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ZOLEDRONIC ACID EFFECT ON BONE OF GROWING RATS

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Abstract

Zoledronic acid (ZA) is an antiresorptive drug used in children with bone diseases like osteogenesis imperfecta, juvenil osteoporosis, fibrous dysplasia and primary bone tumors. The aim of the present study was to evaluate the effects of ZA dose accumulation on growing bone during different periods of treatment in normal rats. Methods: A 4x2 factorial design was used to study the effect of the dose of ZA (D: 0-2.5-12.5-25 μg Z/kg body weight/s.c. weekly) and the length of treatment (T: 15-30 days) in normal female Sprague Dawley rats. Bone morphometric, histomorphometric, densitometric and biomechanical studies were performed.

Results: Femoral length and cross-sectional area were affected by both D and T. A significant interaction between D and T was observed in length with a lower value at higher dose and 30 days of treatment. Growth plate of the tibia showed a decrease in total thickness with D and T. Histomorphometric and connectivity parameters of trabecular bone were significantly increased with D and several parameters were also affected by T. Cortical bone strength was increased only with T. Biomechanical parameters of trabecular bone showed significant interaction with greater effect at higher D and T.

Conclusion: Even though a mild negative effect of the highest dose of ZA on linear and appositional growth was observed, the other bone parameters evaluated were improved. A careful risk/benefit analysis would lead us to conclude that the mild deleterious effects of ZA during growth are outweighed by the benefit obtained with treatment.

Key words: bone growth, bone diseases, bone strength, cartilage growth, zoledronic acid.

Resumen

EFECTO DEL ACIDO ZOLEDRÓNICO EN EL HUESO DE RATAS EN CRECIMIENTO

El ácido zoledrónico (AZ) es un fármaco antirresortivo utilizado en niños con enfermedades óseas como osteogénesis imperfecta, osteoporosis juvenil, displasia fibrosa y tumores óseos primarios. El objetivo del presente estudio fue evaluar los efectos de las dosis acumuladas de AZ en el hueso en crecimiento de ratas hembras normales durante diferentes períodos de tratamiento.

Métodos: se utilizó un diseño factorial de 4x2 para estudiar el efecto de la dosis de AZ (D: 0-2,5-12,5-25 µg Z / kg de peso corporal /sc semanalmente) y el período de tratamiento (T: 15-30 días) en ratas Sprague Dawley. Se realizaron estudios óseos morfométricos, histomorfométricos, densitométricos y biomecánicos.

Resultados: la longitud y el área de sección transversal del fémur se vieron afectadas tanto por D como por T. Se observó una interacción significativa entre D y T en la longitud obteniéndose un valor más bajo a la dosis más alta y a 30 días de tratamiento. El cartílago de crecimiento de la tibia mostró una disminución en el espesor total con D y T. Los parámetros histomorfométricos y de conectividad del hueso trabecular aumentaron significativamente con D y varios parámetros también se vieron afectados por T. La fortaleza ósea cortical aumentó solo con T. Los parámetros biomecánicos del hueso trabecular mostraron una interacción significativa con un mayor efecto a mayor D y T.

Conclusión: a pesar que se observó un leve efecto negativo de la dosis más alta de AZ sobre el crecimiento lineal y aposicional, el resto de los parámetros óseos evaluados mejoraron. Un análisis cuidadoso del riesgo /

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beneficio permite concluir que los efectos negativos leves del AZ durante el crecimiento son superados por el beneficio obtenido con el tratamiento.

Palabras clave: *crecimiento óseo, enfermedades óseas, fortaleza ósea, cartílago de crecimiento, ácido zoledrónico.*

Introduction

Bisphosphonates (BP) are antiresorptive drugs widely used in clinical practice for the treatment of bone diseases associated with high bone turnover in adults[1,2]. BP are structural analogs of pyrophosphate that bind to bone mineral and inhibit bone resorption by acting on osteoclasts[3] and also show positive effects on the viability of osteoblasts and osteocytes[4]. They are increasingly used in pediatric patients with osteogenesis imperfecta, juvenile osteoporosis, fibrous dysplasia and primary bone tumors[5-7].

