A Comparison of Estrogen and Two Different Doses of Calcitonin in Ovariectomized Rats

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The purpose of this study was to investigate the treatment efficacies of salmon calcitonin (SC) and estrogen in a type-I osteoporotic rat model.

Sixty, 3-month-old, female Wistar rats were divided into six groups. The first group was used as the control, and the second a sham, the other four were surgically ovariectomized. 24 hours after the ovariectomy, they were either left untreated (OVX), or treated with an injection of either 17-β estradiol (E) 30 mcg/kg/24 hours, low-dose calcitonin (LDC) 10IU/kg/48 hours or high-dose calcitonin (HDC) 20 IU/kg/48 hours. 6 weeks later, the bone densities were measured by DEXA, the animals sacrificed and the femurs harvested for histomorphometric evaluation.

The bone mineral densities (BMD) of the spine and proximal femur were lower in the OVX group, but only the values of the spine BMD were statistically significant. The BMD of the spine seemed to be preserved with all the treatments. The histomorphometric evaluation revealed that after the OVX the decrease in the trabecular volume was prevented by all the treatments. However, significant changes in the indices of bone formation were not shown.

In conclusion, all the treatments prevented bone lost in the ovariectomized rats. Histopathological measurements of bone formation are unlikely to provide any evidence for the effects of these agents on the osteoblastic function. In the animal model of estrogen depletion, our results suggest that the calcitonin provides an important alternative therapy for postmenopausal osteoporosis.

**Key Words:** Estrogen, calcitonin, rat

INTRODUCTION

Osteoporosis is accepted as one of the most important chronic diseases with regard to death, and its functional dependence and social cost. Postmenopausal bone loss is mainly due to the loss of gonadal functions. Estrogen affects bones by acting directly on its related receptors, which are located on osteoblasts and osteoclasts. Estrogen causes a depletion in the number of the osteoclast cells in bone by inhibiting the maturation at the cell level. It has been suggested that cytokines also play an important role in this mechanism. Estrogen inhibits the maturation of the osteoclasts, via the cytokines, and also enhances the synthesis of the cytokines which play roles in bone formation. The major action of estrogen is in the suppression of bone loss. However, the stimulating role of estrogen in bone formation is still controversial. The use of hormone replacement therapy (HRT) has some restrictions due to the potential risks of breast cancer, venous thromboembolism, migraine, coronary heart disease and strokes. Besides these, its effect on the bone mineral density depends greatly on the time when the therapy is initiated, and the total duration of its use. In women not taking hormone replacement therapy, calcitonin has been shown to be a good alternative in the treatment of postmenopausal osteoporosis. The basic action of calcitonin is in the inhibition of bone resorption, however, it has also been strongly argued that it has an anabolic effect on cartilage forma-
tion, bone matrix synthetic activity and bone growth.\textsuperscript{17} However, in a recent randomized controlled trial, calcitonin was reported to have a dose-dependent effect. A significant reduction in vertebral fractures was seen in osteoporotic women taking a daily dose of 200 IU calcitonin, but not in those taking either a lower (100 IU) or higher (400 IU) dose.\textsuperscript{85}

The ovariectomized (OVX) rat is considered a good animal model of postmenopausal osteoporosis, and has become a very popular model to investigate the effects of estrogen deficiency on the metabolism of bones at various skeletal sites.\textsuperscript{20,21} OVX rats can also serve as an animal model to investigate the effects of therapeutic agents on bone mass, structure and turnover. The loss of bone in OVX rats is usually earlier and more substantial in areas of cancellous bone than cortical bone. In studies trying to evaluate the effects of calcitonin on OVX rats, doses of subcutaneous calcitonin were varied from 5 to 20 IU/kg, with administration on alternate days.\textsuperscript{21-24} In these studies calcitonin was found to be a useful agent in protecting cancellous bone loss. However, the detailed effects of different doses of calcitonin, compared to estrogen, remain to be evaluated. Therefore, the objective of this study was to investigate the efficacies of low and high doses of salmon calcitonin, and their comparison with estrogen treatments, by evaluating bone turnover, bone histomorphometry and bone mass measurements, in OVX rats.

