Int. J. Oral Maxillofac. Surg. 2019; 48: 1494–1503
https://doi.org/10.1016/j.ijom.2019.04.002, available online at https://www.sciencedirect.com

International Journal of Oral & Maxillofacial Surgery

Research Paper
Bone Regeneration

Alendronate induces postnatal maxillary bone growth by stimulating intramembranous ossification and preventing premature cartilage mineralization in the midpalatal suture of newborn rats

J. S. Vieira[1], E. J. Cunha[1], J. F. de Souza[2], R. D. Sant'Ana[1], J. C. Zielak[1], T. A. Costa-Casagrande[1], A. F. Giovanini[1]
[1]Graduate Programme in Clinical Dentistry, Positivo University, Curitiba, Paraná, Brazil; [2]Department of Stomatology, School of Dentistry, Federal University of Paraná, UFPR, Paraná, Brazil

J. S. Vieira, E. J. Cunha, J. F. de Souza, R. D. Sant'Ana, J. C. Zielak, T. A. Costa-Casagrande, A. F. Giovanini: Alendronate induces postnatal maxillary bone growth by stimulating intramembranous ossification and preventing premature cartilage mineralization in the midpalatal suture of newborn rats. Int. J. Oral Maxillofac. Surg. 2019; 48: 1494–1503.

Abstract. Cleft palate is a common malformation of craniofacial development, and postnatal deficiencies in palate formation may occur. The aim of this study was to determine whether alendronate treatment could induce maxillary mineralization and thus reduce the need for surgical procedures. The effects of alendronate on maxillary bone development, the midpalatal suture, and the levels of transforming growth factor beta-1 (TGF-β1), bone morphogenetic protein 2 (BMP-2), collagen I and II, and V-ATPase were evaluated in newborn rats. Thirty newborn rats were placed in a control group and 30 in a group that received intraperitoneal alendronate (2.5 mg/kg/day). The animals were euthanized on day 7 or 12, and the heads were subjected to histological and immunohistochemical analyses. Specimens from rats that received alendronate presented larger bone matrix deposition in areas of intramembranous ossification of the maxillary bone when compared to controls. Furthermore, higher levels of TGF-β1, BMP-2, and collagen I were observed, whereas osteoclasts showed no V-ATPase. The alendronate group also showed higher levels of TGF-β1 and collagen II in the midpalatal suture, whereas BMP-2 levels were lower than in controls. These results coincided with an expansion of the chondroid. In conclusion, alendronate increased the intramembranous ossification in the maxillary bone in association with increased expression of TGF-β1, BMP-2,

0901-5027/01101494 + 010

and collagen I and decreased V-ATPase. The drug induced an expansion of chondrocytes and a decrease in mineral bone deposition despite the high levels of TGF-β1 in this area. Alendronate may therefore be useful in the treatment of diseases affecting bone growth.

Key words: alendronate; bone; development; TGF-β1; BMP-2; collagens; V-ATPase.

Accepted for publication 1 April 2019
Available online 1 May 2019

Growth disorders and likely cleft palate formation constitute the most prevalent deformities of craniofacial development, and affect approximately 1 to 25 per 10,000 newborns worldwide[1].

The disruption of craniofacial development seems to be the invariable result of a reduction or even suppression of the proliferation and migration of cranial neural crest cells (CNCC), due to the mutation of Msh homeobox 1 (Msx-1) and the consequent failure of the mesenchyme derived from CNCC to interact with the craniopharyngeal ectoderm[2]. This is a result of the suppression of bone morphogenetic proteins (BMPs) and of distal-less homeobox 5 (Dlx-5) for osteoblastogenesis of the palate[3], as well as the suppression of sonic hedgehog (Shh) and transforming growth factor beta-3 (TGF-β3), which play an important role in regulating growth and morphogenesis in middle-line formation during the palate[4,5] to posterior palate fusion process, which commonly occurs during week 15 to 16 of embryonic development (E15–E16)[6].

In pathological situations of maxillary growth deficiency or submucous cleft of the hard palate, the use of a substance or drug instead of a surgical approach would appear to be beneficial to minimize complications and morbidity. In this context, it was hypothesized that alendronate, a nitrogen-containing bisphosphonate, could be a likely drug candidate for inducing growth of the maxillary bone in newborn rats[7,8].

The postnatal use of alendronate is not expected to mimic the exact effect of embryonic development, because bisphosphonates inhibit Msx-1 expression and do not promote an excitatory effect on CNCC[9]. Conversely, bisphosphonates stimulate osteoprogenitor mesenchymal cells, increasing the expression of Dlx-5 and BMP-2 and promoting osteoconduction[9–11]. This drug also seems to exert chondroprotective[12] and anti-resorptive effects[9].

The pathways by which alendronate activates these osteoproteins remain unclear. However, there is evidence of alendronate properties that increase the expression and activity of transforming growth factor beta-1 (TGF-β1), which seems to contribute to repair and postnatal osteogenesis[13].

Although TGF-β1 is not a specific protein that works during embryological development of the palate, this cytokine acts in nearly all processes of hard and soft tissue proliferation in postnatal tissue maturation, inducing chemotaxis and the proliferation of progenitor stem cells[14], as well as contributing to the differentiation of bone lineages by inducing the gene expression of specific proteins such as BMP-2[15,16].

The pleiotropic effect of TGF-β1 also contributes to mesenchymal scaffolding formation, and it is required to produce the architecture of the extracellular matrix[17]. Among the extracellular matrix proteins induced by TGF-β1, the production of specific collagen types is considered relevant for differentiated anatomical sites and tissue. Thus, the action of TGF-β1 seems to be crucial to intramembranous ossification (by contributing to the synthesis and deposition of collagen I) and to endochondral ossification (by producing collagen II in areas that require chondroid differentiation and maturation)[18,19].

On the basis of these hypotheses, the aim of the present study was to determine whether the use of alendronate could contribute to bone deposition in the maxillary area of newborn rats. In addition, it was sought to investigate the immunohistochemical presence of TGF-β1, BMP-2, and collagen I and II, and the expression of V-ATPase, a hydrogen pump that may be used as a marker of activated osteoclasts.

Materials and methods

Animals

The Principles of Laboratory Animal Care (NIH Publication 85-23, revised in 1985) and national laws on animal use were observed during this study. The study was approved by the institutional ethics committee for animal research.

