Original article

Additive effects of zoledronic acid and propranolol on bone density and biochemical markers of bone turnover in osteopenic ovariectomized rats

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\textbf{A B S T R A C T}

Objectives: The present study was designed to investigate further the efficacy and safety of zoledronic acid (ZOL) and propranolol (PRO) as monotherapy and combination therapy in a rat model of postmenopausal osteoporosis.

Methods: Female Wistar rats were ovariectomized (OVX) or sham-operated at 3 months of age. Twelve weeks post-surgery, rats were randomized into six groups: (1) sham + vehicle; (2) OVX + vehicle; (3) OVX + ZOL (100 \( \mu \)g/kg, i.v. single dose); (4) OVX + ZOL (50 \( \mu \)g/kg, i.v. single dose); (5) OVX + PRO (0.1 mg/kg, s.c. 5 days per week); (6) OVX + ZOL (50 \( \mu \)g/kg, i.v. single dose) + PRO (0.1 mg/kg, s.c. 5 days per week) for 12 weeks. After treatment, femurs were tested for bone density, porosity and trabecular micro-architecture. Biochemical markers in serum and urine were also determined.

Results: Combined treatment with ZOL plus PRO corrected the decrease in serum calcium and increase in serum alkaline phosphatase and tartarate resistant acid phosphatase level better than single-drug therapy using ZOL or PRO. Moreover, combined treatment with ZOL plus PRO corrected the increase in urine calcium, phosphorous and creatinine level better than single-drug therapy using ZOL or PRO. Combination therapy using ZOL plus PRO also preserved the trabecular micro-architecture and cortical bone porosity.

Conclusion: These data suggest that combined treatment with ZOL plus PRO could be a more effective approach for treating severe osteoporosis in humans.

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Efeitos combinados do ácido zoledrônico e do propranolol sobre a densidade óssea e marcadores bioquímicos de remodelação óssea em ratas osteoporônicas submetidas à ovariectomia

RESUMO

Objetivos: Este estudo foi desenvolvido para investigar a eficácia e a segurança do ácido zoledrônico (ZOL) e do propranolol (PRO) como monoterapia e terapia combinada em um modelo de rato com osteoporose pós-menopáusica.

Métodos: Ratas Wistar fêmeas foram ovariectomizadas (OVX) ou submetidas à cirurgia simulada (placebo) aos três meses de idade. Doze semanas depois da cirurgia, as ratas foram divididas em seis grupos: (1) placebo + veículo; (2) OVX + veículo; (3) OVX + ZOL (100 µg/kg, dose única intravenosa); (4) OVX + ZOL (50 µg/kg, dose única intravenosa); (5) OVX + PRO (0,1 mg/kg, via subcutânea, cinco dias por semana); (6) OVX + ZOL (50 µg/kg, dose única intravenosa) + PRO (0,1 mg/kg, via subcutânea, cinco dias por semana) durante 12 semanas. Depois do tratamento, testou-se a densidade óssea, a porosidade e a microarquitetura trabecular dos fêmures. Também foram avaliados marcadores bioquímicos séricos e urinários.

Resultados: A terapia combinada com ZOL mais PRO foi mais eficaz em corrigir a diminuição do cálcio sérico e o aumento do nível sérico de fosfatase alcalina e fosfatase ácida resistente ao tartarato do que a monoterapia com ZOL ou PRO. Além disso, a terapia combinada com ZOL mais PRO foi mais eficaz em corrigir o aumento dos níveis urinários de cálcio, fósforo e creatinina do que a monoterapia com ZOL ou PRO. A terapia combinada com ZOL mais PRO também preservou a microarquitetura trabecular e a porosidade do osso cortical.

Conclusão: Os resultados sugerem que a terapia combinada com ZOL mais PRO pode ser a abordagem mais eficaz para o tratamento da osteoporose grave em humanos.

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the present study was designed to evaluate further the anti-osteoporotic activity and safety of ZOL and PRO combination therapy in a rat model of postmenopausal osteoporosis. We assessed the parameters as follows: (1) the bone porosity measurement of the right femur; (2) measurement of femoral dry bone weight, volume and density; (3) serum and urine biochemical parameters; and (4) left femurs were used for bone histopathology.

