# A Histological Study on the Short-Term Effects of Zoledronate, A Third Generation Bisphosphonate, on the Trabeculae of Growing Bone of Tibia in Albino Rats

**Original Article**

***Sarah Ralte[1], Kamlesh Khatri[2] and Mahindra Nagar[2]***

*[1]Department of Anatomy, North Eastern Indira Gandhi Regional Institute of Health and Medical Sciences (NEIGRIHMS) - An Autonomous Institute under Ministry of Health and Family Welfare (MoHFW), Govt. of India Shillong, Meghalaya, India*

*[2]Department and Institution - Department of Anatomy, University College of Medical Sciences (UCMS), Delhi, India*

## ABSTRACT

**Introduction:** Zoledronate, a third generation bisphosphonate, is a potent bisphosphonate in inhibiting excessive osteoclastic mediated bone resorption. Bisphosphonates show strong affinity to hydroxyapatite crystals in mineralized bone matrix, where they get incorporated by osteoclast, the primary target cell and inhibit osteoclastic bone resorption through multiple complex mechanisms, and increase the trabecular bone mass. As not many histological studies on the short-term effects of zoledronate on growing bone are available, hence this study was undertaken.

**Materials and Methods:** Twenty days old male albino rats (n=15) were randomly divided into two groups. Control group I (eight animals) were given equal volume of normal saline according to body weight. The experimental group II (seven animals) were given 2.8µg/kg body weight of zoledronate subcutaneous, daily for eleven days. All animals were sacrificed on thirty-first day. Tibiae were dissected out, decalcified in ethylene diamine tetra acetic acid and processed for paraffin sectioning. Seven µm thick sections were stained with hematoxylin and eosin and Masson's trichrome stains. The slides were examined under Zeiss light microscope and the findings were analyzed with Image Pro-Express Analyzer.

**Results:** We observed a significant increase ($p$<0.001) in the number of trabeculae both in the regions of primary spongiosa (zoledronate treated: 14.69±1.14/mm$^2$) and secondary spongiosa (zoledronate treated: 11.96±0.29/mm$^2$) in the experimental treated group. The mean width and mean area of trabeculae were seen to be statistically significant ($p$<0.001) in the zoledronate treated group as compared to control saline treated group.

**Conclusion:** The present study showed a significant increased trabecular bone mass in the metaphyseal region following short-term administration of zoledronate despite presence of numerous large polymorphic osteoclasts, the bone resorbing cells. Short-term administration of zoledronate could be highly beneficial in pediatric metabolic bone disorders such as osteoporosis, osteogenesis imperfecta and fibrous dysplasia.

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**Key Words:** Bisphosphonate; proximal metaphysis; tibia; trabeculae; zoledronate.

**Corresponding Author:** Sarah Ralte, MD, Department of Anatomy, North Eastern Indira Gandhi Regional Institute of Health and Medical Sciences (NEIGRIHMS) - An Autonomous Institute under Ministry of Health and Family Welfare (MoHFW), Govt. of India, Mawdiangdiang, Shillong -793018, East Khasi Hills District, Meghalaya state, India, **Tel.**: +9774289885, **E-mail:** sarahzoremi@gmail.com

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## INTRODUCTION

Zoledronate is a highly potent bisphosphonate in the clinical use[1]. In *vitro* and in *vivo* studies have demonstrated that zoledronate, the latest third generation bisphosphonate (approved for clinical use in United States in 2002)[2] is a more potent inhibitor of osteoclasts than older generation bisphosphonates[3,4]. Bisphosphonates bind with strong affinity to hydroxyapatite crystals in the mineralized bony matrix and following uptake by osteoclasts, the primary target cell, they inhibit osteoclastic bone resorption[1]. Their therapeutic efficacy has been well demonstrated in the treatment of bone diseases involving excessive osteoclastic resorption activity. It is presently considered as one of the treatment of choice in metabolic diseases affecting bone such as Paget's disease, malignancies metastatic to bone, in postmenopausal osteoporosis, multiple myeloma and hypercalcemia of malignancy in adults[5,6]. In *vitro* studies have shown that zoledronate is more potent than other bisphosphonates in maintaining bone mass in estrogen deficient rats[7], ovariectomized adult rhesus monkey[8] and significantly improved the trabecular bone mass in a mouse model suffering from Rett Syndrome induced osteoporosis as a result of methyl CpG binding protein (MeCP2) deficiency[9]. Recently, it