Ten female rats (*Rattus norvegicus albinus*, Holtzman) aged 2–3 months and weighing approximately 242–260 g were used. The animals were maintained at a controlled temperature (22 ± 2 °C) and humidity (55 ± 5%), with a 12 h/12 h light/dark cycle and food and water available ad libitum. Using a cotton swab soaked in saline solution (pH 7.0), vaginal oncotic cytology samples were collected daily from all of these female rats. The cytological material obtained was spread on a glass slide and stained with the Papanicolaou technique to identify the oestrus period of the rats based on characteristics of epithelial cells and the presence or absence of inflammatory cells[20]. Female rats in the oestrus period were mated overnight with male rats (two females per male in one cage). Early the following morning, oncotic cytological vaginal smears were again taken from the female rats, stained using Shorr's method, and observed under a light microscope to verify the presence of sperm. The presence of sperm in the vaginal smears was considered as indicating the first day of pregnancy.

Drug treatment

Pregnant rats were single-housed in polypropylene cages until after the birth of the pups. The newborn rats were randomly allocated to two groups: a control group ($n = 30$) and a test group (alendronate group) ($n = 30$). Rats in the control group received 1 ml of sterile 0.9% saline solution per day, while rats in the test group received 2.5 mg/kg/day of alendronate trihydrate (Biolife, Curitiba, Brazil; lot number: 14042132C). As alendronate exerts its stimulation or impairment of osteoblasts and osteoclasts in a dose-dependent manner, it was decided to use the average of the dose range of 1.0 to 5.0 mg/kg/day (i.e., 2.5 mg/kg/day). According to a study published by Bone et al.[21], this dose was well-tolerated and produced dose-dependent increases in bone density without evidence of a plateau formation. The saline and alendronate solutions were administered intraperitoneally daily until euthanasia, which occurred on day 7 (for 15 rats in each group) and day 12 (for 15 rats in each group) after birth. Euthanasia was performed by brief exposure to isoflurane.

Histological processing

Immediately after euthanasia, the heads of the newborn rats were removed and the surgical pieces obtained were immersed in fixative solution for 48 h at 18–20 °C in 4% formaldehyde (prepared from paraformaldehyde) set to pH 7.2 with 0.1 M sodium phosphate. After decalcification for 3 weeks in 7% disodium ethylenediaminetetraacetic acid (EDTA) containing 0.5% formaldehyde in 0.1 M sodium phosphate (pH 7.2), the specimens were dehydrated in graded concentrations of ethanol, cleared in xylene, and embedded in paraffin. Serial 3-μm-thick histological sections were obtained from each specimen in the anterior–posterior direction according to the coronal anatomical plane. Some sections were stained with haematoxylin and eosin (H&E), Masson trichrome, or Alcian blue to verify the histomorphology and histomorphometry of bone and chondroid matrix, while other sections were adhered to silanized slides (Sigma-Aldrich Chemie, Steinheim, Germany) for the immunohistochemical detection of TGF-β1, BMP-2, collagen I and II, and V-ATPase.

Immunohistochemistry processing

Sections 3 μm thick from each specimen were subjected to immunohistochemical detection of proteins. For antigen retrieval, deparaffinized sections were immersed in 10 mM sodium citrate buffer (pH 6.0) and subjected to 3 × 5-min cycles in a microwave oven for V-ATPase and collagen I and II, whereas 1% pepsin (pH 1.8) was used for TGF-β1 and BMP-2. After cooling and inactivation of endogenous peroxidase with 5% hydrogen peroxide, the sections were incubated for 30 min at room temperature with 2% bovine serum albumin (BSA; Sigma-Aldrich Chemie, Steinheim, Germany). The sections were then incubated overnight at 4 °C with the following primary antibodies: anti-TGF-β1 (200 mg/ml, sc-146; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a dilution factor of 1:200, anti-BMP-2 (0.5 mg/ml, ab6285; Abcam, Cambridge, UK) at a dilution factor of 1:150, anti-collagen I (100 ng, ab88147; Abcam, Cambridge, UK) at a dilution factor of 1:1700, anti-collagen II (250 μl, ab185430; Abcam, Cambridge, UK) at a dilution factor of 1:1200, and anti-V-ATPase (200 mg/ml, sc-166848; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a dilution factor of 1:150. A labelled streptavidin/biotin antibody binding detection system (Universal HRP Immunostaining Kit; Diagnostic Biosystems, Foster City, CA, USA) was employed to detect the primary antibodies. After washing in 0.05 M Tris–HCl buffer (pH 7.2), the sections were incubated for 30 min at room temperature in biotinylated anti-rabbit/mouse/goat immunoglobulin (LSAB-plus Kit; Dako, Copenhagen, Denmark). Sections were counterstained with Harris haematoxylin. For the negative control group, the primary antibodies were omitted, and the sections were incubated with non-immune serum.

Image analysis

Images of both histological and immunohistochemical sections were captured with a digital camera (Samsung, South Korea) under a light microscope with a magnification of ×100 and ×200. The digital images were collected and saved at 300 dpi resolution (image size 115 × 75 cm).

The amount of bone matrix deposited in the body of the maxillary bone and the amount of chondroid tissue in the midpalatal area was determined for each histological slice. Additionally, all measurements of histomorphometric data for bone matrix deposition and of immunopositivity for TGF-β1 and BMP-2 were performed using ImageJ software version 1.31 for Mac (National Institutes of Health, Bethesda, MD, USA; https://imagej.nih.gov/ij).

For the histomorphometric and immunohistochemical measurements, the microscopy images were processed using Adobe Photoshop version 11.0 for Mac. In each image, a line was inserted that was tangent to the lower edge of the nasal septum cartilage. The maxillary area below this line was considered the region of interest for the present study, and this was the region in which all measurements were performed. The lateral borders were established by lines drawn longitudinally along the midline of the molar teeth that were in the histological section, while the lower limit consisted of the epithelial lining of the mucosa. Fig. 1A shows the area that was evaluated in each specimen.

Each micrograph obtained was imported into ImageJ software (https://imagej.nih.gov/ij) and the total area, as well as the area of bone matrix, areas of chondroid tissue and matrix (stained with Masson trichrome and Alcian blue), and areas positive for collagen I and II (brownish colour) were carefully selected and measured. Cells positive for TGF-β1 and BMP-2 were manually counted and tagged. The presence of V-ATPase restricted to multinucleated cells consistent with osteoclasts was also determined. The image of a 1-mm slide micrometer was used to calibrate all measurements. The average of three measurements for each parameter was then calculated for each specimen. After obtaining counts, all data were transformed into percentages.

Statistical analysis

For histomorphological and immunohistochemical analysis, the data were evaluated within the monitoring period. The Shapiro–Wilk test was used to determine data normality, following which the Kruskal–Wallis non-parametric test was used to determine any significant differences among the groups. A P-value of <0.05 was considered to be statistically significant.

