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## Original article

# Effect of combined treatment with zoledronic acid and propranolol on mechanical strength in an rat model of disuse osteoporosis



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## ABSTRACT

**Objectives:** A model that uses right hind-limb unloading of rats is used to study the consequences of skeletal unloading during various conditions like space flights and prolonged bed rest in elderly. This study was aimed to investigate the additive effects of antiresorptive agent zoledronic acid (ZOL), alone and in combination with propranolol (PRO) in a rat model of disuse osteoporosis.

**Methods:** In the present study, 3-month-old male Wistar rats had their right hind-limb immobilized (RHLI) for 10 weeks to induce osteopenia, then were randomized into four groups: (1) RHLI positive control, (2) RHLI plus ZOL (50 µg/kg, i.v. single dose), (3) RHLI plus PRO (0.1 mg/kg, s.c. 5 days per week), (4) RHLI plus PRO (0.1 mg/kg, s.c. 5 days per week) plus ZOL (50 µg/kg, i.v. single dose) for another 10 weeks. One group of non-immobilized rats was used as negative control. At the end of treatment, the femurs were removed and tested for bone porosity, bone mechanical properties, and bone dry and ash weight.

**Results:** With respect to improvement in the mechanical strength of the femoral mid-shaft, the combination treatment with ZOL plus PRO was more effective than ZOL or PRO monotherapy. Moreover, combination therapy using ZOL plus PRO was more effective in improving dry bone weight and preserved the cortical bone porosity better than monotherapy using ZOL or PRO in RHLI rats.

**Conclusions:** These data suggest that this combined treatment with ZOL plus PRO should be recommended for the treatment of disuse osteoporosis.

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## Efeitos da terapia combinada com ácido zoledrônico e propranolol na resistência mecânica em um modelo de rato com osteoporose por desuso

### R E S U M O

#### Palavras-chave:

Osteoporose por desuso  
Estudo com ratos  
Ácido zoledrônico  
Propranolol

**Objetivos:** Investigar os efeitos aditivos do agente antirreabsorção ácido zoledrônico (ZOL), isolado e em combinação ao propranolol (PRO), em um modelo de rato com osteoporose por desuso.

**Métodos:** Usou-se um modelo de pata traseira direita de rato privada de descarga de peso para estudar as consequências da falta de descarga de peso sobre o esqueleto durante várias condições, como missões espaciais e repouso prolongado no leito em idosos. Ratos Wistar machos de três meses de idade foram submetidos à imobilização da pata traseira direita (IPTD) por 10 semanas para induzir à osteopenia; em seguida, foram divididos aleatoriamente em quatro grupos: 1 – IPTD para controle positivo; 2 – IPTD mais ZOL (50 µg/kg, dose única intravenosa); 3 – IPTD mais PRO (0,1 mg/kg, via subcutânea, cinco dias na semana); 4 – IPTD mais PRO (0,1 mg/kg, via subcutânea, cinco dias na semana) mais ZOL (50 µg/kg, dose única intravenosa) por outras 10 semanas. Um grupo de ratos não imobilizados foi usado como controle negativo. No fim do tratamento, os fêmures foram removidos e testaram-se a porosidade do osso e suas propriedades mecânicas, além do peso seco e das cinzas do osso.

**Resultados:** No que diz respeito à melhoria da resistência mecânica da diáfise femoral média, a terapia combinada com ZOL mais PRO foi mais eficaz do que a monoterapia com ZOL ou PRO. Além disso, a terapia combinada com ZOL mais PRO foi mais eficaz na melhoria do peso seco do osso e preservou melhor a porosidade do osso cortical do que a monoterapia com ZOL ou PRO em ratos submetidos à imobilização da pata traseira direita.

**Conclusões:** Esses dados sugerem que a terapia combinada com ZOL mais PRO deve ser recomendada para o tratamento da osteoporose por desuso.

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## Introduction

Osteoporosis is a bone debilitating disease that causes nearly 9 million bone fractures each year.<sup>1</sup> Disuse (unloading) is one of the important causes of osteoporosis.<sup>2</sup> Mechanical loading is essential for the normal functioning of bone tissue. Immobilization results in imbalance of bone metabolism followed by rapid bone loss and impairment of bone mechanical function.<sup>3</sup> This immobilization-induced bone loss is caused by an increased bone resorption and a decreased bone formation. Disuse (unloading) osteoporosis occurs in patients with spinal cord injuries, patients confined to prolonged bed rest, and astronauts exposed to microgravity during space flight.<sup>2</sup> Microgravity induced osteoporosis poses a major threat to the astronaut's health. Microgravity leads to the unloading of the skeleton especially weight bearing bones.<sup>3</sup> Disuse osteoporosis not only increases the susceptibility to fractures in patients with spinal cord injuries and elderly requiring bed rest, but also threatens safety and health of astronauts during spaceflights. Therefore, it is very essential to find relevant countermeasures for disuse osteoporosis to reduce or prevent such bone loss.

