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Original article

Additive effect of zoledronic acid and alfacalcidol in the treatment of disuse osteoporosis in rats

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ABSTRACT

Objectives: Disuse by bed rest, limb immobilization or space flight causes rapid bone loss. We conducted the present study to investigate the therapeutic effects of zoledronic acid (ZOL), alone and in combination with alfacalcidol (ALF) 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 divided into four groups: 1 – RHLI positive control; 2 – RHLI plus ZOL (50 μg/kg, i.v. single dose); 3 – RHLI plus ALF (0.5 μg/kg, oral gauge daily); 4 – RHLI plus ALF (0.5 μg/kg, oral gauge daily) 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 the treatment, the femurs were removed and tested for bone porosity, bone mechanical properties, and bone dry and ash weight.
Results: Combination therapy with ZOL plus ALF was more effective in decreasing bone porosity than each drug administered as monotherapy in RHLI rats. With respect to improvement in the mechanical strength of the femoral mid-shaft, the combination treatment of ZOL plus ALF was more effective than each drug administered as a monotherapy. Moreover, combination therapy using ZOL plus ALF was more effective in improving dry bone and ash weight, than single-drug therapy using ZOL or ALF in RHLI rats.
Conclusions: These data suggest that combination therapy with ZOL plus ALF represents a potentially useful therapeutic option for the treatment of disuse osteoporosis.

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Efeito combinado do ácido zoledrônico e do alfacalcidol no tratamento da osteoporose por desuso em ratos

Palavras-chave:
Osteoporose por desuso
Estudo com ratos
Ácido zoledrônico
Alfacalcidol

RESUMO

Objetivos: O desuso pelo repouso no leito, pela imobilização de membros ou por missões espaciais provoca a perda óssea rápida. Fez-se este estudo para investigar os efeitos terapêuticos do ácido zoledrônico (ZOL), isoladamente e em combinação ao alfacalcidol (ALF), em um modelo de rato com osteoporose por desuso.

Métodos: Ratos Wistar machos de três meses foram submetidos à imobilização da pata traseira direita (IPTD) por 10 semanas para induzir a osteopenia; em seguida, foram divididos em quatro grupos: 1 – IPTD para controle positivo; 2 – IPTD mais ZOL (50 μg/kg, dose única intravenosa); 3 – IPTD mais ALF (0,5 μg/kg, via oral diariamente); 4 – IPTD mais ALF (0,5 μg/kg, via oral diariamente) 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: A terapia combinada com ZOL mais ALF foi mais eficaz em reduzir a porosidade do osso do que a monoterapia com um dos fármacos administrado isoladamente em ratos submetidos à IPTD. No que diz respeito à melhoria da resistência mecânica da diáfise femoral média, o tratamento combinado com ZOL mais ALF foi mais eficaz do que a monoterapia com um dos fármacos administrado isoladamente. Além disso, a terapia combinada com ZOL mais ALF foi mais eficaz na melhoria do peso seco e das cinzas do osso do que a monoterapia com ZOL ou ALF em ratos submetidos à IPTD.

Conclusões: Esses dados sugerem que a terapia combinada com ZOL mais ALF representa uma opção terapêutica potencialmente útil para o tratamento da osteoporose por desuso.

Introduction

Mechanical loading is essential for the normal functioning of bone tissue.[1] Maintenance of skeletal integrity, bone mass and bone formation in weight-bearing limbs are dependent on gravity.[2] Skeletal unloading induced by prolonged cast or splint fixation, stress protection secondary to plate fixation of fractures, incapacitation due to chronic illness or spinal cord injury, or weightlessness associated with orbital space flight causes a decrease in bone mass in both human and animal models.[2,3] Immobilization (disuse) osteoporosis causes net bone loss as a result of an imbalance between bone resorption and bone formation.[3] Hence, we have to keep in mind that bone loss due to prolonged immobilization increases the susceptibility to fractures in patients with spinal cord injuries, elderly requiring bed rest and astronauts during long space missions. Therefore, it is very essential to select optimal treatment for effective management of disuse osteoporosis.

