Research, Society and Development, v. 10, n. 14, e518101422159, 2021
(CC BY 4.0) | ISSN 2525-3409 | DOI: http://dx.doi.org/10.33448/rsd-v10i14.22159

Effects of bisphosphonates on different zones of the epiphyseal growth plate of rats

Efeitos dos bisfosfonatos em diferentes zonas da placa de crescimento epifisária de ratos

Efectos de los bisfosfonatos en diferentes áreas de la placa de crecimiento epifisario de ratas

Received: 10/22/2021 | Reviewed: 10/31/2021 | Accept: 11/08/2021| Published: 11/13/2021

Deise Ponzoni
ORCID: https://orcid.org/0000-0003-2855-7495
Universidade Federal do Rio Grande do Sul, Brazil
E-mail: deponzoni@yahoo.com
Elissa Kerli Fernandes
ORCID: https://orcid.org/0000-0003-2332-3518
Universidade Federal do Rio Grande do Sul, Brazil
E-mail: elissa.kfernandes@yahoo.com.br
Mateus Muller da Silva
ORCID: https://orcid.org/0000-0001-7417-3201
Universidade Federal do Rio Grande do Sul, Brazil
E-mail: mateus18muller@gmail.com
Izabel Cristina Custódio de Souza
ORCID: https://orcid.org/0000-0002-6117-1810
Universidade Federal de Pelotas, Brazil
E-mail: belcustodio20@yahoo.com.br
John Kim Neubert
ORCID: https://orcid.org/0000-0001-6774-3093
University of Florida, USA
E-mail: jneubert@dental.ufl.edu
Alexandre Silva Quevedo
ORCID: https://orcid.org/0000-0001-5613-8015
Universidade Federal do Rio Grande do Sul, Brazil
E-mail: quevedoalexandre@hotmail.com

Abstract
Bisphosphonates (BIS) are indicated for several clinical disorders (e.g., osteoporosis). However, BIS has been associated with osteonecrosis and alterations in osteoclastogenesis and skeletal development. This study aimed to evaluate the effects of BIS (zoledronic acid - ZA and alendronate sodium - AS) on zones of the growth plate of rat femur. Animals (Wistar rats, n = 19) were divided into groups: 1) AS Group: animals received alendronate sodium orally (3 mg/kg per day); 2) ZA Group: ZA was administered intraperitoneally (0.2 mg/kg per week); and 3) Control Group (CG): a vehicle was administered. Animals were euthanized 21 days after the treatment, and femurs were collected for histological analysis. The images of all zones (resting, proliferative, hypertrophic, and calcified) were processed by the Qcapture® software providing a 40 and 400-fold increase. ZA decreased epiphyseal growth plate cell zones (ZA Group vs. CG) in most cases. Likewise, AS diminished the proliferative zone (AS Group vs. CG). Furthermore, ZA increased the calcified zone (ZA Group vs. CG). Previous works demonstrated that BIS decrease the epiphyseal disc. This reduction is probably due to the shortening of the cellular zones that undergoes calcification/ossification. The present results suggest that BIS should be carefully indicated because these drugs might accelerate epiphyseal closure.
Keywords: Alendronate; Zoledronic acid; Growth plate; Animal models; Epiphyses.

Resumo
Os bisfosfonatos (BIS) são indicados para diversas condições clínicas, como a osteoporose. No entanto, os BIS têm sido associado a osteonecrose, alterações na osteoclastogênese e no desenvolvimento esquelético. O presente estudo testou a hipótese de que os BIS (alendronato de sódio - AS e ácido zoledrônico - ZA) modificam a espessura das zonas da placa de crescimento do fêmur de ratos. Ratos Wistar (n = 19) foram divididos em: 1) Grupo AS: alendronato de sódio por via oral (3 mg / kg / dia); 2) Grupo ZA: administração de ácido zoledrônico por via intraperitoneal (0,2 mg / kg / semana); e 3) Grupo Controle (GC): sem administração de medicamentos. Após 21 dias de tratamento, os animais foram eutanasiados e os fêmures coletados para análise histológica. As imagens de diferentes zonas da placa de crescimento (zonas de repouso, proliferativas, hipertróficas e calcificadas) foram capturadas usando o software Qcapture® (aumento de 40 e 400 vezes). ZA diminuiu as zonas de células da placa (Grupo ZA vs. CG) na maioria dos casos. Da mesma forma, o AS causou uma diminuição da zona proliferativa (Grupo AS vs. GC). Além disso, ratos tratados com ZA apresentaram aumento da espessura da zona calcificada (Grupo ZA vs. GC). Estudos anteriores mostraram efeito dos BIS na redução da espessura do disco epifisário. Essa

redução da placa de crescimento provavelmente deve-se ao encurtamento das zonas celulares que sofrem calcificação e ossificação. Nossos resultados sugerem que os BIS devem ser indicados com cautela, pois podem acelerar o fechamento epifisário.
Palavras-chave: Alendronato; Ácido zoledrônico; Placa de crescimento; Modelos animais; Epífises.

Resumen
Los bisfosfonatos (BIS) están indicados para varias afecciones clínicas, como la osteoporosis. Sin embargo, los BIS se han asociado con osteonecrosis, alteraciones en la osteoclastogénesis y desarrollo esquelético. El presente estudio probó la hipótesis de que BIS (alendronato sódico - AS y ácido zoledrónico - ZA) modifica el grosor de las zonas de la placa de crecimiento del fémur de rata. Las ratas Wistar (n = 19) se dividieron en: 1) Grupo AS: alendronato sódico oral (3 mg / kg / día); 2) Grupo ZA: administración intraperitoneal de ácido zoledrónico (0,2 mg / kg / semana); y 3) Grupo Control (GC): sin administración de medicación. Después de 21 días de tratamiento, se sacrificó a los animales y se recogieron los fémures para análisis histológico. Se capturaron imágenes de diferentes zonas de la placa de crecimiento (zonas de reposo, proliferativas, hipertróficas y calcificadas) utilizando el software Qcapture® (aumento 40 y 400 veces). ZA disminuyó las zonas de células de placa (Grupo ZA frente a CG) en la mayoría de los casos. Asimismo, AS provocó una disminución en la zona proliferativa (Grupo AS vs. GC). Además, las ratas tratadas con ZA mostraron un mayor grosor de la zona calcificada (Grupo ZA frente a GC). Estudios anteriores han demostrado el efecto de BIS en la reducción del grosor del disco epifisario. Esta reducción de la placa de crecimiento probablemente se deba al acortamiento de las zonas celulares que sufren calcificación y osificación. Nuestros resultados sugieren que los BIS deben indicarse con precaución, ya que pueden acelerar el cierre epifisario.
Palabras clave: Alendronato; Ácido zoledrónico; Placa de crecimiento; Modelos animales; Epífisis.

