.0 ± 10.5</td>
<td>26.5 ± 1.9</td>
<td>14.3 ± 0.7</td>
<td>379.0 ± 34.0</td>
</tr>
<tr>
<td>Male mice**</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SHAM</td>
<td>2870.6 ± 90.8</td>
<td>33.0 ± 1.1</td>
<td>10.5 ± 0.3</td>
<td>334.5 ± 15.8</td>
</tr>
<tr>
<td>OVX</td>
<td>2870.6 ± 90.8</td>
<td>33.0 ± 1.1</td>
<td>10.5 ± 0.3</td>
<td>334.5 ± 15.8</td>
</tr>
</tbody>
</table>

The pharmacokinetic parameters of calcium were calculated from the increment of plasma calcium concentration over the mean control level of calcium after i.v. or oral administration of calcium, as shown in Figs. 1 and 2. Each value represents the mean ± S.D (n = 5). *Dose means a quantity of calcium in CaCl\(_2\) solution. §HJ refers Hachimi-jio-gan extract. **The pharmacokinetic parameters of calcium in male mice (ddY strain) were obtained from Ueda Y and Taira Z.

All serum markers increased by 1.3 to 31.0% in OVX mice compared with those from SHAM mice (as shown in Table 1), with the increase in BUN, CRE and ALP being significant (p < 0.001). Noticeably, markers for renal function increased (BUN and CRE) more than markers for liver function (ALT, TBIL, TP, ALB and GLOB), excluding ALP (p < 0.001). Although ALP is a marker related to liver function, ALP also is indicative of bone resorption and bile duct function. The increases in serum electrolyte concentration in OVX mice were also relatively small compared with that of SHAM mice, and therefore indicated that electrolyte balance was regulated by renal function to maintain homeostatic normal ranges.

The increase in renal function may indicate that the kidney balances serum electrolytes to a normal level by retaining electrolytes or other elements. Changes in the other markers, AMY and the electrolytes Na\(^+\), K\(^+\), Ca\(^{2+}\) and PHOS, are also small between OVX and SHAM mice. The physiological changes in the bone and serum markers indicated that the OVX mice developed osteoporosis similar to that found in postmenopausal women; that is, disease characterized by decreased bone density from bone loss due to activation of osteoblasts.
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**Effects of Hachimi-jio-gan extract on the stimulation of RANKL-induced osteoclast differentiation in RAW264.7 cells by LPS**

Murine monocytic RAW264.7 cells were cultured in α-MEM medium with 50 ng/ml RANKL for 5 days, and then double-stained with TRAP for osteoclasts and methyl green for nuclei. As shown in Figure 2, the cells (Figure 2a) were found to be differentiated to TRAP-positive cells, using a light microscope. NO production in the cells was measured using the supernatant obtained from the cell culture. The ratio of TRAP-positive cells increased at 1.34- and 1.66-fold for MRT_{oral} and F_{A} values after treatment with Hachimi-jio-gan extract. Thus, the increased F_{A} values of calcium resulted in increased MRT_{oral} values.

**Effect of Hachimi-jio-gan extract on the pharmacokinetic behavior of calcium in OVX mice**

The pharmacokinetic behavior of calcium was examined using pharmacokinetic measurements in SHAM and OVX mice, and the effect of oriental Kampo medicine, Hachimi-jio-gan extract, on the intestinal calcium absorption was also monitored. The results are summarized in Figure 1 and Table 2.

As shown in Figure 1a, the time-course of plasma calcium concentration was similar between SHAM and OVX mice after i.v. administration at 30 mg/kg calcium. The pharmacokinetic parameters of calcium were calculated from the increment of the plasma calcium concentration subtracted from the mean control level of calcium immediately before i.v. administration. As shown in Table 2, the results indicated that all pharmacokinetic parameters, AUC_{oral}, MRT_{oral}, CL and Vd_{ss}, were statistically similar between corresponding parameters in SHAM and OVX mice (p < 0.05). Thus, the results indicate that the metabolic processing of calcium should be similar in SHAM and OVX mice.

