INTRODUCTION

Osteoporosis is a major health problem in the elderly population, particularly females. Osteoporosis related disabilities take an enormous personal and economic toll. It is currently estimated that 200 million women worldwide suffer from osteoporosis.1

Therapeutic drugs for osteoporosis are mostly anti-catabolic agents which decrease bone turnover by inhibiting bone resorption. They have minimal to moderate effect on bone mineralization, hence cannot reverse osteoporosis in patients who have lost a significant amount of bone mass. The nitrogen-containing bisphosphonates blunt the resorptive component of bone homeostasis by inhibiting farnesyl-pyrophosphate synthetase step of the mevalonate pathway.2 A very interesting finding is the observation that HMG-CoA reductase inhibitors (statins) inhibit the same pathway further upstream. Hence they could be equally effective at preventing osteoclast activation.3 Statins, possibly, also enhance osteoblastic activity by increasing synthesis of bone morphogenetic proteins (BMP-2).4 BMP-2 is a growth factor that stimulates growth and maturation of osteoblasts. Discovery of a drug that stimulates bone formation, when used alone or synergistically with drugs that inhibit resorption and be able to restore bone strength, would constitute a major breakthrough in osteoporosis treatment.

In vitro cell cultures and in vivo studies in animals have almost consistently demonstrated that statins stimulate bone formation.5,6 However, clinical trials have yielded discrepant results.7,8 One possible explanation for this inconsistency could be that statins, prescribed for treatment of hyperlipidemia, target the liver and achieve low concentration in peripheral tissues; hence reach the bone micro-environment in low, ineffective concentration. Secondly, therapeutic doses used in animal models of osteoporosis are many times higher than human doses. Moreover, 40 - 75% of oral dose of statins is absorbed from gut. Studies have demonstrated bone promoting effect through bypassing hepatic metabolism, e.g. via local application of trans-dermal patches.9 Lovastatin nanoparticles, delivered as an injection into the site of fractured femur in rats, increased healing rate and decreased fracture gap at 4 weeks.10 The lipophilicity of individual statins might affect their ability to penetrate...
into peripheral cells including bone cells. Hydrophilic molecules like pravastatin may not reach the bone micro-environment and not found effective in stimulating bone formation.\textsuperscript{11} Therefore, the response may vary depending upon the statin property, dose and route of administration. Pitavastatin is lipophilic and having high bioavailability than other statins, could achieve higher blood levels at peripheral target tissue sites.

The purpose of this study was to determine the effect of pitavastatin in attenuating skeletal changes induced by bilateral-ovariectomy in rats.

**METHODOLOGY**

The study was performed in accordance with the Jinnah Postgraduate Medical Centre. Guidelines for use of experimental animals, from January to July 2013. It was approved by the Animal Ethics Committee (F.1-2BMSI-E/012/JPMC, dated 15th October 2012) of the Institute.

Female Sprague Dawley rats, 3 months old, weighing 200 - 250 gms, bred in animal house of Basic Medical Sciences Institute, JPMC, Karachi, were randomly divided into 4 groups. Group C included (n=10) Sham OVX rats receiving 0.5 ml/day of distilled water. Group-OVX included (n=8) OVX rats receiving 0.5 ml/day of distilled water. Group-1P comprised (n=10) OVX rats receiving pitavastatin (0.4 mg/kg/day) orally. This is equivalent to the highest therapeutic dose. Group-2P comprised (n=10) OVX rats receiving pitavastatin (0.8 mg/kg/day) orally. This is equivalent to double the highest therapeutic dose. Body surface area was used for conversion from animal to human dose and vice versa.\textsuperscript{12} Formula for dose translation was based on body surface area as animal dose (mg/kg) = human dose (mg/kg) x human Km/animal Km, human dose of 4 mg pitavastatin/60 kg = 0.06 mg/kg and animal dose in mg/kg = 0.06 x 37/5.9 = 0.376 = approx. 0.4 mg/kg. Pitavastatin suspension (0.4 mg/ml) was prepared by dissolving a tablet and making the volume upto 10 ml in distilled water. For example, a dose of 0.4 mg/kg for a rat weighing 250 gm was 0.1 mg. As 0.4 mg pitavastatin was present in 1 ml solution, therefore, volume given to this rat was 0.25 ml.