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has been demonstrated that treatment with zoledronate significantly prevented bone loss following liver transplantation[10] and was seen to increase the bone mass density in 'highly active antiretroviral therapy' treated human immunodeficiency virus (HAART-treated HIV) patients[11]. The aim of the present study is to observe and analyze the histological effects of zoledronate, on the proximal metaphyseal trabecular bone in albino rat pups as few histomorphological studies are currently available on the short-term effects of zoledronate on the developing bone.

## MATERIALS AND METHODS

Twenty day old male albino rats belonging to the Wistar strain were obtained from the Animal House of University College of Medical Sciences, Delhi, India, after approval for animal experimentation for conduction of this study towards the fulfillment of M.D degree (Anatomy), was granted from the Ethical Committee of University College of Medical Science, Delhi, India. Adult male and female rats were kept in cages for breeding under standard conditions and observed daily. After birth, all adult males were removed from the cages. The pups remained with their mother till 19$^{th}$ day of age. On attaining 20$^{th}$ day of age, fifteen male pups were randomly selected. The newborn rats were then divided into two groups- control saline treated group and zoledronate treated group.

### *Treatment Regime of the newborn male rats*

a. Control Group I - comprised of eight pups (n= 8) that were treated with normal saline.

b. Experimental Group II - comprising of 7 animals (n= 7) that were administered zoledronate (Brand name- Zoldonat, NATCO Pharmaceutical Ltd., Hyderabad, India) 2.8 µg/kg body weight[12] subcutaneously from 20$^{th}$ to 30$^{th}$ day. The weight of the newborn rats selected for the study was recorded daily from 20$^{th}$ day to 30$^{th}$ day of age using an electronic weighing scale with a minimum grading of one gram.

Following treatment, the animals were sacrificed on the 31$^{st}$ day of age by ether inhalation and then perfused with 10% formal saline. The pups were immersed in 10% formalin for few days. Both tibiae were dissected out from each animal, following disarticulation of the hind-limbs. Connective tissues, muscle and fascia covering the tibia were thoroughly removed. The dry weight of each tibia was measured by a digital weighing scale. The length of tibia and the widest breadth of proximal end of tibia were recorded with the help of Vernier caliper. The weight, length and breadth of tibia were recorded in an earlier work of the same study conducted by the present authors[13]. The bones were decalcified in ethylene diamine tetra-acetic acid (EDTA). Tibiae were cut transversely at its middle part into half. The upper half of tibia was taken up for further paraffin sectioning by the process of dehydration in ascending grades of alcohol (70%, 80%, 90% and 100% alcohol) followed by clearing in methylbenzine and benzene solutions. The processed tissues were embedded in heated paraffin wax (60 °C) in the form of Leukhart's L-shaped bars. Longitudinal paraffin sections of seven microns thickness were cut serially with the help of Weswox Optik rotatory microtome. Sections were put in warm water bath (40-42°C temperature) and transferred onto the glass slides (by floatation method) which were already smeared with egg albumin-glycerine mixture. For fixation purpose, the tissue mounted slides were placed in an incubator (maintained at 37°C temperature) and finally taken up for staining with hematoxylin and eosin (HE) and Masson's trichrome stains.