1. Introduction

Bisphosphonates (BIS) are analogous to the pyrophosphate (Fernandes et al., 2005). They may be classified as aminobisphosphonates or non-aminobisphosphonates, depending on the presence or absence of nitrogen in the structure (Lin, 1996). Generally, BIS that contain nitrogen are more potent than non-aminobisphosphonates (Russell et al., 2008). Zoledronic acid (ZA) and alendronate sodium (AS) are the most prescribed aminobisphosphonates (Patntirapong & Poolgesorn, 2018).

These drugs are indicated for disorders of bone metabolism due to the ability to bind to the hydroxyapatite and inhibit osteoclast-mediated bone resorption (Dominguez et al., 2011). Bone remodeling occurs through resorption and formation events mediated by osteoclasts or osteoblasts, respectively (Mackie et al., 2011). Primarily, BIS are used to treat osteoporosis in the elderly population (Lin, 1996). However, BIS are also administered at different growth stages to treat pathological conditions, such as osteogenesis imperfecta (Biggin & Munns, 2017), Paget's disease (de Oliveira et al., 2019), and juvenile osteoporosis (Batch et al., 2003).

The clinical benefits of BIS have been associated with alterations in osteoclastogenesis, bone formation, and skeletal growth (Rezende et al., 2017). Nevertheless, the influence of BIS on bone growth is not fully known (Bianchi, 2005). Therefore, animal models might be a valuable tool to investigate the impact of BIS on the epiphyseal plate and, consequently, on human growth. Particularly, the choice of rats as an animal model may be relevant because similar methodologies have been applied to study different bone diseases (Erdogan et al., 2014; Oyhanart et al., 2015; Özenci et al., 2013). Furthermore, the closure of the growth plate does not usually occur in rats. However, skeletal growth slows down around 7 to 8 months of age in these rodents (Quinn, 2005).

The epiphyseal growth plate causes the longitudinal growth of the bones and is composed of specialized cartilage (Ballock & O'Keefe, 2003). This structure is divided into zones (i.e., resting, proliferative, hypertrophic, and calcified) corresponding to the stage of maturation (Wilsman et al., 1996). Previous studies have shown that BIS may modify the thickness of growth plate zones (Junges, 2013).

Therefore, considering the numerous factors capable of altering bone structure during the BIS administration, the present study aimed to evaluate the effects of aminobisphosphonates (alendronate sodium and zoledronic acid) on the growth plate zones of the rat femur.

2. Methodology

This study had a quantitative approach and a basic experimental design (De Barros Silva et al., 2015; Huang et al., 2005).

2.1 Animals

The sample (n=19) consisted of one hundred-day-old male Wistar rats weighing 250-300 grams from the Hospital de Clínicas de Porto Alegre (Unit of Animal Experimentation). The rats were randomized by weight and allocated into different groups. Rats were kept in cages of polypropylene (49x34x16cm). The animal facility was maintained under controlled conditions: 12:12 light–dark (on at 7.00 a.m. and off at 7.00 p.m.), controlled temperature room (22±2ºC), and food (Nuvilab, Moinhos Purina, Porto Alegre, RS - Brazil) and water ad libitum. The experimental protocol complied with the ethical and methodological standards of the ARRIVE guidelines (Kilkenny et al., 2010). Attempts were made to minimize animal suffering and decrease pain and discomfort, as well as to use the minimum number of animals required to produce reliable scientific data. The Institutional Animal Care and Use Committee (IACUC) of Hospital de Clínicas de Porto Alegre (HCPA) previously had approved the experimental project (protocol N. 09-366). The experiments followed the Guide for the Care and Use of Laboratory Animals 8th edition 2011 and law 11.794 (Brazil), which establishes procedures for the scientific use of animals. The research leading to these results received funding from Institutional Scientific Initiation Scholarship Program (PIBIC) and Incentive Fund for Research and Events (FIPE/Hospital de Clínicas de Porto Alegre - HCPA) – FIPE.

2.2 Treatment protocols

The rats were randomly distributed into groups: control group (CG, n=5), AS group (n=7), and ZA group (n=7). The drug doses were adjusted to the rat's body weight using two protocols that had been already established in the literature (De Barros Silva et al., 2015; Huang et al., 2005). For example, the oral dose of AS in humans is 1mg/kg per week (Porras et al., 1999). However, the bioavailability of AS orally is approximately 1% for humans. On the other hand, AS has low bioavailability in rats, and the elimination rate of this drug is much greater in rats than in humans (Huang et al., 2005). Therefore, due to the pharmacodynamics of AS, doses were adjusted to 3mg/kg per day (gavage) (Alendronato de Sódio®, Eurofarma S.A, RJ, Brazil) for three weeks (Huang et al., 2005).

ZA can be administered intravenously at a dose of 4mg/patient/month in humans (Khosla et al., 2007). Here, animals received intraperitoneal injections of ZA (Ácido Zoledrônico®, Eurofarma S.A, RJ, Brazil) at 0.2mg/Kg per week intraperitoneally for three weeks, which is in accordance with previous studies (De Barros Silva et al., 2015).

After receiving the treatments, animals were euthanized in a carbon dioxide gas (CO2) chamber. The control animals (CG) did not receive any pharmacological intervention.