Although they have been an effective option for childhood bone diseases, as they showed decrease in pain and fractures, their use in children and adolescents continues to be controversial. The most used BP in children is pamidronate but zoledronic acid (ZA) is being increasingly used because of its higher antiresorptive potency and reduced frequency of administration[8]. Moreover, ZA has several antitumor effects on various tissues and prevents skeletal complications in different tumors[9]. It is used with the usual chemotherapy in the treatment of primary tumors, like osteosarcoma and Ewing´s sarcoma, in children and adolescents[10]. However, the information about safety and efficacy of ZA in young patients is limited and there are few published data about long-term use[11-13].

Experimental studies have suggested that BP administration may result in changes in the growing skeleton. A study carried out on healthy rats showed that alendronate causes a significant decrease in femoral and tibial length, tibial growth plate thickness, and longitudinal growth of hemi-mandibles[14]. Also, alendronate prevents the removal of calcified cartilage and maturation of trabeculae[15]. Experiments in rabbits treated with ZA showed bone resorption alterations associated with cartilage matrix retention and a decrease in tibial length[16]. Moreover, ZA has been found to induce marked osteosclerosis in primary and secondary spongiosa in a murine model of metastatic tumor, which generates a bone growth arrest during treatment[17].

Pre-clinical observations are consistent with the case of a patient who showed growth arrest for 10 months during treatment with ZA with normal gain in size once the treatment had ended[18]. Munns et al showed that bone formation rate per bone surface of children with osteogenesis imperfecta treated with pamidronate, was only 17% that of untreated osteogenesis imperfecta patients and 25% of healthy controls[19]. Furhter, endochondral growth was transiently disturbed by high doses of ZA under primary bone tumors treatment in pediatric patients[18]. However, it was found that ZA decreased the number of bone fractures and pain in children with osteogenesis imperfecta and osteoporosis, and improved their functional status[20,21]. In addition, ZA showed higher Z-scores for areal bone mineral density (BMD) in lumbar spine and vertebral reshaping in children with severe osteogenesis imperfecta, but long-bones fracture rates were still high[22]. Moreover, a recent study showed an increase in lumbar spine areal BMD and a higher final height z-score in patients with osteogenesis imperfect after BP treatment during growth[13].

In summary, the data reported suggested that BP may have a negative impact on bone growth. As bone fragility in pediatric patients is different from adults, making difficult the data extrapolation, the clinical use of BP should be carefully evaluated. Studies with antiresorptive agents as BP in children are insufficient to address all the concerns about its efficacy and safety. There is not even a consensus on which BP, dose, and treatment duration to use, although experts recommend their limited use for bone diseases[8,9]. Thus, the aim of the present study was to evaluate the effects of ZA dose accumulation on growing bone during different periods of treatment in normal rats.

Materials and methods

Animals

Twenty-one day old female Sprague Dawley rats (n=24) provided by the School of Medicine, Rosario National University (Argentina) were used. Rats were housed in a room with 12-h light and dark cycles, and constant temperature of 24±1ºC. All the experiments were conducted in accordance with international guidelines for animal care[23] and have been approved by the Bioethics Committee of our Institution.

A 4x2 factorial arrangement consisting of two

factors (ZA dose [D] and length of treatment [T]) and its respective levels [D:0, 2.5, 12.5 y 25 μg/kg body weight, subcutaneously once a week and T: 15 y 30 days] was performed. Animals were randomly assigned into 8 groups (each containing 3 animals). The period of treatment factor had implicit the cumulative dose of ZA and the animal growth. At the end of each period of treatment, rats were euthanized in a CO_2 chamber and tibias and femurs were obtained.

Bone mineral density

At the end of experiment, total BMD (mg Ca/cm^2) was determined on left tibias using X-ray absorptiometry (Work Ray 70 KV, Workman SRL, Argentina) simultaneously with an aluminum step wedge, which was previously calibrated with known calcium concentrations[24]. The total BMD was calculated using Image J 1.48 software (NIH, Maryland, USA).

