Effect of acupuncture at acupoint of Shenshu (BL 23) on the bones of ovariectomized rats

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Abstract

OBJECTIVE: To investigate the effect of acupuncture at acupoint of Shenshu (BL 23) in ovariectomized rats and sham-operated rats.

METHODS: Female Sprague-Dawley rats were divided into the following four groups (n = 12 each): sham operation only (Sham), sham operation with acupuncture (Sham + ACP), ovariectomy only (OVX), and ovariectomy with acupuncture (OVX + ACP). Operations were performed at the 9th week of age. Acupuncture of Shenshu (BL 23) was started at the 11th week of age and conducted 3 times per week until the 26th week of age.

RESULTS: Among ovariectomized rats, the acupuncture-treated rats had significantly lower body weights and cross-linked N-telopeptide of type 1 collagen levels in the urine after 3 weeks of acupuncture treatment, higher bone mineral density of the lumbar vertebra after 7 weeks, and smaller trabecular separation of the sixth lumbar vertebra, measured by micro-computed tomography, after 17 weeks than control rats. These effects were not observed in sham-operated rats. Blood concentrations of estradiol were significantly higher in the OVX + ACP group than in the untreated group. Acupuncture treatment increased plasma testosterone levels in sham-operated rats, while this increase was not observed in OVX rats.

CONCLUSION: Acupuncture of Shenshu (BL 23) prevents bone loss and structural changes by suppressing bone resorption.

Keywords: Acupuncture; Point BL23 (Shenshu); Estrogens; Osteoporosis; Ovariectomy

INTRODUCTION

Osteoporosis is a disease characterized by low bone mass and structural deterioration of bone architecture, leading to an increased risk of bone fracture. Fractures in elderly patients reduce their ability to perform activities of daily living, and osteoporosis adversely affects the prognosis of fracture healing.1,2 The prevalence of osteoporosis and osteoporosis-related fractures is increasing in an ageing society, and particularly in postmenopausal women. A bisphosphonate and a selective estrogen receptor modulator are widely used for the treatment of osteoporosis.3,5 Acupuncture, a form of Traditional Chinese Medicine...
MATERIALS AND METHODS

Animals
The forty-eight 7-week-old female Sprague-Dawley rats weighing (192 ± 9) g were purchased from CLEA Japan, Inc. (Tokyo, Japan) and housed in plastic cages in a room kept at (22 ± 2) °C with 55% ± 10% relative humidity and a 12-h light/dark cycle. The animals were acclimated to their environment for 1 week and had ad libitum access to tap water and a standard rodent diet, which contains 340 kcal/100 g. To prevent the effect of isolation stress (i.e., reduced body weight and food intake), the rats were housed in groups of three rats per cage. All animals in a single cage were assigned to the same treatment group.

Study design
Acupuncture treatment was administered three times per week for 15 weeks. Figure 1 depicts the study design. Forty-eight rats were divided into the following four groups: sham operation only (Sham), sham operation with acupuncture (Sham + ACP), ovariectomy only (OVX), and ovariectomy with acupuncture (OVX + ACP). One rat in the OVX + ACP group died at the 22nd week of age and was excluded from the study. A bilateral ovariectomy or a sham operation was performed at the 9th week of age under pentobarbital anesthesia (50 mg/kg). After the postoperative recovery period, at week 1, rats received acupuncture treatment starting at the 11th week of age and continuing until the 26th week of age. Blood and bone samples were collected at week 27.

Acupuncture treatment
Acupuncture was administered to the Sham + ACP group and the OVX + ACP group three times per week. Beginning at 11 weeks of age, the acupuncture treatment was administered three times per week, starting at the 9th week of age and continuing until the 26th week of age. Blood and bone samples were collected at week 27.

Bone densitometry
Bone mass density (BMD) was measured by the dual-energy X-ray absorptiometry method (DEXA) with a DEXA-based bone densitometry system (DCS-600R; Hitachi Aloka Medical, Ltd., Tokyo, Japan) under pentobarbital anesthesia at the 14th, 18th, 22nd, and 26th week of age. The method of Kasai et al. was modified to measure the regions of interest (ROIs) in rats. Bone mass densities of the lumbar spine, lower body, right hind leg, right femur, and right tibia were measured.