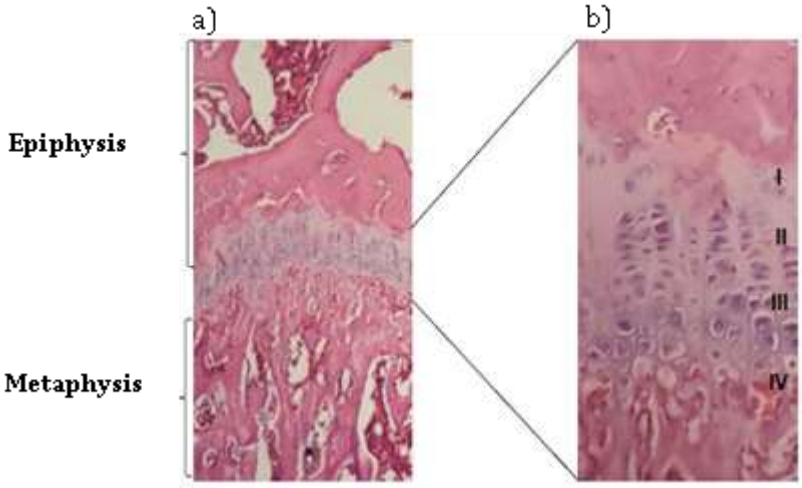
2.3 Histological preparation

Both femurs of each rat were collected and fixed in 10% neutral buffered formalin. After 2 days, bones were decalcified in nitric acid (10%) for 7 days. Afterward, the pieces were dehydrated, and prepared in paraffin blocks. Using microtome, sagittal sections (5µm) were cut and stained using hematoxylin-eosin (H&E) (Fischer et al., 2008).

2.4 Evaluation of epiphyseal disc

Three different areas of distal epiphysis were evaluated: the medial, intercondylar (the region between the condyles), and lateral regions. Two slides (right and left sagittal femoral cut) were prepared for each studied area. The average of the two slices was used for the analysis. Images of those regions were acquired using Qcapture® software (magnification of 400x). The area of the disc (Figure 1a) was determined using the Adobe Photoshop CS3 extended program (Saad et al., 2008). Areas of the epiphyseal disc were classified into four zones: resting, proliferative, hypertrophic, and calcified (Figure 1b). After identification, each zone was delimited and assessed. Finally, the relative area that each zone occupies in the disc was determined relative to the total thickness of the epiphyseal growth plate (i.e., percentage).

Figure 1: Epiphyseal plate.



I = resting zone,
II = proliferative zone,
III = hypertrophic zone,
IV = calcified zone

Source: Authors.

Figure 1 shows representative microphotographs of H&E-stained histological sagittal sections showing the epiphyseal disc. Panel (a) demonstrates the epiphysis, growth plate, and metaphysis (magnification 40 x). Panel (b) shows a magnification (400 x) of the growth plate where different cell layers can be identified (according to the stage of ossification): resting, proliferative, hypertrophic, and calcified zones. This area is formatted by different areas: resting zone, proliferative zone, hypertrophic zone, and calcified zone

2.5 Statistical analysis

The normality of data distribution was assessed by the Kolmogorov-Smirnov test. Each area was expressed by the percentage of the total thickness of the grow plate, according to the following formula:

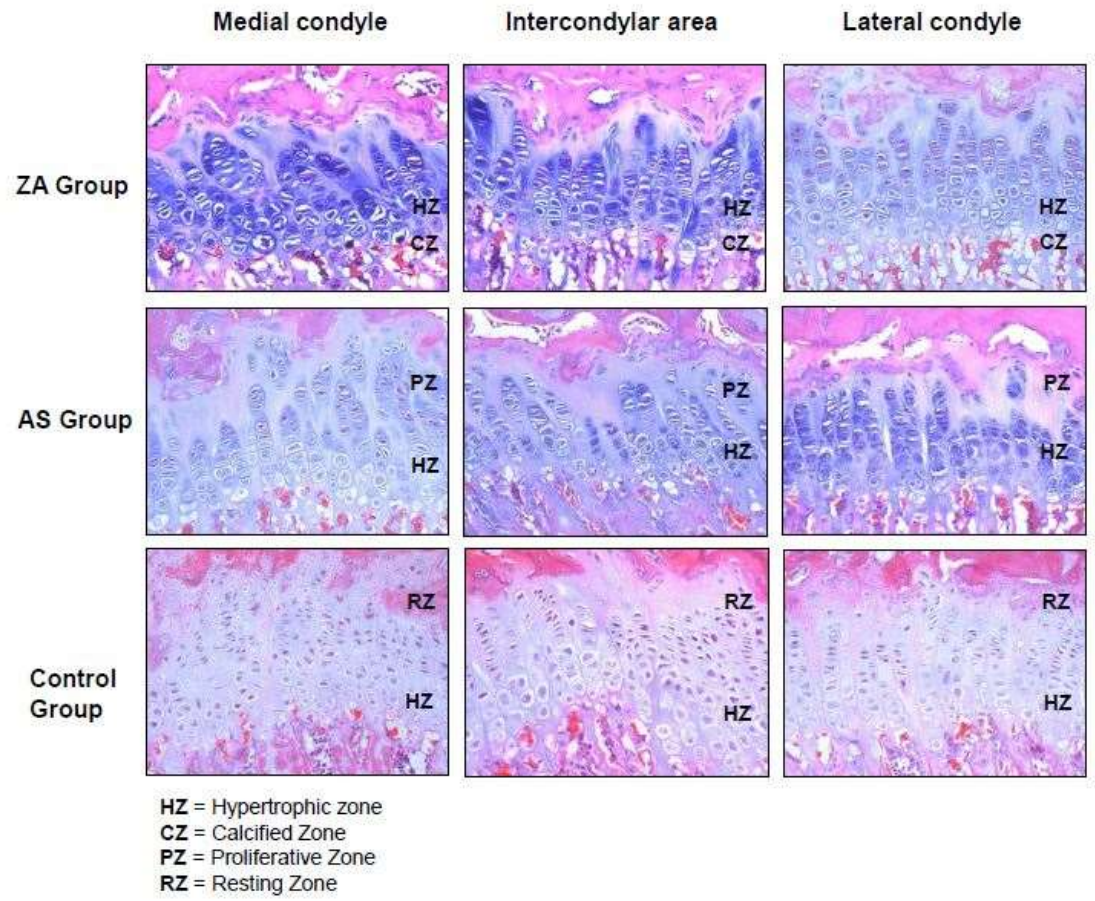
Percentage of a zone = (thickness of the zone*100)/(total thickness of the epiphyseal disc)

Average area (pixel^2) ± standard deviation of the mean (S.E.M.) expressed the data. To compare results from different groups, a one-way analysis of variance ANOVA, followed by Fisher's L.S.D. was used. SPSS 20.0 packages for windows were used to performed all analysis. $P < 0.05$ was considered to be statistically significant.