Bone histomorphometry

The proximal epiphysis of the right tibias were fixed in 10% phosphate buffered formaldehyde and decalcified in 10% EDTA before embedded in paraffin. Five-μm longitudinal sections were stained with hematoxylin and eosin and 3 sections per rat were examined. Digitalized images were obtained with a light microscope (Mikoba 320, China) and a digital camera (Olympus SP-350, China). Analyses were performed in a 2 mm^2 area at 1 mm from growth plate-metaphyseal junction. The following measurements were performed[25]: 1) total tissue volume, TV (μm^2); 2) trabecular bone volume, BV (μm^2); and 3) trabecular bone surface, BS (μm). With these values, histomorphometrical variables were calculated: 1) bone volume, BV/TV (%) = [BV*100/TV]; 2) trabecular thickness, Tb.Th (μm) = [2/(BS/BV)]; 3) trabecular number, Tb.N (1/mm) = [(BV/TV)/(Tb.Th)]; and 4) trabecular separation, Tb.Sp (μm) = [(1/Tb.N)-Tb.Th]. On the same sections, growth plate thickness (GPC.Th, μm), hypertrophic cartilage thickness (HpZ.Th) and resting and proliferative cartilage thickness (R&PZ.Th) were measured on digital images. Measurements were performed at three location (25, 50 and 75 % of cartilage width) within the growth plate (3 measurements per location, see figure 1)[26]. All the measurements were carried out by the same operator in a blind fashion using the software Image J 1.48 and they were averaged to obtain a single value per tibia.

The analysis of trabecular interconnectivity was performed as previously published[24,27], and the following parameters were measured using ImageJ 1.48 software: total number of nodes (Nd), number of node-to-node branches (NNd), number of node-to-termini branches (NNdTm), number of trees (T.N), number of terminals (Tm), and number of branches with two terminals (NTm). With these parameters, we calculate the interconnectivity parameters: interconnectivity index [ICI = Nd*NNd) / T.N* (NNdTm+1)]; node-to-termini ratio [R= Nd/Tm] and mean size of branches [Dist (mm) = ∑ branches size / (NNdTm+NNd+NTm)].

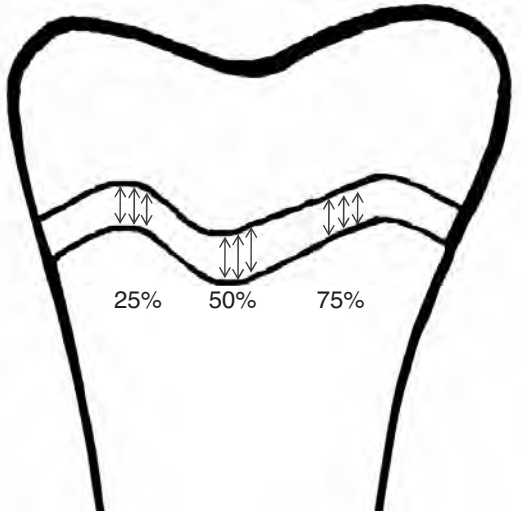

Figure 1. Scheme of measurement sites on cartilage.

Bone measurements

Longitudinal growth was assessed by measuring the femoral and tibia length using a digital caliper (accurate ±0.01 mm). In order to assess bone appositional growth, cross sections -500 μm in thickness- of right femoral diaphyses were cut with a low speed saw (IsoMet. Buehler Ltd. Illinois, USA). A digital image was obtained at 40x magnification and the following measurements were performed using Image J 1.48 software: 1) cross-sectional area (CS.Ar: the area of bone tissue bounded by the periosteal surface, mm^2), 2) medullary area (Me.Ar: the area delineated by the endocortical surface, mm2), 3) Cortical width (Ct.W, mm) and 4) Cross sectional moment of inertia (CSMI, mm^4) was calculated as ([periosteal diameter/2]^4 – [medullary diameter/2]4) * π/64[24].

Mechanical testing

Femurs were stored at -20 ºC wrapped in saline-soaked gauze. For mechanical testing, they were thawed at 37°C. Cortical bone strength at the femoral mid-diaphysis was determined using a three-point bending test and

trabecular bone strength was evaluated by a compression test on a 2.5-mm thickness transversal section from distal epiphysis[28,29]. Mechanical testing was performed on a machine designed at the engineering department of the Bone Biology Laboratory, with a 300 N load cell with 0.01 N of discrimination and an accuracy of 10 μm in displacements. The two-bar distance for the three-point bending test was 12 mm at T15 and 13 mm at T30. The compression test was performed with a compression cone of 7.07 mm^2 and a speed of 0.01 mm/s was used for both tests. Load vs. displacement plots were recorded by a software (Biomedical Data Acquisition Suite 1.0, Argentina, 2011) to determine bone structural properties. The software data acquisition rate was 10 Hz. Ultimate load (N) was defined as the highest load and the fracture load (N) was recorded just before the first decline in load. The stiffness (N/mm) was determined as the slope of the linear portion of the load vs. displacement curve. Absorbed energy (mJ) was defined as the area under the curve until the fracture load point.