Analysis of urine cross-linked N-telopeptide of type 1 collagen (NTx)
A 24-h urine sample was collected using metabolic cages at the 14th, 18th, 22nd, and 26th week of age. Urine samples were tested for NTx, a marker of bone resorption, using an enzyme-linked immunosorbent assay (ELISA) kit (Osteocheck, TCI Holdings, Inc., Tokyo, Japan). The samples were collected at 22 weeks of age, corresponding to the start of the acupuncture treatment period.
resorption, using a quantitative enzyme-linked immunosorbent assay (ELISA) kit (Osteomark™ NTx; Inverness Medical Innovations Inc., Walther, MA, USA).

**Analysis of blood estradiol and testosterone levels**

At the 27th week of age, animals were anaesthetized with pentobarbital (50 mg/kg), and intracardiac blood samples were collected into tubes containing ethylene-diaminetetraacetic acid (EDTA). Blood plasma was harvested by centrifugation at 1000 × g for 15 min. Samples were at −80℃ stored until analysis. Commercially available ELISA kits were used to measure the plasma concentrations of estradiol (Estradiol EIA Kit; Cayman Chemical Company, Ann Arbor, MI, USA) and testosterone (Testosterone EIA Kit; Cayman Chemical Company, Ann Arbor, MI, USA).

**Morphological analysis**

At the 27th week of age, the sixth lumbar vertebra was used for trabecular bone structure analysis. The sixth lumbar vertebra was scanned in the range between 500 and 600 µm, from the caudal side toward the rostral side, with a voxel size of 15 µm, by using a bench-top micro-CT system (InspeXio SMX-90CT; Shimadzu Corp., Kyoto, Japan). The trabecular volume/total volume (BV/TV) ratio, which is the volume ratio of the bone marrow and bone; trabecular thickness (Tb.Th), which is the width of trabecular bone; trabecular number (Tb.N), which is the number of trabeculae per unit length (1 mm); and trabecular separation (Tb.Sp), which is the distance between trabeculae, were analyzed with TRI/3D-BON software (Ratoc System Engineering Co., Ltd., Tokyo, Japan).

**Statistical analysis**

All data are presented as the mean ± standard error (SE). Data were analyzed with the analysis of variance (ANOVA) test for multiple comparisons, followed by Tukey’s test; the Tukey-Kramer test was used when data were missing. A P value less than 0.05 was considered significant. Statistical analysis was performed with commercially-available statistical software (Statistica ver.9.0; StatSoft Japan Inc., Tokyo, Japan).

**Ethical considerations**

The present study was performed with the approval and permission of the Suzuka University of Medical Science Animal Experimentation Ethics Committee (Approved number: 124). Breeding and processing of laboratory animals were conducted according to the regulations of Suzuka University of Medical Science.

**RESULTS**

**Bone mineral density**

The BMD of OVX rats was significantly lower than that of Sham rats at the 14th week of age and all subsequent time points. The BMD in the lumbar spine in OVX + ACP rats was higher at the 18th week of age than in OVX rats (P < 0.01); this difference was significant at the 18th week of age, which was 7 weeks after the start of the acupuncture treatment (Table 1).

**Trabecular bone structure analysis**

In OVX rats, the BV/TV and Tb.N were significantly lower, while Tb.Th and Tb.Sp were significantly higher (P < 0.01) than those of Sham rats or Sham + ACP rats. The Tb.Sp of OVX + ACP rats was significantly lower than that of the OVX group (P < 0.01, Table 2).

**NTx in urine**

Sham levels were considered as the control levels. Urinary NTx levels in OVX rats at 14 weeks of age (3 weeks of acupuncture treatment) were significantly higher than those of Sham rats. Furthermore, NTx levels of OVX + ACP rats were significantly lower than those of the OVX group. No significant differences were observed at the 18th, 22nd, or 26th week of age (Figure 2).