Animals were fasted overnight with free access to water only. Using all aseptic precautions, bilateral ovariectomy was performed under ether anesthesia using the double dorso-lateral approach method.\textsuperscript{13} Sham surgery was performed in Group C following all the above steps but the ovaries were replaced in the abdominal cavity following visualization without clamping or excision. Treatment was started on the fifth postoperative day and continued for 8 weeks. Drugs were given by oral-feeding tube. There were two deaths in OVX Group during this period. The animals were killed by exsanguination. Ovariectomy was confirmed at necropsy by the absence of ovaries and presence of markedly atrophic uterine horns. In the Sham group both ovaries and uterine horns were normal in size and vascularity.

Blood samples were collected and allowed to coagulate for 20 minutes at room temperature and then centrifuged at 3000 rpm for 10 minutes. Serum was separated, portioned in two, and kept frozen at -20°C until analysis. Using kits from Randox Laboratories, UK, the ALP and ACP levels were determined spectrophotometrically by colorimetric method. Femora and tibiae were removed, cleaned of all attached muscles and tissues, and preserved in ethanol for further studies.

Bone density of the right femur and tibia was measured according to method of Broulik et al. with slight modification.\textsuperscript{14} The right femora and tibiae were put in unstoppered glass bottles completely submerged in de-ionized water and left in a desiccator for 24 hours. The bone volume was determined using IITC Life Science Plethysmometer, USA (sensitivity (0.01 ml). The bone was attached to a fine silk and just submerged completely in distilled water (density 1 gm/cm\textsuperscript{3}) and volume read from the display. The bones were then blotted on a tissue and weighed using a Mettler Balance with a sensitivity of 0.1 mg.

Density was calculated by the formula (A / B) x P, where A = weight of hydrated bone out of water, B = volume of hydrated bone in water, and P = density of distilled water at room temperature.

The femora and tibiae were preserved in 70% alcohol till CT scan using Toshiba Aquilon (16 slice) Model. The bones were scanned with a voltage of 120 kV, a tube current of 30 mAs and 0.5 mm slice thickness. The HU numbers were obtained at the lower metaphysis of femur and the upper metaphysis of tibia. An average of three readings was taken for each end. HU obtained by CT scan has been shown to correlate strongly with Dual Energy X-ray Absorptiometry (DEXA) scores and provide an alternative method in determining regional bone mineral density for diagnosis and evaluation of osteoporosis treatment.\textsuperscript{15}

All data were presented as mean ± standard deviation. Comparison of data for effect of pitavastatin on Hounsfield index, bone density, acid phosphatase, and alkaline phosphatase values were analyzed by one-way ANOVA and multiple comparisons were performed by Scheffe’s post hoc test. P-values < 0.05 were considered significant. The data was analyzed using SPSS version 19.

**RESULTS**

As shown in Table I and Figure 1, HU at the distal femoral metaphysis decreased from 610.60 ±110.55 in Sham to 458.50 ±82.28 in OVX (p=0.004). At 0.4 mg/kg/day, the HU value of 454.60 ±65.10, remained significantly lower than Sham (p = 0.002) and there was
Pitavastatin prevents osteopenia in rats


At the proximal tibial metaphysis, OVX resulted in significant reduction in HU from 595.70 ±80.87 in Sham to 358.63 ±77.48 (p < 0.001). At the lower dose of pitavastatin, HU value of 412.80 ±37.95 remained significantly lower than Sham (p < 0.001). 0.8 mg/kg produced significantly increased HU value 461.50 ±41.83 in relation to OVX (p=0.014); but it remained significantly lower than Sham level (p < 0.001).