Statistical analysis

Data were expressed as mean ± SD. Two-way analysis of variance (ANOVA) was performed with D of ZA and T as analyzed factors. The multiple comparison Bonferroni post-test was performed if significant interaction was found. If there was no interaction between variables, it was indicated only if there was D or T effect. Differences were considered significant if $p<0.05$. Statistical analyses were performed using the software R 2.14.0.

Results

Bone and growth plate growth

Femoral length was affected by both D and T. As expected, a significant increase in femoral length was observed at 30 days compare to 15 days at all doses. However, a significant interaction between D and T was observed and after 30 days of treatment, femur length was lower at D25 than D0, D2.5 and D12.5. In addition, tibial length and most cartilage parameters were significantly affected by both D and T (Figure 2). Although no interaction between D and T was found, tibial length and GPC.Th showed lower values at D25 than D0, D2.5 and D12.5 after 30 days of treatment. Moreover, GPC.Th showed the lowest value at D25 also after 15 days of treatment. The R&PZ.Th and HpZ.Th was affected by D and T in a similar way as GPC (data not shown).

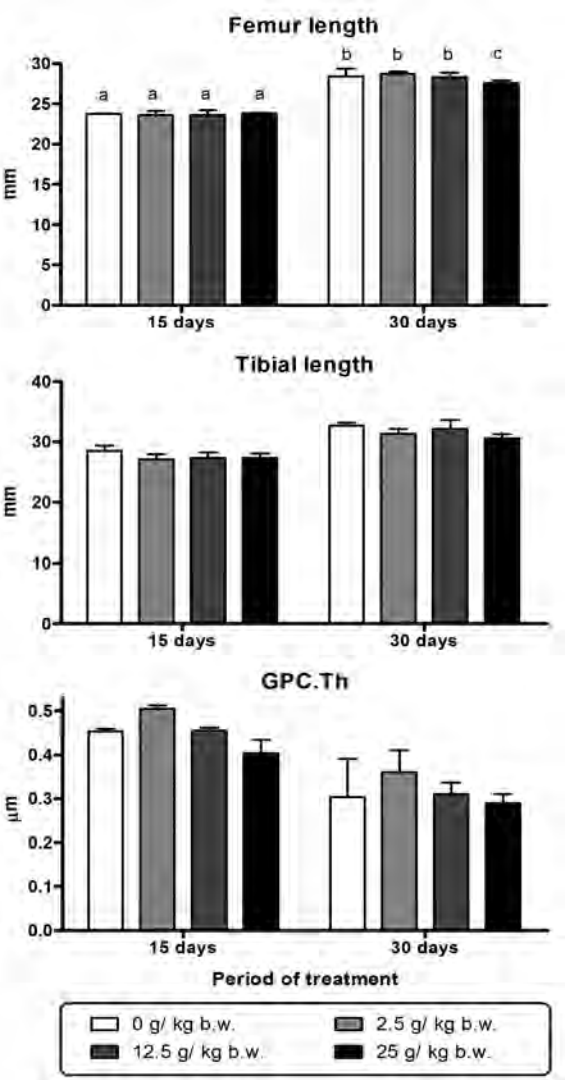

Figure 2. Effects of ZA dose accumulation on bone and growth plate growth. All parameters were significantly affected by the period of treatment. Two-way ANOVA, $p<0.05$.Values bearing different letters between bars are significantly different (Bonferroni post-test, showed in case of interaction only).

Table1 shows that all parameters that evaluate appositional growth were affected by the period of treatment. Moreover, the D also affected CS. Ar, Me.Ar and Ct.W. Interaction between D and T was observed only for Me. Ar where lower values were found at the highest dose in both periods of treatment. Even though there was no significant interaction between D and T in CS.Ar lower values were also observed at D25. However, the Ct.W was not negatively affected since higher values were found at all ZA doses than D0 at both period of treatment.