**Concentration of plasma estradiol and testosterone**

At the 27th week of age, estradiol concentrations in OVX rats were significantly lower than those of Sham rats (P < 0.05) and Sham + ACP rats (P < 0.01). Estradiol concentrations of OVX + ACP rats were significantly higher than those of OVX rats. In contrast, testosterone concentrations were not significantly different between Sham rats and OVX or OVX + ACP rats. However, testosterone concentrations of Sham + ACP rats were significantly higher than those of the other three groups (P < 0.01) (Figure 3).
At the 12th week of age (P < 0.05), the difference persisted until the end of this study. In addition, acupuncture treatment significantly decreased the weight of OVX + ACP rats at the 14th week of age (P < 0.01), and the effect continued thereafter. Acupuncture did not affect the body weight of Sham + ACP rats (Figure 4).

Food intake
At the 13th and 14th week of age, OVX rats had a higher caloric intake than Sham and Sham + ACP rats (P < 0.01). The caloric intake of OVX rats exceeded

<table>
<thead>
<tr>
<th>Item</th>
<th>Before ovariectomy</th>
<th>Acupuncture treatment (start of treatment is at 11 weeks of age)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>8 weeks</td>
<td>14 weeks</td>
</tr>
<tr>
<td>Lumber vertebra</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>144.4±1.9</td>
<td>183.5±2.9*</td>
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<tr>
<td>Sham ACP</td>
<td>146.0±2.4</td>
<td>186.5±2.9*</td>
</tr>
<tr>
<td>OVX</td>
<td>143.1±2.5</td>
<td>160.1±2.2</td>
</tr>
<tr>
<td>OVX+ACP</td>
<td>145.9±1.7</td>
<td>162.7±2.6</td>
</tr>
<tr>
<td>Hind quarter</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>124.5±1.2</td>
<td>154.1±1.7*</td>
</tr>
<tr>
<td>Sham ACP</td>
<td>124.4±1.2</td>
<td>159.2±1.3*</td>
</tr>
<tr>
<td>OVX</td>
<td>124.8±1.0</td>
<td>144.7±0.7</td>
</tr>
<tr>
<td>OVX+ACP</td>
<td>125.2±0.9</td>
<td>140.8±1.3</td>
</tr>
<tr>
<td>Hind leg (right)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>151.3±2.1</td>
<td>193.1±2.6*</td>
</tr>
<tr>
<td>Sham ACP</td>
<td>150.2±1.7</td>
<td>197.5±1.7*</td>
</tr>
<tr>
<td>OVX</td>
<td>149.5±1.5</td>
<td>179.1±1.9</td>
</tr>
<tr>
<td>OVX+ACP</td>
<td>151.2±1.1</td>
<td>180.4±1.2</td>
</tr>
<tr>
<td>Femur (right)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>151.3±2.1</td>
<td>193.1±2.6*</td>
</tr>
<tr>
<td>Sham ACP</td>
<td>150.2±1.7</td>
<td>197.5±1.7*</td>
</tr>
<tr>
<td>OVX</td>
<td>149.5±1.5</td>
<td>179.1±1.9</td>
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<tr>
<td>OVX+ACP</td>
<td>151.2±1.1</td>
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</tr>
<tr>
<td>Tibia (right)</td>
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<tr>
<td>Sham</td>
<td>112.9±1.2</td>
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</tr>
<tr>
<td>Sham ACP</td>
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<td>144.7±1.7*</td>
</tr>
<tr>
<td>OVX</td>
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</tr>
<tr>
<td>OVX+ACP</td>
<td>113.7±0.8</td>
<td>134.6±1.1</td>
</tr>
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</table>

Notes: data from rats at 8 weeks before ovariectomy. Ovariectomy was performed at 9 weeks. Acupuncture treatment for Shen shu (BL 23) was administered three times per week between 11 and 26 weeks (105 days in 15 weeks). Data from rats aged 14-26 weeks show a change in bone mineral density with acupuncture treatment. Sham group: Sham operation only; OVX group: ovariectomy only; Sham ACP group: sham operation and acupuncture treatment; OVX + ACP group: ovariectomy and acupuncture treatment. The bone mineral density in the lumbar spine in OVX + ACP rats was higher at 18 weeks compared to Sham or OVX rats. All data are given as means ± standard error. Data were analyzed with the analysis of variance test for multiple comparisons, followed by the Tukey-Kramer test. Compared with OVX, *P < 0.01, †P < 0.05; compared with OVX + ACP, ‡P < 0.01; and compared with Sham, ‡P < 0.05.