Several animal models have been suggested for studying immobilization induced bone loss including neurectomy, tail suspension, plaster casting, and elastic bandaging. In this study, right hind-limb immobilization (RHLI) was achieved by a new procedure which was developed to avoid the problems caused by most widely used methods for the immobilization (e.g., plaster cast, bandaging or tail suspension) of rats.

The smaller weight of the framework compared with a plaster cast kept the difficulty in movement and locomotion to a minimum, with a consequent minimal body weight loss throughout the period of immobilization. Moreover, no skin ulceration or foot swelling was found in the animals when the immobilization was removed. The immobilization procedure proposed was effective in producing long-term disuse in the hind-limbs of rats and is a good alternative to the traditional methods of immobilization.<sup>2</sup>

Zoledronic acid (ZOL) is a third generation nitrogen containing bisphosphonate that binds to hydroxyapatite with the highest affinity and inhibits osteoclasts with the highest potency of all licensed bisphosphonates.<sup>4,5</sup> Therefore, ZOL needs only to be injected once annually in patients, while still efficiently inhibiting osteoclastic activity and thereby reducing the risk of fracture.<sup>6</sup> Although anti-resorptive agents such as bisphosphonates are effective in reducing bone loss, they are not able to induce formation of a new one.<sup>5</sup>

Propranolol (PRO), a non-selective  $\beta$ -adrenergic antagonist, is now considered to be a potential drug under investigation for fracture healing and more specifically for osteoporosis therapy. In rat model of postmenopausal osteoporosis treatment with PRO improves bone properties by increasing bone formation and decreasing bone resorption.<sup>7-10</sup> Moreover, various preclinical studies have demonstrated that treatment by PRO mitigated the bone loss induced by unloading.<sup>2,11,12</sup> Furthermore, results of some prior epidemiological studies confirm the hypothesis that  $\beta$ -blockers use is associated with a decrease in fracture risk.<sup>13-15</sup> Combination therapy is now the subject of extensive investigation because, in some cases, it

can increase the effectiveness of treatment. Teriparatide is an analog of human parathyroid hormone (PTH). It is an anabolic agent that reduces the risk of fracture in osteoporotic patients. Combined PTH and bisphosphonate treatment resulted in more pronounced improvements of the bone architecture than either PTH or bisphosphonate treatment alone.<sup>16-18</sup> Rodrigues et al. demonstrated that low doses of PRO suppress bone resorption by inhibiting receptor activator of nuclear factor kappa-B ligand (RANKL)-mediated osteoclastogenesis as well as inflammatory markers without affecting hemodynamic parameters.<sup>19</sup> This result is supported by a previous finding, which showed that propranolol stimulates osteoprotegerin (OPG) on its own in osteoblast cells.<sup>20</sup> The ability to stimulate osteoblast, while also damping osteoclasts makes PRO an attractive and unique alternative to antiresorptive therapy for osteoporosis. PRO, which could directly prevent bone loss and biomechanical alteration by increasing bone formation and decreasing bone resorption, may be the next anabolic agent for osteoporosis treatment after PTH.<sup>7,8,10,19,20</sup>

As immobilization induced bone loss involves both increased bone resorption and decreased bone formation, it seems to be obvious to target the immobilization induced bone loss with a combined antiresorptive and bone anabolic treatment regimen, such as ZOL and PRO. The effects of a combined PRO and ZOL treatment have previously been studied in ovariectomized rats,<sup>7</sup> whereas this treatment regimen has not previously been investigated in immobilization-induced osteopenia. Consequently, the aim of the present study was to investigate the efficacy of a bone anabolic agent PRO, a bone antiresorptive agent ZOL, and the combination of these two in the treatment of immobilization-induced osteopenia in rats. Owing to the different mechanisms of action of ZOL and PRO, our hypothesis was that the combination of ZOL and PRO would facilitate greater improvements in bone properties than either intervention alone. We assessed the parameters as follows: (1) the mechanical properties in immobilized (right) and non-immobilized (left) femoral mid-shaft; (2) the bone porosity measurement of the immobilized (right) and non-immobilized (left) femur; (3) measurement of immobilized (right) and non-immobilized (left) dry bone and ash weight.

## 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), Neon Pharma (Mumbai, India), Indian Immunologicals (Hyderabad, India), and S.D. Fine chemicals (Mumbai, India), respectively.