Table 1. *Morphometric parameters*

Period of treatment	15 days				30 days				Two-way ANOVA		
Dose	*0*	*2.5*	*12.5*	*25*	*0*	*2.5*	*12.5*	*25*	*D*	*T*	*D-T*
CS.Ar (mm^2)	5.61±0.59	5.07±0.57	5.34±0.50	4.94±0.42	7.47±1.52	7.53±0.64	7.63±0.36	6.65±0.44	p<0.05	p<0.05	ns
Me.Ar (mm^2)	$2.87±0.47^{a}$	$2.26±0.23^{bc}$	$2.39±0.25^{bc}$	$2.07±0.18^{c}$	$2.70±0.47^{ab}$	$2.98±0.49^{a}$	$2.93±0.25^{a}$	$2.39±0.25^{c}$	p<0.05	p<0.05	p<0.05
Ct.W (mm)	0.61±0.10	0.62±0.14	0.80±0.21	0.68±0.08	1.00±0.15	1.05±0.23	1.17±0.16	1.15±0.25	p<0.05	p<0.05	ns

Two-way ANOVA, Bonferroni post-test (for interaction only). Values bearing different letters between columns are significantly different (p<0.05). Abbreviation = D: ZA doses; T: period of treatment; D-T: dose and time interaction; CS.Ar: cross-sectional area; Me.Ar: medullary area; Ct.W: Cortical width.

BMD measurement

Radiographs showed the characteristic radiodense lines in the metaphyses of ZA treated rats that corresponded to the number of administered doses (2 or 4). BMD was only affected by T (Two-way ANOVA, p<0.05) and no interaction between D and T was observed (T15 days:D0=10.34±3.67 mg Ca/cm^2, D2.5=7.84±2.97, D12.5=11.56±2.39, D25=11.10±2.88; T30 days: D0=14.31±2.14, D2.5=15.02±2.54, D12.5=16.93±5.26, D25=17.89±4.15).

Bone histomorphometry

D but not T had effect on most histomorphometric parameters (Table 2). A significant effect of T was found only on Tb.N. The higher BV/TV in D12.5 and D25 after 15 and 30 days of treatment was due to an increased in Tb.Th and Tb.N.

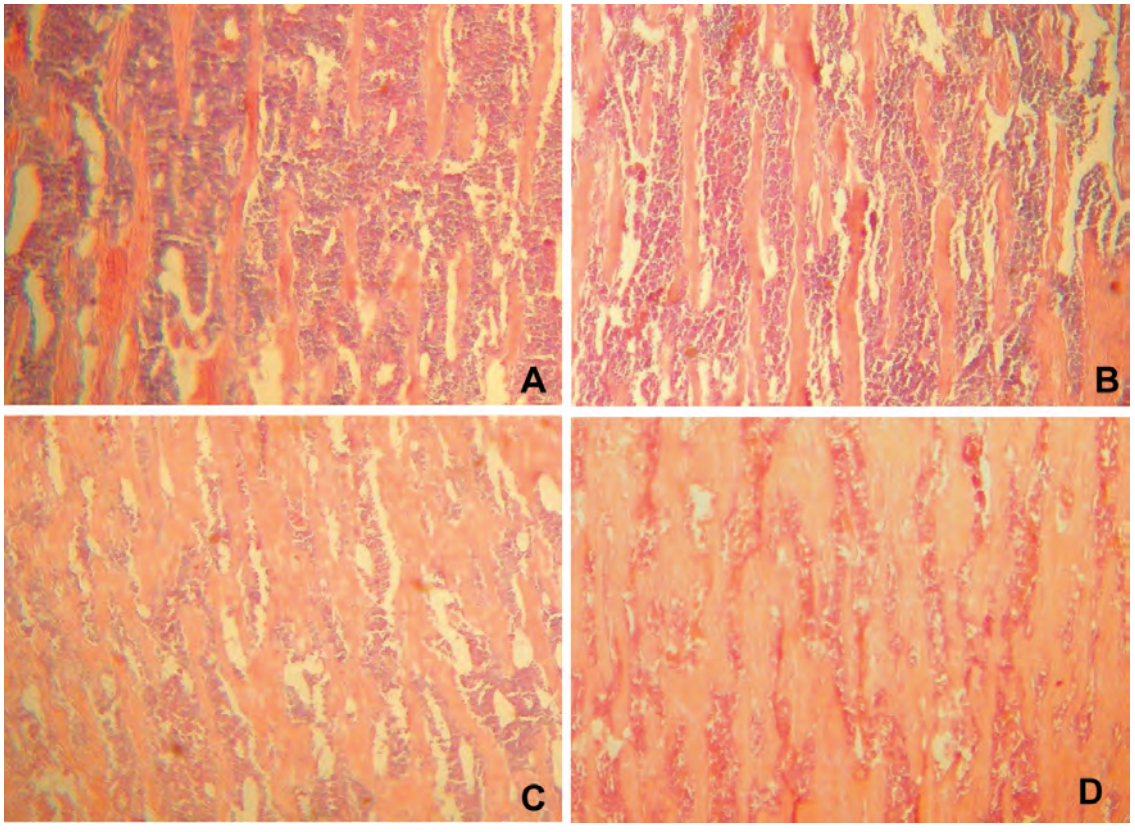

Figure 3. *Representative images (40x) of trabecular bone of tibiae showing the effect of dose at 30 days of treatment. A: D0, B: D2.5, C: D12.5 and D: D25.*