Abercrombie (1946) method was used for linear measurements wherein the ocular micrometer was calibrated with the standard stage micrometer[14]. These calibrations were done under x10 objective lens. Observations were taken from every 20th tissue section. The number, width and area of trabeculae were recorded under Zeiss light microscope. Keeping the particular eye piece and particular objective, these readings were constant. The number of trabeculae was counted at 100x magnification in two fields. One grid field was placed randomly near the growth cartilage metaphyseal junction (GCMJ) while the other grid was placed further away from the GCMJ, towards the diaphyseal region. The number of trabeculae falling within the grid was counted. The width of the trabeculae, area of the trabeculae, area of the marrow spaces and relative proportion of trabeculae to marrow spaces were analyzed with Image Pro-Express Analysis System software. Mann-Whitney's test was the statistical test used to analyze the results of both the groups where probability *(p) value* < 0.001 was considered significant (S).

## RESULTS

In the present study, there were no significant differences in final body weights of the rat pups as well as the weight, length and breadth of tibia among the experimental and control groups. The metaphysis of proximal end of tibia was described as the area lying close to the inferior margin of the growth plate being bounded on both sides by the periosteum. The upper portion of the metaphysis adjacent to the epiphyseal plate was broader as compared to its distal narrower part. It was observed that the length of both lateral parts of the metaphysis was slightly longer than the central region of the metaphysis. Two distinct regions of the metaphysis were observed in both groups stained with HE at 100x magnification. The primary spongiosa was identified as the richly vascular region of new bone growth lying adjacent to the growth cartilage metaphyseal junction (GCMJ) characterized by numerous, slender, irregular trabeculae, composed largely of calcified cartilage spicules covered with a fine layer of bone. A large number of osteoblasts and occasional osteoclasts were seen (Figure 1). Under HE stain, the calcified cartilage (cc) stained dark eosinophilic, bony matrix light eosinophilic and hemopoietic tissue stained deep purple. Red blood cells (RBCs) stained pinkish red.

The authors of this study observed the region of secondary spongiosa lying adjacent to diaphysis characterized by numerous longitudinal thick bars of bony trabeculae interconnected to each other. This region appeared to be more vascular than primary spongiosa as abundant blood vessels were seen here. Numerous osteoblasts and occasional osteoclasts were seen in contact with trabecular bone. Red blood cells present in large marrow spaces were seen in between the trabeculae especially more towards the diaphyseal region (Figure 2). We observed the osteoblasts, the bone forming cells, as a single layer of cuboidal, spindle shaped or polygonal basophilic cells in contact with trabeculae. On the other hand, the osteoclasts, the bone resorbing cells, were seen as large, polymorphous multi-nucleated cells having closely packed rounded nuclei, scattered within the foamy eosinophilic cytoplasm under HE stain at higher magnification (Figures 4,8). The osteoclasts are described as lying in 'Howship's lacunae' which are depressions or pits resorbed against the bone surfaces. When stained with Masson's trichrome, the nuclei appeared brownish black in color and the cytoplasm stained reddish brown color. The collagen fibres in bony trabeculae stained dark blue whereas the calcified cartilage spicules took on a light blue color. Nucleoli were prominently seen in the nuclei of osteoblast and osteoclast cells. Osteocytes appeared to be lying in clear spaces known as lacunae within the bony trabeculae (Figures 5,6,7).

In zoledronate treated group, the amount of trabecular bone in the metaphyseal region and the longitudinal extent of the metaphysis extending into the diaphyseal region were seen to be greatly increased under HE and Masson's trichrome stain (Figures 3,6) as compared to the control group (Figures 2,5). We observed a significant increase ($p$<0.001) in the number of trabeculae in the region of secondary spongiosa (zoledronate treated: 11.96 ± 0.29/mm2, control: 5.60 ± 0.57/mm$^2$) in the experimental group (Table 1). There was a significant increase ($p$<0.001) in the mean width of trabeculae in the zoledronate treated rats (20.40 ± 3.14µm) as compared to control group (10.32 ± 0.87µm) (Table 2 and Figures 6,5 respectively). The mean area of trabeculae was seen to be statistically significant ($p$<0.001; Table 3) in the zoledronate treated group (27674.92 ± 1049.82µm$^2$) while the mean area of marrow spaces was significant ($p$<0.001) in the control saline treated group (67410 ± 929.12 µm$^2$) from the zoledronate treated group (Table 3). The relative proportion of the trabeculae to the marrow spaces was found to be significant ($p$<0.001) in the zoledronate treated group as compared to the control group (Table 4). The authors also observed that in zoledronate treated rats, numerous large elongated osteoclasts were seen along the margins of the bony trabeculae (Figures 3,4). Some osteoclasts were quite large containing numerous nuclei hanging from bone spicules (Figures 4,8), whereas osteoclasts from saline treated control group were relatively smaller in size and had fewer nuclei (Figures 2,7).