3. Results

The summary of the findings (optical image of H&E strain) is presented in Figure 2. The present results corroborate the literature showing that chondrocytes are distributed differently depending on their location. Therefore, chondrocytes are irregularly scattered in resting zone and arranged in columns in hypertrophic and proliferative zones. The deepest cartilage layer has a calcified matrix. The results showed alterations in the cartilage zones in animals treated to BIS (see details below).

Figure 2: Histological changes in the epiphyseal growth plate by bisphosphonate treatments.



Source: Authors.

Resting Zone had cells that are irregularly scattered, whereas, in Hypertrophic and Proliferative Zones, chondrocytes are arranged in columns parallel to the larger axis of the bone. After proliferation, these cells undergo hypertrophy and die initiating ossification by bone cell invasion.

Intercondylar and Medial regions: The CG showed the greater thickness of cell zones than the ZA (RZ, PZ, HZ) and AS (PZ) groups. The ZA group had the largest calcified zone.

Lateral region: The CG had larger cell zones than both bisphosphonate groups, except for the HZ of AS group. The ZA and AS groups had larger calcified zones than CG.

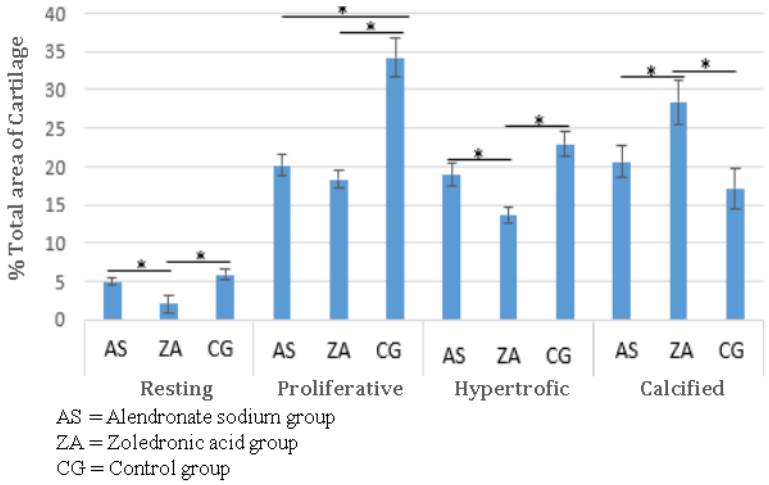
3.1 Medial condyle region

Figures 2 and 3 show the effect of ZA on different zones, showing a decrease of the resting (CG vs. ZA, $F_{(2\text{-}20)}$=6.15, $p<0.05$), proliferative (CG vs. ZA, $F_{(2\text{-}20)}$=24.04, $p<0.05$), and hypertrophic zones (CG vs. ZA, $F_{(2\text{-}20)}$=10.42, $p<0.05$).

Furthermore, ZA increased the length of the calcified zone compared to the control group (CG vs. ZA, $F_{(2\text{-}20)}$=4.91, p<0.05). However, AS only caused a decrease in the proliferative zone (CG vs. AS, $F_{(2\text{-}20)}$=24.04, p<0.05). In addition, there was a trend of AS group having a smaller hypertrophic zone than the control animals (AS vs. CG, $F_{(2\text{-}20)}$=10.42, p=0.059).

Animals that received AS presented higher thickness than animals treated with ZA in the hypertrophic ($F_{(2\text{-}20)}$=10.42, p<0.05) and resting (AS vs. ZA, $F_{(2\text{-}20)}$=6.15, p<0.05) zones. Furthermore, the calcified zone was thicker in ZA group than AS group (AS vs. ZA, $F_{(2\text{-}20)}$=4.91, p<0.05).

Figure 3: Medial condyle region



Source: Authors.

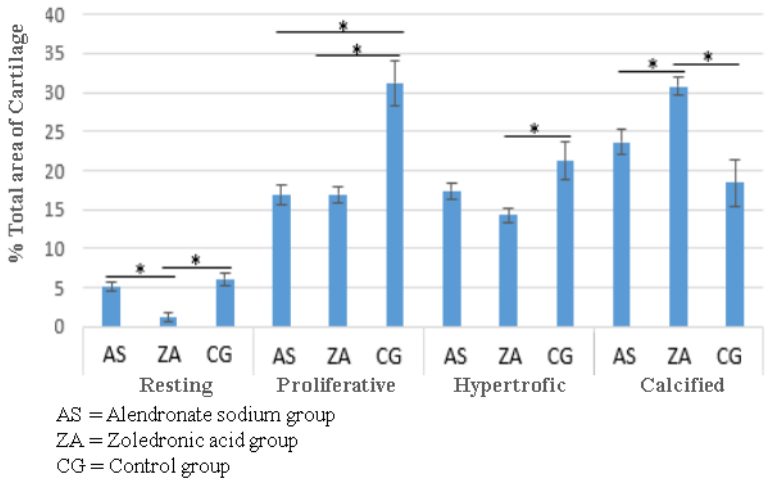
Figure 3 summarizes the findings in the medial condyle. Compared to control animals, ZA reduced in the thickness of the resting, proliferative, and hypertrophic zones (ZA vs. CG). Moreover, ZA animals had a greater thickness of calcified zone than AS and Controls groups (ZA vs. AS and ZA vs. CG). The AS caused a shrinkage of the proliferative zone (AS vs. CG). Comparing the two drugs, ZA showed a more significant decrease in the resting and hypertrophic (AS vs. ZA) zones.

