Institute of Physiology, Physiological Chemistry and Nutrition Physiology, Veterinary Faculty, Ludwig-Maximilians-University, Munich, Germany, and
Research Department, Hoffmann-La Roche Ltd, Basel, Switzerland

Osteopenia Caused by Ovariectomy in Young Female Rats and Prophylactic Effects of 1,25-dihydroxyvitamin D₃

B. Kohn, R. G. Erben, H. Weiser, W. A. Rambeck and H. Zucker

Address for correspondence: Dr. W. A. Rambeck, Institute of Physiology, Physiological Chemistry and Nutrition Physiology, Veterinary Faculty, Ludwig-Maximilians-University, Veterinarstr. 13, W-8000 München 22, Germany

(Received for publication May 5, 1990)

Summary

Young female rats were subjected to either bilateral ovariectomy or sham operation. One group of ovariectomized (ovx) animals was treated with the vitamin D metabolite 1,25(OH)₂D₃ after surgery. The effects of ovariectomy and 1,25(OH)₂D₃ treatment on different markers of bone formation (serum alkaline phosphatase, serum bone gla protein) and bone resorption (fasting urinary hydroxyproline) were determined. All rats were euthanized at 7 weeks post ovariectomy and their first lumbar vertebra were processed undecalcified for quantitative bone histomorphometry. A significant decrease in cancellous bone mass was noted in ovx compared with sham-operated rats. This bone loss was associated with increased biochemical markers of bone formation and bone resorption. Furthermore, ovariectomy led to elevated osteoblast perimeter and osteoid parameters as well as an increased osteoclast number. These data indicate that young growing rats develop osteopenia 7 weeks after ovariectomy which goes along with an accelerated bone turnover. Bone gla-protein, alkaline phosphatase and hydroxyproline can be useful markers in studies with ovx rats. Treatment with 1,25(OH)₂D₃ led to a lowered urinary hydroxyproline excretion and a significant decrease of osteoclast number whereas histomorphometrical indices of bone formation were nearly unchanged. Cancellous bone mass increased significantly in ovx rats treated with 1,25(OH)₂D₃ compared with non-treated rats. These results suggest that 1,25(OH)₂D₃ has a prophylactic effect with regard to bone loss in ovx rats which refers to a diminished bone resorption in the high bone turnover condition of ovx animals.

Introduction

Loss of ovarian function is an important factor in the etiology of osteoporosis in women after menopause, and therefore ovariectomy in animals has been used as a model for postmenopausal osteoporosis (6, 15, 20). Numerous studies have shown that bone loss occurs in ovariectomized rats (8, 10, 14, 20, 24). This phenomenon is well documented by chemical, roentgenological and histomorphometrical bone analysis. Less is known about

U.S. Copyright Clearance Center Code Statement: 0931-184X/91/3801-0054$02.50/0
the changes of parameters of bone metabolism in serum and urine after ovariectomy in rats. Studies in human medicine have shown that these biochemical markers correlate with bone resorption (fasting urinary hydroxyproline/creatinine [13]), and bone formation (serum alkaline phosphatase [13] and the bone-specific bone gla-protein [BGP] [3]). The purpose of this study was to investigate which changes of these bone markers occur in rats after ovariectomy. Moreover it was of interest to compare the biochemically measured parameters of bone turnover with histomorphometrical bone parameters.

The most important vitamin D metabolite 1,25-dihydroxycholecalciferol (1,25(OH)2D3) is required for mineralization of the organic bone matrix by providing calcium and phosphate (11), by influencing the bone forming osteoblasts (16) and by modulating bone resorption (4). This steroid hormone seems to have a key position in bone metabolism and that is why the efficacy of 1,25(OH)2D3 in prophylaxis and therapy of postmenopausal osteoporosis has been examined for several years. Some investigators were able to demonstrate positive therapeutic effects of different vitamin D metabolites on bone mass in ovariectomized rats (8, 10, 14). The present study employed a wide range of biochemical markers together with histomorphometrical bone analysis in order to evaluate possible prophylactic effects of 1,25(OH)2D3 with regard to bone loss in ovariectomized rats.