Materials and methods

Drugs, chemicals and other materials

ZOL was obtained from Naprod Life Sciences, Maharashtra, India. PRO, ketamine, xylazine and xylene was obtained from Aurobindo Pharma (Hyderabad, India), Glaxo SmithKline Pharmaceuticals (Mumbai, India), Neon Pharma (Mumbai, India), Indian Immunologicals (Hyderabad, India), and S.D. Fine chemicals (Mumbai, India), respectively. Ethicon chromic sutures-3/0, and Ethicon mersilk sutures-3/0 were obtained from Johnson & Johnson Ltd., Baddi, Himachal Pradesh, India.

Experimental animals

In-house laboratory-bred healthy female Wistar rats with 12 weeks age were included for the study. Animals were maintained under controlled temperature at 25 ± 2°C with 12 h light/dark cycle with food and water and provided ad libitum. The experiments were conducted as per the CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals) guidelines after obtaining ethical clearance from the Institutional Animal Ethical Committee.

Pre-clinical study design

At three months of age, ovariectomy was performed under ketamine and xylazine anesthesia (80 and 10 mg/kg; i.p.) according to the method described by Khajuria et al.17 The SHAM-operation rats were subject to SHAM surgery exposure without removing the ovaries. At 3 months after ovariectomy (age: 6 months), animals were divided into six groups of six animals each. A group of ovariectomized (OVX) rats was left untreated (untreated OVX). Both the untreated OVX and the sham OVX groups were used as controls. Two OVX groups were treated with single intravenous dose of ZOL at 50 µg/kg (ZOL 50 group) and 100 µg/kg (ZOL 100 group), administered into tail vein as a slow intravenous injection over 30 s under light inhalation anesthesia. One OVX group was treated with PRO at a dose of 0.1 mg/kg, injected subcutaneously 5 days per week, for 12 weeks. Treatment on the remaining OVX group was initiated with a 50 µg/kg ZOL and 0.1 mg/kg PRO combination-treated (ZOL 50 + PRO) group. Subcutaneous injections five days per week in case of OVX groups treated with PRO and ZOL 50 + PRO require some animal handling and create some stress to the animals. Therefore, apart from positive and negative control groups, OVX groups treated with ZOL (100 and 50 µg/kg, intravenous single dose) were also subcutaneously administered vehicle (normal saline, 5 days per week) for 12 weeks. The medication dosages used in this experiment were selected from previous studies on rat osteoporosis model.4

At the end of an experiment (animals aged approximately 9 months), rats were anesthetized with ether and blood was collected from retro-orbital plexus. After, centrifugation serum was harvested and kept at −20°C until analysis. At the end of the study, all groups were euthanized by an overdose of anesthesia. In all rats, femurs were excised and cleared of fat and connective tissues. Right femurs were soaked in saline solution gauze and frozen at −20°C for the bone porosity and density tests. In all rats, left femurs were excised and cleared of fat and connective tissues, and immediately fixed in 10% formaldehyde for 48 h at 4°C.

Serum and urine biochemical analysis

The levels of serum and urine calcium, serum and urine inorganic phosphorous, urine creatinine and alkaline phosphatase activity were estimated by commercially available kits (Autospan, Span diagnostics Ltd., Surat, India) using autoanalyzer (Artos semi-autoanalyzer, Swemed Biomedicals Pvt. Ltd., Bangalore, India). Serum tartarate resistant acid phosphatase (TRAP) activity was determined by a nitrophenol-based method as described by Janckila et al.18

Measurement of bone porosity by X-ray imaging

The right femurs of all animals were scanned with foX-Rayzor, which is a portable X-ray inspection system equipped with “Calculate histogram” tool software, according to the method described by Khajuria et al.8 Briefly, for X-ray analysis, whole femur was divided into four equal fields, which includes distal femoral epiphysis (R1), femoral shaft (R2 and R3) and proximal femur (R4).