Results

Histological and histomorphometric effects of alendronate (Fig. 1)

Quantitative data for the histomorphometric analysis of the percentage bone matrix deposited are given in Fig. 1B. Relevant changes were detected on day 7, especially in the area of intramembranous ossification that forms the maxillary bone, when the control group (Fig. 1C) and alendronate group (Fig. 1D) specimens were compared. In specimens that received the drug, the maxillary bone showed integral intramembranous fusion throughout the range analyzed, whereas only three of 15 specimens in the control group demonstrated total intramembranous fusion; however, this fusion was unilateral (two on the left side and one on the right side). In contrast, neither the control group nor the test group showed mineralization in the midpalatal suture area. In this topography, all specimens were composed of evident chondroid tissue (Fig. 1G–I) (Masson trichrome), a matrix rich in glycoproteins (Alcian blue-positive) (Fig. 1L–N), and a thin layer of dense connective tissue in the midpalatal suture area.

On day 12, all groups showed only integral intramembranous fusion throughout the range analyzed (Fig. 1E, F). However, the specimens that received alendronate showed a larger bone matrix deposition that also exhibited compact and haversian characteristics, whereas the control group showed bone matrix forming a diploë surrounding a clear medullar area (not shown). In the midpalatal suture area, the control group contained a few chondrocytes (Fig. 1J) and chondroid matrix

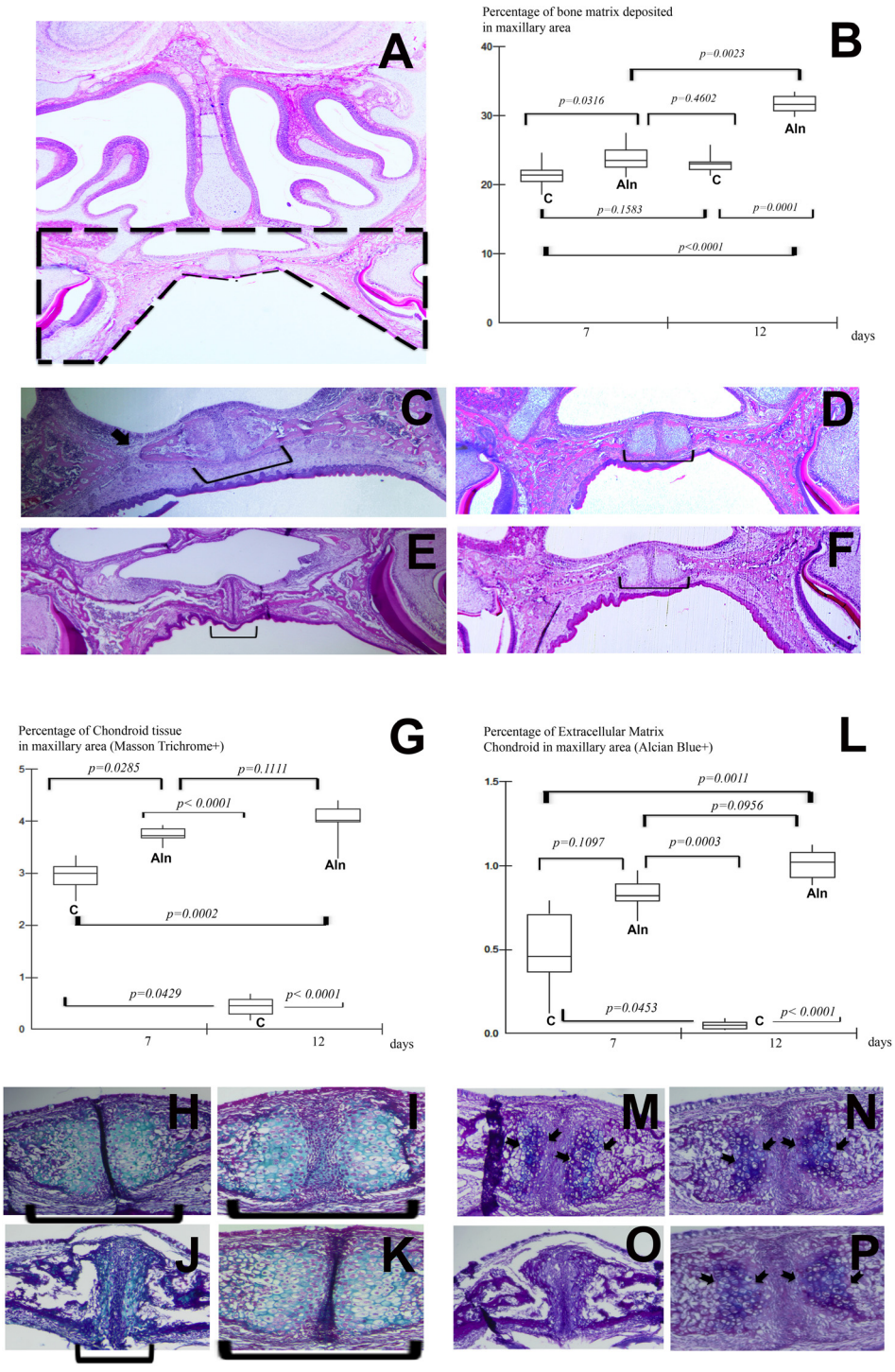

Fig. 1. (A) The region enclosed within the dashed line indicates the area that was used to determine all histomorphometric measurements in this study, as described in the Materials and methods section (H&E, original magnification ×40). (B) Quantitative assessment of the percentage of bone matrix deposited in the delimited area. Histological analysis of deposited bone matrix in day 7 samples: (C) control group and (D) alendronate group; note the lack of bone integration in the region of membranous ossification in the control group (arrow), as well as in the area of the midpalatal suture (bracket) in both groups (H&E, original magnification ×100). Histological analysis of deposited bone matrix in day 12 samples: (E) control group and (F) alendronate group (H&E, original magnification ×100). (G) Quantitative assessment of the percentage of chondroid tissue deposited in the delimited area (specifically in the midpalatal suture). Histological analysis of chondroid in day 7 samples: (H) control group and (I) alendronate group (Masson trichrome, original magnification ×200). Histological analysis of chondroid in day 12 samples: (*J*) control group and (K) alendronate group (Masson trichrome, original magnification ×200). (L) Quantitative assessment of the percentage of chondroid matrix in the delimited area. Histological analysis of chondroid matrix in day 7 samples: (M) control group and (N) alendronate group; chondroid matrix indicated by arrows (Alcian blue, original magnification ×200). Histological analysis of chondroid matrix in day 12 samples: (O) control group and (P) alendronate group; chondroid matrix indicated by arrows (Alcian blue, original magnification ×200). C = control group, Aln = alendronate group; $P < 0.05$ indicates a statistically significant difference.

with a larger area of mineralization separated by a thin line of connective tissue (Fig. 1O). In contrast, the amount of chondroid tissue and matrix (containing Alcian blue) was increased in specimens that received alendronate (Fig. 1K, P).