**MATERIALS AND METHODS**

Sixty, 3-month-old, female Wistar rats, weighing an average of 182g (150-210), were used as the experimental animals in this study. The rats were housed in special plastic rat cages, four per cage, with a 12 h light/dark cycle. They were fed a standard rat diet, with a 3.36% calcium content, and allowed water \textit{ad libitum}. 24 hours after the ovariectomy they rats were divided into six groups. The first group was used as the control, and the second was a sham group, operated on under ketamine and rhompun anesthesia. The other four groups were surgically ovariectomized, with a dorsal approach, and left untreated, or treated with either 17-β estradiol (E2) 30 mcg/kg/24 hours, (Sigma, St. Lois, MO, USA) or calcitonin (Miacalcic\textsuperscript{25} Amp. by Novartis-Pharma) at a low-dose (LDC) 10IU/kg/48 hours, or a high-dose (HDC) 20IU/kg/48 hours, as shown in Table 1. The rats were weighed at the end of every week, and drug dosages adjusted according to the weight gains.

After 6 weeks, all the animals were anesthetized 12 hours after the cessation of feeding. Bone density measurements were then performed using a dual-photon x-ray absorptiometry (DEXA) bone densitometry (Lunar, Madison, WI, USA) and the Small Animal Software, version 4.6d, which has previously been stated to be a useful tool in the study of small animals.\textsuperscript{25} The rats were positioned on the middle of the measurement tables, and a series of transverse scans obtained from the tip of the nose, to the midpoint of the tail, of the rat. The high-middle resolution mode was selected as the scanning, with each measurement completed in approximately 20 minutes. During the measurements, 150\textmu Amp of power, and a 0.24 mRem radiation dose, were produced by the scanner. All measurements were performed by the same technician, and analyzed by the same method, to minimize operational errors. Boxes

**Table 1.** Grouping and Treatment Protocols of Rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Number</th>
<th>Operation</th>
<th>Treatment</th>
<th>Dose and administration of rats route of the drug</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>10</td>
<td>none</td>
<td>untreated</td>
<td></td>
</tr>
<tr>
<td>Group II</td>
<td>10</td>
<td>sham</td>
<td>untreated</td>
<td></td>
</tr>
<tr>
<td>Group III</td>
<td>10</td>
<td>Ovariectomy</td>
<td>untreated</td>
<td></td>
</tr>
<tr>
<td>Group IV</td>
<td>10</td>
<td>Ovariectomy</td>
<td>OVX + E2</td>
<td>30 mcg/kg/24 hours, SC injected</td>
</tr>
<tr>
<td>Group V</td>
<td>10</td>
<td>Ovariectomy</td>
<td>OVX + LDC</td>
<td>10 IU/kg/48 hours, SC injected</td>
</tr>
<tr>
<td>Group VI</td>
<td>10</td>
<td>Ovariectomy</td>
<td>OVX + HDC</td>
<td>20 IU/kg/48 hours, SC injected</td>
</tr>
</tbody>
</table>

OVX, Ovariectomy; E2, 17-β estradiol; LDC, low-dose calcitonin; HDC, high-dose calcitonin.

