Prevention of bone loss in calcium deficient ovariectomized rats by OST-6, a herbal preparation

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ABSTRACT
OST-6, a herbomineral preparation was studied for its inhibitory effects on the progress of bone loss induced by ovariectomy (OVX) and concurrent calcium deficiency in rats. Calcium deficient ovariectomized rats were administered with OST-6 at 250 mg/kg b.wt. twice a day orally for 16 weeks. Compared with sham operated animals, OVX animals showed an increase in serum ALP, urinary excretion of calcium and phosphorus, which were significantly prevented in OST-6 administered rats. Evaluation of cortical bone morphometric indices by CT-Scanning technique showed an increased medullary width and cross-sectional medullary area (MA), decreased periosteal area (PA), combined cortical thickness (CCT), cortical area/periosteal area (CA/PA) and maximal metaphyseal radial density (MMRD) in OVX animals when compared with sham operated. OST-6 treatment significantly prevented these bone resorption variables. Scanning Electron Microscopy (SEM) study revealed porous and erosive appearance of femur bone at the epiphyseal region and decreased calcium to phosphorus ratio (Ca:P) in the OVX rats when compared with sham operated rats. The treatment with OST-6 prevented the epiphyseal bone resorption and maintained Ca:P ratio. The results of ash analysis indicated a reduced bone mineral content (calcium and phosphorus) and ash weight and percent ash in OVX animals when compared with sham operated animals. All results are statistically significant at P<0.05. These finding suggest the usefulness of OST-6 in the prevention of bone loss in a natural way through utilization of herbal resources.

Keywords: Osteoporosis; Ovariectomy; OST-6 (Osteocare); CT-scanning; Scanning electron microscopy; Herbal preparation

1. INTRODUCTION
Osteoporosis is the most frequent metabolic condition experienced by elderly individuals. It is a systemic skeletal disease characterized by low bone mass and microarchitectural deterioration of bone tissue with a consequent increase in bone fragility and susceptibility to fracture (Peak et al., 1993). Osteoporosis that is associated with ovarian hormone deficiency following menopause is by far the most common cause of age related bone loss (Albright et al., 1941). Menopause results in elevated bone turnover, an imbalance between bone formation and bone resorption and net bone loss (Riggs and Melton, 1986). Postmenopausal osteoporosis has become a major problem with significant morbidity and mortality (Cummings et al., 1990).
Estrogen replacement therapy is approved for the prevention of bone loss in postmenopausal women and is efficacious in reducing the incidence of skeletal fractures (Turner et al., 1994). However, estrogen use and compliance are limited due to its numerous undesirable side effects such as uterine and breast cancer (Lindsay and Cosman, 1992). Hence, it would be most helpful to explore naturally occurring substances especially of plant origin that could prevent bone loss and free from any adverse effects. Many consumers would also prefer to avoid synthetic molecules in favor of natural products.

Ayurveda, an ancient system of Indian medicine mentions several plants that are useful in the correction of bone metabolic disorders such as osteoporosis. OST-6 is a herbomineral preparation formulated with such plants and had been reported earlier for beneficial effects in various bone disorders (Mitra et al., 2000, 2001). The formulation consists of Terminalia arjuna W&A, Withania somnifera Dunal, Commiphora mukul Hook Ex stock and Praval bhasma.

The main objectives of the proposed study are to evaluate the preparation (OST-6) for its usefulness in preventing bone loss in calcium deficient ovariectomized rats. The OVX model was chosen for the study because OVX rat has been particularly useful to study the efficacy of various agents for the prevention and for reversal of bone loss (Kalu et al., 1989). The rate of bone loss following ovariectomy can be increased by concurrent calcium deficiency (Hodgkinson et al., 1978). Since Praval bhasma, a rich natural source of calcium (Asundi and Dixit, 1978) is also one of the main ingredients in the formulation (OST-6), the study was designed to investigate the effects in calcium deficient ovariectomized rats.

2. MATERIALS AND METHODS

2.1 Preparation and standardization of OST-6
Each gram of OST-6 contains Terminalia arjuna (bark, 250 mg), Withania somnifera (root, 250 mg), Commiphora mukul (gum resin, 280 mg) and Praval bhasma (220 mg). The plant constituents were powdered and mixed in appropriate proportion and standardized by using HPTLC as reported earlier (Mitra et al., 2000, 2001).