### Experimental animals

In-house laboratory bred healthy male Wistar rats with 12 weeks of age were included in the study. Animals were maintained under controlled temperature at  $25 \pm 2^\circ\text{C}$  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, right hind-limb of the rats were immobilized against the abdomen under ketamine (80 mg/kg) and xylazine (10 mg/kg) anesthesia, intraperitoneally according to a new method of right hind-limb immobilization (RHLI) described previously.<sup>2</sup>

Rats were divided into 5 groups (6 rats per group): (1) non-immobilized (negative control) group; (2) RHLI (positive control) for 20 weeks; (3) RHLI for 10 weeks, and then RHLI plus ZOL (50  $\mu\text{g}/\text{kg}$ , single intravenous dose) for another 10 weeks; (4) RHLI for 10 weeks, and then RHLI plus PRO (0.1 mg/kg, injected subcutaneously 5 days per week) for another 10 weeks; (5) RHLI for 10 weeks, then RHLI plus PRO (0.1 mg/kg, injected subcutaneously 5 days per week) plus ZOL (50  $\mu\text{g}/\text{kg}$ , single intravenous dose) for another 10 weeks. Subcutaneous injections five days per week in case of immobilized groups treated with PRO and ZOL plus PRO require some animal handling and create some stress to the animals. Therefore, non-immobilized (negative control) and RHLI (positive control) and RHLI plus ZOL groups were subcutaneously administered vehicle (normal saline, 5 days/week) for 10 weeks. The medication dosages used in this experiment were selected from previous studies on rat on rat model of postmenopausal osteoporosis.<sup>7</sup> At the end of treatment study, all groups were euthanized by an overdose of anesthesia. In all rats, immobilized (right) and non-immobilized (left) were excised and cleared of fat and connective tissues. Femurs were soaked in saline solution gauze and frozen at  $-20^\circ\text{C}$  till further analysis. Both immobilized (right) and non-immobilized (left) femurs were used for measurement of bone porosity, biomechanical properties, femoral length, femoral dry weight and ash weight.

### Final body weight and femoral length

Body weight (expressed in grams) was monitored at the start and the end of the experiments. Femoral length was measured with a precision caliper.

### 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 previously.<sup>8,21</sup> Briefly, for X-ray analysis of rat femur, whole femur was divided into four equal fields, which includes distal femoral epiphysis (R1), femoral shaft (R2 and R3) and proximal femur (R4).

### Biomechanical bone strength testing

The mechanical properties of the femoral mid-shaft were measured using three-point bending, using a universal testing machine (BISS Makron, Bangalore, India). Femur strength was assessed by three-point bending as previously described.<sup>8</sup>

**Table 1 – Effects of the different treatments on body weight.**

Group	Body weight	
	Pre-treatment (g)	Post-treatment (g)
Normal control	253.2 ± 10.65 <sup>a</sup>	320.1 ± 13.39 <sup>a</sup>
RHLI positive control	230.1 ± 18.22	216.6 ± 9.01
RHLI + ZOL	229.4 ± 11.22	218.0 ± 10.19
RHLI + PRO	222.4 ± 11.07	217.0 ± 10.21
RHLI + ZOL + PRO	227.4 ± 19.88	219.6 ± 14.14

Pre-treatment shows the data on the day prior to the start of treatment. Post-treatment shows data on the final day of the treatment. Data are expressed as the mean ± S.D. (n=6), evaluated by one-way ANOVA followed by Tukey's multiple comparison test.

<sup>a</sup> p < 0.001, compared to RHLI (positive control) group. All groups except normal group underwent right hind-limb immobilization (RHLI).

Briefly, femurs were removed from the -20 °C freezer and rehydrated in a saline solution for 4 h at room temperature. Hydrated weight of the bones was determined using a four decimal place digital scale. Length of the bones was measured with calipers. Specimens were placed on two supports that were separated by a distance of 12 mm and bent until fracture by lowering the crosshead positioned at the mid-shaft at a constant speed of 0.033 mm/s. From the load-displacement curve, the peak load (N), the ultimate stiffness (N/mm), and the toughness (mJ) were obtained. Ultimate stress (strength) and Young's modulus were derived from load-deformation curves obtained by using equations described by Khajuria et al.<sup>8</sup>

#### Measurement of femoral dry weight and ash weight

After conducting three-point bending test, the femurs of all animals were 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. Next, the dried femurs were burned to ash at 900 °C for 5 h, and their ash weight was measured.

#### 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, and differences between the immobilized side and the non-immobilized side were compared with the Wilcoxon signed-rank test. 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

### Effect of different treatments on body weight and femoral length

Ten weeks after RHLI, the body weights were significantly lower for animals in the RHLI (positive control) and RHLI treatment groups compared with the non-immobilized normal group. This difference became greater at the end of the experiment (RHLI for another 10 weeks). However, there were no statistically significant differences in weights observed between any of the active treatment groups and that of the RHLI (positive control) group (Table 1).

The length of the immobilized femurs was not significantly different from that of the non-immobilized femurs of the same rats in the RHLI (positive control) group and RHLI treatment groups (Table 2).