Effects of alendronate on selected immunohistochemical markers

A detailed description of the quantitative immunohistochemistry data is given in Figs 2–4.

TGF-β1 (Fig. 2)

All groups were positive for TGF-β1 (Fig. 2A). On day 7, the control group presented positive signals along the deposition of bone matrix and weak signals in areas adjacent to the developing bone (Fig. 2B), whereas in the alendronate group, positive signals were seen diffusely and in cells surrounding the neoformed bone fragment in areas of intramembranous ossification (Fig. 2C). In the midpalatal suture, a few positive signals for TGF-β1 (Fig. 2F) were detected in the control group, restricted to chondrocytes (Fig. 2G), whereas in specimens that had received alendronate, the signals were seen in both chondrocytes and fusiform cells in fibrous tissue in the suture area (Fig. 2H). On day 12, the distribution pattern of TGF-β1 was similar to that on day 7 for both groups, although the number of cells positive for TGF-β1 decreased in the control group (Fig. 2I) and increased in specimens treated with alendronate (Fig. 2J).

BMP-2 (Fig. 3)

All groups showed positive signals for BMP-2 (Fig. 3A–E). On day 7, the specimens from the control group presented positive signals for BMP-2 in bone tissue. Furthermore, discrete signals adjacent to neoformed bone matrix, as well as in front of the growing maxillary bone, which remained disconnected in the intramembranous area, were observed (Fig. 3B). In the alendronate group, positive signals were observed in the deposited bone matrix. In addition, the signals were observed diffusely in cells that surrounded the neoformed

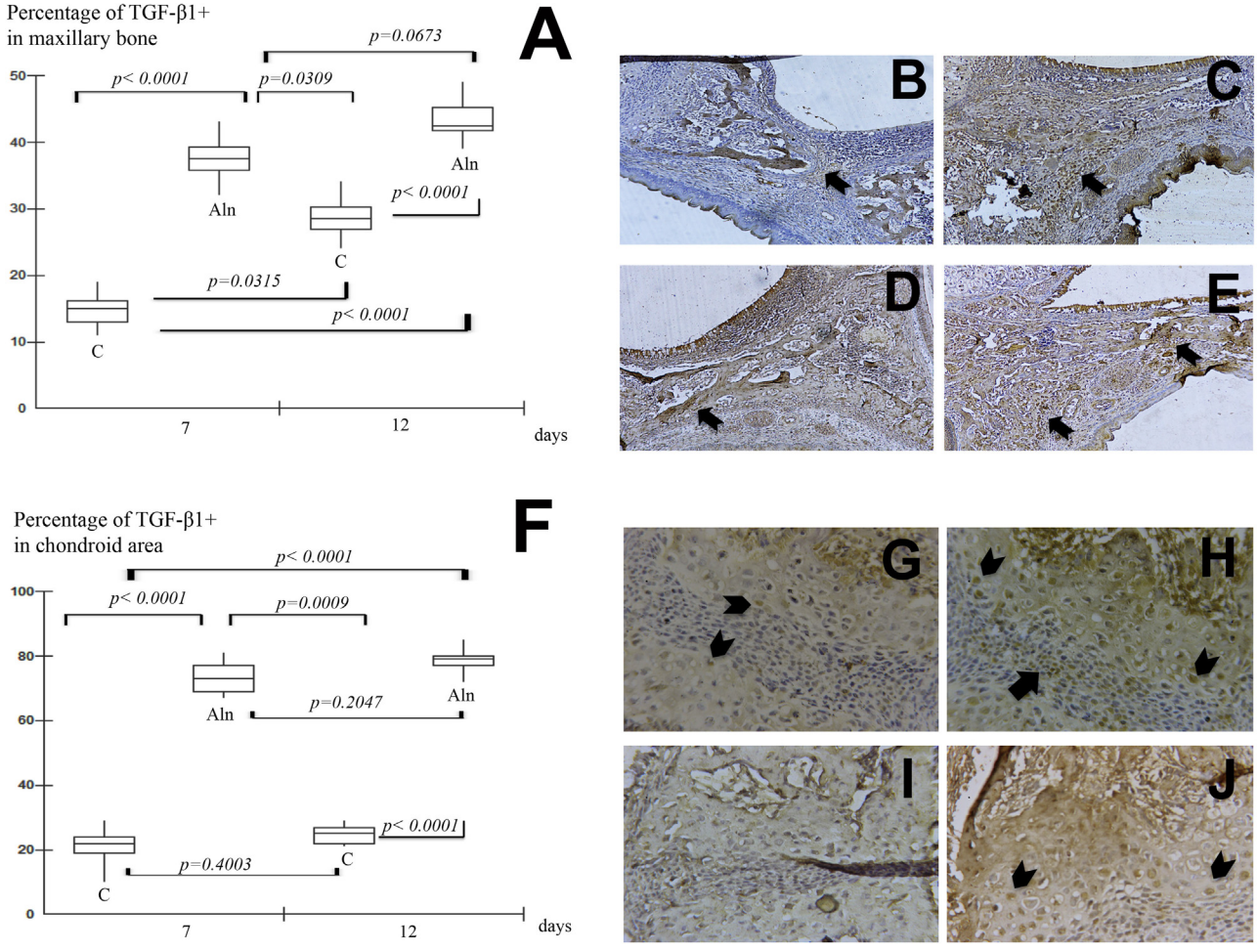

Fig. 2. (A) Quantitative assessment of the percentage of TGF-β1-positive cells in the area of study; note the higher positivity for the alendronate group as compared to the control group. The pattern of distribution of TGF-β1 in cells (arrows) in day 7 samples: (B) control group and (C) alendronate group; and day 12 samples: (D) control group and (E) alendronate group (original magnification ×100). (F) Quantitative assessment of the percentage of TGF-β1-positive cells specifically in the chondroid area (midpalatal suture). TGF-β1 positivity in the chondroid area in day 7 samples: (G) control group and (H) alendronate group (original magnification ×400); positivity both in chondrocytes (arrow heads) and connective tissue (arrows). Note the higher positivity for TGF-β1 in the alendronate group specimens. This condition remained in day 12 samples: (I) the control group and (*J*) alendronate group (original magnification ×400). C = control group, Aln = alendronate group; $P < 0.05$ indicates a statistically significant difference.

