The beneficial effect of OST-6 (OsteoCare), a herbomineral formulation, in experimental osteoporosis

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SUMMARY
OST-6 (OsteoCare), a herbomineral formulation, was evaluated for its inhibitory effect on the progress of bone loss induced by ovariectomy in rats. Ovariectomized (Ovx) rats were administered with OST-6 at 250 and 500 mg/kg b.wt., orally daily for 90 days. On 91st day, ovariectomized rats showed reduced bone mineral content and increased serum alkaline phosphatase levels, excretion of urinary calcium and pyridinium cross-links levels. Histologically, bone sections revealed narrowed and disappearance of trabeculae and widened medullary spaces. The total numbers of Tartrate-resistant acid phosphatase (TRAP) positive cells were significantly increased both in-vivo and in-vitro methods. OST-6, at a dose of 500 mg/kg, significantly improved bone mineral contents, serum alkaline phosphatase levels, reduced the elevated urinary calcium and pyridinium cross-links excretion, number of TRAP positive cells and reversal of the above mentioned histological features. These results indicate the usefulness of OST-6 in the management of osteoporosis in a natural way through herbal resources.

Key words: Osteoporosis, Herbomineral formulation, OST-6 (OsteoCare), Bone histomorphometry, Osteoclasts, Pyridinium Cross-links.

INTRODUCTION
Osteoporosis is now widely recognized as a public health problem since this disease, which increases bone fragility and thereby the risk of fractures, is associated with high mortality, morbidity and medical expenses throughout the world. The most common type of osteoporosis is the bone loss associated with ovarian hormone deficiency at menopause. The ovariectomized rat is the most appropriate model for studying the mechanism as well as potential treatments of post-menopausal osteoporosis in humans and are useful model to study the efficacy of various pharmaceutical candidates for their prevention and/or reversal of bone loss (Kalu et al., 1989; Wronski and Yen, 1991; Miller, 1997).

Osteoporosis, a heterogeneous group of syndromes where the total skeletal mass will be decreased due to imbalance between resorption of bone by osteoclasts (OCs) and subsequent formation of bone by osteoblasts (OBs). To increase bone mass, treatments of osteoporosis should stimulate bone formation and/or inhibit bone resorption, the aim being to produce a positive net balance in bone remodeling. In the orthodox medicine, osteoporosis treatment
involves hormone replacement therapy, calcium, calcitonin, androgens, parathormone and growth hormones. Conventional treatment results in varied effects like an increased incidence of endometrial and breast cancer with hormonal replacement therapy, pseudo-arthritis with fluoride treatment, extra skeletal effects in cases treated with growth factors such as insulin like growth factor I and II and transforming growth factor (Joshi and Paramar, 1997; Mary Wheeler, 1976).

Considering the broad spectrum effect of osteoporosis in the medical system, currently an increasing demand is sought in the alternative system of medicine to design strategies to prevent and cure this devastating ailment. Ayurveda, an ancient system of Indian medicine cited several plants, which are useful in bone disorders including bone fractures and metabolic disorders with no adverse effects. OST-6 is a herbomineral formulation comprising mainly *Terminalia arjuna*, *Withania somnifera* and *Commiphora mukul* that are well known for their bone remineralization.

*Terminalia arjuna* is extensively used for the treatment of osteoporosis and other bone related disorders as it improves the synthesis and secretion of female hormones (Nadkarni, 1996a). *Withania somnifera* is considered as a rejuvenator in Ayurveda. It helps in relieving the pains associated with osteoporosis frequently encountered in old age and is also useful in cases of general debility, nervous exhaustion and muscle pains (Nadkarni, 1996b). *Commiphora mukul* helps in remineralization of the bones especially in old age, thus reversing the process of osteoporosis (Nadkarni, 1996c).