3.2 Intercondylar region

Compared to control animals, ZA decreased the resting (CG vs. ZA, $F_{(2\text{-}20)}$=17.17, p<0.05), proliferative (CG vs. ZA, $F_{(2\text{-}20)}$=20.49, p<0.05), and hypertrophic (CG vs. ZA, $F_{(2\text{-}20)}$=6.03, p<0.05) zones (Figures 2 and 4). Moreover, length of the calcified zone was greater in animals that received ZA (CG vs. ZA, $F_{(2\text{-}20)}$=10.50, p<0.05). Furthermore, the AS treatment induced a shrinkage of the proliferative zone (CG vs. AS, $F_{(2\text{-}20)}$=20.49, p<0.05).

Comparing ZA animals to those that received AS, there was a decrease in the resting zone in the ZA group (ZA vs. AS, $F_{(2\text{-}20)}$=17.17, p<0.05). However, the calcified zone was thicker in animals treated with ZA (AS vs. ZA, $F_{(2\text{-}20)}$= 10.50, p<0.05).

Figure 4: Intercondyle region



Source: Authors.

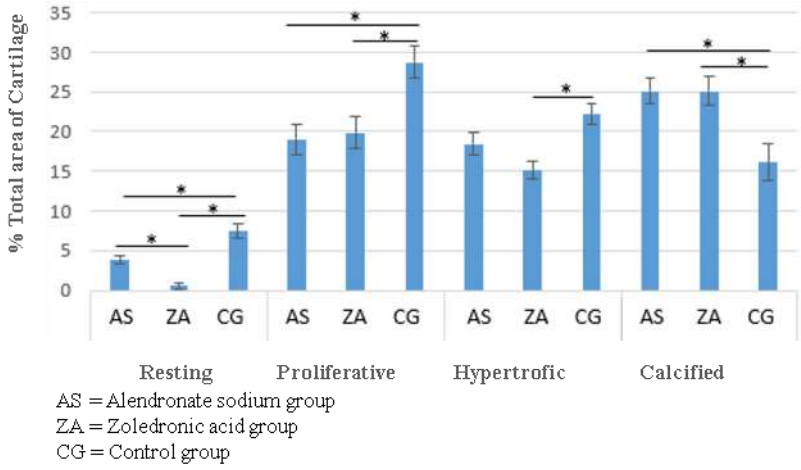
Figure 4 indicates that ZA decreased the hypertrophic, proliferative, and resting (ZA vs. CG) zones. However, ZA animals showed greater calcified zone when compared to control (ZA vs. CG) and AS (ZA vs. AS) animals. The AS treatment reduced the proliferative zone (AS vs. CG) but showed a smaller effect than ZA treatment in the resting zone (AS vs. AZ).

3.3 Lateral region

In Figures 2 and 5, ZA shows a reduction in the resting (CG vs. ZA, $F_{(2\text{-}20)}$=29.60, p<0.05), proliferative (CG vs. ZA, $F_{(2\text{-}20)}$=6.50, p<0.05), and hypertrophic (CG vs. ZA, $F_{(2\text{-}20)}$=6.50, p<0.05) zones. Furthermore, ZA treatment increased the calcified zone (CG vs. ZA, $F_{(2\text{-}20)}$=6.88, p<0.05). On the other hand, AS animals showed a decrease of the resting (CG vs. AS, $F_{(2\text{-}20)}$=29.60, p<0.05) and proliferative (CG vs. AS, $F_{(2\text{-}20)}$=6.51, p<0.05) zones. Moreover, the calcified zone was thicker in the AS group than in control animals (CG vs. AS, $F_{(2\text{-}20)}$=6.88, p<0.05).

The resting zone was reduced in animals that used ZA compared to AS group (AS vs. ZA, $F_{(2\text{-}20)}$=29.60, p <0.05).

Figure 5: Lateral condyle region



Source: Authors.

Figure 5 shows that ZA was able to reduce the proliferative, resting, and hypertrophic zones, but increase the calcified zone compared to controls (ZA vs. CG). Likewise, AS induced a reduction in the resting and proliferative zones (CG vs. AS). However, the calcified zone was thicker in the AS animals compared to control group (CG vs. AS). The resting zone was decreased in the ZA animals compared to those treated with AS.

4. Discussion

Overall, the present data showed that BIS are able to alter the morphology of the epiphyseal growth plate in rats. For instance, AS was able to reduce the proliferative zone, while ZA decreased most of the cellular zones but increased the calcified zone. The shrinking the cell zone size did not compensate the increase of the calcified zone. This unbalance between reduction/increase between cellular and calcified zones may explain the decrease of the total growth plate thickness during BIS treatment (Junges, 2013).

The long bones growth occurs by endochondral ossification, where the embryonic cartilaginous skeleton is replaced by mature bone tissue (Mackie et al., 2008; Russell et al., 2008). Some drugs may disrupt (i.e., delay, abolish, or decrease) bone formation. For example, BIS, which are antiresorptive drugs, inhibit bone resorption by their effects on clastic cells. These bone cells are involved in the reabsorption of calcified cartilage septa and in the invasion of osteogenic cells. During the endochondral ossification process, the osteogenic cells secrete bone matrix over the calcified cartilage (Li et al., 2006).

Differences between humans and rats might be considered when age is critical. Epiphyseal disc Closure varies according to each bone and between individuals in humans. However, all bone epiphyses finish growing around 20 years of age. In rats, epiphyseal disc closure does not usually occur. Moreover, skeletal growth reduces around 7-8 months in Sprague-Dawley rats (Quinn, 2005). Here, this study used 120-day-old rats that did not reach musculoskeletal maturity. Therefore, the data were collected during growth and can be associated to human beings during the skeletal growth phase.

The epiphyseal growth plate is composed of specialized cartilage and is related to the growth of long bones (Ballock & O'Keefe, 2003). Based on the ossification stage, growth plate is divided into several zones: calcified, hypertrophic, proliferative, and resting. (Wilsman et al., 1996). Chondrocytes secrete and maintain a highly specialized matrix. This cellular environment contributes to ossification induced by a sequence of events that cause changes in chondrocyte morphology. (Mackie et al., 2011).