2.2 Protocol
Thirty, 3 month old female, virgin Sprague-Dawley rats weighing between 225 and 250 g were obtained from The Animal Facility, R&D Center, The Himalaya Drug Company, Bangalore and maintained in standard laboratory conditions. After 3 weeks of adaptation, they were randomized by weight into three groups of 10 each. Group 1 rats were sham operated and fed with commercial pelleted diet containing normal calcium and ordinary drinking water ad libitum. Groups 2 and 3 rats were bilaterally ovariectomized under pentobarbitone anesthesia (30 mg/kg b.wt.) and fed with casein based synthetic diet containing a low quantity of calcium (Ca, 0.04%) and distilled water ad libitum. The ovariectomy was confirmed by observing the atrophic uterus at the beginning of the experiment. The animals were allowed a week to recover and then treatment was started as follows:

Group 1: Sham control and received vehicle.
Group 2: Ovariectomized control and received vehicle.
Group 3: Ovariectomized and treated with OST-6 (250 mg/kg b.wt. twice a day per oral). This dose was selected on the basis of earlier studies (Mitra et al., 2001).

All experimental designs and procedures had received the approval of the institutional ethics committee. The treatment was continued for 16 weeks and at the end of experimental period total urine excreted over 24 h period was collected from overnight fasted rats by housing each rat individually in a metabolic cage. The animals were killed and blood collected from carotid bleeding was centrifuged to separate serum and preserved (-20°C) for analysis of calcium, phosphorus and alkaline phosphatase.

The left femurs were cleared of soft tissue by autoclaving at 110°C for 20 minute and preserved for measurement of cortical bone indices (using CT-scanning) and ash analysis. The right femurs were also removed at necroscopy, cleaned of soft tissues and frozen till SEM and QXA tests could be performed.

### 2.2.1 Serum and urine analysis

Total calcium and inorganic phosphorus in serum and urine were determined by colorimetry using commercially available test kits (SIGMA) in an automatic analyzer (Hitachi BM 704). Serum alkaline phosphatase activity was also measured by the same technique using commercially available diagnostic reagent kit (DIA-LAB).

### 2.2.2 Measurement of cortical bone morphometric indices and radial density

The left femurs that were harvested from all three groups were scanned using a spiral CT-scanning machine (Somatom Plus-4, Siemens). For each bone sample two axial sections, one at 40% of the length from the distal end in the diaphysis (cortical) and other at the distal part of the metaphysis (cancellous) were taken.

Using an image analysis system available with the scanner, the axial sections were magnified (X64) to distinguish the cortex from medullary canal. Keeping the window centering and window width values common, a minimum of 2 diameters (two each for total width ($W$) and medullary canal) were drawn using a cursor on the magnified image to calculate the medullary canal width ($m$) and $W$. Combined cortical thickness (CCT), periosteal area (PA), medullary area (MA) and the ratio of cortical area to periosteal area (CA/PA) were calculated from these value (Wegener, 1983). Cortical width was obtained by subtracting medullary width from $W$. Cortical area (CA) was derived from the formula,

$$CA = \Pi \left[ \left( \frac{W}{2} \right)^2 - \left( \frac{m}{2} \right)^2 \right]$$

on the assumption that the cross section at the midshaft region was circular (Armstrong et al., 1972) at 40% length from distal end. Maximal radial density measurements were also carried out at the metaphyseal region of distal femur (Schering, 1983). The density values are expressed as Hounsfield units (HU).

### 2.2.3 Scanning electron microscopy and quantitative X-ray analysis

The frozen right femurs were placed in 5% sodium hypochlorite solution (Commercial Bleach) for 4 hour. The bones were then dehydrated in ethanol and dried, mounted on stubs
and coated with gold using a sputter coater (Miller and Bowman, 1998). The bones were examined on a JEOL JSM-840 A scanning electron microscope. In addition to the observation of qualitative bone resorption at the epiphyseal edges, the calcium to phosphorous ratio at the central metaphysis region in the distal femur was also determined. These measurements were carried out using Quantitative X-ray analysis system attached to SEM and with the aid of a software system (LINK ISIS, Oxford, UK).