OST-6 was reported to have bone remineralization property when administered to ricketic rats (Mitra et al., 2000). This study was designed to elucidate OST-6, a herbomineral formulation, for the prevention of rapid bone loss occurring after ovariectomy in adult female rats and, if it is, to gain insight into the mechanisms of this effect both *In vivo* and *In vitro*.

**MATERIALS AND METHODS**

**Preparation 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), Praval bhasma (220 mg). Preparation and standardization of OST-6 was done as reported earlier (Mitra et al., 2000).

**Experimental design**

Thirty two female rats (16 weeks old) of the Sprague-Dawley strain, weighing between 250-275g, were selected and randomized into 4 groups of 8 animals each. The animals were housed in standard laboratory conditions under a temperature of 22 ± 3°C, relative humidity 50-55% and 12 hr light/dark cycle. Drinking water and nutritionally balanced synthetic pelleted diet containing 0.8% calcium and 0.8% inorganic phosphorus and 45 ng vitamin D₃ per gram dry diet (Lipton India Ltd., Mumbai) were supplied *ad-lib* throughout the study period.

The rats from all the groups were ovariectomized except for rats from Group I, which served as sham operated control. Rats from Group-II received the vehicle and served as ovariectomized control. Groups-III and IV were maintained on normal diet, along with oral
administration of OST-6 at 250 and 500 mg/kg b.i.d., respectively. The treatment of respective groups was started 7 days after the ovariectomy and continued for 90 days. Ovariectomy was performed by ligation and excision of the ovaries along the upper horns, under general anesthesia with pentobarbital sodium (35 mg/kg b. wt., i.p.), through aseptic incisions of the dorsal skin and muscle layers. In the sham operation the ovaries were exposed as above and gently manipulated but not excised.

**Necropsy and processing of the tissues**

On the 91st day, urine was collected from the overnight fasted rats and subjected for the estimation of pyridinoline (Pyr), deoxypyridinoline (dPyr) (Abbiati et al., 1994), hydroxyproline (Neuman and Logan, 1950) and calcium levels (Sarkar and Chauhan, 1967). The rats of all the five groups were anesthetized by using diethyl ether for collection of blood samples and then euthanized. Serum samples were subjected for the estimation of calcium (Sarkar and Chauhan, 1967), inorganic phosphorus (IP) (Varley, 1980) and bone specific alkaline phosphatase (ALP) (Horn, 1972).

The animals of the respective groups were then systematically necropsied and both femur bones were collected and divided into two sets. One set was taken for bone mineral estimation after ashing in an electric furnace at 700°C for 8 hours. The other set was fixed in 10% neutral buffered formalin (NBF) for 12 h at 4°C, decalcified in 5% ethylenediamine tetraacetic acid (EDTA, pH 7.4) for 7 days, embedded in paraffin, and cut into longitudinal sections of 5µ thickness. The sections were stained with hematoxylin and eosin (H&E) and tartrate-resistant acid phosphatase (TRAP), a cytochemical marker for osteoclasts, and finally counter-stained with hematoxylin (Bancroft and Cook, 1988; Drury, 1980). The number of positively stained cells in sections of the median portion of whole femora was enumerated for the four groups.

**In-vitro bone resorption studies**

**Formation of OCLs:** Co-culture with mouse bone marrow cells and osteoblast like cells was carried out by the method of Takahashi et al. (1988). Osteoblast-like cells were prepared from day old mouse calvariae and plated at 10^4 cells/well in α-MEM containing 10% fetal calf serum, penicillin (100 IU/ml) and streptomycin (100 µg/ml). Bone marrow cells were prepared from six-week-old Swiss mice. The mice were euthanized by cervical dislocation and tibiae were aseptically removed. The bone ends were cut and the marrow cavity was flushed with 1 ml of α-MEM medium. Cells were washed and plated at 10^5 cells/well on cultures of osteoblast like cells. Medium was replaced every 2 d with 1α,25-(OH)2D3 (10^-8 M). Different concentration of OST-6 extract was added to the medium at each media change. All the cultures were maintained at 37°C in a humidified atmosphere of 5% CO2 in air. After 8 days, cells were washed with PBS (pH 7.4) and fixed with ethanol : acetone (1:1 v/v) for 1 min. Culture plates were dried at room temperature for 10 min, the cells were stained for TRAP cells and counter stained with methyl green. The cells containing three or more nuclei were counted as multinucleated cells. The results are expressed as the Mean ± SEM of triplicate culture.