The present data demonstrated that the administration of ZA (short-term period) in rats was able to decrease the resting zone in all condyle regions: lateral, intercondylar, and medial (Figures 3, 4, and 5). Likewise, AS decreased the resting zone of the lateral condyle (Figure 5). Furthermore, the reduction of the intercondylar and lateral regions was found to be more significant in animals that received ZA than those treated with AS (Figures 4 and 5, respectively). The resting zone is a contoured region (narrow and irregular) consisting of chondrocytes organized in cell lines (single or paired).

These chondrocytes are in a relatively latent state where there is a high relationship between extracellular matrix and the cell volum (Ballock & O'Keefe, 2003; Ernst B. Hunziker, 1994). The resting zone plays a key role supplying chondrocytes to the proliferative zone (Pfeil & DeCamp, 2009). In all studied condyle areas, ZA showed a more significant effect on the resting zone when compared to AS. The difference in drug effects may be due to the higher potency of ZA (Russell et al., 2008). Over time, the chondrocytes mature, enlarge to a hypertrophic state, and achieve a proliferative phenotype. Therefore, these cells acquire a flattened morphology, organize themselves longitudinally, and form the proliferative zone (E. B. Hunziker et al., 1987).

The use of ZA decreased the proliferation zone in the lateral, intercondylar, and medial regions. The AS had a similar effect in all three areas (Figures 3, 4, and 5). Previous studies had reported that chondrocytes go through a series of rapid

divisions, along the long bones (proximodistal axis) in the proliferation zone (Pfeil & DeCamp, 2009). Furthermore, there is the formation of intracellular matrix and synthesis of collagen and proteoglycan (E. B. Hunziker et al., 1987). The decrease of the proliferative zone by BIS can involve the reduction of the number of cells originating from the resting zone (Pfeil & DeCamp, 2009). Moreover, the diminution of vascular endothelial growth factor (VEGF) expression would regulate the turnover of chondrocytes contributing to reducing this cell zone (Gerber et al., 1999). Furthermore, the low levels of growth factors may also decrease angiogenesis in long bones (Oyhanart et al., 2015).

After a number of divisions, the chondrocytes stop to divide and become hypertrophic (Brighton, 1984). Therefore, cell division ends, and the chondrocytes start to differentiate terminally (Pfeil & DeCamp, 2009). Here, ZA caused a reduction in the hypertrophic zone in all studied regions of the epiphyseal disc (Figures 3, 4, and 5). However, the AS had a more discreet effect. It has been seen that the decrease of the hypertrophy zone by BIS is dose-dependent (Zhu et al., 2014). Therefore, the dosage of AS used in the present work may not have been sufficient to induce more remarkable tissue changes. Furthermore, the hypertrophic chondrocytes are involved in cartilage calcification that is used as model for bone formation (Ballock & O'Keefe, 2003). The lower thickness of the hypertrophic zone caused by BIS treatment may be due to the less number of hypertrophic chondrocytes per column (Oyhanart et al., 2015).

In a previous study, our group has shown lower thickness of the epiphyseal disc after a BIS treatment (Junges, 2013). Corroborating previous work, the present data indicate that BIS may cause a reduction in the hypertrophic, proliferative and resting zones (Oyhanart et al., 2015). It has been seen that AS (0.2 mg/kg/week) for growing animals caused a decrease in the thickness of the epiphyseal cartilage. This effect may be explained by two different reasons: a) a reduction of different cartilage areas or, b) changes in the morphology or number of osteoclasts (Oyhanart et al., 2015).

In this study, the distal femur growth plate showed apparently different responses to ZA and AS. Alternatively, other factors (e.g., dosages, potency, and posology) may induce distinct responses in bone physiology. For example, when 2.5 mg/kg was administered once a day, for 21 consecutive days, BIS cause expansion of cartilage zones (Rezende et al., 2017). Moreover, a short-term ZA treatment (0.1mg/kg 3 times per week for up to 8 weeks) caused interruption of the development of the proximal tibia growth plate of rats. The authors reported non-physiological alterations in chondrocytes, cell alignment disruption, and decreased physical height (Erdogan et al., 2014).

The variability of treatment response using different BIS can be associated with the drug potency and its affinity to bone hydroxyapatite (Lawson et al., 2005). The highest mineral-binding capacity is related to more significant inhibition of bone turnover, which can influence the drug potency and clinical relevance of different BIS (Lawson et al., 2005). Another factor that contributes to the bisphosphonates potency is the inhibition of the enzyme farnesyl pyrophosphate synthase (FPPS) found in osteoclasts (Harrington et al., 2004). These drug characteristics may justify the more significant effects of ZA on cell zones, which have higher FPPS activity and affinity for hydroxyapatite (Lawson et al., 2005). The ZA potency may justify the enlargement of the calcified cartilage zone in all studied femoral areas (Figures 3, 4, and 5). Therefore, the effect of AS was more restricted than ZA in reducing the calcified zone (Lawson et al., 2005). Even though, in the lateral region of the femoral condyle, the thickness of the calcified zone was narrower in animals that received AS compared to the control group (Figure 5).

The use of BIS has been associated with bone alterations such as sclerotic lines in the metaphysis (Silva et al., 2010) and spontaneous (Tan et al., 2019) or atypical (Leclerc et al., 2019) fractures. The present findings indicated that BIS might alter the epiphyseal growth plate. While cell areas were reduced, there was an increase in the calcified zone in animals that received BIS. Therefore, the reduction in the epiphyseal growth plate found in earlier studies is probably due to the decrease of the resting, proliferative, hypertrophic zones.

5. Final Considerations

The use of BIS in clinical treatment should be carefully indicated in patients prior to epiphyseal closure because there are alterations in the process of endochondral ossification and, consequently, a possible reduction in long bone growth. Future studies should address a possible relationship between the histological findings reported in the present study and the possible disruption in growth. For example, differences between sexes or ages are factors that can potentially influence the responses of the femoral growth plate to the use of bisphosphonates. Furthermore, different concentrations of these drugs may have varied results from those found here.

Acknowledgments

We thank the staff of the Animal Experimentation Unit (UEA), headed by Marta Justina Giotti Cioato, DNP for the professionalism and credibility in the service provided, ensuring the quality of research.