2.2.4. Ash weights and mineral content of bone
At ashing, the samples were placed in tared fused silica crucibles, weighed, dried to a constant weight at 110°C and ashed for 24 hour at 650°C (Rude, et al., 1999). The ash weights were determined and the samples were suitably diluted with deionized water to assay for calcium, phosphorus and magnesium.

3. STATISTICS
All statistical analysis was done by using one way analysis of variance (ANOVA) followed by Dunnet multiple comparison test to determine any significant difference between the groups using INSTANT GRAPHPAD PRIM software. Differences between means at the 5% confidence level \( (P<0.05) \) were considered to be statistically significant.

4. RESULTS
4.1 Serum and urine analysis
The effect of OST-6 on serum calcium, phosphorus and alkaline phosphatase (ALP) and urine calcium and phosphorus are presented in Table 1. The results indicate a significant reduction \( (P<0.01) \) in the serum calcium in OVX rats when compared with sham and OST-6 treated animals. However, there was no significant difference in the serum phosphorus levels among any groups. Statistically significant \( (P<0.01) \) elevated levels of serum ALP was observed in the OVX animals when compared with sham operated animals. Treatment with OST-6 reduced the serum ALP levels \( (P<0.01) \).

<table>
<thead>
<tr>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Calcium (mmol/l)</strong></td>
<td>2.58 ± 0.014</td>
<td>2.35 ±0.036( ^a )</td>
</tr>
<tr>
<td><strong>Phosphorus (mmol/l)</strong></td>
<td>1.54 ± 0.020</td>
<td>1.50 ± 0.026</td>
</tr>
<tr>
<td><strong>ALP</strong></td>
<td>109.25 ± 2.11</td>
<td>147.50 ± 2.97( ^a )</td>
</tr>
<tr>
<td><strong>Calcium (mg/24 h)</strong></td>
<td>0.51 ± 0.01</td>
<td>0.81 ±0.04( ^c )</td>
</tr>
<tr>
<td><strong>Phosphorus (mg/24 h)</strong></td>
<td>4.92 ± 0.21</td>
<td>7.13 ± 0.22( ^c )</td>
</tr>
</tbody>
</table>

All values are expressed as Mean ± S.E.M.
\( ^a \) \( P<0.01 \) as compared with sham operated rats (Group 1)
\( ^b \) \( P<0.01 \) as compared with ovariectomized rats (Group 2)
\( ^c \) \( P<0.001 \) as compared with sham operated rats (Group 1)
\( ^d \) \( P<0.001 \) as compared with ovariectomized rats (Group 2)

Daily urinary excretion of calcium was significantly increased in OVX animals when compared with sham animals. Treatment with OST-6 resulted in a significant reduction of urinary calcium and phosphorus excretion.
4.2 Cortical bone morphometric indices and radial density measurements

The effect of OST-6 on various cortical bone morphometric indices measured at 40% length from the distal end of femur is presented in Table 2. Ovariectomy and calcium deficiency resulted in a significant reduction ($P<0.01$) in $W$, CCT, PA, CA, CA/PA and maximal metaphyseal radial density (MMRD). The results also indicate an increase in medullary width and cross-sectional MA in these animals (Fig. 1a, b and c) when compared with sham animals. Treatment with OST-6 had increased the CCT, PA, CA, CA/PA, MMRD and prevented the decrease in medullary width and cross sectional MA.