Bisphosphonates inhibit bone resorption as they are selectively incorporated into osteoclasts and interfere with the resorptive action of osteoclasts.[4] Zoledronic acid (ZOL) is a third generation nitrogen containing bisphosphonate, and is widely used for postmenopausal and glucocorticoid-induced osteoporosis in humans.[5–8] The effect of a single treatment of ZOL for immobilization-induced osteoporosis has been shown in animal studies.[9,10] Although anti-resorptive agents such as bisphosphonates are effective in reducing bone loss, they are not able to induce formation of a new bone.[7,10]

Alfacalcidol (1 alpha-hydroxy Vitamin D_3 – ALF) is a synthetic Vitamin D analog, a calcium regulating hormone, and is frequently used in several countries to treat osteoporosis.[9,11] ALF reduces parathyroid hormone levels, as a result of both increased calcium absorption and an inhibition of the proliferation of the parathyroid gland, and also decreases the release of pro-inflammatory cytokines, which contribute to osteoclast activation. Moreover, ALF stimulates the formation and action of osteoblasts, leading to increased bone formation.[12–15] It has been demonstrated previously that the administration of ALF diminished the effect of immobilization in the development of osteoporosis.[9,16]

ZOL and ALF are commercially available in India. ZOL is known to inhibit osteoclast-mediated bone resorption,[9,12,17] while ALF exerts both anabolic and anti-resorptive effects on the skeleton.[13,18,19] A combination of two different drugs is believed to be a more effective treatment than a single treatment for osteoporosis; the combination of bisphosphonate and a bone anabolic drug has been used clinically for postmenopausal osteoporosis.[20,21] As immobilization-induced bone loss involves both increased bone resorption and decreased bone formation,[3] it seems obvious to target the immobilization-induced bone loss with a combined anti-resorptive and bone anabolic treatment regimen, such as ZOL and ALF. The effects of a combined ZOL and ALF treatment have previously been studied in ovariectomized rats,[17] whereas this treatment regimen has not previously been investigated in rat model of disuse osteoporosis. Consequently, the aim of the present study was to investigate the efficacy of a bone anabolic agent ALF, a bone anti-resorptive

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 ALF, our hypothesis was that the combination of ZOL and ALF 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 and ALF were obtained from Naprod Life Sciences (Maharashtra, India) and GlaxoSmithKline Pharmaceuticals (Mumbai, India), respectively. Ketamine, xylazine and xylene were obtained from Neon Pharma (Mumbai, India), Indian Immunologicals (Hyderabad, India), and S.D. Fine chemicals (Mumbai, India), respectively.

Experimental animals

Twelve-week-old male Wistar rats weighing 170–180 g were included in 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, 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 hind-limb immobilization described by Khajuria et al.[9]

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 μg/kg, single intravenous dose) for another 10 weeks; 4 – RHLI for 10 weeks, and then RHLI plus ALF (0.5 μg/kg, oral gauge daily) for another 10 weeks; 5 – RHLI for 10 weeks, then RHLI plus ALF (0.5 μg/kg, oral gauge daily) plus ZOL (50 μg/kg, single intravenous dose) for another 10 weeks. Oral gauge daily in case of RHLI groups treated with ALF and ZOL plus ALF required some animal handling and created some stress to the animals. Therefore, non-immobilized (negative control) and RHLI (positive control) and RHLI plus ZOL groups were orally administered vehicle (normal saline) for 10 weeks. All RHLI rats were single housed in polypropylene cages (size: 421 mm × 290 mm × 190 mm with gap of 7 mm between wires) during the experimental period. The medication dosages used in this experiment were selected from our previous study conducted on rat model of postmenopausal osteoporosis.[17]

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 °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 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 previously.[22] Briefly, for X-ray analysis of rat femur, whole femur was divided into four equal fields, which included 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.[22] 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 previously.[22]

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 had become 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).

There were no significant differences in immobilized (right) femoral length between positive control group (39.89 ± 1.3 mm), ZOL group (40.23 ± 4.7 mm), ALF group (39.78 ± 8.4 mm), ZOL + ALF group (40.69 ± 10.4) and normal control group (41.09 ± 2.2 mm). Moreover, there were no significant differences between the immobilized (right) side and non-immobilized (left) side (data not shown).

Effect of different treatments on bone porosity

The X-ray image shows the difference in the X-ray transmission intensity depending on the porosity of the sample. The X-ray transmission intensity is directly proportional to the porosity. The effects of RHLI and subsequent treatment with all therapeutic interventions on the porosity of the right femur were measured by X-ray imaging, as shown in Fig. 1. X-ray intensity for the RHLI (positive control) group at R1 (distal epiphysis), R2 (mid-shaft: distal) R3 (mid-shaft: proximal) and R4 (proximal epiphysis) was significantly higher than those for the non-immobilized normal group, which indicates an immobilization elicited increase in bone porosity in these areas.

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[a]	320.1 ± 13.39[a]
RHLI positive control	230.1 ± 18.22	216.6 ± 9.01
RHLI + ZOL	229.4 ± 11.22	218.0 ± 10.19
RHLI + ALF	232.8 ± 17.15	223.4 ± 12.18
RHLI + ZOL + ALF	225.8 ± 13.34	212.6 ± 11.13

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.