As shown in Table I and Figure 2, there was a significant reduction in density of whole femur in OVX Group in comparison to Sham Group 1.50 ±0.10 vs. 1.75 ±0.09 (p=0.049). Low dose of pitavastatin did not produce a significant increase in femoral density above OVX level 1.52 ±0.14 vs. 1.50 ±0.10 (p=0.995). Eight weeks of pitavastatin (0.8 mg/kg/day) administration, restored femoral density to Sham level 1.75 ±0.09 vs. 1.86 ±0.28 (p=0.621). The tibial density showed a significant decline at 8th week post-ovariectomy from 1.91 ±0.17 to 1.44 ±0.14; (p < 0.001). 0.4 mg/kg did not produce a significant elevation in density in comparison to OVX value 1.44 ±0.14 vs. 1.54 ±0.11; (p=0.565). Although 0.8 mg/kg/day significantly increased tibial density in OVX rats from 1.44 ±0.14 to 1.66 ±0.23; (p=0.010); but this remained significantly lower than Sham OVX i.e. 1.66 ±0.23 vs. 1.91 ±0.17; (p=0.029).

As shown in Table I and Figure 3, comparison at 8 weeks post-ovariectomy showed significant increase in bone formation marker ALP (90.55 ±13.46 vs. 109.76 ±12.42; p=0.039) and bone resorption marker ACP (2.38 ±0.18 vs. 2.88 ±0.21; p=0.011) in Sham compared with OVX Group. Treatment with pitavastatin (0.4 mg/kg) did not produce significant improvement in ALP (109.76 ±12.42 vs. 109.39 ±10.64; p=1.000) and ACP (2.88 ±0.21 vs. 2.90 ±0.42; p=0.999) levels over the OVX Group values. However, 0.8 mg/kg/day administered for 8 weeks, the bone markers were restored to levels comparable to Sham, i.e. ALP (90.55 ±13.46 vs. 88.87 ±12.93; p=0.995) and ACP (2.38 ±0.18 vs. 2.38 ±0.19; p=0.995).

### Table I: Comparison of tibial and femoral densities and bone turnover markers from ovariectomized and Sham-ovariectomized rats and ovariectomized rats given 8 weeks of pitavastatin treatment.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>OVX (n=8)</th>
<th>Sham (n=10)</th>
<th>Pitavastatin 0.4 mg/kg/day (n=10)</th>
<th>Pitavastatin 0.8 mg/kg/day (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HU Lower femur</td>
<td>458.50 ±82.28</td>
<td>610.60 ±110.55*</td>
<td>454.60 ±65.10*</td>
<td>574.20 ±51.34*</td>
</tr>
<tr>
<td>HU Upper tibia</td>
<td>358.63 ±77.48</td>
<td>596.70 ±80.87*</td>
<td>412.80 ±37.95*</td>
<td>461.50 ±41.83*</td>
</tr>
<tr>
<td>Density femur (g/cm³)</td>
<td>1.50 ±0.10</td>
<td>1.75 ±0.09*</td>
<td>1.52 ±0.14</td>
<td>1.86 ±0.28*</td>
</tr>
<tr>
<td>Density tibia (g/cm³)</td>
<td>1.44 ±0.14</td>
<td>1.91 ±0.17*</td>
<td>1.54 ±0.11^</td>
<td>1.70 ±0.15^</td>
</tr>
<tr>
<td>ALP (IU/L)</td>
<td>109.76 ±12.42*</td>
<td>90.55 ±13.46*</td>
<td>109.39 ±10.64*</td>
<td>88.87 ±12.93*</td>
</tr>
<tr>
<td>ACP (IU/L)</td>
<td>2.88 ±0.21</td>
<td>2.38 ±0.18*</td>
<td>2.90 ±0.42^</td>
<td>2.38 ±0.19^</td>
</tr>
</tbody>
</table>

* Statistically significant as compared to OVX p < 0.05
^ Statistically significant as compared to Sham p < 0.05
∆ Statistically significant as compared to IP p < 0.05
DISCUSSION

The ovariectomized rat is a classical model used to mimic osteoporotic bone loss in post-menopausal women. It is a very commonly used in vivo model for investigating osteoporosis and its response to treatment. In this study, the ovariectomized rats were found to have marked osteoporotic bone changes as evidenced by a significant decrease in Hounsfield indices of the distal femoral and proximal tibial metaphyses. These sites were chosen because statistically significant bone loss is found in proximal tibial metaphysis at 4 weeks post-ovariectomy. The distal metaphysis of femur has also been shown to be exquisitely sensitive to estrogen deprivation. The shafts of long bones are composed mostly of compact bones; whereas, trabecular bone dominates in metaphysis and epiphysis.