Taken together, these data indicate a role for acupuncture treatment increased the levels of testosterone in Sham + ACP rats. However, acupuncture in OVX + ACP rats. These effects were not observed in Sham + ACP rats. However, acupuncture increased the levels of testosterone in Sham + ACP rats. Taken together, these data indicate a role for acupuncture treatment at Shenshu (BL 23) in preventing bone fragility in ovariectomized rats.

**DISCUSSION**

In the present study, acupuncture of Shenshu (BL 23) was evaluated to determine if it inhibits the changes in bone induced by ovariectomy. Acupuncture partially, but significantly, reversed the decrease in bone density in the lumbar vertebrae after 7 weeks of acupuncture treatment in 18-week-old rats. Micro-CT analysis showed that the distance between trabecular bones was smaller in OVX + ACP rats than in OVX rats. Acupuncture treatment also inhibited an increase in NTx, implying a decrease in bone resorption. In addition, the acupuncture treatment increased blood estradiol levels in OVX + ACP rats. These effects were not observed in Sham + ACP rats. However, acupuncture increased the levels of testosterone in Sham + ACP rats. Taken together, these data indicate a role for acupuncture treatment at Shenshu (BL 23) in preventing bone fragility in ovariectomized rats.

Data from rats at 8 weeks, before ovariectomy. Ovariectomy was performed at 9 weeks. Acupuncture was started from 11 weeks. Data from rats aged 8-26 weeks show a change in body weight with acupuncture treatment. Acupuncture treatment significantly decreased the weight of OVX + ACP rats at 14 weeks of age ($P < 0.01$), and the effect continued thereafter. Sham group: sham operation only; OVX group: ovariectomy only; Sham ACP group: sham operation and acupuncture treatment; OVX + ACP group: ovariectomy and acupuncture treatment. Data were analyzed with the analysis of variance test for multiple comparisons, followed by the Tukey-Kramer test. Compared with Sham, $P < 0.01$; $P < 0.05$; compared with OVX, $P < 0.01$.

The preventive effects of acupuncture on bone mass loss and bone structure deterioration in ovariectomized rats have been reported by several groups, including our laboratory. Zhang et al have reported that acupuncture at the Zusanli (ST 36) and Sanyinjiao (SP 6) points inhibited a decrease in the bone density of the tibia and overall bone resorption; the microarchitecture and bone morphometry of the lumbar vertebrae and tibiae showed improvement in rats receiving acupuncture compared to those without the treatment. Zhou et al have reported that electroacupuncture treatment at Zusanli (ST 36) and Sanyinjiao (SP 6) acupoints in-
hibited the loss in bone density of the fifth lumbar vertebral body and proximal metaphysis of the femur in ovariectomized rats; increased serum bone-specific alkaline phosphatase levels, an indicator of osteoclast activity, were observed. In our study, OVX + ACP rats were treated with acupuncture at only Shenshu (BL 23), which is different from previous studies. The effect of single-point acupuncture at Shenshu (BL 23) has been reported in a senescence-accelerated mouse strain P6 (SAMP6), demonstrating its effectiveness in promoting bone formation, restoring bone volume, improving bone architecture, and reversing osteoporosis to a degree. In conclusion, acupuncture of acupoint of Shenshu (BL 23) prevents bone loss by suppressing bone resorption in ovariectomized rats. Comparisons of the acupuncture effect between ovariectomized rats treated with acupuncture and sham-operated rats treated with acupuncture indicate that this effect may be associated with increased estrogen levels. Bone structure analysis by micro-CT supports the beneficial effect of acupuncture.

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