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

After 10 weeks of therapy, all active treatments (single and combined) succeeded in decreasing bone porosity in RHLI rats. X-ray transmission intensity values at R1 (distal femoral epiphysis) for ZOL, ALF and ZOL + ALF groups was lower than those of RHLI (positive control) group ($p < 0.001$). Similarly, the X-ray transmission intensity values at R2 (distal femoral shaft) for ZOL, ALF and ZOL + ALF groups was lower than those of RHLI (positive control) group ($p < 0.01$; $p < 0.05$ and $p < 0.001$ respectively). Likewise, the X-ray transmission intensity values at R3 (proximal femoral shaft) for ZOL, ALF and ZOL + ALF groups was lower than those of RHLI (positive control) group ($p < 0.01$; $p < 0.001$ and $p < 0.001$ respectively). Furthermore, the X-ray transmission intensity values at R4 (proximal femoral epiphysis) for ZOL, ALF and ZOL + ALF groups was lower than those of RHLI (positive control) group ($p < 0.01$; $p < 0.01$ and $p < 0.001$ respectively). In contrast, the X-ray transmission intensity of the ZOL + ALF group was significantly lower than that of the ZOL and ALF groups at R1, R2, R3 and R4 regions ($p < 0.05$).

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 caused significant reductions in the peak load, ultimate stiffness, toughness, ultimate strength and Young's modulus compared with those in non-immobilized normal group ($p < 0.001$).

In ZOL, ALF and ZOL + ALF groups, the peak load of the femur was significantly higher than in the RHLI (positive control) group ($p < 0.05$; $p < 0.01$ and $p < 0.001$, respectively). Similarly, ZOL, ALF and ZOL + ALF groups, the ultimate stiffness of the femur was significantly higher than in the RHLI (positive control) group ($p < 0.01$; $p < 0.01$ and $p < 0.001$ respectively). The toughness of the femur in the ZOL, ALF and ZOL + ALF groups was significantly higher than in the RHLI (positive control) group ($p < 0.01$; $p < 0.05$ and $p < 0.001$ respectively). In all single treatments, the toughness was significantly lower than that in the ZOL + ALF group ($p < 0.05$). Moreover, ZOL, ALF and ZOL + ALF groups, the ultimate strength of the femur was significantly higher than in the RHLI (positive control) group ($p < 0.001$). In ZOL and ALF groups, the ultimate strength was significantly lower than that in the ZOL + ALF group ($p < 0.01$; $p < 0.001$, respectively). Furthermore, the Young's modulus of the ZOL, ALF and ZOL + ALF groups was significantly increased when compared with the RHLI (positive control) group ($p < 0.05$; $p < 0.01$; $p < 0.01$ and $p < 0.001$, respectively). In all single treatments, the Young's modulus was significantly lower than that in the ZOL + ALF group ($p < 0.05$).

Comparison of the effect of different treatments on dry and ash weights of immobilized and non-immobilized femurs in rats

RHLI induced a significant decrease ($p < 0.001$) in dry and ash weights in immobilized control rat femurs compared to non-immobilized normal control rats (Table 2). The RHLI femur of rats treated with single and combined treatments, dry and