Table 2. The Means and Standard Deviations of the Biochemical Parameters for Each Group, with Their Statistical Significance

<table>
<thead>
<tr>
<th></th>
<th>Estrogen (pg/ml)</th>
<th>Parathormon (pg/ml)</th>
<th>Osteocalcin (ng/ml)</th>
<th>Calcium (mg/ml)</th>
<th>Inorganic Phosphate (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>514.2 ± 259.9&lt;sup&gt;ad&lt;/sup&gt;</td>
<td>0.85 ± 0.16</td>
<td>1.8 ± 1.1</td>
<td>10.1 ± 0.2</td>
<td>7.3 ± 1.0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Group II</td>
<td>298.4 ± 216.6&lt;sup&gt;ad&lt;/sup&gt;</td>
<td>1.79 ± 1.85</td>
<td>1.4 ± 0.3</td>
<td>10.0 ± 0.4</td>
<td>7.6 ± 1.7&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Group III</td>
<td>56.3 ± 69.9&lt;sup&gt;bd&lt;/sup&gt;</td>
<td>1.87 ± 2.82</td>
<td>0.9 ± 0.8</td>
<td>9.8 ± 0.6</td>
<td>10.8 ± 3.9&lt;sup&gt;bd&lt;/sup&gt;</td>
</tr>
<tr>
<td>Group IV</td>
<td>249.6 ± 193.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.02 ± 0.73</td>
<td>1.1 ± 1.2</td>
<td>9.8 ± 0.5</td>
<td>7.4 ± 2.6&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Group V</td>
<td>53.8 ± 35.7&lt;sup&gt;bd&lt;/sup&gt;</td>
<td>0.9 ± 0.1</td>
<td>1.7 ± 1.0</td>
<td>9.7 ± 0.6</td>
<td>8.6 ± 1.6&lt;sup&gt;ad&lt;/sup&gt;</td>
</tr>
<tr>
<td>Group VI</td>
<td>100.0 ± 140.0&lt;sup&gt;bd&lt;/sup&gt;</td>
<td>1.04 ± 0.35</td>
<td>2.0 ± 1.5</td>
<td>9.7 ± 0.6</td>
<td>7.9 ± 1.3</td>
</tr>
</tbody>
</table>

<sup>a</sup>p<0.001, <sup>b</sup>p<0.05 (Kruskal Wallis one-way analyses of variance test).
<sup>c</sup>significantly different from group-I, <sup>d</sup>significantly different from group-II, <sup>e</sup>significantly different from group-III, <sup>f</sup>significantly different from group-IV, <sup>g</sup>significantly different from group-V, <sup>h</sup>significantly different from group-IV (Mann-Whitney U test).

were drawn to limit the area of concern, and the Bone Mineral Density (BMD), of the lumbar vertebrae and the proximal femur, obtained in g/cm². All the animals were sacrificed by a cardiac puncture before they awaken. After the centrifugation at 2500 rpm for 10 minutes, the serum samples were stored, deep frozen at -20°C, until required. The samples were analyzed for calcium (Ca) and inorganic phosphorus (Pi), with an Olympus AU 600 model autoanalyzer. The plasma levels of parathormon (PTH) and estrogen (E2) were measured by chemiluminescent method, with an Immulyte hormone analyzer (DPC, Washington, USA). The plasma osteocalcin levels were measured using a micro ELISA method, with Biotech test kits (Trinity Biotech, plc, Dublin Ireland).

The right femurs of the rats were harvested and kept in 1.5% silver nitrate (AgNO₃), followed by 5% sodium thiosulphate solutions for 30 and 2 hours, respectively. Finally, the bone samples were left in 10% formic acid solution for 20 hours. Longitudinal sections were cut in 4-μm thickness, stained with hematoxylin-eozin, and assessed in duplicate, at different times, using a Visopan-Reichart microscope employing Zeiss-1 and -2l graters, with the aim of blinding the assessments. The osteoid surface, an index of bone turnover, was stained pink, and the mineralized cancellous bone to a gradually reducing black-brown colour towards inner surface. Histopathological analyses were carried out on the BV/TV for the bone volume (trabecular bone volume to tissue volume), OV/BV for the osteoid volume (osteoid volume to trabecular volume), Ob.S/BS for the osteoblast surface (osteoblast surface to trabecular surface) and the Oc.S/BS for the osteoclast surface (osteoclast surface to trabecular surface), ratios.