To examine intestinal calcium absorption across the digestive tract, we studied the pharmacokinetic parameters of calcium in SHAM and OVX mice using the time-course of plasma calcium concentration after oral administration of calcium (150 mg/kg body weight; Figure 1b). The pharmacokinetic parameters of calcium in SHAM and OVX mice were calculated using the time-course of plasma calcium concentration subtracted from the mean control level of blood calcium immediately before the oral administration of calcium. The results are summarized in Table 2. Even though the MRT_{oral} value of OVX mice was 26.3% lower than that of SHAM mice, the AUC_{oral} values of SHAM and OVX mice were similar, as were the F_{A} values. The plasma C_{max} at a T_{max} of 30 min was also similar for every parameter (9.3 and 9.4 µg/ml, respectively). This indicates that the rate of intestinal calcium absorption in OVX mice was faster than that in SHAM mice. Furthermore, we examined the effect of Hachimi-jio-gan extract on the pharmacokinetic behavior of SHAM and OVX mice after oral administration of calcium (150 mg/kg of body weight). As shown in Figure 1b and Table 2, the results indicated that all MRT_{oral} and F_{A} values were similar between SHAM and OVX mice (p < 0.05). However, those values increased at 1.34- and 1.66-fold for MRT_{oral} and 1.86- and 1.96-fold for F_{A} after treatment with Hachimi-jio-gan extract. Thus, the increased F_{A} values of calcium resulted in increased MRT_{oral} values.
positive cells in RAW264.7 cells (Figure 3a) and NO production (Figure 3b) increased with LPS concentration in a dose-dependent manner. Furthermore, to measure the effects of Hachimi-jio-gan extract, RAW264.7 cells were cultured in α-MEM medium with Hachimi-jio-gan extract, LPS, and 50 ng/ml RANKL for 5 days. The results showed that Hachimi-jio-gan extract suppressed the activity of LPS on the ratio of TRAP-positive cells (Figure 3a) and NO production (Figure 3b).

Discussion

We studied the effect of Hachimi-jio-gan extract on calcium metabolism in an OVX mouse model using pharmacokinetic parameters. The OVX mice were shown to develop osteoporosis in bone mass and NTx studies. The pharmacokinetic parameters for calcium after i.v. administration showed that the metabolic behavior of calcium is similar between OVX and SHAM mice, and the absolute calcium bioavailability (Fₐ) after oral administration showed that the intestinal calcium absorption is similar between OVX and SHAM mice, even though MRTₐ of OVX mice is 37.5% lower than that of SHAM mice. That is, the period of intestinal absorption of calcium in OVX mice shortened compared to SHAM mice. However, these results in OVX and SHAM female mice were significantly different from that in male mice. That is, the CLᵢ values of calcium showed that calcium metabolism in OVX and SHAM female mice is 1.36-fold greater in male mice. Furthermore, the MRTᵢ of calcium indicated that the period of intestinal calcium absorption in OVX and SHAM female mice were 1.38- and 2.21-fold more prolonged than that of male mice, respectively. This might mean that calcium metabolism in OVX mice is closer to that of male mice. This observation corresponds to that of the laborious method in rats observed by Brommaga et al., in which intestinal calcium absorption in OVX rats was identical to the mean value for the estrous cycling rats. Morris et al. and Song et al. also reported gender-related differential intestinal calcium absorption.

Treatment with Hachimi-jio-gan extract increased Fₐ and MRTᵢ after oral administration to a similar extent in OVX and SHAM mice. That is, as shown by the MRTᵢ of calcium, the rate
of intestinal calcium absorption in OVX mice was 26.3% lower than that in SHAM mice. Hachimi-jio-gan extract improved the extent of intestinal calcium absorption by 19.9 and 20.2% in SHAM and OVX mice, respectively. MRT was equivalent between SHAM and OVX mice, therefore Hachimi-jio-gan extract increased intestinal calcium absorption time in OVX compared with SHAM mice. These findings indicate that Hachimi-jio-gan could be effective for treatment and/or prevention of bone loss and is in accordance with previous clinical usage and studies. For example, showed that Unkei-to, Hachimi-jio-gan, and Juzen-taiho-to are as effective as 17β-estradiol in preventing the development of bone loss in OVX rats, while determined that Hachimi-jio-gan and Juzen-taiho-to are effective in preventing bone loss in SAMP6 mice.

Although it is well known that LPS stimulates the production of inflammatory mediators, such as NO, and causes various systemic diseases, diabetes mellitus caused by LPS from periodontitis bacteria. This should be a subject of further investigation as the mechanism of action of Hachimi-jio-gan extract currently remains elusive.

In conclusion, Hachimi-jio-gan extract may increase intestinal calcium absorption and suppress the acceleration of RANKL-induced osteoclast differentiation in RAW264.7 cells. Thus, treatment with Hachimi-jio-gan extract may treat or prevent osteoporosis. Furthermore, Hachimi-jio-gan extract might reduce risk factors for other systemic problems including cardiovascular diseases, and diabetes mellitus caused by LPS from periodontitis bacteria. This should be a subject of further investigation as the mechanism of action of Hachimi-jio-gan extract currently remains elusive.

Acknowledgments

The study was supported in part by research grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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