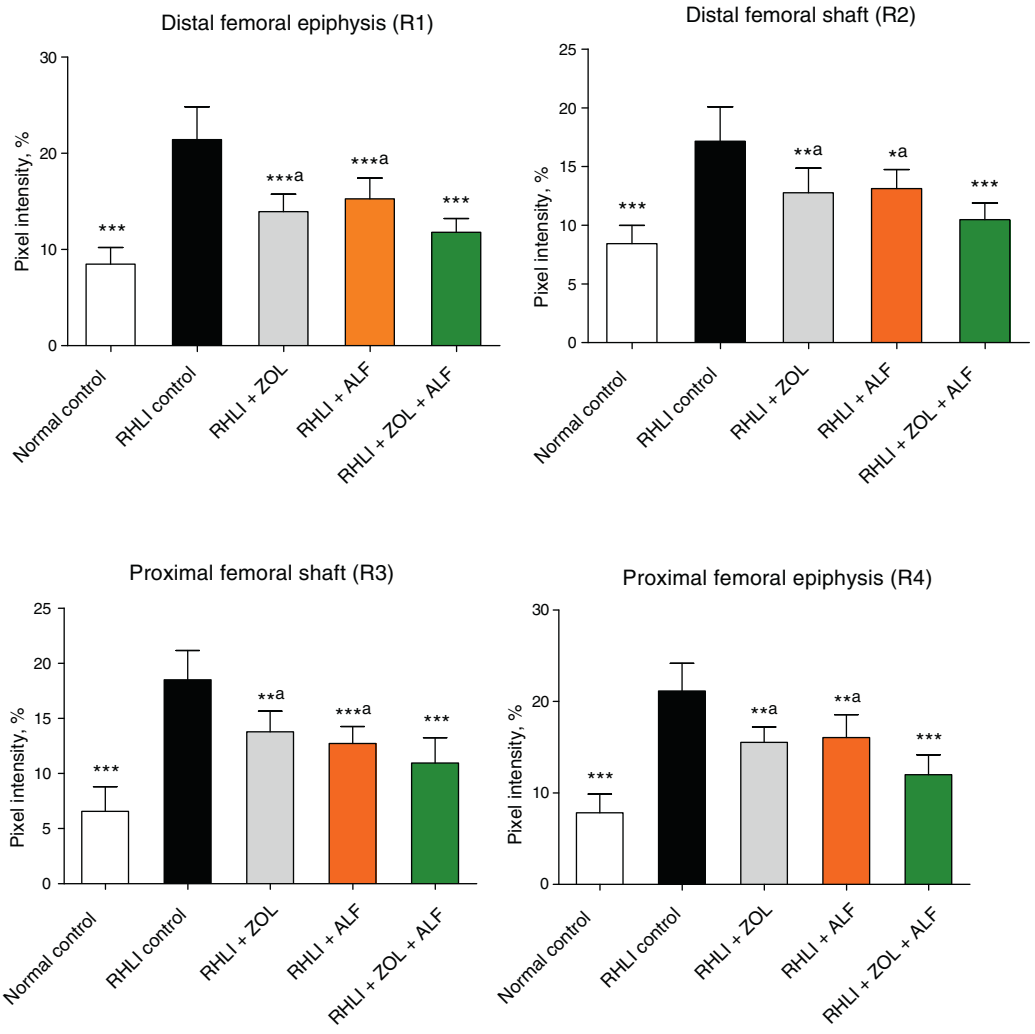

Fig. 1 – Effect of zoledronic acid and alfacalcidol, alone or in combination on femoral porosity. Bone porosity of R1: distal femoral epiphysis, R2: distal femoral shaft, R3: proximal femoral shaft, R4: proximal femoral epiphysis. Data are shown as the mean ± SD ($n = 6$), evaluated by Tukey's multiple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

Table 2 – 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)[a]	Bone ash weight (mg/bone)[a]
Normal control	630.7 ± 8.1	381.4 ± 7.7	639.8 ± 12.2[b]	385.7 ± 9.4[b]
RHLI positive control	620.5 ± 10.9	368.9 ± 4.3	543.9 ± 15.5	321.5 ± 11.5
RHLI + ZOL	622.1 ± 18.1	379.1 ± 7.2	593.7 ± 13.7[b,c]	351.7 ± 9.8[b,c]
RHLI + ALF	627.5 ± 12.1	382.4 ± 9.6	583.5 ± 9.4 [b,c]	349.5 ± 7.6[b,c]
RHLI + ZOL + ALF	640.7 ± 14.8	392.2 ± 13.6	619.1 ± 12.1[b]	369.9 ± 9.4[b]

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

[a] Indicates, for the parameter, a significant difference of the active treatments between the two.

[b] $p < 0.001$, compared to immobilized RHLI (positive control) group; legs.

[c] $p < 0.05$ compared to ZOL + ALF group.