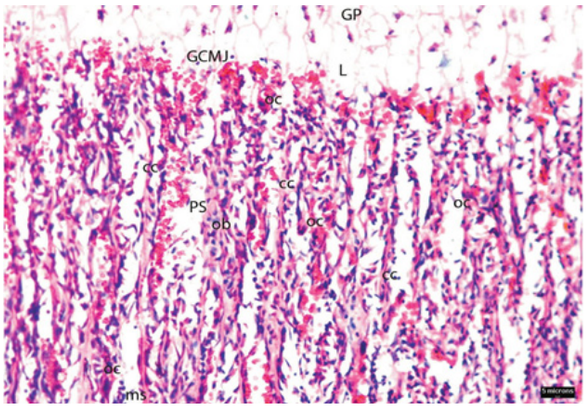


**Fig. 1:** Photomicrograph of primary spongiosa (PS) from proximal tibial metaphysis of control rat showing numerous, irregular, slender longitudinally oriented trabeculae (trab) with narrow marrow spaces (ms) between them. The calcified cartilage (cc) is dark eosinophilic, bone matrix light eosinophilic and hemopoietic tissue deep purple. Red blood cells (RBCs) appear pinkish red. 100x (HE).

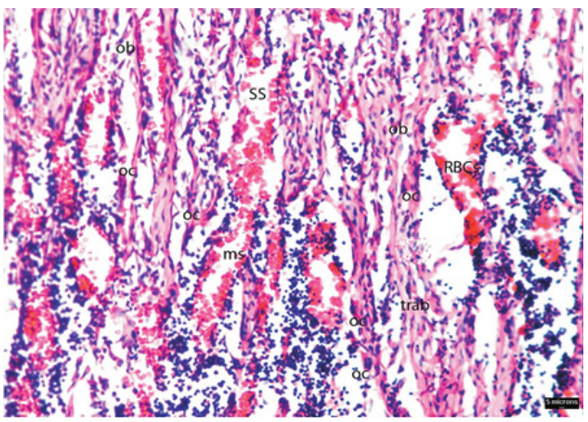


**Fig. 2:** Photomicrograph of secondary spongiosa (SS) from proximal tibial metaphysis of control group depicting elongated bars of bony trabeculae (trab) extending into the medullary cavity (mc). Large marrow spaces (ms) containing hemopoietic tissue are seen in between the adjacent trabeculae.100x (HE).

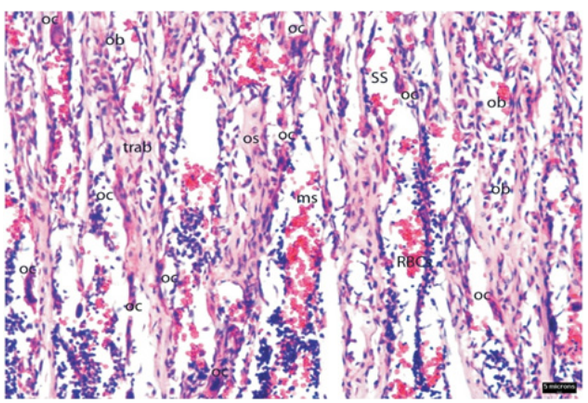


**Fig. 3:** Photomicrograph of secondary spongiosa (SS) of proximal metaphysis from zoledronate treated rat depicting large trabeculae (trab) with narrow interconnecting marrow spaces (ms) filled with hemopoietic tissue stained purple. Numerous large polymorphous osteoclasts (oc) are seen lining the trabecular surfaces.100x (HE).