| Table 2: The effect of OST-6 on various cortical bone morphometric indices measured at 40% length from the distal end of femur bone and radial density using CT-scanning technique |
|----------------------------------|-----------------|-----------------|-----------------|
|                                  | Group 1          | Group 2          | Group 3          |
| (1) $W$ (mm)                    | 3.64 ± 0.007     | 3.54 ±0.005$^a$ | 3.59 ± 0.013$^b$|
| (2) Medullary width (mm)        | 2.21 ± 0.005     | 2.69 ± 0.005$^a$ | 2.41 ± 0.010$^b$|
| (3) CCT (mm)                    | 1.43 ± 0.005     | 0.85 ± 0.001$^a$ | 1.18 ± 0.014$^b$|
| (4) PA (mm$^2$)                 | 10.42 ± 0.045    | 9.84 ± 0.032$^a$ | 10.17 ± 0.074$^b$|
| (5) MA (mm$^2$)                 | 3.84 ± 0.019     | 5.68 ± 0.025$^a$ | 4.59 ± 0.039$^b$|
| (6) CA (mm$^2$)                 | 6.57 ± 0.033     | 4.16 ± 0.011$^a$ | 5.60 ± 0.074$^b$|
| (7) CA/PA                       | 0.63 ± 0.002     | 0.42 ± 0.001$^a$ | 0.55 ± 0.004$^b$|
| (8) Radial density (HU)         | 1132.87 ± 2.33   | 988.5 ±1.721$^a$ | 1234.15 ± 16.78$^b$|

All values are expressed as Mean ± S.E.M.

$^a P<0.01$ as compared with sham operated rats (Group 1)

$^b P<0.01$ as compared with ovariectomized rats (Group 2)

Fig. 1: Computed tomographic images of femur metaphysis of a rat belonging to sham operated group (Fig. 1a), OVX group (Fig. 1b) and OST-6 treated group (Fig. 1c). The scans were taken at 40% length from the distal end. Note the increased width of the marrow cavity and the concomitant reduction in cortical thickness in the OVX rat compared with sham rat. Treatment of OVX rats with OST-6 prevented cortical bone loss at the endosteal surface of the femoral shaft.

4.3 Scanning electron microscopy and quantitative X-ray analysis

The epiphyseal edges of the distal femur bone were observed to study the resorption pattern in all groups. The scanning electron photomicrographs (Fig. 2a, b and c) clearly indicate an extensive resorption in ovariectomized animals when compared with sham operated animals. Observation of the number of resorption pits and depth on the surface clearly demonstrates the beneficial effects of OST-6 in inhibiting bone resorption.

The results of QXA (Table 3) shows a reduced elemental composition of calcium, phosphorus and calcium to phosphorus (Ca:P) ratio at the distal femur region in the calcium deficient ovariectomized animals when compared with sham operated. Treatment with OST-6
prevented the resorption of calcium and phosphorus from this region and, thus maintained a normal Ca:P ratio.

Table 3: The effect of OST-6 on composition of calcium and phosphorus and Ca:P ratio in the metaphyseal region of distal femur as determined by SEM and QXA

<table>
<thead>
<tr>
<th>Elemental composition</th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium elemental (%)</td>
<td>71.18 ± 0.117</td>
<td>69.17 ± 0.197</td>
<td>72.07 ± 0.095</td>
</tr>
<tr>
<td>Phosphorus elemental (%)</td>
<td>29.16 ± 0.126</td>
<td>30.18 ± 0.295</td>
<td>30.02 ± 0.210</td>
</tr>
<tr>
<td>Ca:P ratio</td>
<td>2.440 ± 0.007</td>
<td>2.295 ± 0.027</td>
<td>2.401 ± 0.017</td>
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</table>

All values are expressed as Mean ± S.E.M.

4.4 Ash analysis

The results of ash analysis are presented in the Table 4. Extremely significant reduction in the femur ash weight, ash Ca, ash P and ash Mg was observed in the OVX animals when compared with sham operated rats. Treatment with OST-6 significantly improved the ash weight (%) ash and mineral composition (calcium and phosphorus) but did not show any effect on magnesium levels.

Table 4: The effect of OST-6 on the mineral composition of ash in femoral bone

<table>
<thead>
<tr>
<th>Ash parameters</th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ash weight (g)</td>
<td>0.28 ± 0.13</td>
<td>0.21 ± 0.10</td>
<td>0.25 ± 0.19</td>
</tr>
<tr>
<td>Ash (%)</td>
<td>72.94 ± 1.07</td>
<td>66.03 ± 1.03</td>
<td>69.76 ± 1.29</td>
</tr>
<tr>
<td>Calcium (mg)</td>
<td>99.93 ± 1.49</td>
<td>75.12 ± 1.19</td>
<td>88.08 ± 1.23</td>
</tr>
<tr>
<td>Phosphorus (mg)</td>
<td>49.19 ± 1.10</td>
<td>39.80 ± 1.15</td>
<td>42.07 ± 1.04</td>
</tr>
<tr>
<td>Magnesium (mg)</td>
<td>2.26 ± 0.13</td>
<td>1.88 ± 0.19</td>
<td>1.89 ± 0.23</td>
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</tbody>
</table>

All values are expressed as Mean ± S.E.M.