References

Ballock, R. T., & O'Keefe, R. J. (2003). Physiology and pathophysiology of the growth plate. In *Birth Defects Research Part C - Embryo Today: Reviews*. 69(Issue 2), 123–43. Birth Defects Res C Embryo Today. https://doi.org/10.1002/bdrc.10014

Batch, J. A., Couper, J. J., Rodda, C., Cowell, C. T., & Zacharin, M. (2003). Use of bisphosphonate therapy for osteoporosis in childhood and adolescence. In *Journal of Paediatrics and Child Health* . 39(Issue 2), 88–92. J Paediatr Child Health. https://doi.org/10.1046/j.1440-1754.2003.00083.x

Bianchi, M. L. (2005). How to manage osteoporosis in children. In *Best Practice and Research: Clinical Rheumatology*. 19(Issue 6), 991–1005. Best Pract Res Clin Rheumatol. https://doi.org/10.1016/j.berh.2005.06.006

Biggin, A., & Munns, C. F. (2017). Long-Term Bisphosphonate Therapy in Osteogenesis Imperfecta. In *Current Osteoporosis Reports*. 15(Issue 5), 412–418. Current Medicine Group LLC 1. https://doi.org/10.1007/s11914-017-0401-0

Brighton, C. T. (1984). The growth plate. In *Orthopedic Clinics of North America*. 15(Issue 4), 571–595. Elsevier. https://doi.org/10.1016/s0030-5898(20)31257-8

Barros Silva, P. G., Ferreira Junior, A. E. C., Teófilo, C. R., Barbosa, M. C., Lima Júnior, R. C. P., Sousa, F. B., Mota, M. R. L., De Albuquerque Ribeiro, R., & Alves, A. P. N. N. (2015). Effect of different doses of zoledronic acid in establishing of bisphosphonate-related osteonecrosis. *Archives of Oral Biology*. 60(Issue 9), 1237–1245. https://doi.org/10.1016/j.archoralbio.2015.05.015

Oliveira, F. A. K., Pinto, F. F. E., Sardenberg, T., Pereira, G. J. C., Curcelli, E. C., & Penna, V. (2019). Diagnosis and management of paget's disease of bone - series of 8 cases. *Acta Ortopedica Brasileira*. 27(Issue 1), 31–32. https://doi.org/10.1590/1413-785220192701161107

Dominguez, L. J., Bella, G. Di, Belvedere, M., & Barbagallo, M. (2011). Physiology of the aging bone and mechanisms of action of bisphosphonates. *Biogerontology*. 12(Issue 5), 397–408. https://doi.org/10.1007/s10522-011-9344-5

Erdogan, M., Bereket, C., Ozkan, N., Alici, O., Sener, I., Desteli, E. E., & Ilkaya, F. (2014). The effect of zoledronic acid on growth plates and high turnover bones. *Bratislava Medical Journal*.115(Issue 3), 131–135. https://doi.org/10.4149/BLL_2014_028

Fernandes, C., Leite, R. S., & Lanças, F. M. (2005). Bisfosfonatos: Síntese, análises químicas e aplicações farmacológicas. In *Quimica Nova* . 28(Issue 2), 274–280. Sociedade Brasileira de Quimica. https://doi.org/10.1590/S0100-40422005000200019

Fischer, A. H., Jacobson, K. A., Rose, J., & Zeller, R. (2008). Hematoxylin and eosin staining of tissueand cell sections. *Cold Spring Harbor Protocols*. 3(Issue 5). https://doi.org/10.1101/pdb.prot4986

Gerber, H. P., Vu, T. H., Ryan, A. M., Kowalski, J., Werb, Z., & Ferrara, N. (1999). VEGF couples hypertrophic cartilage remodeling, ossification and angiogenesis during endochondral bone formation. *Nature Medicine*. 5(Issue 6), 623–628. https://doi.org/10.1038/9467

Harrington, J. T., Ste-Marie, L. G., Brandi, M. L., Civitelli, R., Fardellone, P., Grauer, A., Barton, I., & Boonen, S. (2004). Risedronate Rapidly Reduces the Risk for Nonvertebral Fractures in Women with Postmenopausal Osteoporosis. *Calcified Tissue International*. 74(Issue 2), 129–135. https://doi.org/10.1007/s00223-003-0042-4

Huang, R. C., Khan, S. N., Sandhu, H. S., Metzl, J. A., Cammisa, F. P., Zheng, F., Sama, A. A., & Lane, J. M. (2005). Alendronate inhibits spine fusion in a rat model. *Spine*. 30(Issue 22), 2516–2522. https://doi.org/10.1097/01.brs.0000186470.28070.7b

Hunziker, E. B., Schenk, R. K., & Cruz-Orive, L. M. (1987). Quantitation of chondrocyte performance in growth-plate cartilage during longitudinal bone growth. *Journal of Bone and Joint Surgery - Series A*. 69(Issue 2), 162–173. https://doi.org/10.2106/00004623-198769020-00002

Hunziker, Ernst B. (1994). Mechanism of longitudinal bone growth and its regulation by growth plate chondrocytes. *Microscopy Research and Technique*,

28(Issue 6), 505–519. https://doi.org/10.1002/jemt.1070280606

Junges, A. C. (2013). Avaliação das características microscópicas do fêmur de ratos. https://lume.ufrgs.br/handle/10183/152822

Khosla, S., Burr, D., Cauley, J., Dempster, D. W., Ebeling, P. R., Felsenberg, D., Gagel, R. F., Gilsanz, V., Guise, T., Koka, S., McCauley, L. K., McGowan, J., McKee, M. D., Mohla, S., Pendrys, D. G., Raisz, L. G., Ruggiero, S. L., Shafer, D. M., Shum, L., … Shane, E. (2007). Bisphosphonate-associated osteonecrosis of the jaw: Report of a Task Force of the American Society for Bone and Mineral Research. In *Journal of Bone and Mineral Research* . 22(Issue 10), 1479–1491. https://doi.org/10.1359/jbmr.0707onj