Material and Methods

Experimental animals

For this study 18 ten week old Sprague-Dawley rats at a mean initial body weight of 200 g were used. 12 of them were ovariectomized and the remaining 6 were sham-operated. They were divided into the following 3 groups, consisting of 6 rats per group:

I. sham-operated (sham)
II. ovariectomized (ovx)
III. ovx + 0.05 μg 1,25(OH)2D3/animal and day

The animals were kept in individual cages on a standard laboratory diet (0.9 % calcium, 0.75 % phosphorus, 600 IU vitamin D3/kg diet) for 7 weeks. Food and tap water were available ad libitum. 1,25(OH)2D3 was dissolved in ethanol/propyleneglycol 1:10 and added to the diet, starting the day after surgery. During the experimental period the animals were weighed every 2 weeks. Urine was collected in metabolic cages 4 weeks after starting the experiment. Blood was obtained via orbital sinus puncture under ether anesthesia at the end of the trial. At 7 weeks postovariectomy all rats were euthanized by an overloading ether dosis. Success of ovariectomy was confirmed at autopsy by failure to detect ovarian tissue and observation of marked atrophy of the uterine horns.

Analytical procedures

Calcium in serum and urine was determined by flame photometry, serum phosphate by a testkit of Boehringer, Mannheim, FRG and the activity of serum alkaline phosphatase by a testkit of Sigma Diagnostics. Bone gla protein (BGP) or osteocalcin was measured by a radioimmunoassay according to the method of Price and Nishimoto (18). The test kit for determining BGP in rat serum was provided by Dr. P. V. Hauschka (Children’s Hospital, Boston, MA). Urinary hydroxyproline was determined by a modified method of Stegemann (21). Fasting calcium and hydroxyproline excretion in urine were expressed as a ratio to creatinine excretion.

Bone histomorphometry

Histomorphometrical bone analysis was carried out using the first lumbar vertebrae. They were carefully defleshed and fixed in 40 % ethanol at 4°C for 2 days. The vertebra were then dehydrated and embedded undecalcified in methylmethacrylate as described previously (2). 5 μm thick longitudinal sections in the median plane of the vertebrae were performed on a Reichert Jung Polycut sledge microtome (Reichert-Jung, FRG) and stained with toluidine blue and von Kossa (2). All measurements were made on cancellous bone. The area within 1 mm from the epiphyseal growth plate was excluded (12). A semiautomatic system (Zeiss Videoplan, C. Zeiss, FRG) was used for the measurements. At a magnification of 250 x, 30 fields were evaluated in every section, which was equivalent to an area of 3.6 mm². The following data were measured and calculated according to the nomenclature scheme approved by the American Society for Bone and Mineral Research (17).
1. Bone area = bone area / tissue area * 100 (%)
2. Bone perimeter = bone perimeter / tissue area (mm/mm²)
3. Trabecular width = bone area / bone perimeter * 2,000 (µm)
4. Osteoid area = osteoid area / bone area * 100 (%)
5. Osteoid perimeter = osteoid perimeter / bone perimeter * 100 (%)
6. Osteoid width = osteoid area / osteoid perimeter * 1,000 (µm)
7. Osteoblast perimeter = osteoblast perimeter / bone perimeter * 100 (%)
8. Osteoclast number = osteoclast number / mineralized perimeter (/mm)

Since the cancellous bone of rat lumbar vertebrae is markedly anisotropic we used only two-dimensional terms.

**Statistical analysis**

Data are expressed as means ± SD; differences between the groups were evaluated with the Wilcoxon-Mann-Whitney U-test. P values of less than 0.05 were considered to be significant.

**Results**

The average weight gain per day of the ovx rats was significantly higher than that of the sham-operated controls (p < 0.01) (Table 1). Vitamin D metabolite treatment had no influence on this higher weight gain in ovx rats.