### Effect of different treatments on bone porosity

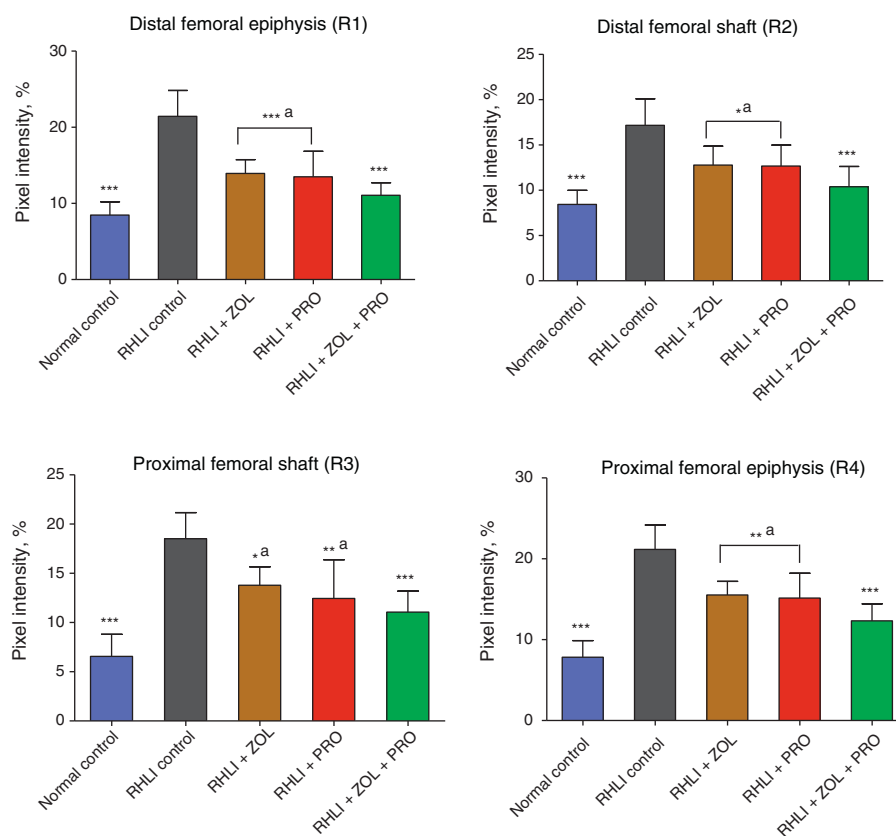
X-ray transmission intensity for the RHLI (positive control) group at R1, R2, R3 and R4 regions of rat femoral bone was significantly higher than those for the non-immobilized normal control group, which indicates an immobilization elicited increase in porosity in these areas.

After 10 weeks of therapy, all active treatments (single and combined) succeeded in decreasing bone porosity in RHLI rats.

**Table 2 – Average femoral length in control and experimental groups of rats.**

Group	Femur length (mm)	
	Left (non-immobilized)	Right (immobilized)
Normal control	40.80 ± 2.1	41.09 ± 2.2
RHLI positive control	39.99 ± 3.4	39.89 ± 1.3
RHLI + ZOL	40.31 ± 4.7	40.23 ± 4.7
RHLI + PRO	40.23 ± 3.3	40.14 ± 8.7
RHLI + ZOL + PRO	40.51 ± 7.6	40.48 ± 2.9

Data are expressed as the mean ± S.D. (n=6). All groups except normal group underwent right hind-limb immobilization (RHLI).



**Fig. 1 – Effect of zoledronic acid and propranolol, alone or in combination on femoral porosity.** Data are shown as the mean  $\pm$  SD ( $n = 6$ ), evaluated by Tukey's multiple comparison test. Bone porosity of R1: distal femoral epiphysis, R2: distal femoral shaft, R3: proximal femoral shaft, R4: proximal femoral epiphysis \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ , compared to RHLI (positive control) group; <sup>a</sup> $p < 0.05$ , compared to ZOL plus PRO group. All groups except normal group underwent right hind-limb immobilization (RHLI).

The X-ray transmission intensity of ZOL, PRO and ZOL plus PRO groups was significantly lower as compared with RHLI (positive control) group at R1, R2, R3 and R4 regions of rat femoral bone. The X-ray transmission intensity of ZOL plus PRO groups was significantly lower than that of the ZOL and PRO group at R1, R2, R3 and R4 regions. These results indicate that as compared to monotherapy with ZOL or PRO, the combination therapy with ZOL plus PRO is more beneficial for the mass of both trabecular and cortical bones that were decreased by immobilization (Fig. 1).