Kilkenny, C., Browne, W. J., Cuthill, I. C., Emerson, M., & Altman, D. G. (2010). Improving Bioscience Research Reporting: The ARRIVE Guidelines for Reporting Animal Research. *PLoS Biology*. 8(Issue 6), e1000412. https://doi.org/10.1371/journal.pbio.1000412

Lawson, M., Triffitt, J., Ebetino, F., Barnett, B., Phipps, R., & Locklin, R. (2005). *Potential bone mineral binding differences among bisphosphonates can be demonstrated by the use of hydroxyapatite column chromatography. - ORA - Oxford University Research Archive*. https://ora.ox.ac.uk/objects/uuid:37e8be6d-f3f0-49aa-8b1f-ccbc78ea2f07

Leclerc, J. T., Michou, L., Vaillancourt, F., Pelet, S., Simonyan, D., & Belzile, E. L. (2019). Prevalence and Characteristics of Atypical Periprosthetic Femoral Fractures. *Journal of Bone and Mineral Research*. 34(Issue 1), 83–92. https://doi.org/10.1002/jbmr.3584

Li, Z., Kong, K., & Qi, W. (2006). Osteoclast and its roles in calcium metabolism and bone development and remodeling. In *Biochemical and Biophysical Research Communications*. 343(Issue 2), 345–350. https://doi.org/10.1016/j.bbrc.2006.02.147

Lin, J. H. (1996). Bisphosphonates: A review of their pharmacokinetic properties. In *Bone*. 18(Issue 2), 75–85. Elsevier Inc. https://doi.org/10.1016/8756-3282(95)00445-9

Mackie, E. J., Ahmed, Y. A., Tatarczuch, L., Chen, K. S., & Mirams, M. (2008). Endochondral ossification: How cartilage is converted into bone in the developing skeleton. In *International Journal of Biochemistry and Cell Biology*. 40(Issue 1), 46–62. https://doi.org/10.1016/j.biocel.2007.06.009

Mackie, E. J., Tatarczuch, L., & Mirams, M. (2011). The skeleton: A multi-functional complex organ. The growth plate chondrocyte and endochondral ossification. In *Journal of Endocrinology*. 211(Issue 2), 109–121. https://doi.org/10.1530/JOE-11-0048

Oyhanart, S. R., Escudero, N. D., & Mandalunis, P. M. (2015). Effect of alendronate on the mandible and long bones: An experimental study in vivo. *Pediatric Research*. 78(Issue 6), 618–625. https://doi.org/10.1038/pr.2015.163

Özenci, A. M., Aslan, T., Şahin, Z., Özbey, Ö., acar, N., & Üstünel, I. (2013). Protective effect of zoledronic acid on corticosteroid-induced chondrocyte apoptosis in rat articular cartilage. *Acta Orthopaedica et Traumatologica Turcica*. 47(Issue 6), 430–435. https://doi.org/10.3944/AOTT.2013.3136

Patntirapong, S., & Poolgesorn, M. (2018). Alteration of macrophage viability, differentiation, and function by bisphosphonates. *Oral Diseases*. 24(Issue 7), 1294–1302. https://doi.org/10.1111/odi.12908

Pfeil, D. J. F., & DeCamp, C. E. (2009). The epiphyseal plate: physiology, anatomy, and trauma - PubMed. *Compend Contin Educ Vet.*, *31*(8), E1-11.

Porras, A. G., Holland, S. D., & Gertz, B. J. (1999). Pharmacokinetics of alendronate. In *Clinical Pharmacokinetics*. 36(Issue 5), 315–328). Adis International Ltd. https://doi.org/10.2165/00003088-199936050-00002

Quinn, R. (2005). Comparing rat's to human's age: How old is my rat in people years? In *Nutrition*. 21(Issue 6), 775–777). https://doi.org/10.1016/j.nut.2005.04.002

Rezende, E., Bradaschia-Correa, V., Siviero, F., Ambrosio, L. M. B., & Arana-Chavez, V. E. (2017). Effects of bisphosphonates on osteogenesis and osteoclastogenesis signaling during the endochondral ossification of growing rats. *Cell and Tissue Research*. 368(Issue 2), 287–300. https://doi.org/10.1007/s00441-017-2574-3

Russell, R. G. G., Watts, N. B., Ebetino, F. H., & Rogers, M. J. (2008). Mechanisms of action of bisphosphonates: Similarities and differences and their potential influence on clinical efficacy. In *Osteoporosis International*. 19(Issue 6), 733–759. https://doi.org/10.1007/s00198-007-0540-8

Saad, H. A., Terry, M. A., Shamie, N., Chen, E. S., Friend, D. F., Holiman, J. D., & Stoeger, C. (2008). An easy and inexpensive method for quantitative analysis of endothelial damage by using vital dye staining and adobe photoshop software. *Cornea*. 27(Issue 7), 818–824. https://doi.org/10.1097/ICO.0b013e3181705ca2

Silva, É. C. C., Terreri, M. T. R. A., Castro, T. C. M. de, Barbosa, C. P. L., Fernandes, A. R. C., & Hilário, M. O. E. (2010). Linhas escleróticas metafisárias em crianças e adolescentes em uso de alendronato. *Revista Brasileira de Reumatologia*. 50(Issue 3), 283–290. https://doi.org/10.1590/s0482-50042010000300008

Tan, J., Sano, H., & Poole, K. (2019). Antiresorptive-associated spontaneous fractures of both tibiae, followed by an atypical femur fracture during the sequential treatment with alendronate, denosumab then teriparatide. *BMJ Case Reports*. 12(Issue 7). https://doi.org/10.1136/bcr-2019-229366

Wilsman, N. J., Farnum, C. E., Leiferman, E. M., Fry, M., & Barreto, C. (1996). Differential growth by growth plates as a function of multiple parameters of chondrocytic kinetics. *Journal of Orthopaedic Research*. 14(Issue 6), 927–936. https://doi.org/10.1002/jor.1100140613

Zhu, E. D., Louis, L., Brooks, D. J., Bouxsein, M. L., & Demay, M. B. (2014). Effect of bisphosphonates on the rapidly growing male murine skeleton. *Endocrinology*. 155(Issue 4), 1188–1196. https://doi.org/10.1210/en.2013-1993