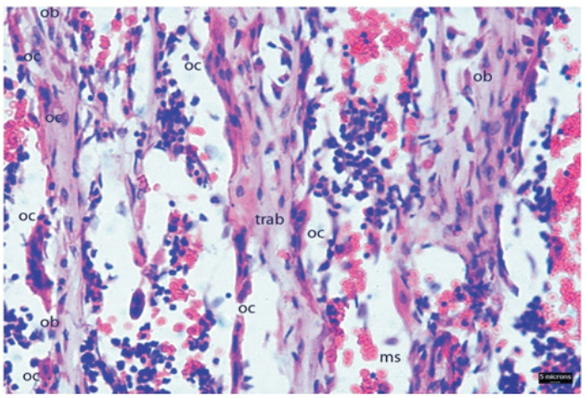


**Fig. 4:** Photomicrograph of seconday spongiosa (SS) of proximal tibial metaphysis from zoledronate treated group showing polymorphous well extended osteoclasts (oc) lining the contours of trabeculae (trab). 200x (HE).

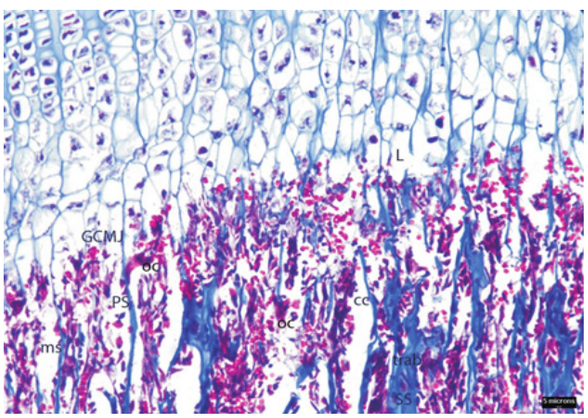


**Fig. 5:** Photomicrograph of primary spongiosa (PS) of proximal tibial metaphysis from control group showing predominantly calcified cartilage (cc) spicules stained light blue and bone matrix dark blue. The trabeculae (trab) are lined by numerous osteoblasts (ob) and occasional osteoclasts (oc). The lacunae (L) at the growth cartilage metaphyseal junction (GCMJ) are seen to be partially eroded and invaded by brightly stained orange red hemopoietic tissue. 100x (Masson's trichrome).

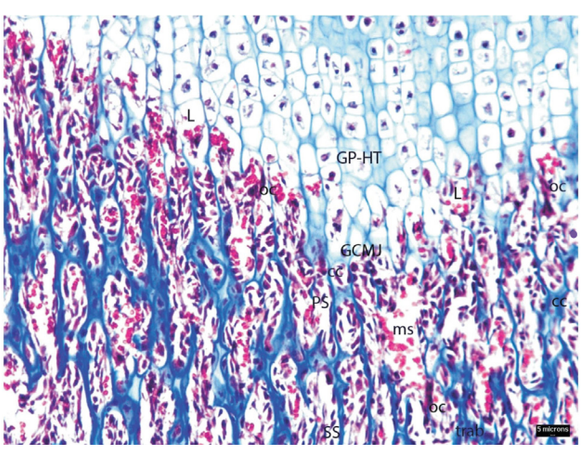


**Fig. 6:** Photomicrograph of primary spongiosa (PS) from proximal tibial metaphysis of zoledronate treated group showing large newly formed trabeculae (trab) composed mainly of calcified cartilage (cc) stain light blue and a thin layer of dark blue bone matrix. Marrow spaces (ms) are seen between trabeculae. 100x (Masson's trichome).