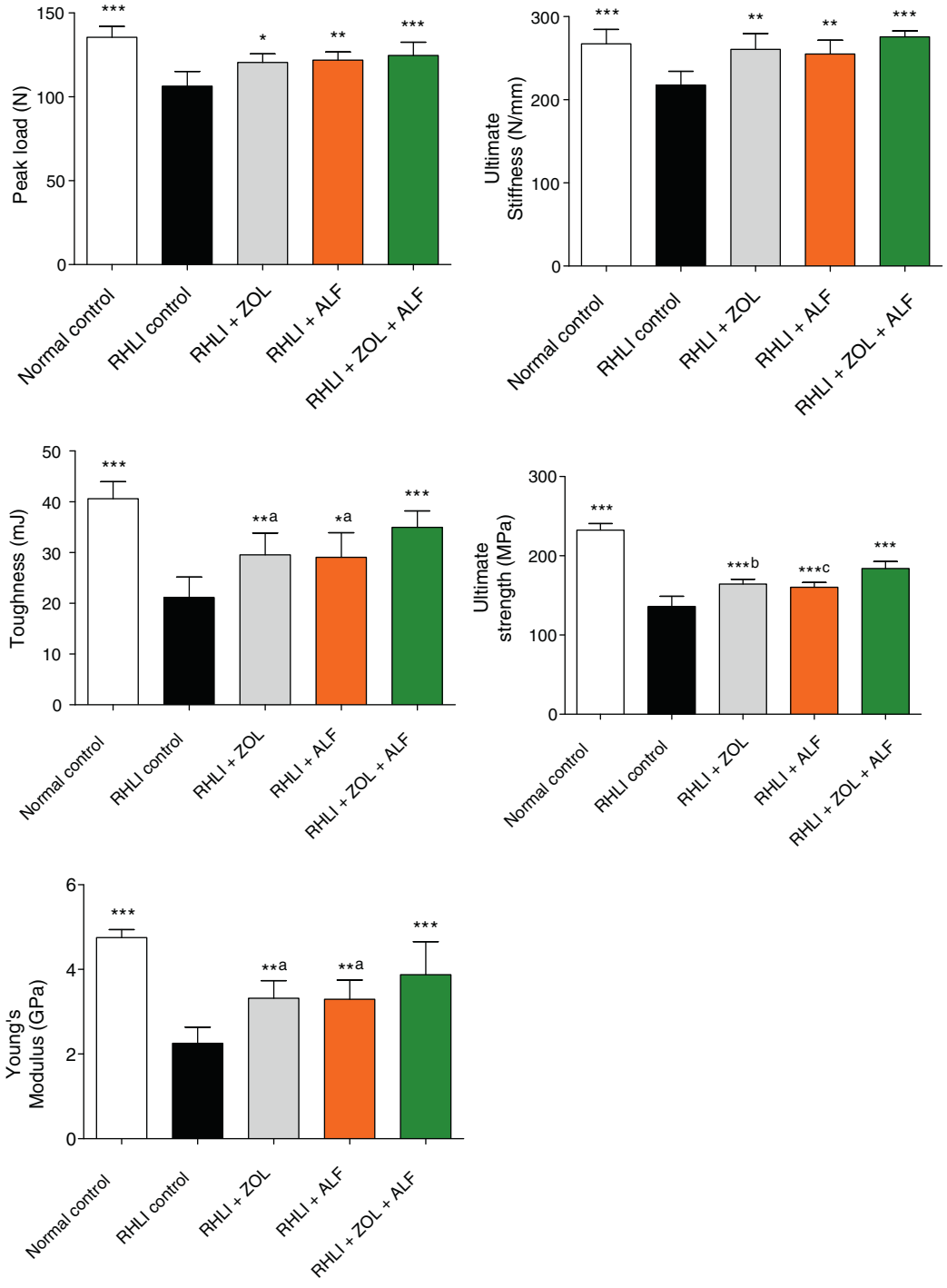

Fig. 2 – Effects of zoledronic acid, alfacalcidol, or zoledronic acid plus alfacalcidol on the mechanical strength of the femoral diaphysis. The diaphysis was subjected to three-point bending to failure, which provided data on peak load, ultimate stiffness, toughness, ultimate strength, and Young's modulus. 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 RHLI (positive control) group; ^{a} $p < 0.05$; ^{b} $p < 0.01$, ^{c} $p < 0.001$; compared to ZOL + ALF group. All groups except normal group underwent right hind-limb immobilization (RHLI).

ash weights were significantly heavier than those in the RHLI (positive control) group. The RHLI femur of the rats treated with ZOL + ALF, the dry weight was significantly heavier than those in ZOL or ALF treated groups ($p < 0.05$ in the ALF group). Moreover, the RHLI femur of the rats treated with ZOL + ALF group, the ash weight was significantly heavier than those in ZOL and ALF treated groups ($p < 0.05$ in the ALF group). In the left non-immobilized femurs, no significant difference was observed between single and combined treated and non-immobilized normal groups.

Comparison between non-immobilized (left) leg and immobilized (right) leg with in a same group

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 significant difference between the left and right leg within the same group. 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 RHLI (positive control) group ($p < 0.001$). 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 RHLI groups treated with ZOL or ALF ($p < 0.05$). In contrast, the RHLI group treated with ZOL + ALF showed full protection against disuse osteoporosis at R1, R2, R3 and 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) ($p < 0.001$). Similarly, in the RHLI groups treated with ZOL or ALF, the immobilized side (right) had significantly ($p < 0.01$) 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 + ALF showed full protection against immobilization (Fig. 4).