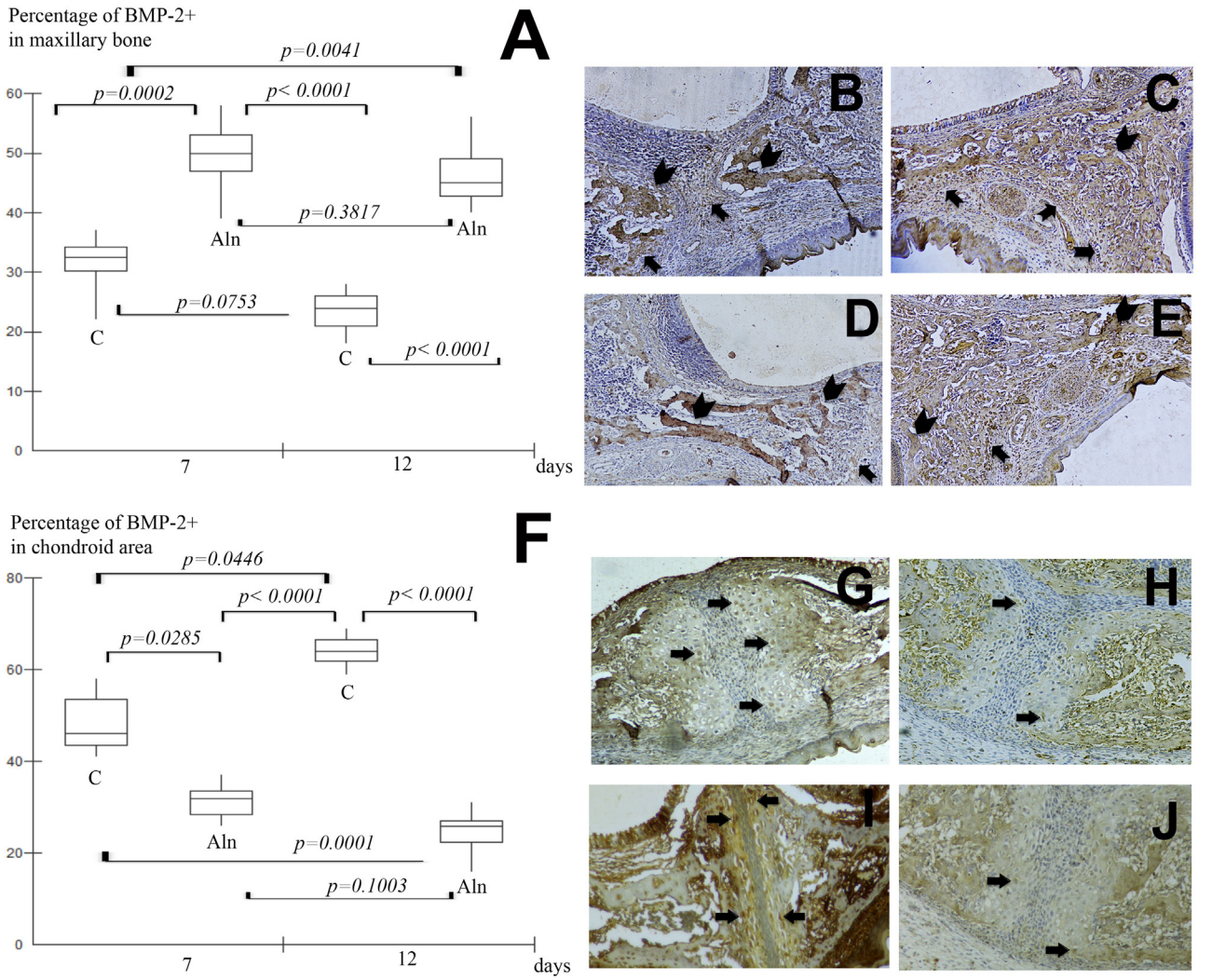

Fig. 3. (A) Quantitative assessment of the percentage of BMP-2-positive cells in the area of study; note the higher positivity in the maxillary bone (the region of intramembranous ossification) for the alendronate group as compared to the control group. The pattern of distribution of BMP-2 in cells (arrow heads) and bone matrix (arrows) in day 7 samples for (B) the control group and (C) the alendronate group, and in day 12 samples for (D) the control group and (E) the alendronate group (original magnification ×40). (F) Quantitative assessment of the percentage of BMP-2-positive cells specifically in the chondroid area (midpalatal suture); a higher percentage of BMP-2 was found for the control group, while immunopositivity was scarce in the alendronate group. The pattern of positivity for BMP-2 in chondrocytes (arrows) in day 7 samples for (G) the control group and (H) the alendronate group, and in day 12 samples for (I) the control group and (*J*) the alendronate group (original magnification ×100). C = control group, Aln = alendronate group; $P < 0.05$ indicates a statistically significant difference.

bone fragment in areas of intramembranous ossification (Fig. 3C). In the midpalatal suture, positive signals for BMP-2 were detected in chondrocytes in both groups, although the protein levels were clearly lower in chondrocytes of the group that had received alendronate (Fig. 3H, *J*). The BMP-2 immunolocalization pattern on day 12 was similar to that on day 7 in both groups, although the clear presence of BMP-2 was observed in the midpalatal suture in the control group (Fig. 3I), whereas in the specimens that had received alendronate, the BMP-2-positive cells were concentrated in the maxillary bone (Fig. 3E).

Collagen I (Fig. 4)

Collagen I was present in the body of the maxillary bone in areas of intramembranous ossification (Fig. 4A–C). The pattern in the control and alendronate groups was similar on days 7 and 12 (Fig. 4B–E). However, a clearly larger amount of this protein was observed in the group that had received the drug.

Collagen II (Fig. 4)

Collagen II was assessed only in areas where chondroid tissue was observed (Fig. 4F). Low levels of this protein were detected in the control group, exhibiting fine and delicate fibre morphology surrounding the chondrocyte area (Fig. 4G, I). In the alendronate group, high levels of collagen II were detected, filling the entire inter-chondrocyte area, both on day 7 (Fig. 4H) and day 12 (Fig. 4J). In contrast, on day 12, the protein was not observed in the median palate suture area in the control group.