**Statistical analysis**

The values are expressed as Mean ± SEM. The results were analyzed statistically using one way ANOVA followed by Post Bonferroni’s multiple comparison tests using Prism software.
package to find out the level of significance. The minimum level of significance was fixed at $p<0.05$.

**RESULTS**

**Effect of OST-6 on $1\alpha$, 25-(OH)$_2$D$_3$-induced OCLs formation**

Figure 1 shows the effect of OST-6 on the formation of TRAP-positive OCLs induced by $1\alpha$, 25-(OH)$_2$D$_3$ at a concentration of $10^{-8}$ M. No cytotoxicity was observed with OST-6 at this concentration.

**Serum and urine biochemistry**

Ovariectomized rats (Group-II) showed significantly increased levels of serum alkaline phosphatase ($142.13 \pm 6.80$), urinary excretion of hydroxyproline ($126.05 \pm 13.09$), pyridinoline ($18.88\pm3.38$) and deoxypyridinoline ($6.42 \pm 1.87$) levels compared to sham operated control (Group-I). It was noticed that there was significant reduction in the levels of bone calcium ($75.21 \pm 6.15$) and inorganic phosphorus ($16.12 \pm 1.05$). However there were no significant changes in the levels of serum calcium and inorganic phosphorus levels. Treatment with OST-6 in Group- IV significantly prevented the elevation of serum alkaline phosphatase levels. There was a significant increase in bone calcium and inorganic phosphorus levels in animals treated with OST-6 at 250 and 500 mg/kg dose (Group-III and IV) compared to ovariectomized rats (Group-II). Elevation of urinary excretion of osteoclastic activity markers viz., hydroxyproline, pyridinoline and deoxypyridinoline levels were prevented significantly in Group-IV (Table 1).

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<th>Table 1: The effect of OST-6 on serum, bone and urinary biochemical markers in ovariectomized rats (n=8)</th>
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* $p<0.01$ as compared GII vs GI; • $p<0.01$ as compared GII vs GIII; * $p<0.01$ as compared GII vs GIV

**Histomorphology**

Histologically the sections of distal third of femur in the region proximal to the epiphyseal growth plate were examined for the changes. Animals of Group-I showed normal compactness of diaphysis and competent trabeculae (Figs. 2a and 2b). Group-II animals showed sparse, uniform thinning of trabeculae resulting in widened inter trabecular spaces.
(Figs. 3a and 3b). Cartilaginous proliferates in the areas of softened plates of focal to restricted islets were observed. Animals of Groups-III and IV showed minimum number of thin trabeculae and less frequent cartilaginous proliferation (Figs. 4a and 4b). Femur sections stained for reddish TRAP positive cells were more noticed in Group-II (Fig. 5b) compared to Group-I (Fig. 5a). In Group-III the TRAP positive nuclei were less in their number (Fig. 5c).

**DISCUSSION**

Both estrogen and dietary calcium deficiencies are important risk factors in the pathogenesis of osteoporosis. Post-menopausal osteoporosis is considered to result from ovarian exhaustion. Age related bone loss is greatly accelerated in women after the menopause and women lose approximately 30% of their cortical bone during their life time (Ettinger et al., 1985; Nuki 1998). The present study creating experimental model of menopause by ovariectomy indicated that the model provided the needs of the study.