#### Effect of different treatments on mechanical properties in the femoral mid-shaft

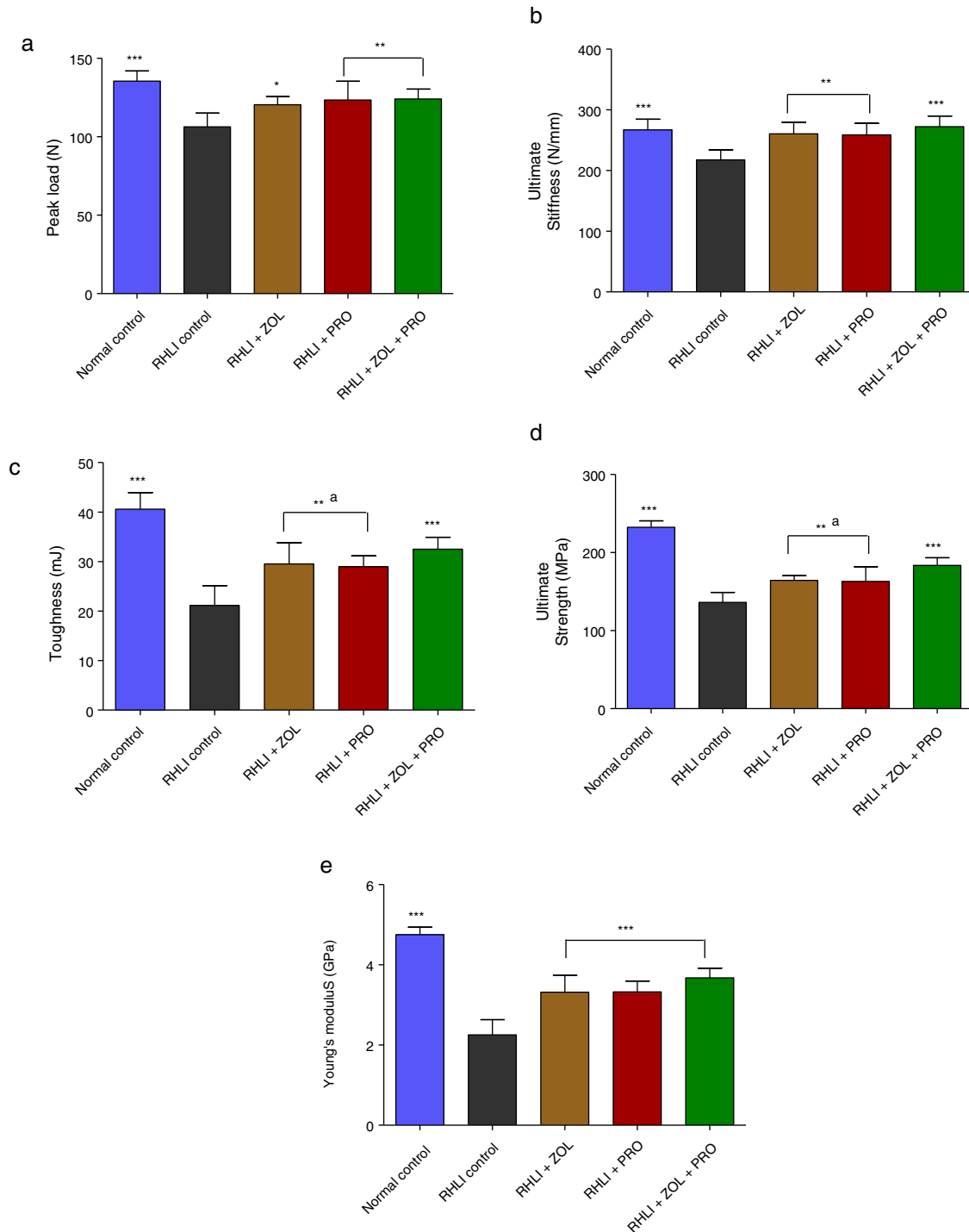
Fig. 2 shows the peak load, ultimate stiffness, toughness, ultimate strength and Young's modulus in the femoral mid-shaft, respectively. Three-point bending tests of the right femur indicated that RHLI (positive control) group caused significant reductions in the peak load, ultimate stiffness, toughness, ultimate strength and Young's modulus compared with those in non-immobilized normal group. In the ZOL, PRO and ZOL plus PRO groups, the peak load, ultimate stiffness, toughness, ultimate strength and Young's modulus of the femur was

significantly higher than in the RHLI (positive control) group. In ZOL and PRO groups, the ultimate strength and Young's modulus was significantly lower than that in the ZOL plus PRO group.