**Blood and urine chemical findings (Table 1)**

Serum calcium and phosphate levels were higher in ovx compared to sham-operated animals. The group given 1,25(OH)₂D₃ showed elevated serum calcium (p < 0.01) and phosphate concentrations. The activity of total alkaline phosphatase was significantly higher in the ovx than in the sham group (p < 0.01). Treatment with 1,25(OH)₂D₃ led to a

<table>
<thead>
<tr>
<th>Table 1. Weight gain, blood and urine chemical findings in sham-operated rats, ovx rats and ovx rats orally treated with 1,25(OH)₂D₃ (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parameter</td>
</tr>
<tr>
<td>------</td>
</tr>
<tr>
<td>average weight gain per day (g)</td>
</tr>
<tr>
<td>serum calcium (mmol/l)</td>
</tr>
<tr>
<td>serum phosphate (mmol/l)</td>
</tr>
<tr>
<td>serum alkaline phosphatase (U/l)</td>
</tr>
<tr>
<td>serum BGP* (ng/ml)</td>
</tr>
<tr>
<td>urinary calcium/creatinine</td>
</tr>
<tr>
<td>urinary hydroxyproline/creatinine</td>
</tr>
</tbody>
</table>

* Significantly different from sham (p < 0.01); † significantly different from ovx (p < 0.01); ‡ significantly different from ovx (p < 0.05). * BGP (bone gla protein) was determined in pooled serum.

Fig. 1. Representative median sections of the first lumbar vertebra (5 µm) thick, von Kossa stain, magnification (16 ×) from sham-operated rats (a), ovx rats (b), and ovx rats orally treated with 1,25(OH)₂D₃ (c)
marked decrease of this enzyme activity ($p < 0.01$). Ovariectomy caused a distinct increase of serum BGP levels when compared with sham-operated rats. The vitamin D metabolite treatment led to a further increase of serum BGP concentrations in the ovx animals.

There was no difference in urinary calcium excretion between sham-operated and ovx animals. In the group given $1,25(\text{OH})_2\text{D}_3$ a sixfold increase of urinary calcium excretion was obtained ($p < 0.01$). The hydroxyproline excretion in urine was significantly higher in the ovx than in the sham-operated group ($p < 0.01$), whereas the ovx rats treated with the vitamin D metabolite had lower urinary hydroxyproline excretion than untreated ovx rats.

**Histomorphometrical results (Table 2)**

Ovx rats showed an about 20% decrease in bone area ($p < 0.05$) and a statistically non-significant reduction of trabecular width when compared with sham-operated controls. Osteoid area, osteoid perimeter and osteoblast perimeter were severalfold higher in the ovx than in the sham-operated group ($p < 0.01$). Osteoid width doubled from about 3 μm in sham-operated animals to about 6 μm in ovx animals ($p < 0.05$). Ovariectomy in rats led to a twofold increase in osteoclast number in comparison to sham-operated controls ($p < 0.01$).

The most prominent effects of vitamin D metabolite treatment in ovx rats were increases in bone area, bone perimeter and trabecular width ($p < 0.01$), even above the level of sham-operated controls. Furthermore, ovx rats treated with $1,25(\text{OH})_2\text{D}_3$ showed a highly significant reduction in osteoclast number compared with non-treated ovx rats ($p < 0.01$). Osteoid area, osteoid perimeter and osteoid width as well as osteoblast perimeter remained nearly unchanged under $1,25(\text{OH})_2\text{D}_3$ treatment.

**Discussion**

Ovariectomy was followed by a highly significant and rapid gain in body weight as the study progressed confirming an observation by previous investigators (8, 9, 14, 24). The reason for this difference in weight has not been established, but studies with pair-feeding of rats after ovariectomy still revealed higher body weights in ovx animals

<table>
<thead>
<tr>
<th>Parameter</th>
<th>sham</th>
<th>ovx</th>
<th>ovx + 0.05 μg 1,25(OH)_2D_3/animal and day</th>
</tr>
</thead>
<tbody>
<tr>
<td>bone area (%)</td>
<td>37.38a</td>
<td>5.61</td>
<td>29.97</td>
</tr>
<tr>
<td>bone perimeter (mm/mm²)</td>
<td>6.52</td>
<td>0.63</td>
<td>5.87</td>
</tr>
<tr>
<td>trabecular width (μm)</td>
<td>115.2</td>
<td>18.7</td>
<td>102.5</td>
</tr>
<tr>
<td>osteoid area (%)</td>
<td>0.39a</td>
<td>0.26</td>
<td>2.22</td>
</tr>
<tr>
<td>osteoid perimeter (%)</td>
<td>4.16a</td>
<td>1.90</td>
<td>18.27</td>
</tr>
<tr>
<td>osteoid width (μm)</td>
<td>3.04b</td>
<td>1.67</td>
<td>6.23</td>
</tr>
<tr>
<td>osteoblast perimeter (%)</td>
<td>5.12a</td>
<td>1.18</td>
<td>15.60</td>
</tr>
<tr>
<td>osteoclast number (mm)</td>
<td>1.21a</td>
<td>0.41</td>
<td>2.31</td>
</tr>
</tbody>
</table>