V-ATPase and number of osteoclasts (Fig. 4)

Similarities were found in the number of osteoclasts in the control and alendronate groups. On day 7, the median number of osteoclasts was 18 multinucleated cells/mm^2 (range 13–21 cells/mm^2) in the con-

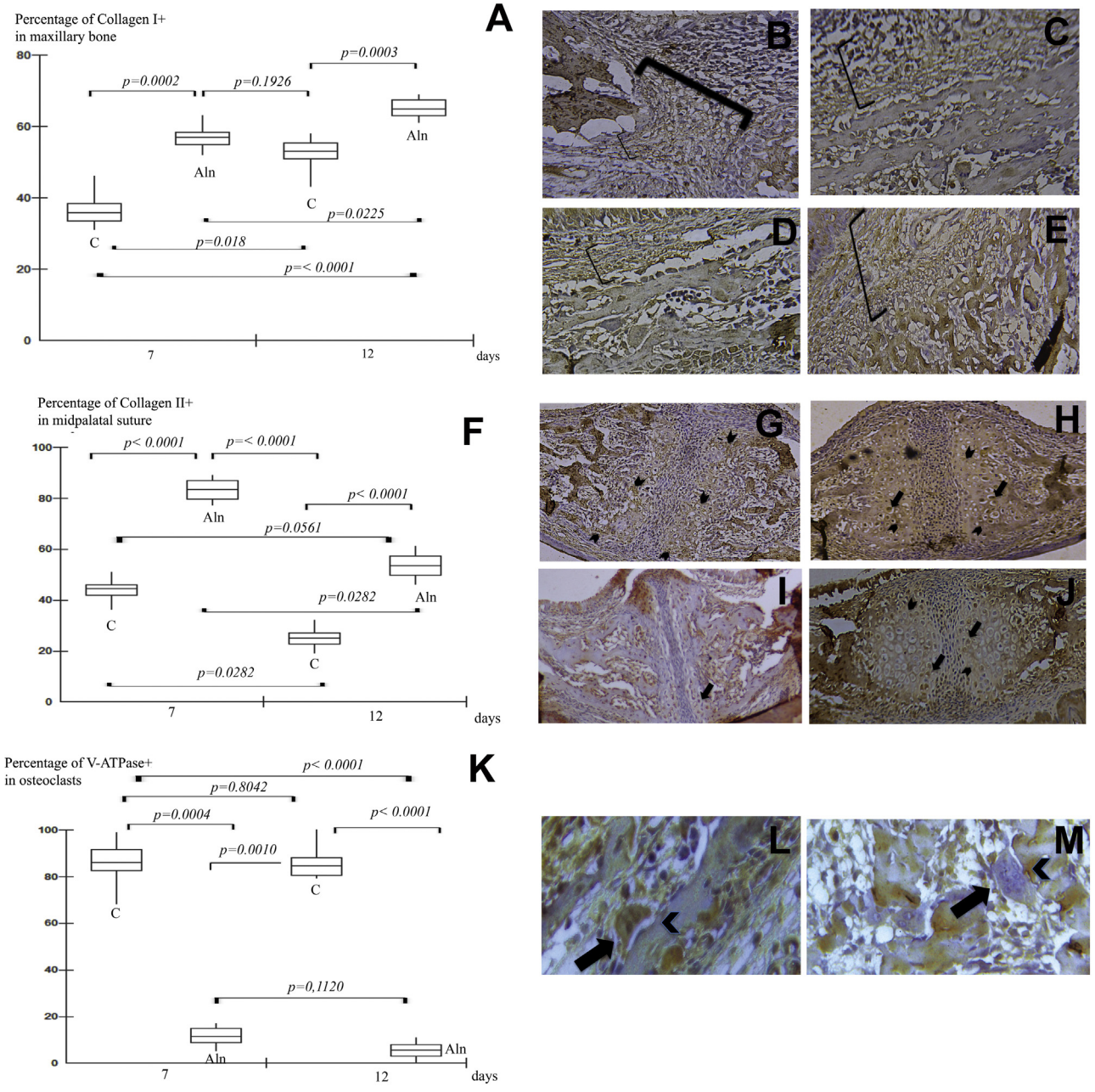

Fig. 4. (A) Quantitative assessment of the percentage area of collagen I measured in the body of the maxillary bone (intramembranous ossification); note the higher positivity of collagen I in the alendronate group as compared to the control group. The area of distribution of collagen I (bracket) in day 7 samples from (B) the control group and (C) the alendronate group, and in day 12 samples from (D) the control group and (E) the alendronate group (original magnification ×200). (F) Quantitative assessment of the percentage of collagen II specifically in the chondroid area (midpalatal suture). The distribution of collagen II in day 7 samples from (G) the control group and (H) the alendronate group (original magnification ×100); note the lower and more fibrillar collagen II deposition in the control group (arrow heads), while the deposition of collagen II in the alendronate group was extensive both in chondrocytes (arrows) and chondroid matrix (arrow heads). A similar pattern of collagen II is seen in chondrocytes (arrows) in (I) the control group and (*J*) the alendronate group in the day 12 samples (original magnification ×100). (K) Quantitative assessment of the percentage of V-ATPase in osteoclasts. (L) V-ATPase positivity (arrow) in the control group and the presence of a Howship lacuna (arrow head) in the adjacent bone (original magnification ×400). (M) In contrast, note the negative V-ATPase in osteoclasts in the alendronate group (arrow) adjacent to intact and linear bone tissue (arrow head) (original magnification ×400). C = control group, Aln = alendronate group; $P < 0.05$ indicates a statistically significant difference.

trol group and 16 cells/mm^2 (range 12–19 cells/mm^2) in the alendronate group ($P = 0.0642$). On day 12, the median number of osteoclasts was 21 multinucleated cells/mm^2 (range 16–23 cells/mm^2) in the control group and 19 cells/mm^2 (range 17–22 cells/mm^2) in the alendronate group ($P = 0.0588$).

However, the presence of V-ATPase proton pump was observed in most osteoclasts surrounding the bone trabeculae in the group control (Fig. 4K) on both days 7 and 12 (Fig. 4L). In contrast, limited V-ATPase was detected in osteoclasts in the alendronate group (Fig. 4M).

Discussion

A likely genetic therapy that may promote CNCC migration, reorganization of the interaction between the mesenchyme derived from CNCC and the craniopharyngeal ectoderm, and activation of the intrinsic pathways for the cellular differentiation that occurs in the embryonic stage has not been established thoroughly in the medical literature[22]. However, new strategies or alternative therapies with the aim of promoting new bone formation and the restructuring of facial harmony are consistently sought.

Although some evidence suggests that alendronate does not exert an effect on CNCC[9], this drug may promote an excitatory effect on osteoconduction and osteodifferentiation[13]. This study assumed the hypothesis that the postnatal use of alendronate could help maxilla and palate development when applied in newborn rats.

The results of this study show that alendronate induced a larger bone matrix deposition. On the other hand, the drug delayed bone matrix formation in the midpalatal suture area, where a larger expansion of chondrocytes occurred. These changes occurred concomitantly with a significantly increased number of TGF-β1-positive cells in these anatomical areas. The presence of a higher number of TGF-β1-positive cells in specimens that received alendronate is supported by previous studies, which have shown that this drug may be responsible by increasing and maintaining higher levels[23], as well as higher expression of this cytokine in bone sites[24]. Thus, TGF-β1 may explain the growth of the maxillary bone in some pathological situations during development.