Descriptive statistics were performed on the continuous variables, with the results presented as the mean ± standard deviation, at 95% confidence intervals. The Kruskal-Wallis test, a nonparametric equivalent to a one-way ANOVA, was used for comparing the continuous variables of several independent samples, and the Mann-Whitney test if a significant difference was found on post-hoc tests.

RESULTS

Weight gains were observed in all the rats during the course of the study, but with no differences seen between the groups. The final mean body weights in groups-I to VI were 201 ± 10.5, 204.5 ± 18.8, 198.5 ± 18.7, 200 ± 17.9, 198.5 ± 11.3 and 204.5 ± 11.6, respectively.

The mean estrogen levels in groups I and II were higher than in groups III, V and VI, (p<0.001 and p<0.01), (p<0.01) and (p<0.01), respectively, but did not differ significantly between groups II and IV (p>0.05) (Table 2). In group-IV, the estrogen level was higher than groups III and
V ($p<0.01$). Based on these results, the OVX procedure, and the effect of E2 treatment on increasing the estrogen levels were proved. There were no statistical differences in the effects of parathormon, osteocalcin and calcium levels between the groups ($p>0.05$). The iPTH levels tended to be rise after the ovariectomy ($p<0.05$) in group III compared to those in groups I and II, while those in groups IV and VI were close to that of group I ($p>0.05$), but those in group V were higher than those in groups I and IV ($p<0.05$).

There were reduction in the spine BMD values after the ovariectomy ($p=0.001$) in group III compared with groups I and II (Table 3), whereas in groups IV, V and VI, the values seemed to be preserved ($p<0.01$, $p<0.01$ and $p<0.001$, respectively) compared to those in group III. However, there were no statistical differences between these groups. On the other hand, despite the slightly low BMD in group III in the proximal femur, no statistically significant differences were found to exist between the groups ($p>0.05$).

In the histopathological evaluation the trabecular bone volume was observed to differ significantly between the groups (Fig. 1), with the bone volume being decreased after the OVX process. In group III, the value was significantly lower than those in groups I and II ($p<0.01$), but those in groups IV, V and VI were similar to groups I and II ($p>0.05$). Thus, it can be stated that the bone loss due to the OVX procedure might be prevented completely with E2 and calcitonin treatment. Treatment with E2, LDC or HDC, decreased the rise in the osteoclast surface in O VX rats. No significant changes in indices of bone formation, such as new osteoid tissue formation and osteoblast surface, were shown in this study, with no statistical significant differences found for the OV/BV and Ob.S/BS values between the groups.

**DISCUSSION**

A measure of the efficacy of a therapeutic agent in preventing osteoporosis is its capability to depress bone turnover; on the other hand, an increase in the BMD and decrease in the fracture rate are more important in assessing the efficiency of a treatment. Cancellous bone loss, after an OVX procedure in the female rats, has been shown in previous studies. The mechanism for bone loss is a function of the negative imbalance of bone resorption and formation. Soon after the OVX procedure, 400 and 270% rate increases in bone resorption and formation occurs, respectively, and consequently a detectable bone loss appeared on the 14th day. Animals have been used in previous studies as a reliable vehicle for assessing the efficacy of different agents in preventing bone loss, hence the OVX rat model was used in this study. In studies that try to evaluate the effects of the antiresorptive agents, the question is, 'what is the most appropriate time to initialize the treatment'. Histopathological changes have been reported as early as the 5th day following an ovariectomy in rats. Therefore, the treatment has to be initialized as soon as possible in early period.