#### Comparison between non-immobilized (left) leg and immobilized (right) leg within a same group

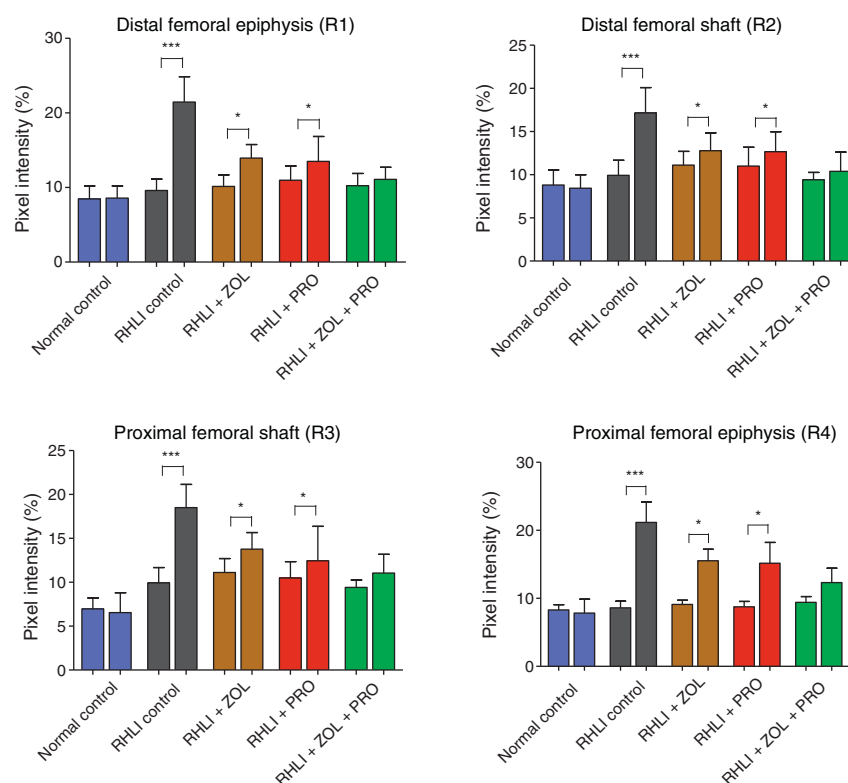
RHLI induced a significant decrease in dry and ash weights in RHLI (positive control) rat femurs compared to non-immobilized normal control rats (Table 3). In the RHLI rats treated with all active treatments (single and combined), dry and ash weights were significantly heavier than those in the RHLI (positive control) group. The RHLI femur of the rats treated with ZOL plus PRO, the dry and ash weight was significantly heavier than those in ZOL or PRO treated groups. In the left non-immobilized femurs, no significant difference was observed between RHLI (positive control) group, non-immobilized normal group and all active treatments (single and combined) groups.

The bone porosity and mechanical properties of the left and right legs are plotted as "split-bar" diagrams in Figs. 3 and 4, respectively. An asterisk indicates that there was a



**Fig. 2 – Effects of zoledronic acid, propranolol, or zoledronic acid plus propranolol on the mechanical strength of the femoral diaphysis. The diaphysis was subjected to three-point bending to failure, which provided data on peak load (a), ultimate stiffness (b), toughness (c), ultimate strength (d), and Young's modulus (e). Data are shown as the mean  $\pm$  SD ( $n = 6$ ), evaluated by Tukey's multiple comparison test. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ , compared to RHLI (positive control) group; <sup>a</sup> $p < 0.05$ , compared to ZOL + PRO group. All groups except normal group underwent right hind-limb immobilization (RHLI).**





**Fig. 3 – Femoral porosity for the non-immobilized (left bar) and the immobilized (right bar) side within the same group. Asterisk denotes significant difference between the non-immobilized side and the immobilized side (mean  $\pm$  SD). All groups except normal control group underwent right hind-limb immobilization (RHLI).**

significant difference between the left and right leg within the same group. At R1, R2, R3 and R4 regions of rat femoral bone, the X-ray transmission intensity for the immobilized side (right) seemed significantly higher than those from the non-immobilized side (left) in the RHLI (positive control) group.

Similarly, at R1, R2, R3 and R4 regions, the X-ray transmission intensity for the immobilized side (right) seemed significantly higher than those from the non-immobilized side (left) in the immobilized groups treated with ZOL or PRO. In contrast, the RHLI group treated with ZOL plus PRO showed full protection against disuse osteoporosis at R1, R2, R3 and

**Table 3 – Effect of immobilization and different treatments on dry bone and ash weight.**

Group	Left non-immobilized femur		Right immobilized femur	
	Dry bone weight (mg/bone)	Bone ash weight (mg/bone)	Dry bone weight (mg/bone) <sup>a</sup>	Bone ash weight (mg/bone) <sup>b</sup>
Normal control	630.7 $\pm$ 8.1	381.4 $\pm$ 7.7	639.8 $\pm$ 12.2 <sup>c</sup>	385.7 $\pm$ 9.4 <sup>c</sup>
RHLI positive control	620.5 $\pm$ 10.9	368.9 $\pm$ 4.3	543.9 $\pm$ 15.5	321.5 $\pm$ 11.5
RHLI + ZOL	622.1 $\pm$ 18.1	379.1 $\pm$ 7.2	593.7 $\pm$ 13.7 <sup>c,d</sup>	351.7 $\pm$ 9.8 <sup>b,d</sup>
RHLI + PRO	619.3 $\pm$ 12.5	372.9 $\pm$ 8.5	592.4 $\pm$ 12.3 <sup>c,d</sup>	346.1 $\pm$ 12.3 <sup>b,d</sup>
RHLI + ZOL + PRO	638.2 $\pm$ 9.2	388.9 $\pm$ 15.6	617.9 $\pm$ 15.5 <sup>c</sup>	365.6 $\pm$ 6.2 <sup>e</sup>

Data are expressed as the mean  $\pm$  S.D. (n = 6), evaluated by one-way ANOVA followed by Tukey's multiple comparison test.