TGF-β1 is a multifunctional cytokine that may regulate many biological processes during development, including cell proliferation and differentiation, as well as the inhibition of proliferation at other sites[25]. In bone tissue, TGF-β1 is one of the most abundant cytokines stored in the matrix, and when activated it promotes osteoprogenitor cell proliferation and mesenchymal stem cell recruitment to active bone remodelling sites[26] in intramembranous ossification. However, in endochondral areas, TGF-β1 seems to have different effects.

It should be noted that the effects of TGF-β1 on skeletal cells are controversial. Some studies have demonstrated that TGF-β1 may compromise the differentiation of osteoprogenitor cells into functional osteoblasts, because this cytokine seems to have an antagonistic effect on BMP-2[27]. In contrast, the present study demonstrated a direct correlation between increased numbers of BMP-2-positive cells and TGF-β1-positive cells in specimens that received alendronate. In addition, these results are consistent with a larger area of deposition of collagen I, the prevalent type of collagen in bone extracellular matrix. In this regard, the study results are consistent with those of some studies that have indicated that TGF-β1, in response to binding to its receptor, may be directly or indirectly responsible for the transcription of other genes, among them the BMP-2 gene[28]. This activation and the consequent higher number of BMP-2-positive cells appear to be responsible for the increased osteoconductive and osteoinductive properties[29], and may underlie the histological results of the present study.

Another factor that may contribute to an increase in bone deposition in the maxillary bone is the presumed inhibition of osteoclast activity by alendronate that culminates in decreased bone resorption[30]. However, no differences in the numbers of osteoclasts detected (multinucleated cells peripherally located to the bone matrix) were observed. These results are consistent with the findings of Cheng et al.[8], who described not only similarities in the number of osteoclasts in their specimens, but also found no difference in tartrate-resistant acid phosphatase (TRAP) signals in a similar bisphosphonate-free animal model.

In an attempt to improve our understanding in this context, it was assumed that the function of osteoclasts could be associated with the presence or absence of the endogenous ATP-dependent electrogenic proton pump known as V-ATPase. Evaluation of the presence of this protein seems to be relevant, because V-ATPase is highly expressed during the formation of osteoclasts and is involved in distinct physiological stages of osteoclast maturation and function, including polarization as well as extracellular acidification[31]. As expected, in contrast to the control group, a significant reduction in V-ATPase expression in specimens that had received alendronate was observed, indicating that the inhibition of osteoclast activity also contributed to the large bone matrix deposition in this study.

In different circumstances, alendronate seems to delay the chondro-substitution for bone matrix in the midpalatal suture area, because an expansion of the chondroid area occurred in specimens that received the drug. This increase in cartilage may be attributed to the use of alendronate, and a hypothesis that should be taken into consideration is the likely close relationship between chondrocytes and increases in TGF-β1 levels[32,33] promoted by alendronate. In fact, cartilage has limited self-formation potential, which requires both chondrocyte differentiation and expansion and subsequent mineralization in cases of endochondral ossification[34]. Of note, the deposition of calcium in chondrocytes requires previous differentiation into hypertrophic chondrocytes, which may contribute to delays in mineralization of cartilage and of the suture.

Corroborating the hypothesis suggested herein, Tekari et al. revealed that the expansion of chondrocytes seems to be strictly associated with functional endogenous TGF-β signalling[35]. They demonstrated that the TGF-β receptor inhibited the cytokine-dependent pathway, a condition that coincided with a cessation of cartilaginous growth and chondroid differentiation.

The chondroblastic effect of TGF-β1 signalling may be suggested here, as the specimens that received alendronate presented a larger cellular chondroid (demonstrated by Masson trichrome) and an increase in collagen II levels and in the proteoglycan-rich matrix (area Alcian blue-positive), which are the fundamental types of collagen and protein found in chondroid matrix[36]. In addition, collagen II has no mineralization effect and requires the transition to collagen I, a condition that seems to be delayed when alendronate is used.

In conclusion, the results of this study demonstrated that alendronate inhibited bone resorption, because it reduced V-ATPase levels and increased the expression of TGF-β1 and BMP-2. These changes enhanced the peripheral levels of collagen I and induced the growth of maxillary bone in an intramembranous ossification. Concomitantly, alendronate impaired the early mineralization of endochondral ossification through chondrocyte expansion induced by TGF-β1 and the deposition of collagen II. These conditions are required for orthopaedic growth of the maxilla, providing new evidence that alendronate may be a likely candidate for the treatment of patients who present maxillary growth disorders.

Funding

There was no source of funding. The study was fully funded by the authors.

Competing interests

None.

Ethical approval

Approval was obtained from the institutional animal care committee of Universidade Positivo (protocol #266-2014).

Patient consent

Not required.