HU are standard numbers derived from CT imaging which represent relative density of body tissues according to a calibrated gray-level scale based on 1000 HU for air and zero HU for water. HU has been found strongly correlated to tissue density; hence, CT scan bone densitometry is deemed a reliable method to analyze bone density.

Very significant decrease in bone density of whole femora and tibiae in OVX rats was also reflected in data calculated by the Archimedes principle. Application of the Archimedes equation is a relatively simple method and does not require expensive instruments. It is equally effective to DEXA in determining differences in bone density, as densities obtained by both these methods are highly and significantly correlated.

Bone mass measurements are often combined with bone turnover markers to improve assessment of osteoporosis. Plasma ACP is one of the parameters for assessment of bone resorption and ALP for bone formation. Decrease in bone markers during therapy reflects increase in bone mineral density. The obtained results show significant elevation of bone turnover markers in OVX rats in comparison to Sham OVX animals indicating a high rate of metabolism, i.e. resorption and formation.

In order to elucidate the effect of pitavastatin on the development of osteopenic changes, ovariectomized rats were administered doses of 0.4 mg/kg and 0.8 mg/kg corresponding to maximum therapeutic and supra-therapeutic doses, respectively. These doses were chosen keeping in mind that bioavailability and systemic concentration of most statins are less than 5% and they achieve low levels in peripheral targets including bones. This has been cited as one of the reasons for failure of statins to produce reproducible biologic effects on bones in clinical studies. However, the distinctive feature of pitavastatin is its high bioavailability and good lipid solubility which makes pitavastatin highly suitable for investigating its effects on bones.

These results showed that therapeutic dose of pitavastatin, given orally to OVX rats, did not significantly improve HU value of distal femur, proximal tibia or the whole bone density measurements above the OVX group values. In contrast administration of supra-therapeutic doses, resulted in highly significant increase in HU values at lower metaphysial ends and whole femur density, approximately comparable to the Sham OVX animals. There was a difference in response of femora and tibiae to the higher dose of pitavastatin. Although significant improvement in these parameters was observed in tibia when compared to OVX rats, but it remained significantly lower than the Sham OVX animals even after 8 weeks of administration of pitavastatin. This is possibly because following estrogen depletion, bone loss is not uniform with the cancellous bone showing greater depletion than cortical bone. This is followed by a slower rate of bone formation. Osteoporotic changes appear to be site specific. The proximal tibia metaphysis which is rich in trabecular bone loses significant amount of bone at 14 days postsurgery and reaches steady state at 90 days. Therefore, osteoporotic changes at this site are more intense and would require therapy for a longer duration or a higher dose in order to full magnitude of effect to be apparent. The biomarkers of bone remodelling were notably elevated in OVX and low dose groups, which indicated a high bone turnover in these animals. Administration of high dose pitavastatin produced a very significant decrease in level of these bone metabolic markers back to a level approximating the Sham Group. This shows that the higher dose of pitavastatin is able to decrease rate of bone metabolism and increase osteogenic activity. Other studies have also shown that osteogenic effect of statins is dose-related. Pytlík et al. showed that simvastatin (3 and 6 mg/kg) administered to ovariectomized rats intensified bone formation, decreased bone resorption, and improved mechanical properties with stronger activities produced with the higher dose. Gutierrez et al. demonstrated lovastatin applied transdermally accelerated fracture healing. However, the oral dose required to have an effect on bone was ten times higher. A study using simvastatin coated titanium wire to stabilize rat tibial fracture verified accelerated healing at high doses. Normal therapeutic doses have been found to have minimal efficacy in osteoporosis treatment. This would require enhanced delivery to bones.
CONCLUSION

Pitavastatin is a bone anabolic agent which prevented estrogen-depletion-induced osteoporosis in rats, when orally administered in high doses.

REFERENCES