* Significantly different from ovx ($p < 0.01$); b significantly different from ovx ($p < 0.05$).
Osteopenia Caused by Ovariectomy in Young Female Rats

compared with those in sham-operated controls (22, 25). Thus it seems likely that factors other than food intake accounted for the postovariectomy weight gain.

Fasting urinary hydroxyproline excretion can be useful as a marker of bone resorption (13) while alkaline phosphatase (13) and BGP (3) in serum reflect osteoblastic activity (13). BGP is more expressive because it is a bone specific marker whereas different isoenzymes of alkaline phosphatase are produced in liver, kidney, red and white blood cells and so on. Different authors reported that women after menopause show increased activity of alkaline phosphatase (5) as well as elevated serum BGP levels (23) and hydroxyproline excretion in urine (19). Ovariectomy in the present study was accompanied by a pronounced rise of these three bone markers. This suggests that there is an increased bone resorption and formation, i.e. an accelerated rate of bone turnover 7 weeks postovariectomy in rats.

Osteoclastic, osteoblastic and osteoid parameters were significantly higher in ovx rats than in sham-operated controls. These data support our biochemical results of an increased bone turnover rate after ovariectomy.

Similar histological observations have been reported in the proximal tibia and lumbar vertebra of rats 5 and 10 weeks after ovariectomy (24, 26). We used the first lumbar vertebra for our histomorphometrical analysis because the spinal column is mechanically less affected by the difference in weight between ovx and sham-operated rats than long bones of the legs. Furthermore BARON et al. (1) could show that there is at least some remodelling activity in the cancellous bone of the rat vertebra. As a result of the altered bone metabolism after ovariectomy there was a remarkable loss of lumbar cancellous bone.

Treatment with the vitamin D metabolite 1,25(OH)2D3 had a prophylactic effect with regard to bone loss in ovx rats. Osteoid and osteoblast parameters were nearly unchanged as a result of vitamin D metabolite treatment whereas osteoclast number was markedly diminished. These results together with a decrease of urinary hydroxyproline excretion suggest that the bone preserving effect of 1,25(OH)2D3 in ovx rats refers to a decrease of bone resorption. The biochemical markers of bone formation, alkaline phosphatase and BGP reacted differently on 1,25(OH)2D3 application. While enzyme activity fell, BGP levels showed a further increase. BGP and alkaline phosphatase might reflect various aspects of osteoblastic activity which are differently influenced by 1,25(OH)2D3. The precise mode of action of estrogen on the skeleton has yet to be determined but osteoporosis may actually represent the end result of a number of different disorders. Recently ERIKSON et al. (7) detected estrogen receptors in human osteoblast-like cells and suggested that estrogen acts directly on human bone cells through a classical receptor-mediated mechanism. Possibly estrogen protects against excessive bone resorption, therefore loss of this hormone causes a higher resorption and — as bone formation and resorption are coupled — leads to an increased bone turnover rate. The efficacy of treating ovx rats with 1,25(OH)2D3 may be a consequence of an improved dietary calcium absorption and an accordingly decreased serum level of parathyroide hormone (PTH). PTH is a potent stimulator of osteoclast-mediated resorption in bone tissue, both in vitro and in vivo (16).

Our study revealed that young growing rats develop a high bone turnover osteopenia after ovariectomy. The bone markers BGP, alkaline phosphatase and hydroxyproline can be useful in studies with ovx rats and may provide a means of accessing the effects of various therapies on conditions of increased bone turnover. Our observation of a prevention of bone loss associated with estrogen deficiency by 1,25(OH)2D3 could be interesting in the treatment of women who are at risk to develop a high bone turnover osteoporosis.

Acknowledgements

We would like to thank Mr. FAMULA, C.ZEISS, FRG, for providing the Videoplan semiautomatic system. This work was supported by the Deutsche Forschungsgemeinschaft.
References