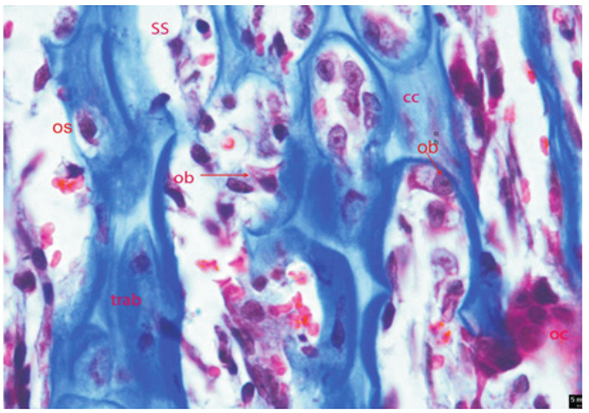


**Fig. 7:** Photomicrograph of proximal metaphysis from control group showing trabeculae (trab) with occasional central cores of calcified cartilage (cc) stained light blue and bone matrix dark blue. An osteoclast (oc) is seen at the right bottom margin. Osteoblasts (ob) showing a clear area at one end of the cell are seen (arrow). Osteocytes (os) lying in a clear lacunae are also seen. 400x (Masson's trichrome).

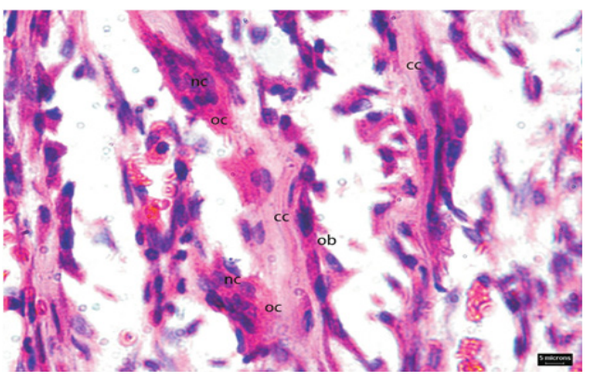


**Fig. 8:** Photomicrograph of osteoclasts (oc) from proximal tibial metaphysis of experimental group showing a large polymorphous osteoclast (on the upper left) containing numerous nuclei. 400x (HE).

**Table 1:** Mean number of trabeculae in the proximal metaphysis of tibia of albino rats

| Group | Mean number of trabeculae / $mm^2$±SD | |
|---|---|---|
| | Primary spongiosa (Area near GCMJ) | Secondary spongiosa (Area away from GCMJ) |
| Control Group I (Saline Treated) | 10.30 ± 0.72 | 5.60 ± 0.57 |
| Experimental Group II (Zoledronate Treated) | 14.69 ± 1.14* | 11.96 ± 0.29* |

*$p$<0.001 (significant), SD-Standard deviation

**Table 2:** Mean width of trabeculae in the proximal tibial metaphysis of rats

| Group | Mean width of trabeculae (µm)±SD |
|---|---|
| Control Group I (Saline Treated) | 10.32 ± 0.87 |
| Experimental Group II (Zoledronate Treated) | 20.39 ± 3.14* |

*$p$<0.001 (significant), SD-Standard deviation

**Table 3:** Mean area of trabeculae and marrow spaces in the proximal tibial metaphysis of rats

| Group | Mean area of trabeculae ($\mu m^2$)±SD | Mean area of marrow spaces ($\mu m^2$)±SD |
|---|---|---|
| Control Group I (Saline Treated) | 10375.0 ± 755.64 | 67410.0 ± 929.12* |
| Experimental Group II (Zoledronate Treated) | 27674.92 ± 1049.82* | 49950.52 ± 1556.81 |

*$p$<0.001 (significant), SD-Standard deviation

**Table 4:** Relative proportion of the trabeculae to the marrow spaces in proximal tibial metaphysis of rats

| Group | Relative proportion of the trabeculae to the marrow spaces |
|---|---|
| Control Group I (Saline Treated) | 1.0: 6.7 |
| Experimental Group II (Zoledronate Treated) | 2.7 : 4.9* |