Table 2. *Bone histomorphometry measurements*

Period of treatment	15 days				30 days				Two-way ANOVA		
Dose	*0*	*2.5*	*12.5*	*25*	*0*	*2.5*	*12.5*	*25*	*D*	*T*	*D-T*
BV/TV (%)	29.02±11.68	30.96±9.63	41.08±0.65	49.90±9.48	31.36±7.10	39.20±6.27	50.20±7.41	50.14±1.98	p<0.05	ns	ns
Tb.Th (um)	36.50±8.02	35.50±5.99	44.80±6.33	47.30±9.11	37.30±1.85	39.40±1.17	43.00±2.65	44.00±6.03	p<0.05	ns	ns
Tb.N (mm^{-1})	7.81±1.44	8.63±1.40	9.26±1.16	10.57±0.56	8.43±1.94	9.90±1.57	11.69±1.51	11.49±1.05	p<0.05	p<0.05	ns
Tb.Sp (um)	94.90±29.30	82.80±25.30	64.10±7.39	47.40±9.37	85.20±24.7	65.30±21.4	43.60±12.4	43.70±2.10	p<0.05	ns	ns

Two-way ANOVA. Abbreviation = D: ZA doses; T: period of treatment; D-T: dose and time interaction; BV/TV: bone volume; Tb.Th: trabecular thickness; Tb.N: trabecular number; Tb.Sp: trabecular separation

Table 3. *Trabecular connectivity assessment*

Period of treatment	15 days				30 days				Two-way ANOVA		
Dose	*0*	*2.5*	*12.5*	*25*	*0*	*2.5*	*12.5*	*25*	*D*	*T*	*D-T*
ICI	0.35±0.11	0.63±0.40	0.41±0.01	1.05±0.06	0.59±0.39	0.78±0.28	1.64±0.51	2.70±1.18	p<0.05	p<0.05	ns
R	0.31±0.09	0.40±0.11	0.36±0.07	0.58±0.01	0.34±0.07	0.54±0.13	0.61±0.11	0.72±0.13	p<0.05	p<0.05	ns
Dist (mm)	0.13±0.05	0.15±0.01	0.22±0.01	0.20±0.04	0.14±0.01	0.17±0.01	0.18±0.01	0.17±0.02	p<0.05	ns	ns
NTm	17.5±8.85	6.33±1.15	6.25±6.01	4.00±3.61	16.8±2.47	9.50±6.06	6.67±0.76	5.67±3.51	p<0.05	ns	ns

Two-way ANOVA. Abbreviation = D: ZA doses; T: period of treatment; D-T: dose and time interaction; ICI: interconnectivity index; R: node-to-termini ratio; Dist: mean size of branches; NTm: number of branches with two terminals.

Connectivity parameters were affected by D and T except for Dist which only was affect by the D (Table 3). Despite no interaction between both factors, higher ICI and R values were found in groups with D12.5 and D25 after 15 and 30 days of treatment showing an increase in trabecular connectivity. Moreover, the NTm parameter, which provides information about loss of connectivity in the bone architecture, was decreased in all groups treated with ZA independently of the length of treatment.

Mechanical testing

Biomechanical parameters of cortical bone, analyzed with three-point bending test and the CSMI were affected only by length of treatment (Table 4), except for absorbed energy, which was affected by both factors. On the other hand, trabecular biomechanical parameters such as fracture load and stiffness were affected by the D and T. Also, significant interaction between D and T was observed, with higher values in fracture load and stiffness at all ZA doses compared with D0 after 30 days of treatment. No effect on the absorbed energy was found in the compression test (Table 4).