Measurement of dry bone weight, volume and density

After X-ray, the right femur of all animals was dehydrated with ethanol, and fat was removed with diethyl ether. After the bones were allowed to air-dry, the dry bone weight was measured with a digital weighing balance (Contech CA-124, Contech Instruments Ltd., Mumbai, India). The volume and density of the bones were measured according to the procedure reported by Khajuria et al.6

Bone histopathology

The left femurs were cleaned from soft tissue, placed in decalciﬁng solution [8% hydrochloric acid (37%, v/v) and 10% formic acid (89%, v/v) in phosphate-buffered saline] for about 24 h at 37°C, dehydrated in 95% (v/v) ethanol and embedded in parafﬁn. 5 mm-thick parafﬁn-embedded horizontal bone sections were cut from the proximal end of the diaphysis, stained with hematoxylin–eosin and examined by light microscopy. Femur heads (the area between the hip joint cartilage and metaphyseal cartilage) were assessed for the quality of bone and trabecular density, according to the score shown in Table 1. Cartilage integrity is considered as an additional index of
bone quality, because osteoporosis is also responsible for cartilage deterioration and treatments that restore bone integrity are also able to preserve a good trophism of the cartilage indirectly.\textsuperscript{19}

**Safety assessment of different treatments**

General toxicity study was conducted in accordance with good laboratory practices (GLP) and the OECD (Organization for Economic Co-operation and Development) guidelines for the Testing of Chemicals with some modifications. Changes in behavior, physical parameters (body weight, food and water intake) and local injury (tissue damage or necrosis at site of injection, inflammation and any other abnormal signs) were studied throughout the treatment period. Mortality if any, in all the groups, during the course of treatment was also recorded. At the end of treatment hematological, biochemical (liver function tests and renal function tests) were studied. Serum biochemistries were analyzed with a semi automated chemistry analyzer using kits for serum glutamic oxaloacetic transaminase (SGOT) and serum glutamic pyruvate transaminase (SGPT) from Agappe Diagnostics Ltd., Kerala, India. Other parameters were estimated such as blood urea nitrogen (Autospan, Span Diagnostics Ltd., Surat, India), total protein and creatinine (Agappe Diagnostics Ltd., Kerala, India) as signs of nephrotoxicity.

**Statistical analysis**

All data were expressed as the mean ± standard deviation (SD). For all the data, comparisons between different treatments were analyzed by one-way ANOVA followed by Tukey’s multiple comparison tests. In all cases, a probability error of less than 0.05 was selected as the criterion for statistical significance. Graphs were drawn using Graph Pad Prism (version 5.0 for Windows).

**Results**

**Effects on serum biochemical parameters**

Serum calcium concentration was significantly lower in the OVX group than in the SHAM group (p<0.05). Significant differences were not observed in the single treatment groups relative to the OVX group. However, the serum calcium level in the ZOL 50+PRO group was significantly higher than that in the OVX group (p<0.05). The final serum phosphorus levels were not significantly different between the SHAM and the OVX animals. Likewise, there were no changes in the serum phosphorus levels observed among the several pharmacological treatments (Table 2).

At the end of experiment, serum level of alkaline phosphatase was significantly higher in the OVX group than in the SHAM group (p<0.001). In contrast, the serum level of alkaline phosphatase was significantly lower in all single treatment groups as compared to OVX group (p<0.05). Likewise, the serum alkaline phosphatase level in the ZOL 50+PRO group was significantly lower than that in the OVX group (p<0.01). Moreover, the serum alkaline phosphatase levels in the ZOL 50+PRO group were significantly lower than those in the ZOL 100, ZOL 50 and PRO groups (p<0.05, Table 2). Ovariectomy induced high bone turnover in rats and increased serum TRAP levels in the OVX control group in comparison with the SHAM control group (p<0.001). The ZOL 100, ZOL 50, PRO and ZOL 50+PRO groups had significantly decreased serum TRAP levels compared to the OVX group (p<0.01, p<0.001, p<0.001 and