*$p$<0.001 (signficant)

## DISCUSSION

Bisphosphonates are synthetic analogues of the naturally occurring pyrophosphate molecule characterized by two P-C-P bonds and are potent inhibitors of osteoclastic mediated bone resorption[1,5]. Zoledronate, a third generation nitrogen containing bisphosphonate, is the most potent bisphosphonate inhibitor of osteoclast-mediated bone resorption introduced for therapeutic use in 2002[2-4]. Past studies have described the inhibitory effect of bisphosphonates on the osteoclasts of bone, the chief bone resorbing cell, since the late 1960s[12,13,15-17].

The authors observed there were no significant changes in the mean body weight of rats as well as in the mean weight, length and breadth of tibia as compared to the control group in an earlier work of the present study[13]. However, Wronski *et al*. had reported a 10% increase in the weight of rats treated with etidronate[18]. No significant difference in the mean weight, length and breadth of tibia was reported by Ma *et al*. (1995)[19]. In contrast, a significant shortening of radius (1.96±0.005 cm; $p$<0.001) was observed by Reitsma *et al*. on administration of highest dose of bisphosphonate (APD) as compared to controls (2.01±0.007 cm). They also observed a significant increase (55.20±0.5 mg; $p$<0.01) in the radius weight despite its shortening[20]. Koivukangas *et al*. (2001) also observed significantly shorter tibia (39.70±0.6 mm, $p$<1.01) in the high clodronate treated group than in the control group (40.30±0.40 mm) after long term administration in growing rats although no significant differences were reported in the final body weights or tibial weight[21].

Our observations were similar to the descriptions of the metaphysis noted by other authors[22,23] and the present authors in an earlier study of the same[13] had observed numerous osteoclasts exhibiting varying degrees of polymorphism.

In the current study, we observed an overall thickening of the metaphysis in the zoledronate treated animals as compared to control group and this is most probably due to an increase in the number of trabeculae. The authors noted a significant increase (11.96 ± 0.29 per mm2; $p$<0.001) in the mean number of trabeculae in the region of secondary spongiosa in zoledronate treated rats (Table 1). The long, thick trabeculae were seen to extend into the medullary cavity towards the diaphyseal region (Figures 3,6) as has also been reported by others[12,16,21].

The mean width of trabeculae showed a statistically significant ($p$<0.001) increase in the zoledronate treated group (Table 2) and thereby, a significant increase ($p$<0.001) in the mean area of trabeculae was observed (Table 3; Figures 3,6) throughout the metaphysis. Our findings were in concordance with the observations of Schenk *et al*. (1973); Miller and Jee, (1979); Pataki *et al*. (1997) and Mayahara and Sasaki (2003) who all reported a marked increase in the number and thickness of trabeculae following treatment with bisphosphonates and attributed it to a dose-dependent suppression of cancellous bone turnover and inhibition of resorption of mineralized cartilage septae resulting in an increase in the amount and connectivity of cancellous bone in the proximal tibial metaphysis of rat[12,16,17,24]. Zoledronate was found to be 100-fold more potent than pamidronate in increasing cancellous bone in the proximal tibial metaphysis of rat and this was attributed to an increase in the number, width and area of the trabeculae[12]. Similar findings were described by Lin *et al*. (1994) who observed a significant increase ($p$<0.05) in the density of both the primary and secondary spongiosa in combined PGE2 and risedronate treatment compared with that of age-related controls on the rat skeleton[25]. Matos *et al*. (2007) conducted a study to observe the effect of zoledronate on bone remodelling during healing process following treatment with 0.04 mg/kg of zoledronate in rabbits which had undergone shaft osteotomy of the cranial end of the fibula[26]. They reported that there was marked bone formation in the metaphyseal region, which accounted for a significant increase in the trabecular bone mass in the zoledronate treated group as compared to the control animals, which were similar to our findings in the present study. Long-term administration of bisphosphonate in ovariectomized rats was found to establish numerous bony bar connections between longitudinal and transverse bony trabeculae in the secondary spongiosa of metaphysis, which were seen to extend further into the medullary cavity resulting in a broad plate-like trabecular bony formation[19] which was similar to the findings in our present short-term study (Figures 3, 4, 6).