In a previous study using a rat model, the effects on the bones of a hysterectomy and ovar-

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**Table 3. The Comparison of the BMD Values in the Spine and Proximal Femur Regions of the Rats**

<table>
<thead>
<tr>
<th>Group</th>
<th>Lumbar spine (g/cm²)</th>
<th>Proximal femur (g/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>0.237 ± 0.01</td>
<td>0.310 ± 0.02</td>
</tr>
<tr>
<td>Group II</td>
<td>0.238 ± 0.01</td>
<td>0.311 ± 0.03</td>
</tr>
<tr>
<td>Group III</td>
<td>0.218 ± 0.01*</td>
<td>0.276 ± 0.03</td>
</tr>
<tr>
<td>Group IV</td>
<td>0.241 ± 0.01</td>
<td>0.299 ± 0.03</td>
</tr>
<tr>
<td>Group V</td>
<td>0.239 ± 0.02</td>
<td>0.301 ± 0.03</td>
</tr>
<tr>
<td>Group VI</td>
<td>0.247 ± 0.02</td>
<td>0.304 ± 0.05</td>
</tr>
</tbody>
</table>

*p<0.01, (Kruskall Wallis one-way analyses of variance test).

*z=0.001, p=0.001, p<0.01, p<0.01 and p<0.001 for the group III versus groups I, II, IV, V and VI, respectively (Mann-Whitney U test).
Fig. 1. The histomorphometric evaluation of the rat femur; A) Trabecular bone volume to trabecular volume ratios, B) Osteoid volume to trabecular volume ratios, C) Osteoid surface to trabecular surface ratios, D) Osteoblast surface to trabecular surface ratios. Boxplots representing the interquartile ranges, containing 50% of the values. The lines extend from the boxes to the highest and lowest values (excluding extreme values). The circles represent individual values for the rats within each group. The BV/TV ratio for the rats in Group III was lower than for those in groups I and II (p<0.01). With all the treatment groups, the values did not differ significantly from those of groups I and II (p>0.05). No statistically significant differences were found between the OV/BV, OS/BS and Ob.S/BS values for the groups.

Ovariectomy were investigated. In relevant studies, resorption markers were found to be increased, and the total calcium and BMD decreased, which were reversed by estrogen treatment. An increase in the levels of calcium excreted in the urine in the 1st and 3rd weeks after an ovariectomy became
normal in the 6th week. On the other hand, serum phosphate levels were elevated as a consequence of the increased renal tubular re-absorption. In a previous study, while no significant change in the serum calcium levels was recorded, the phosphorus levels were found to be increased from the 6th day following an ovariectomy, but this increase was slowed down by estrogen treatment. Changes in the levels of PTH mostly occur due to changes in serum calcium levels. Secondary rises in the levels of PTH and 1,25 (OH)2D have been reported when the serum calcium levels were significantly decreased after a tiludronate injection. In our study, no changes were observed in the serum calcium, PTH or osteocalcin levels. Calcitonin may induce hypocalemia, which can sequentially induce a pulsed PTH secretion, which is a strong bone anabolic hormone. This study has shown that calcitonin treatment does not affect the secretion of PTH, but the possibility still exists that the calcitonin indirectly induced the PTH secretion, with the time lag between the dosing and measurement, making such pulses undetectable. iP seemed to be a more sensitive resorption marker, and more useful in the monitoring of the effects of antiresorptive treatments. Calcitonin was found to suppress the iP in the OVX rats in a dose-dependent manner.

There are still no reference values or standard sites available for BMD measurements in rats. Therefore, previous studies have performed measurements at different sites, such as the vertebrae, proximal-distal femur, femur shaft, proximal-distal tibia or tibia shaft. Bagi, et al. published that the proximal femur was the most appropriate site in OVX rats. In contrast; another study suggested the lumbar spine and the metaphysis of the tibia for reflecting the changes in OVX rats. For our study, the lumbar spine and proximal femur regions were selected as the measurement sites. Further studies on the precision, measurement sites and reference values for BMD measurements in rats may further clarify the situation.