<table>
<thead>
<tr>
<th>Group</th>
<th>Ca (mg/dL)</th>
<th>Pi (mg/dL)</th>
<th>ALP (IU/L)</th>
<th>TRAP (IU/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHAM</td>
<td>9.03 ± 0.13\textsuperscript{a}</td>
<td>6.77 ± 0.41</td>
<td>74.85 ± 3.88\textsuperscript{c}</td>
<td>0.31 ± 3.02\textsuperscript{c}</td>
</tr>
<tr>
<td>OVX</td>
<td>7.99 ± 0.29</td>
<td>6.30 ± 0.38</td>
<td>155.7 ± 14.47</td>
<td>1.18 ± 10.12</td>
</tr>
<tr>
<td>OVX + ZOL 100</td>
<td>8.03 ± 0.58</td>
<td>6.43 ± 0.25</td>
<td>108.2 ± 9.65\textsuperscript{b,d}</td>
<td>0.72 ± 9.05\textsuperscript{b,d}</td>
</tr>
<tr>
<td>OVX + ZOL 50</td>
<td>8.31 ± 0.51</td>
<td>6.55 ± 0.42</td>
<td>105.01 ± 8.13\textsuperscript{b,d}</td>
<td>0.66 ± 10.18\textsuperscript{b,d}</td>
</tr>
<tr>
<td>OVX + PRO</td>
<td>8.11 ± 0.21</td>
<td>6.53 ± 0.44</td>
<td>103.36 ± 6.14\textsuperscript{b,d}</td>
<td>0.63 ± 14.99\textsuperscript{b,d}</td>
</tr>
<tr>
<td>OVX + ZOL 50+PRO</td>
<td>9.13 ± 0.82\textsuperscript{a}</td>
<td>6.90 ± 0.32</td>
<td>80.03 ± 10.13\textsuperscript{c}</td>
<td>0.41 ± 10.1\textsuperscript{c}</td>
</tr>
</tbody>
</table>

Data are shown as the mean ± SD (n = 6), evaluated by Tukey’s multiple comparison test.\textsuperscript{a} p < 0.05.\textsuperscript{b} p < 0.01.\textsuperscript{c} p < 0.001, compared to OVX group.\textsuperscript{d} p < 0.05, compared to ZOL 50+PRO group. All groups except SHAM group undergo ovariectomy.
Table 3 – Effects of zoledronic acid and propranolol, alone or in combination on urine calcium (Ca), inorganic phosphorous (Pi) and creatinine (Cr) levels in OVX rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>Ca (mg/dl)</th>
<th>Pi (mg/dl)</th>
<th>Creatinine (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHAM</td>
<td>20.10 ± 7.16*c</td>
<td>3.86 ± 0.33*c</td>
<td>1.22 ± 0.81*</td>
</tr>
<tr>
<td>OVX</td>
<td>48.33 ± 11.07</td>
<td>5.58 ± 0.88</td>
<td>3.14 ± 0.97</td>
</tr>
<tr>
<td>OVX + ZOL 100</td>
<td>34.03 ± 17.58*a,b,d</td>
<td>4.36 ± 0.56*a,b,d</td>
<td>1.82 ± 0.49*a,b,d</td>
</tr>
<tr>
<td>OVX + ZOL 50</td>
<td>36.31 ± 11.98*a,b,d</td>
<td>4.15 ± 0.59*a,b,d</td>
<td>1.67 ± 0.46*a,b,d</td>
</tr>
<tr>
<td>OVX + PRO</td>
<td>33.22 ± 12.81*a,b,d</td>
<td>4.05 ± 0.74*a,b,d</td>
<td>1.76 ± 0.39*a,b,d</td>
</tr>
<tr>
<td>OVX + ZOL 50 + PRO</td>
<td>28.15 ± 9.02*a,b,d</td>
<td>3.68 ± 0.42*a,b,d</td>
<td>1.13 ± 0.34*a,b,d</td>
</tr>
</tbody>
</table>

Data are shown as the mean ± SD (n = 6), evaluated by Tukey’s multiple comparison test.

*p < 0.05.
**p < 0.01.
***p < 0.001, compared to OVX group.
*dp < 0.05; compared to ZOL 50 + PRO group.
All groups except SHAM group undergo ovariotomy.

p < 0.001, respectively). Moreover, significant reductions in levels of serum TRAP levels were observed in the ZOL 50 + PRO group compared to the ZOL 100, ZOL 50 and PRO groups (p < 0.05).