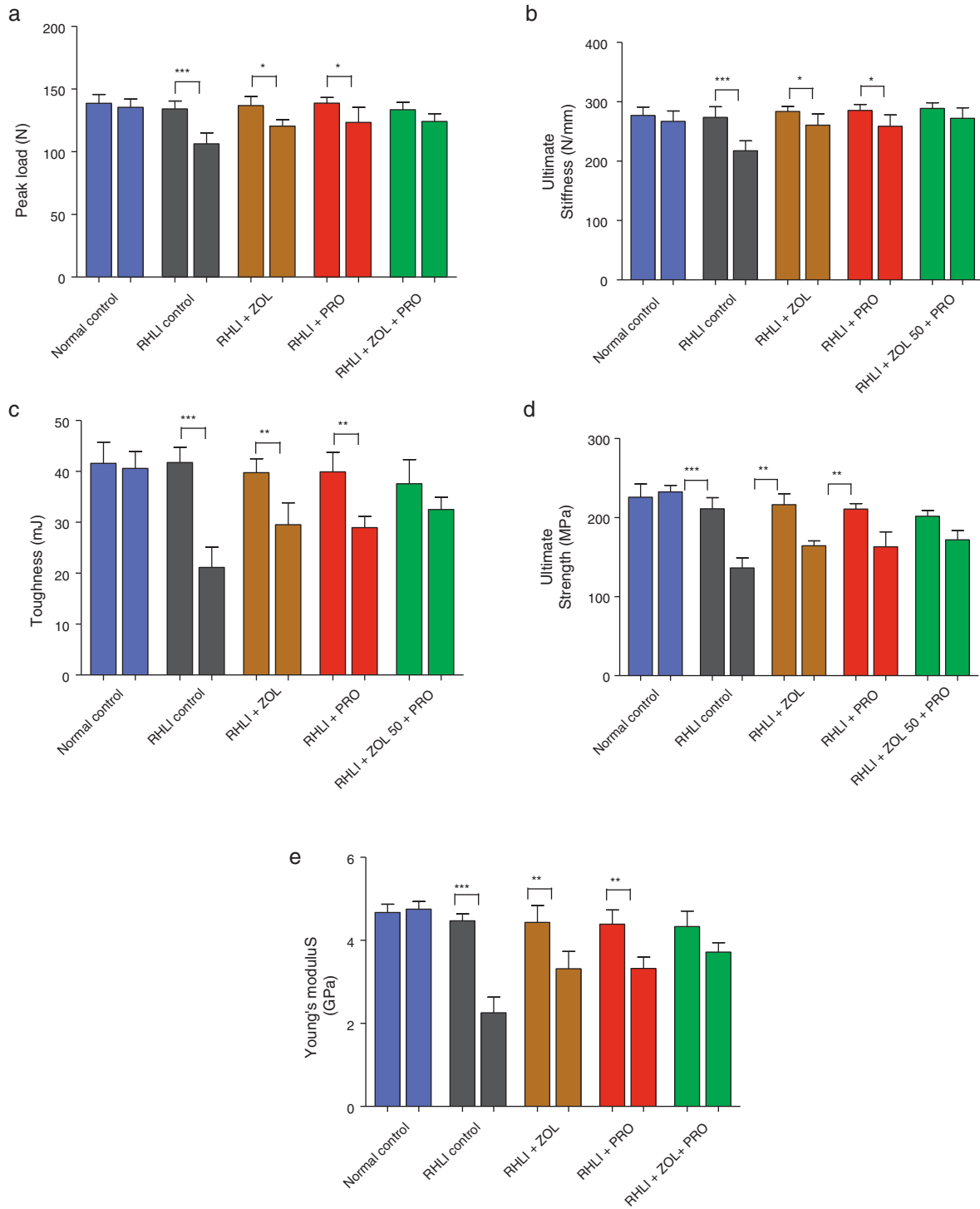
<sup>a</sup> Indicates, for the parameter, a significant difference of the active treatments between the two legs.

<sup>b</sup> p < 0.05.

<sup>c</sup> p < 0.001, compared to RHLI (positive control) group;

<sup>d</sup> p < 0.05 compared to ZOL plus PRO group. All groups except normal group underwent right hind-limb immobilization (RHLI).

<sup>e</sup> p < 0.01.



**Fig. 4 – Mechanical properties for the non-immobilized (left bar) and the immobilized (right bar) side with in the same group. The femoral mid-shaft was subjected to three-point bending to failure, which provided data on peak load (a), ultimate stiffness (b), toughness (c), ultimate strength (d), and Young’s modulus (e). Asterisk denotes significant difference between the non-immobilized side and the immobilized side (mean ± SD). All groups except normal group underwent right hind-limb immobilization (RHLI).**



R4 regions, as indicated by X-ray transmission intensity values (Fig. 3).

At the femoral mid-diaphysis (three-point bending test), the effect of immobilization was very pronounced in RHLI (positive control) group; that is, the immobilized side (right) had significantly lower values of strength parameters including peak load, ultimate stiffness, fracture toughness, ultimate strength and Young's modulus than the non-immobilized side (left). Similarly, in the RHLI groups treated with ZOL or PRO, the immobilized side (right) had significantly lower values of strength parameters, including peak load, ultimate stiffness, fracture toughness, ultimate strength and Young's modulus than the non-immobilized side (left). In contrast, the RHLI group treated with ZOL plus PRO showed full protection against immobilization (Fig. 4).