Discussion

To our knowledge, the combined effects of anti-resorptive and anabolic agents on the skeleton have not specially been assessed in rat model of disuse osteoporosis. The present study was conducted to clarify the efficacy of combined administration of ZOL and ALF on overall quality of bone in RHLI rats. The results of this study showed that ZOL and ALF monotherapy was able to counteract the bone loss in a rat model of disuse osteoporosis. The anti-osteoporotic property of ZOL plus ALF combination therapy appeared to be more effective when compared to ZOL or ALF monotherapy. Thus, we confirmed the beneficial effects of combined administration of ZOL and ALF for treatment of disuse osteoporosis in rats.

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.[9,23] 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.

Prominent increase in bone porosity was observed at R1, R2, R3 and R4, after immobilization of right hind-limb. 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 treatment with ZOL, ALF and ZOL + ALF. In the analysis of the bone porosity of rat femur using X-ray imaging, it was found that combination therapy with ZOL + ALF was statistically superior to ZOL or ALF monotherapy in suppressing the increase in bone porosity due to RHLI. This indicates that combination therapy with ZOL + ALF thickens and strengthens cortical bone.

It should be noted that, in rats treated with ZOL, ALF and ZOL + ALF, 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 + ALF, dry and ash weights in the right immobilized femur were significantly greater than those of the ZOL or ALF groups. These results showed that the combined treatment with ZOL + ALF is beneficial for increasing the mass of rat femoral bones that was decreased due to RHLI.

Bone mechanical properties are related to bone density, architecture, connectivity and mineralization.[23] Immobilization results in decreased mechanical properties[9,24] and the same was observed in the present study. In this experiment, the mechanical properties of the rat femoral bone decreased in RHLI (positive control) group when compared to non-immobilized normal control group, suggesting an increase in the fragility of the cortical bone of RHLI rats. Results of the bending test in rats treated with ZOL, ALF and ZOL + ALF indicate higher ultimate peak load, stiffness, energy, bending stress and Young's modulus compared to RHLI (positive control) group rats. Combination therapy with ZOL 50 + ALF was statistically superior to ZOL or ALF monotherapy at increasing femoral mid-shaft toughness, ultimate strength, and Young's modulus.

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 was not completely corrected with ZOL or ALF monotherapy. In contrast, combined treatment with ZOL + ALF showed full protection against disuse osteoporosis, suggesting that the combination therapy has a therapeutic advantage over each drug monotherapy for the treatment of disuse osteoporosis.

Osteoporosis therapeutics has been dominated by anti-resorptive agents, mainly bisphosphonates (risedronate, alendronate, ibandronate, pamidronate, zoledronic acid, etc.) which prevent further bone loss in established osteoporosis but do not change the bone mass or replenish the already lost bone.[7,10,25] In the present experimental research we preferred ZOL over other bisphosphonates available in India. ZOL has several advantages over other bisphosphonates: (1) the obvious advantage of ZOL over other bisphosphonates is the high level of adherence that is possible under the controlled environment of a once yearly infusion administered under medical supervision. Considering the low rates of adherence to oral bisphosphonates, this is a significant medical advance[26]; (2) oral bisphosphonates are not easily absorbed by the intestine and exhibit variable bioavailability. Therefore, high doses are required to be administered orally which, cause adverse