Effects on urinary calcium, phosphorous and creatinine levels

The urinary calcium, phosphorous and creatinine excretion profiles of the SHAM group, OVX group and OVX rats treated with various therapeutic interventions are shown in Table 3. A significant increase in urinary calcium excretion was observed in the OVX control in comparison to the SHAM group (p < 0.001). The ZOL 100, ZOL 50, PRO and ZOL 50 + PRO groups had significantly decreased urinary calcium excretion (p < 0.01, p < 0.01, p < 0.01 and p < 0.001, respectively). Similarly, a significant increase in urinary phosphorous excretion was observed in the OVX control compared to the SHAM group (p < 0.001). The ZOL 100, ZOL 50, PRO and ZOL 50 + PRO groups had significantly decreased urinary phosphorous excretion (p < 0.01, p < 0.01, p < 0.01 and p < 0.001, respectively). Likewise, a significant increase in urinary creatinine excretion was observed in the OVX control in comparison to the SHAM group (p < 0.001). The ZOL 100, ZOL 50, PRO and ZOL 50 + PRO groups had significantly decreased urinary creatinine excretion (p < 0.01, p < 0.01, p < 0.01 and p < 0.001, respectively). Moreover, significant reductions in the levels of urine calcium, phosphorous and creatinine were observed in the ZOL 50 + PRO group compared to the ZOL 100, ZOL 50 and PRO groups (p < 0.05; Table 3).

Measurement of dry bone weight, volume and density

Table 4 summarizes the effects of OVX and treatment of OVX rats with ZOL 100, ZOL 50, PRO and ZOL 50 + PRO groups on dry bone weight, volume and the dry bone weight per bone volume (density) of the right femur. Dry bone weight of OVX control animals was found to be significantly less than SHAM control animals (p < 0.001). ZOL 100, ZOL 50 and PRO groups exhibited higher values for dry bone weight than the OVX group (p < 0.05, p < 0.01 and p < 0.01, respectively). Similarly, ZOL 50 + PRO group exhibited higher values for dry bone weight than the OVX group (p < 0.001). The bone volume of femur in the OVX group was significantly higher than that in the SHAM group (p < 0.05). This conclusion is based on the gain in body weight observed in the OVX group. No significant differences were observed in the bone volume of all therapeutic interventions as compared to the OVX group (Table 4). With respect to bone mass, the OVX group exhibited significantly lower values for density of the femur than did the SHAM group animals (p < 0.001). This indicates that the bone mass density is decreased by ovariotomy. Values in both bisphosphonate groups (ZOL 100 and ZOL 50) and PRO group were higher than those in the OVX group (p < 0.01, p < 0.001 and p < 0.001, respectively). Similarly, the values were significantly higher in the ZOL 50 + PRO group as compared to the OVX group (p < 0.001). The dry bone weight and density in the ZOL 50 + PRO group were significantly higher than that in the ZOL 100, ZOL 50 and PRO groups (p < 0.05).

Bone porosity

The effects of OVX and subsequent treatment with ZOL 100, ZOL 50, PRO and ZOL 50 + PRO groups on the porosity of the right femur were measured by X-ray imaging. X-ray transmission intensity for the OVX group at R1 (distal epiphysis), R2 (mid-shaft: distal), R3 (mid-shaft: proximal), and R4 (proximal epiphysis) was significantly higher than those for the SHAM group, which indicates an OVX-elicted increase in porosity in these areas. After 12 weeks of therapy, all the active treatments succeeded in decreasing bone porosity in OVX animals. X-ray transmission intensity values at R1 for ZOL 100, ZOL 50, PRO and ZOL 50 + PRO groups were lower than those of OVX group (p < 0.05, p < 0.01, p < 0.01 and p < 0.001, respectively). The X-ray transmission intensity of the ZOL 50 + PRO group was significantly lower than that of the ZOL 100, ZOL 50, and PRO groups at R1 (p < 0.01, p < 0.05 and p < 0.05, respectively). Moreover, the X-ray transmission intensity values at R2, R3 and R4 regions for ZOL 100, ZOL 50, PRO and ZOL 50 + PRO groups were lower than those of OVX group (p < 0.05, p < 0.05, p < 0.05 and p < 0.001, respectively). The X-ray transmission intensity of the ZOL 50 + PRO group was significantly lower than that of all single treatments at R2, R3 and R4 region (p < 0.05). No significant differences were observed among single treatment groups at R1, R2, R3 and R4 regions. These results indicate that the combined treatment with ZOL 50 + PRO is beneficial for the mass
of both trabecular and cortical bones that were decreased by OVX (Fig. 1).