5. DISCUSSION AND CONCLUSIONS

The present study clearly demonstrates the usefulness and beneficial effects of OST-6 in the prevention of bone loss induced by ovariectomy and concurrent calcium deficiency. It is well known the both estrogen and calcium deficiencies are important risk factors in the pathogenesis of osteoporosis. Ovariectomy plus calcium deficiency results in great decrease in bone volume, femoral weight, femoral ash weight and cortical cross-sectional area than did the calcium alone (Mazzeo et al., 1988). These changes are partly due to hyperparathyroidism
secondary to calcium deficiency and exacerbated by estrogen deficiency (Riggs and Melton, 1986).

OST-6 treatment had been reported earlier for its beneficial effects in rickets and osteoporosis in rats. The present study was undertaken to investigate its usefulness in treating osteoporosis associated with calcium deficiency.

In the present study, calcium deficient OVX rats developed similar changes to those seen in postmenopausal women as indicated by increased serum ALP and urinary excretion of calcium and phosphorus. Serum ALP is an important biochemical marker of bone formation. The levels of this enzyme is increased in osteoporosis and other bone metabolic disorders (Victor, 1993). Treatment with OST-6 restored the increased levels of serum ALP to a normal value indicating its usefulness in osteoporosis. Fasting urinary calcium excretion could also be used as an important variable for estimating net bone resorption. Again, the treatment with OST-6 reduced the urinary excretion of calcium to a statistically significant level.

The distal metaphyseal region of femur that contains both cortical and trabecular bone is very sensitive to estrogen deprivation and results in rapid and profound osteopenia (Westerlind et al., 1997). Previous publications have described the need to study both cancellous and cortical bone resorption for osteoporosis agents (Mazess, 1990). It is well established that the pattern of bone loss from the cortex in osteoporosis begins from the endosteal surface of the cortex, where there is an enlargement of medullary canal at the expense of inner cortex. Bone loss usually does not occur at the periosteal surface (Higdon et al., 2001). The results of CT-Scanning further confirm this and the above changes were prevented by OST-6 indicating the anti-osteoporotic property. The results of MMRD values were also encouraging in OST-6 treated animals.

SEM with the aid of QXA is being used extensively in understanding the elemental composition of metal and alloys. We utilized the same technique to determine the pattern of bone resorption and calcium to phosphorus ratio (QXA) at the epiphyseal region of distal femur, which predominantly contains the areas on bone resorption. Porous and erosive appearance of femur at the epiphyseal edges was more pronounced and prevalent in OVX animals when compared with sham operated. Treatment with OST-6 decreased the resorption of minerals and maintained the intactness and integrity of the surface indicating its usefulness in the prevention of bone loss.

The long-term consequence of ovariectomy in mature rats is a reduction in femoral mineral content (Ca and P) which was also observed in the present study. Treatment with OST-6 prevented the reduction of ash, % ash, ash Ca and ash P to an appreciable level, ascertaining its usefulness in the prevention of bone loss.

In conclusion, OST-6 treatment in an adult rat model of post menopausal bone loss in severe calcium deficiency had shown promising and beneficial effects on the progress of bone loss and, thus, indicating its usefulness as a potential therapeutic agent in humans as bone remineralization agent.
ACKNOWLEDGEMENTS
The technical assistance of Gurulinga, SEM facility, Department of Metallurgy, Indian Institute of Science, Bangalore-12, India and M. Lokanath, CT-Scanning Facility, KMIO, Bangalore is gratefully acknowledged. We are also grateful to Dr. S.K. Mitra, The Director, R&D, The Himalaya Drug Company, Bangalore for kindly providing the investigative drug and financial assistance.

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