The oral administration of OST-6 to ricketic rats restored bone mineral content (Mitra et al., 2000). This indicates that OST-6 could prevent the progress of bone loss induced by ovariectomy. In the present study, ovariectomized rats developed bone changes similar to those seen in osteoporotic women as indicated by decrease in bone mineral content. OST-6 treatment demonstrated a dose dependent increase in the bone mineral content and bone mass in ovariectomized rats. The unchanged levels of calcium and phosphorous in plasma indicates that homeostatic mechanisms were able to maintain plasma levels of these minerals despite
ovariectomy. Fasting urinary calcium excretion is also a useful variable for estimating net bone resorption. OST-6 treatment showed decreased urinary calcium excretion suggesting that more calcium was deposited in bone. Serum alkaline phosphatase is an important biochemical marker of bone formation. The levels of this enzyme are increased in osteoporosis and other bone metabolic disorders due to increased bone turnover (Victor, 1993). OST-6 treatment restored the increased levels of alkaline phosphatase indicating its beneficial effect in bone formation.

Fig. 4: Epiphyseal region showing moderately thick elongated trabeculae and narrowed inter trabecular spaces in Group III [H&E, (4a) x 100; (4b) x 250]

Fig. 5: Femur sections showing reddish stained TRAP positive osteoclasts (↑) (TRAP, x 1000) (5a) Group-1 (5b), Group II (5c) Group-III

In osteoporosis, depending on the exact site of cleavage, the cross-links found in the urine include free pyridinoline, deoxypyridinoline, N-telopeptides and C-telopeptides (Rosen and Tenenhouse, 1998). Pyridinium cross-links are most specific and sensitive markers available for bone resorption (Eyre 1992). Pyridinoline (Pyr) is the major pyridinium cross-link and is widely distributed in skeletal tissue (Ogawa et al., 1982). Deoxypyridinoline (dPyr), is a minor cross-link and found predominantly in bone and dentin (Robins 1990). Due to slow
turn over of dentin, dPyr is considered to be bone specific (Borggreven 1979). Urinary excretion of hydroxyl proline is also one of the commonly used indices of bone resorption. OST-6 treatment revealed a dose-dependent decrease in the urinary excretion of pyridinoline, deoxypyridinoline and hydroxyproline in ovariectomized rats, indicating that the drug inhibits bone resorption.

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Osteoclastic bone resorption is thought to occur by the formation of new osteoclasts (OCL’s) and activation of mature OCL’s. It is reported that calcitropic hormones like 1α,25-(OH)₂D₃ stimulates the formation of OCL’s (Li et al., 1999). OST-6 dose dependently inhibited the formation of osteoclast like cells in in-vitro studies in the presence of 1α,25-(OH)₂D₃. This is further correlated with in-vivo studies as indicated by less TRAP-positive multinucleated cells in OST-6 treated group as compared to ovariectomized rats.

Like humans, rats have cancellous bone that undergoes remodeling once longitudinal growth has essentially ceased. In adult rats, oophorectomy is followed by an increase in bone turnover associated with bone loss and a permanent deficit of bone mass at several skeletal sites rich in trabecular bone, such as the vertebral bodies, the proximal femur, and the metaphyses of long bones such as the distal femur and proximal tibia. The micro-architectural alteration in cancellous bone is as similar to those observed in postmenopause and age dependent (Bonjour et al., 1999). There is also a transient increase in the endochondral growth, periosteal apposition and cancellous bone turn over with resorption exceeding formation (Miller et al., 1995). The histological findings are in concurrence with the earlier reports (Bonjour et al., 1999). The restoration of trabecular bone with less number of TRAP positive multinucleated OCL’s was noticed in OST-6 treated rats indicates its inhibition of bone resorption and elution of bone calcium. These observations are well correlated with serum ALP and bone mineral content.

In conclusion, OST-6 treatment in the adult rat model of osteoporosis showed desired effects on the inhibitors of bone resorption and stimulators of bone formation, thereby indicating a potential therapeutic usefulness as an anti-osteoporotic agent.

REFERENCES