**Effect of the treatments on bone histopathology**

The sections of the femur head were examined for any histopathological changes. The animals of the SHAM group showed normal compactness of the diaphysis and competent trabeculae (Fig. 2a). The OVX animals showed sparse, uniform thinning of the trabeculae with tendency of disappearance and loss of connectivity resulting in widened intertrabecular spaces (Fig. 2b). Cartilaginous proliferates in the area of softened plates of focal to restricted islets were also observed. Bone histopathology (Fig. 2c–f) revealed a marked effect of ZOL 100, ZOL 50, PRO and ZOL 50+PRO in ovariectomy-induced osteoporosis. The histological score (Fig. 3) of all groups evaluated following the criteria is shown in Table 1. Histopathological assessment of animals treated with all therapeutic interventions showed a restored architecture

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### Table 4 - Effects of zoledronic acid and propranolol, alone or in combination on dry bone weight, volume and density in OVX rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>Dry weight (mg)</th>
<th>Volume (mL)</th>
<th>Density (g/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHAM</td>
<td>615.2 ± 26.20&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.31 ± 0.03&lt;sup&gt;*&lt;/sup&gt;</td>
<td>1.98 ± 0.02&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>OVX</td>
<td>488.7 ± 25.20</td>
<td>0.37 ± 0.04</td>
<td>1.31 ± 0.05</td>
</tr>
<tr>
<td>OVX + ZOL 100</td>
<td>601.6 ± 33.40&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.37 ± 0.03</td>
<td>1.63 ± 0.03&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>OVX + ZOL 50</td>
<td>626.6 ± 20.90&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.37 ± 0.02</td>
<td>1.69 ± 0.04&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>OVX + PRO</td>
<td>630.7 ± 19.40&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.36 ± 0.04</td>
<td>1.75 ± 0.03&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>OVX + ZOL 50 + PRO</td>
<td>674.2 ± 22.30&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.35 ± 0.03</td>
<td>1.90 ± 0.06&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data are expressed as the mean ± SD (n = 6), evaluated by one-way ANOVA followed by Tukey’s multiple comparison test.

*<sup>a</sup> p < 0.05.

<sup>b</sup> p < 0.01.

<sup>c</sup> p < 0.001, compared to OVX group.

<sup>d</sup> p < 0.05, compared to ZOL 50+PRO group.

All groups except SHAM group undergo ovariectomy.

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Fig. 1 - Effect of zoledronic acid and propranolol, alone or in combination on femoral porosity of ovariectomized rats. Bone porosity of R1: distal femoral epiphysis (panel a), R2: distal femoral shaft (panel b), R3: proximal femoral shaft (panel c), R4: proximal femoral epiphysis (panel d). Data are shown as the mean ± SD (n = 6), evaluated by Tukey’s multiple comparison test. *p < 0.05; **p < 0.01; ***p < 0.001, compared to OVX group; *p < 0.05; **p < 0.01, compared to ZOL + PRO group.
Fig. 2 – Effect of zoledronic acid and propranolol, alone or in combination on the femoral trabecular micro architecture in ovariectomized rats: (a) SHAM group; (b) OVX group; (c) OVX + ZOL 100 group; (d) OVX + ZOL 50 group; (e) OVX + PRO group; and (f) OVX + ZOL 50 + PRO group. H&E staining; scale bar = 100 μm. Images are taken at magnification 50×.

Safety assessment

All follow-up animals survived during the study period. In addition, no adverse clinical signs or symptoms were observed during the experimental period. There were no significant differences in food and water consumption between control and treated groups (data not shown). No significant differences in body weights between OVX controls and treated groups were observed during the study period (data not shown). No significant differences in hematological parameters were detected; hemoglobin, total erythrocyte count and total leukocyte count were similar across all treatment groups (Table 5).