In contrast, Schenk *et al*. reported a decrease in the trabecular diameter despite an increase in the number of trabeculae at high doses of various bisphosphonates. The decrease in the trabecular diameter was thought to be due to a mild inhibition of bone formation at highest doses[27]. Shapiro *et al*. (2017) evaluated the effects of zoledronate on a mouse model of Rett syndrome (RTT) induced

osteoporosis, caused by deficiency of methyl CpG binding protein 2 (MeCP2). Following treatment with weekly injections of 20µg/kg zoledronate for a period of six weeks, the authors observed that zoledronate significantly increased trabecular bone mass in the affected RTT mice[9]. In contrast in a micro-CT imaging study of the femoral heads by Ma S *et al*. (2017), the authors reported that in bisphosphonate-treated fracture patients, the trabecular bone developed fewer perforations. The bony trabeculae however showed numerous, large microcracks than the non-treated bisphosphonate fracture group and non-fracture controls. These findings indicated that bisphosphonate therapy was effective at reducing perforations but also caused microcrack accumulation, resulting in decreased mechanical strength. The authors noted that the duration of bisphosphonate treatment had to be carefully considered while treating fracture of head of femur[28].

Different theories regarding the mechanisms of action of bisphosphonates have been suggested by various authors occurring at both tissue and cellular level. We know that bone remodelling has to strike a fine balance between excessive bone synthesis and excessive bone resorption, which is mediated at molecular pathways. Literature studies have shown that bisphosphonates get internalized at the ruffled border of the osteoclast and interfere with various biochemical reactions such as alteration of the osteoclast cytoskeleton, suppression of maturation of osteoclast cells, interference with chemotaxis and binding of osteoclast to bone, disrupting ruffled border formation and actin ring filament in osteoclast resulting in abnormal vesicle transport within osteoclast thus preventing osteoclast from forming a tight sealing zone[1,3].

As few literature studies are currently available on the histomorphological effect following short-term administration of zoledronate, a third generation bisphosphonate, in growing bone, hence, the present study would give some useful insight on the histological effects of zoledronate on trabecular bone in rat pups.

## CONCLUSION

In conclusion, we observed a significant increase in the number, width and area of trabeculae following the short-term administration of the latest third generation bisphosphonate, zoledronate, which would likely account for the increased cancellous bone mass in the metaphyseal region despite the presence of numerous large polymorphic osteoclasts observed by the present authors in an earlier study of the same[13]. The osteoclasts are likely the main site of action of zoledronate, resulting in decreased osteoclastic mediated bone resorption activity as literature studies have shown that bisphosphonates inhibit bone resorption by selectively binding to the matrix of the mineralized bony trabeculae and targeting the primary bone cell, the osteoclast, the chief bone cell responsible for bone resorption. So, zoledronate is highly recommended to treat children with metabolic bone disorders such as osteogenesis imperfecta and fibrous dyspasia. However, further detailed studies are required to determine the potential harmful side effects for the pediatric dosage of zoledronate.

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## ABBREVIATIONS

**L**-lacuna; **GCMJ**-growth cartilage metaphyseal junction; **GC-HT**-growth cartilage hypertrophic zone; **RBC**-red blood cells; **PS**-primary spongiosa; **SS**-secondary spongiosa; **cc**-calcified cartilage; **mc**- medullary cavity; **ms**-marrow spaces; **nc**-nucleus; **ob**-osteoblast; **oc**-osteoclast; **trab**-trabeculae

## CONFLICT OF INTERESTS

There are no conflicts of interest.

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