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## Discussion

This study was aimed at clinical application of the combination therapy with ZOL plus PRO as a curative treatment of established disuse osteoporosis. Due to the difficulty and high cost of conducting experiments during a space flight on astronauts, a number of *in vivo* ground-based experimental models have been established by researchers to simulate the conditions experienced during a space mission. The present study showed that PRO and ZOL monotherapy was able to counteract the bone loss in a rat model of disuse osteoporosis. Furthermore, this study showed that combination therapy with ZOL plus PRO had a therapeutic advantage over ZOL or PRO monotherapy for treatment of disuse osteoporosis.

Body weight in the normal group was greater than in the RHLI (positive control) group. This may have been due to the anesthesia administered during the RHLI procedure. Reduced eating and overall reduced mobility are other possible factors that may have contributed in a minor way to the development of lower body weight and bone loss. Earlier studies have shown a similar decrease in body weight after RHLI.<sup>2,22,23</sup> The length of the femur of the immobilized limb was not significantly different from that of the non-immobilized intact femur of the same rat, suggesting that the longitudinal growth of the bone is not retarded in these animals. It is therefore more likely that we are here dealing with immobilization osteoporosis rather than simple growth retardation.

The increase in the bone porosity at R1, R2, R3, R4 regions of rat femoral bone, due to unloading of right hind-limb was suppressed by all active treatments (single and combined). Moreover, the bone porosity of the ZOL plus PRO group at R1, R2, R3 and R4 regions of rat femoral bone was significantly lower than that of the ZOL or PRO monotherapy. This indicates that combination therapy with ZOL plus PRO thickens and strengthens cortical bone. It is interesting that, in animals treated with single and combined therapy, dry and ash weights in the right immobilized femur were significantly greater than those of the RHLI (positive control) group. Moreover, in the animals treated with combined therapy of ZOL plus PRO, dry and ash weights in the right

immobilized femur 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 RHLI.

Mechanical load is crucial for the maintenance of bone strength. The bone strength is determined by the bone mass and the intrinsic properties of the bone material. Physical inactivity due to spinal cord injuries, prolonged bed rest in elderly and astronauts exposed to microgravity during space flight would accelerate the bone microarchitecture deterioration and demineralization.<sup>2,7</sup> Combination therapy with ZOL plus PRO was statistically superior to monotherapy with ZOL and PRO at increasing femoral mid-shaft toughness and ultimate strength. The current data correlate with findings from our previous studies, demonstrating effects of ZOL plus PRO on the mechanical properties of ovariectomized rat bone.<sup>7</sup> Therefore, it is of high possibility that combination therapy with ZOL plus PRO is capable of treating/preventing weightlessness-induced bone loss.

This study has several limitations. It should be noted that extrapolation to humans of the data from rat studies should be undertaken with caution because (1) rats are quadrupeds, and therefore experience a different loading pattern from that of humans; and (2) the remodeling pattern in rats is different from that of humans. However, the advantage of using this preclinical rat model is that it allows assessment not only of bone turnover and bone mass, but also of bone mechanical properties.

Comparison made between non-immobilized (left) leg and immobilized (right) leg within a same group showed that bone properties were improved by all therapeutic interventions, but the marked osteopenia induced by RHLI were not completely corrected with single treatments like ZOL or PRO alone. In contrast, combined treatment with ZOL plus PRO showed full protection against disuse osteoporosis, suggesting that the combination therapy has a therapeutic advantage over each monotherapy for the treatment of disuse osteoporosis.

With regard to the clinical situation and the knowledge gained from the aforementioned studies concerning immobilization and from the present study, it would seem advisable to shorten immobilization periods as much as possible<sup>24,25</sup> and perhaps to consider the use of combination therapy of ZOL plus PRO as protection against loss of bone density and strength even during short-term immobilization. Moreover, the combined therapy with ZOL plus PRO may prevent or reduce the risk of atrial fibrillation, one of the serious adverse drug reactions of ZOL.<sup>7</sup> In other clinical situations with very long-term immobilization (paraplegia or tetraplegia, both after spinal cord lesions, or hemiplegia after cerebrovascular accident, long term space travel) preventive treatment with ZOL plus PRO may also be recommended based on findings of the present preclinical study.

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## Conclusions

In conclusion, current study provides evidence that PRO and ZOL, when given as monotherapy, were able to reverse the

inhibitory effect of immobilization on bone formation. This study firstly demonstrates that combination therapy with ZOL plus PRO therapy is highly effective in improving the bone properties in an animal model of disuse osteoporosis, suggesting that the combination therapy has a therapeutic advantage over ZOL or PRO monotherapy for the prevention and treatment of disuse osteoporosis induced by mechanical inactivity. The findings are consistent with the effects of ZOL plus PRO on estrogen deficiency-induced bone loss and extend our knowledge regarding the effects of this therapy in immobilization-induced bone loss. As such, this combined regimen may be of interest for further evaluation in clinical studies. 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.

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