Discussion

The use of BP in children and adolescents with bone diseases is increasing since they have provided significant clinical improvements such as decreased of pain, lower incidence of fractures, and better mobility[5,6,13,20]. However, their use in childhood is still controversial because there are very few preclinical experiments and clinical trials[30]. Despite the pamidronate intravenous infusion is being the most used treatment in children and adolescents with bone diseases, ZA is being used more frequently because of its higher potency and reduced frequency of infusion. However, the ZA efficacy and safety have not been thoroughly evaluated[31], raising concerns on whether the drug may interfere with normal bone growth[18,32].

Here, we investigate the effects of ZA on bone properties in growing female normal rats using increasing doses, equivalent to different therapeutic situations in humans[18,30,33] after two

Table 4. *Mechanical testing*

Period of treatment	15 days				30 days				Two-way ANOVA		
Dose	*0*	*2.5*	*12.5*	*25*	*0*	*2.5*	*12.5*	*25*	*D*	*T*	*D-T*
	Three-point bending test										
Fracture load (N)	32.87±3.55	34.34±5.38	34.85±3.29	35.97±1.70	57.18±5.49	56.00±13.89	64.17±6.93	64.39±10.37	ns	p<0.05	ns
Ultimate load (N)	37.24±2.68	37.73±2.06	38.64±3.64	37.49±1.70	59.70±6.45	62.94±5.33	65.85±5.34	66.45±10.89	ns	p<0.05	ns
Stiffness (N/mm)	71.39±19.06	71.20±7.23	102.50±38.47	85.28±37.47	158.40±38.4	133.50±39.10	149.70±60.74	289.90±239.60	ns	ns	
Absorbed energy (mJ)	29.48±7.16	27.37±5.73	30.13±6.05	27.44±1.98	48.07±9.35	55.66±11.85	62.72±6.01	44.27±13.82	p<0.05	p<0.05	ns
CSMI (mm^4)	1.27±0.62	0.97±0.49	1.41±0.58	1.11±0.58	2.23±0.62	2.68±1.30	2.84±0.50	3.14±0.36	ns	p<0.05	ns
	Compression test										
Fracture load (N)	25.45±5.94^{a}	37.61±35.12^{a}	53.49±22.91^{a}	37.75±25.49^{a}	35.75±7.96^{a}	106.90±35.07^{b}	111.20±34.12^{b}	126.40±33.57^{b}	p<0.05	p<0.05	p<0.05
Stiffness (N/mm)	162.00±99.75^{a}	197.00±141.90^{a}	254.80±132.80^{a}	154.80±109.40^{a}	209.40±84.13^{a}	572.00±291.30^{ab}	885.10±534.40^{bc}	1085.80±813.30^{c}	p<0.05	p<0.05	p<0.05
Absorbed energy (mJ)	12.63±18.62	11.46±10.53	9.12±10.80	6.85±11.54	5.55±3.79	12.83±7.59	16.98±12.85	14.82±9.08	ns	ns	ns

Two-way ANOVA, Bonferroni post-test (for interaction only). Values bearing different letters between columns are significantly different (p<0.05). Abbreviation = D: ZA doses; T: period of treatment; D-T: dose and time interaction.

periods of treatment in order to analyze the effect of dose accumulation. In spite of the increasing cumulative dose of ZA, all bone parameters were improved according to the expected growth through the experiment. However, linear and appositional growth were negatively affected under the treatment with the highest cumulative dose of ZA. Femoral and tibial length were lower at D25 compared to D0, D2.5 and D12.5 after 30 days of treatment, an effect that was not found after 15 days of treatment indicating that the cumulative dose of ZA could affect bone growth. On the other hand, GPC.Th was affected by the highest ZA dose from 15 days of treatment. These early effects on the growth plate could explain the ZA effect on femur and tibia growth after 30 days of treatment. These findings are consistent with previous studies. Thus, Oyhanart et al showed that alendronate interferes with long-bone and mandibular growth[14]. In addition, a study in mice with osteosarcoma treated with ZA showed a decrease in tibial length[18]. Moreover, a decrease in tibial longitudinal growth rate was observed after pamidronate or ZA administration in growing rats[34]. The effect on longitudinal growth might be due to the ZA action on osteoclasts since they play an important role in the process of endochondral ossification.