There were no significant changes observed in serum total protein, creatinine and blood urea nitrogen levels in all single and combined treatment groups when compared to control group. No significant increases were observed in serum SGPT and SGOT levels in all therapeutic interventions when compared to control group (Table 6).
Table 5 - Effects of zoledronic acid and propranolol, alone or in combination on various biochemical parameters in OVX rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>Hemoglobin (g %)</th>
<th>Total RBC (×10⁶/mm³)</th>
<th>Total WBC (×10³/mm³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHAM</td>
<td>15.17 ± 1.42</td>
<td>5.98 ± 0.89</td>
<td>5.92 ± 0.96</td>
</tr>
<tr>
<td>OVX</td>
<td>13.98 ± 1.96</td>
<td>5.87 ± 0.97</td>
<td>5.81 ± 1.11</td>
</tr>
<tr>
<td>OVX + ZOL 100</td>
<td>14.44 ± 2.11</td>
<td>6.12 ± 1.13</td>
<td>6.09 ± 1.22</td>
</tr>
<tr>
<td>OVX + ZOL 50</td>
<td>14.85 ± 2.01</td>
<td>5.73 ± 0.68</td>
<td>5.89 ± 0.50</td>
</tr>
<tr>
<td>OVX + PRO</td>
<td>15.01 ± 1.63</td>
<td>5.83 ± 1.17</td>
<td>5.77 ± 0.88</td>
</tr>
<tr>
<td>OVX + ZOL 50 + PRO</td>
<td>14.11 ± 1.36</td>
<td>5.79 ± 0.70</td>
<td>5.97 ± 1.09</td>
</tr>
</tbody>
</table>

Data are expressed as the mean ± SD (n = 6). All groups except SHAM group undergo ovariectomy.

Table 6 - Effects of zoledronic acid and propranolol, alone or in combination on total serum protein, creatinine, blood urea nitrogen, serum glutamic oxaloacetic transaminase (SGOT) and serum glutamic pyruvate transaminase (SGPT).

<table>
<thead>
<tr>
<th>Group</th>
<th>Total serum protein (g/dl)</th>
<th>Creatinine (mg/dl)</th>
<th>Blood urea nitrogen (mg/dl)</th>
<th>SGPT (U/L)</th>
<th>SGOT (U/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHAM</td>
<td>6.90 ± 0.18</td>
<td>0.66 ± 0.01</td>
<td>18.03 ± 1.42</td>
<td>24.81 ± 1.70</td>
<td>34.76 ± 1.45</td>
</tr>
<tr>
<td>OVX</td>
<td>6.17 ± 0.04</td>
<td>0.73 ± 0.03</td>
<td>17.73 ± 2.09</td>
<td>25.63 ± 1.67</td>
<td>33.22 ± 1.78</td>
</tr>
<tr>
<td>OVX + ZOL 100</td>
<td>6.32 ± 0.15</td>
<td>0.71 ± 0.01</td>
<td>17.38 ± 0.95</td>
<td>24.25 ± 1.71</td>
<td>33.19 ± 2.25</td>
</tr>
<tr>
<td>OVX + ZOL 50</td>
<td>6.03 ± 0.67</td>
<td>0.69 ± 0.01</td>
<td>16.45 ± 3.64</td>
<td>23.49 ± 2.80</td>
<td>32.17 ± 2.85</td>
</tr>
<tr>
<td>OVX + PRO</td>
<td>6.47 ± 0.13</td>
<td>0.73 ± 0.01</td>
<td>18.60 ± 2.42</td>
<td>25.15 ± 1.46</td>
<td>34.80 ± 3.48</td>
</tr>
<tr>
<td>OVX + ZOL 50 + PRO</td>
<td>6.41 ± 0.12</td>
<td>0.74 ± 0.04</td>
<td>18.84 ± 2.88</td>
<td>25.27 ± 2.70</td>
<td>33.81 ± 0.44</td>
</tr>
</tbody>
</table>

Discussion

The results of the present preclinical study clearly show that ZOL plus PRO combination therapy was able to counteract the bone loss in a rat model of postmenopausal osteoporosis. These findings support our previous research indicating that the addition of PRO to antiresorptive therapy with ZOL is clearly superior to either intervention alone.