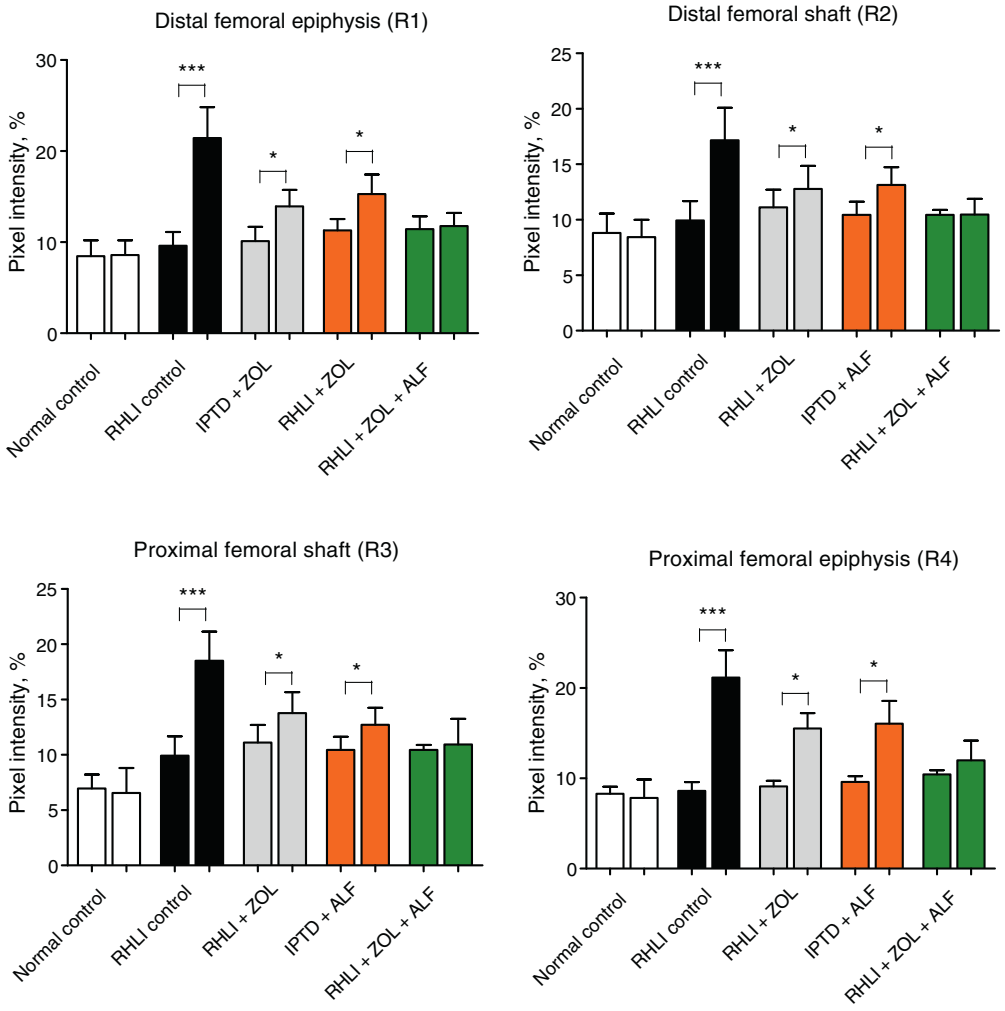

Fig. 3 – Femoral porosity for the non-immobilized (left bar) and the immobilized (right bar) side with in the same group. Asterisk denotes significant difference between the non-immobilized side and the immobilized side (mean ± SD). All groups except normal group underwent right hind-limb immobilization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

events like esophagitis[7]; (3) it was found that under conditions likely to stimulate bisphosphonate binding onto bone, ZOL has higher binding affinity among all other bisphosphonates for bone hydroxyapatite[27]; (4) the cost of ZOL is comparable to the annual cost of oral bisphosphonates and less than the cost of the other intravenous bisphosphonate (ibandronate), which is administered every 3 months.[27] However, parenteral administrations of bisphosphonates have shown acute phase response, hypocalcemia and secondary hyperparathyroidism, musculoskeletal pain, renal complications, osteonecrosis of the jaw and ocular events in some cases.[7,27,28] The results of our previous study showed that a lower dose of ZOL (50 μg/kg, single intravenous dose) had showed similar promising and beneficial effects in treating osteoporosis when compared with the therapeutic dose of ZOL (100 μg/kg, single intravenous dose) in estrogen deficient rats.[29] Therefore, to reduce the risk of known adverse drug reactions by ZOL, we chose a lower dose of ZOL (50 μg/kg, single intravenous dose).

ALF is used to treat osteoporosis because it exerts both anabolic and antiresorptive effects on the skeleton.[13,18,19] However, higher doses of ALF may increase the risk of hypercalciuria or hypercalcemia. On the other hand, treatment with ZOL may cause hypocalcemia by suppression of bone resorption.[17] The different mechanisms of ZOL and ALF suggest that a combination therapy using ZOL with ALF may eliminate or minimize the risk of known adverse drug reactions previously seen by these two drugs when administered alone. Based on the results of our previous and present findings, we propose that combination therapy with ZOL + ALF acts by increasing bone formation (by stimulating the formation and action of osteoblast cells) and decreasing bone resorption (by inducing osteoclast apoptosis), thus rebalancing bone turnover in favor of bone formation, an effect that results in increased bone mass and strength. This can possibly explain how combination therapy with ZOL + ALF was more effective than ZOL or ALF monotherapy in increasing bone mass and strength in RHLI rats.