Nevertheless, studies in pediatric patients with osteoporosis treated with ZA showed that longitudinal growth was unaffected during treatment[33,21]. This is consistent with our results because at low doses -similar to those used for osteoporosis- no longitudinal growth alteration was observed, independently of the length of treatment evaluated in this study. However, the effect of BP on bone length might depend on type, dose, and frequency of BP and age or medical history of the patient and the possibility of a decrease in children height should be considered and monitored.[9]

Linear growth has been a concern in pediatrics patients treated with BP, but the appositional growth has received less attention, although it is important for the development of the skeleton, being one of the determinants of bone strength[35]. During bone growth, bone formation is uncoupled to resorption to shape the bone through modeling. Consistent with this action of osteoclasts, it has been observed in patients with osteogenesis imperfecta treated with pamidronate that this drug interferes with periosteal resorption, process that is normally responsible for the shape of the distal femoral metaphysis[36]. Also BP inhibit endosteal resorption but not periosteal apposition resulting in an increased cortical thickness and a higher

strength[37]. In our study all morphometric parameters -used to evaluate appositional growth- were affected by D and T. Only D25 showed a mild negative effect on CS. Ar but that was counteracted by a decreased of Me. Ar avoiding affect Ct.W and achieving a better distribution of material as shown by CSMI after 30 days of treatment. At the shorter period of treatment (15 days), an 11.8% decrease on Cs. Ar and a 27% decrease on Me. Ar were found indicating a rapid inhibition of bone resorption. This led to an increase of Ct.W indicating that bone resorption was inhibited by ZA therapy but activity on periosteal surfaces was less affected as determined in modeling. The mechanical properties of cortical bone were increased by the period of treatment and only the absorbed energy was negatively affected by the ZA dose. However, this negative effect of the highest ZA accumulated dose on absorbed energy and CS. Ar was not enough to decrease bone strength. This could be due to the greater CSMI shown at D25 after 30 days of treatment indicating a better spatial distribution of material that is highly correlated with bone strength as mentioned above[38].

The bone mass acquired during the growth was evaluated by BMD and the percentage of bone volume. BMD was only affected by the period of treatment. The lack of significant dose effect could be due to the low sensitivity of the technique and would be a limitation of this study. However, ~20% increase in a BMD was observed at D25 after 30 days of treatment which could be explain by the increase in trabecular bone volume. Furthermore, the trabecular bone volume was increased with all ZA doses after 15 and 30 days of treatment as a consequence of increase in both trabecular thickness and, mainly, in trabecular number. The latter could be due to the fact that antiresorptive therapy led to a lesser resorption of primary trabeculae that, necessarily, became secondary trabeculae[39]. In addition, the connectivity indexes also increased with dose and the length of treatment, indicating an improved bone architecture, which could impact on the biomechanical evaluation. These finding matched the observations by Pataki et al.[34] but were different to those conducted in children with osteogenesis imperfecta where the increase in bone volume was due only to an increased in trabecular number but without any effect on trabecular thickness[39].

On the other hand, the compression test, which evaluates the trabecular mechanical properties, showed a favorable effect of dose and the length of treatment on fracture load and stiffness –without changes in absorbed energy. In addition, an interaction of both factors was found in fracture load and stiffness. The improved trabecular mechanical properties could be attributable not only to the increased bone volume but also to the bone microarchitecture since better connectivity parameters were found. Although not significant, a borderline p value (p=0.07) for interaction was found in ICI parameter indicating that the trabecular connectivity would be greater at the highest dose of ZA. In summary, our results indicate that ZA treatment would not have significant negative effects on the mechanical properties of the growing bone. Moreover, an improved on trabecular parameters were observed in all ZA doses.

Some limitations of this study must be pointed out. The experiment was carried out with healthy animals and these findings should be confirmed using experimental models of bone diseases. On the other hand, the conversion of equivalent doses between human and rats, and the possible different pharmacodynamics of ZA between them should be highlighted. However, in spite of limitations, this work carried out a complete bone analysis assessment, including morphometric, densitometric, histomorphometric and biomechanical analysis. Therefore it contributes to current knowledge on the effects of ZA dose accumulation on bone growth.

In conclusion, even though a mild negative effect of the highest dose of ZA on linear and appositional growth were observed; bone volume, trabecular connectivity and trabecular mechanical properties of growing bone were improved also at the highest cumulative ZA dose. As the highest dose was comparable to those used in models of bone pathologies related to primary tumors or bone metastasis, a careful risk/benefit analysis would lead us to conclude that the mild deleterious effects of ZA during growth are outweighed by the benefit obtained with treatment.

Conflict of interest: None to declare

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