It is established that the rate of bone loss after estrogen deficiency may be indirectly assessed with the use of established biochemical markers of bone turnover. Reduction in bone mass, altered calcium metabolism and significant lower values of serum calcium level had been reported in the postmenopausal women. In this study, combination treatment with ZOL plus PRO completely corrected the decrease in serum calcium levels, indicating that the combination therapy ameliorated the changes in calcium.

ZOL plus PRO group was statistically superior in decreasing the serum alkaline phosphatase in the OVX rats. Moreover, combined treatment with ZOL plus PRO was statistically superior to all single treatments in suppressing the increase in serum amounts of TRAP. These results suggest that ZOL plus PRO treatment ameliorated the bone loss due to ovariectomy by both stimulation of bone formation and inhibition of bone resorption. Fast bone losers have elevated concentrations of bone resorption markers, compared with slow bone losers. OVX rats in the present study had an increase loss of urinary calcium, phosphorous and creatinine levels, compared to the SHAM group. But these responses were significantly lowered in OVX rats on receiving ZOL plus PRO therapy. This suggests that this significant decrease in urinary excretion of calcium, phosphorous and creatinine levels could be attributed to decreased bone resorption and/or increased bone formation or both. It is noteworthy to mention that combined treatment with ZOL plus PRO was statistically superior to all other therapeutic interventions in suppressing the increase in urinary amounts of calcium, phosphorous and creatinine.

In the analysis of the bone porosity of rat femur using X-ray imaging, it was found that combined therapy with ZOL plus PRO was statistically superior to ZOL or PRO monotherapy in suppressing the increase in bone porosity, which is due to ovariectomy. This indicates that combined therapy with ZOL plus PRO thickens and strengthens cortical bone. The results of the femoral dry weight and density values estimated directly were consistent with those on femoral porosity found by X-ray imaging. In this study, OVX animals showed a significant decrease in femoral dry weight and density similar to those seen in estrogen deficient osteoporotic women. It is interesting to note that, in animals treated with single and combined therapy, femoral dry weight and density values were significantly greater than those of the OVX group. Moreover, in the animals treated with combined therapy of ZOL plus PRO, femoral dry weight and density values were significantly greater than those of the ZOL or PRO groups. These results showed that the combined treatment with ZOL plus PRO is beneficial for increasing the mass of rat femoral bones that was decreased due to ovariectomy.

Bone histopathology analysis of the OVX rats showed decrease in number and thickness of trabecular and widened intra-trabecular spaces suggesting that bone loss was induced in OVX rats by decreasing bone formation and increasing bone resorption. Histopathological examination also revealed the anti-osteoporotic property of combination therapy as demonstrated by the restoration of trabecular bone in ZOL plus PRO treated group compared with the OVX group. As an outcome, the combined therapy with ZOL plus PRO may act both on collagen structure and bone resorption. This in turn may reduce non-vertebral fractures (example hip fracture), which are the most clinically relevant type of fracture in the elderly population.
Testing for general toxicity using appropriate models can help to define risks of adverse events and to design clinical monitoring protocols with clear endpoints. To investigate possible toxic outcomes of single and combined treatments, physical, hematological and biochemical parameters were estimated. There were no signs of toxicity observed at any dose level of all therapeutic interventions used in this study. No mortality was seen in any of the treatment groups. Hematological and biochemical parameters were unaltered at all single and combined dose levels of treated groups as compared to control. It was inferred from results of our study that the ZOL and PRO as a monotherapy and combination therapy are non-toxic to experimental animals. The present study has proven the safety profile of single and combined therapies used for treatment of osteoporosis.

Conclusions

The result of the present investigation demonstrates that combination therapy using ZOL (antiresorptive agent) and PRO (anabolic agent) may represent a powerful novel approach for treating or reversing severe osteoporosis in humans. As such, this combined regimen can be of interest for further evaluation in clinical studies to represent a potentially useful therapeutic option for patients with osteoporosis. Moreover, PRO might be a new potential bone anabolic agent for prevention/treatment of osteoporosis, and it can be used either alone or in conjunction with bisphosphonate drugs.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

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References