This study has several limitations: (1) rats are quadrupeds, and therefore experience a different loading pattern from that of humans; (2) the remodeling pattern in rats is different from

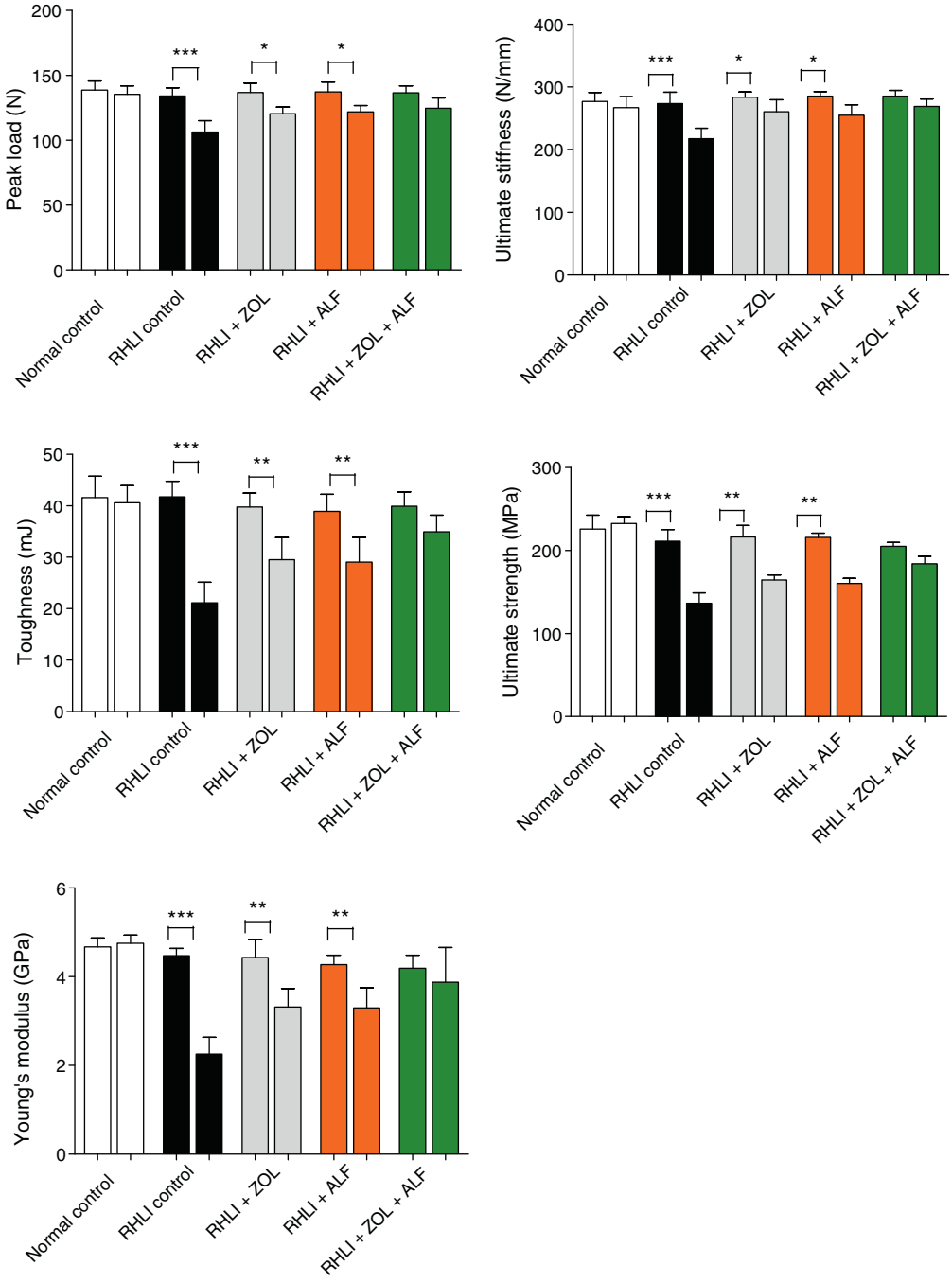

Fig. 4 – Mechanical properties for the non-immobilized (left bar) and the immobilized (right bar) side with in the same group. The femoral mid-shaft was subjected to three-point bending to failure, which provided data on peak load, ultimate stiffness, toughness, ultimate strength, and Young's modulus. 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).

that of humans; (3) in the present study, a growing rat model was used. Thus, maturation-related gains in bone mass could not be ignored in RHLI rats; (4) furthermore, the effect of ZOL and ALF on cortical porosity, which could be observed in aged RHLI rats was not assessed. Therefore, the results of present study cannot be translated into human with primary disuse osteoporosis. Thus, further studies are needed to confirm the beneficial effects of combined administration of ZOL and ALF on the bone mass in RHLI rats.

Conclusions

The current in vivo study firstly demonstrated that combination therapy with ZOL + ALF therapy is highly effective

in improving the bone properties in an rat model of disuse osteoporosis, suggesting that the combination therapy has a therapeutic advantage over ZOL or ALF monotherapy for the treatment of disuse osteoporosis induced by mechanical inactivity. As such, this combined regimen may be of interest for further evaluation in clinical studies.

Conflicts of interest

The authors declare no conflicts of interest.

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