In this study, the bone densitometry measurements showed that E2 and calcitonin treatments prevented the bone loss caused by an ovariectomy, but no significant increase in the cancellous bone volume was observed. With estrogen deficiency, the cancellous bone is more affected than the cortical bone, which may explain why, in this study, the BMD values in the OVX rats were decreased in the spine, but not in the proximal femur. The prevention of bone loss has previously been demonstrated with estrogen treatment, which diminishes the osteoclast number and depresses bone turnover. However, the effects of estrogen on osteoblasts still remain unclear. An increase in the cancellous bone volume has been seen in the first 6 days, but this returned to the initial values by the 22nd day. The effects of calcitonin on the bone mass in rats, with artificial osteoporosis due to various conditions, has been studied previously. In two of the studies, the decrease in the bone mass in OVX or glucocorticoid-induced osteoporosis groups was reversed with calcitonin treatment, as follows: Eel calcitonin, 5 IU/kg, 4 times a week for 12 weeks, and salmon calcitonin, 20 IU/kg every other day for 4 weeks. Two negative studies employed relatively short-term calcitonin treatments, involving salmon calcitonin, 7.5 IU/kg; twice a day for 2 weeks, and eel calcitonin, 1 IU/kg, daily for 4 weeks.

Previous histomorphometric studies have been performed mostly on the proximal tibia, femur and lumbar vertebrae regions of rats, but no site for the optimal analysis of therapeutic agents for osteoporosis in the OVX rat skeleton has been specified. Different sites in OVX rat skeletons may respond to therapeutic interventions in differing manners. For instance, the bone turnover rate was found to be different between the proximal tibia and lumbar vertebrae following OVX. In a recent review, the different affects of antiresorptive therapies on vertebral and non-vertebral fractures were emphasized. We performed a histopathological analysis on the proximal femur only of the rats, which is one of the limitations in our study.

Following OVX, the trabecular bone volume has previously been reported to decrease by 14 to 92%. The histomorphometric indices of bone turnover were suppressed, and trabecular bone losses prevented by treatments with estrogen and calcitonin, however, Li and co-workers failed to confirm these effects. The reduction in the activation found with antiresorptive therapies is associated with a transient increase in the
bone mass, due to the filling-in of the resorption cavities. This is more prominent in cases with a low baseline bone mass and high bone turnover. However, it is unclear whether these agents action involve new cancellous bone formation, or have an independent anabolic effect. In some studies, estrogen\(^{51,52}\) and calcitonin\(^{18}\) were shown to have an independent anabolic effect on the osteoblastic function. However, clinical studies investigating the effects of HRT\(^{84}\) and calcitonin\(^{18}\) have failed to show a marked increase in the BMD, even though a significant decrease in osteoporotic fractures was observed. We found the bone volume was decreased by 33% following an ovariectomy. Whereas, the bone volume was significantly lower in the OVX group compared with the base group, but the BMD values were not significantly different at the proximal femur. Initially, this may appear to be a discrepancy, but it should be remembered that the DXA measured the amount of mineral per unit area, and not the trabecular bone mass. Since the bone loss in the cortical area is slower, a significant decrease in the BMD might not be noticed in DXA scans, where all the cortical and trabecular bone in the scan area was measured. All the treatments seemed to be effective in preserving the bone volume losses, but none had a direct effect on new bone formation. This contrasts with previous studies that have reported the suppression of bone formation by these agents, but these effects were generally attributed to the suppression of bone resorption. This may be related with the lack of, or the delayed, effect of antiresorptive treatments on bone formation. The complete protection of the trabecular bone was provided by the estrogen and the two different doses of calcitonin.

In conclusion, the effects of estrogen and calcitonin on the skeleton are consistent with an antiresorptive action of these agents. The histopathological measures indicated the effects of these agents were mainly on the osteoclastic function, however, they have unlikely have an effect on the osteoblastic function. The effect of calcitonin on rat skeletons does not seem to be dose dependent. These results for the estrogen depleted animal model suggest that calcitonin could provide an important alternative therapy for postmenopausal osteoporosis.

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Skeletal Effects of the Calcitonin in Ovariectomized Rats