References

1. Burg ML, Chai Y, Yao CA, Magee 3rd W, Figueiredo JC. Epidemiology, etiology, and treatment of isolated cleft palate. *Front Physiol* 2016;**7**:67.
2. Han J, Mayo J, Xu X, Li J, Bringas Jr P, Maas RL, Rubenstein JL, Chai Y. Indirect modulation of Shh signaling by Dlx5 affects the oral-nasal patterning of palate and rescues cleft palate in Msx1-null mice. *Development* 2009;**136**:4225–33.
3. Levi G, Mantero S, Barbieri O, Cantatore D, Paleari L, Beverdam A, Genova F, Robert B, Merlo GR. Msx1 and Dlx5 act independently in development of craniofacial skeleton, but converge on the regulation of Bmp signaling in palate formation. *Mech Dev* 2006;**123**:3–16.
4. Xavier GM, Seppala M, Barrell W, Birjandi AA, Geoghegan F, Cobourne MT. Hedgehog receptor function during craniofacial development. *Dev Biol* 2016;**41**:198–215.
5. Zhang Z, Song Y, Zhao X, Zhang X, Fermin C, Chen Y. Rescue of cleft palate in Msx1-deficient mice by transgenic Bmp4 reveals a network of BMP and Shh signaling in the regulation of mammalian palatogenesis. *Development* 2002;**129**:4135–46.
6. Jiang R, Bush JO, Lidral AC. Development of the upper lip: morphogenetic and molecular mechanisms. *Dev Dyn* 2006;**235**:1152–66.
7. Reginster JY, Neuprez A, Beaudart C, Lecart MP, Sarlet N, Bernard D, Disteche S, Bruyere O. Antiresorptive drugs beyond bisphosphonates and selective oestrogen receptor modulators for the management of postmenopausal osteoporosis. *Drugs Aging* 2014;**31**:413–24.
8. Cheng N, Park J, Olson J, Kwon T, Lee D, Lim R, Ha S, Kim R, Zhang X, Ting K, Tetradis S, Hong C. Effects of bisphosphonate administration on cleft bone graft in a rat model. *Cleft Palate Craniofac J* 2017;**54**:687–98.
9. Xuan B, Yang P, Wu S, Li L, Zhang J, Zhang W. Expression of Dlx-5 and Msx-1 in craniofacial skeletons and ilia of rats treated with zoledronate. *J Oral Maxillofac Surg* 2017;**75**. 994.e1–994.e9.
10. Camati PR, Giovanini AF, de Miranda Peixoto HE, Schuanka CM, Giacomel MC, de Araújo MR, Zielak JC, Scariot R, Deliberador TM. Immunoexpression of IGF1, IGF2, and osteopontin in craniofacial bone repair associated with autogenous grafting in rat models treated with alendronate sodium. *Clin Oral Investig* 2017;**21**:1895–903.
11. Vieira JS, Giovanini A, Görhinger I, Gonzaga CC, Costa-Casagrande TA, Deliberador TM. Use of low-dose alendronate improves cranial bone repair and is associated with an increase of osteocalcin: an experimental study. *J Oral Maxillofac Surg* 2017;**75**:1873–81.
12. Lehmann HJ, Mouritzen U, Christgau S, Cloos PA, Christiansen C. Effect of bisphosphonates on cartilage turnover assessed with a newly developed assay for collagen type II degradation products. *Ann Rheum Dis* 2002;**61**:530–3.
13. Göhringer I, Muller CLS, Cunha EJ, Passoni GNS, Vieira JS, Zielak JC, Scariot R, Deliberador TM, Giovanini AF. Would be prophylactic administrations of low concentration of alendronate an alternative for improving the craniofacial bone repair? A preliminary study focused in the period of cellular differentiation and tissue organization. *J Craniofac Surg* 2017;**28**:1869–73.
14. Iwata J, Parada C, Chai Y. The mechanism of TGF-beta signaling during palate development. *Oral Dis* 2011;**17**:733–44.
15. Zhang YD, Zhao SC, Zhu ZS, Wang YF, Liu JX, Zhang ZC, Xue F. Cx43- and Smad-mediated TGF-beta/ BMP signaling pathway promotes cartilage differentiation of bone marrow mesenchymal stem cells and inhibits osteoblast differentiation. *Cell Physiol Biochem* 2017;**42**:1277–93.
16. Ozkaynak E, Schnegelsberg PN, Jin DF, Clifford GM, Warren FD, Drier EA, Oppermann H. Osteogenic protein-2. A new member of the transforming growth factor-beta superfamily expressed early in embryogenesis. *J Biol Chem* 1992;**267**:25220–7.
17. Hinz B. The extracellular matrix and transforming growth factor-beta1: tale of a strained relationship. *Matrix Biol* 2015;**47**:54–65.
18. Ehnert S, Baur J, Schmitt A, Neumaier M, Lucke M, Dooley S, Vester H, Wildemann B, Stöckle U, Nussler AK. TGF-beta1 as possible link between loss of bone mineral density and chronic inflammation. *PLoS One* 2010;**5**:e14073.
19. Lee JY, Kim KH, Shin SY, Rhyu IC, Lee YM, Park YJ, Chung CP, Lee SJ. Enhanced bone formation by transforming growth factor-beta1-releasing collagen/chitosan microgranules. *J Biomed Mater Res A* 2006;**76**:530–9.
20. de Souza JF, Gramasco M, Jeremias F, Santos-Pinto L, Giovanini AF, Cerri PS, Cordeiro Rde C. Amoxicillin diminishes the thickness of the enamel matrix that is deposited during the secretory stage in rats. *Int J Paediatr Dent* 2016;**26**:199–210.
21. Bone HG, Downs Jr RW, Tucci JR, Harris ST, Weinstein RS, Licata AA, McClung MR, Kimmel DB, Gertz BJ, Hale E, Polvino WJ. Dose–response relationships for alendronate treatment in osteoporotic elderly women. Alendronate Elderly Osteoporosis Study Centers. *J Clin Endocrinol Metab* 1997;**82**:265–74.
22. Lee JM, Kim HY, Park JS, Lee DJ, Zhang S, Green DW, Okano T, Hong JH, Jung HS. Developing palatal bone using human mesenchymal stem cell and stem cells from exfoliated deciduous teeth cell sheets. *J Tissue Eng Regen Med* 2019;**13**:319–27.
23. Jia J, Yao W, Amugongo S, Shahnazari M, Dai W, Lay YA, Olvera D, Zimmermann EA, Ritchie RO, Li CS, Alliston T, Lane NE. Prolonged alendronate treatment prevents the decline in serum TGF-beta1 levels and reduces cortical bone strength in long-term estrogen deficiency rat model. *Bone* 2013;**52**:424–32.
24. Giovanini AF, de Sousa Passoni GN, Göhringer I, Deliberador TM, Zielak JC, Storrer CLM, Costa-Casagrande TA, Scariot R. Prolonged use of alendronate alters the biology of cranial repair in estrogen-deficient rats' associated simultaneous immunohistochemical expression of TGF-beta1+, alpha-ER+, and BMPR1B. *Clin Oral Investig* 2018;**22**:1959–71.
25. Sun M, Gewirtz JC, Bofenkamp L, Wickham RJ, Ge H, O'Connor MB. Canonical TGF-beta signaling is required for the balance of excitatory/inhibitory transmission within the hippocampus and prepulse inhibition of acoustic startle. *J Neurosci* 2010;**30**:6025–35.
26. Robey PG, Young MF, Flanders KC, Roche NS, Kondaiah P, Reddi AH, Termine JD, Sporn MB, Roberts AB. Osteoblasts synthesize and respond to transforming growth factor-type beta (TGF-beta) in vitro. *J Cell Biol* 1987;**105**:457–63.
27. Spinella-Jaegle S, Roman-Roman S, Faucheu C, Dunn FW, Kawai S, Galléa S, Stiot V, Blanchet AM, Courtois B, Baron R, Rawadi G. Opposite effects of bone morphogenetic protein-2 and transforming growth factor-beta1 on osteoblast differentiation. *Bone* 2001;**29**:323–30.
28. Chen G, Deng C, Li YP. TGF-beta and BMP signaling in osteoblast differentiation and bone formation. *Int J Biol Sci* 2012;**8**:272–88.
29. Rosen V. BMP2 signaling in bone development and repair. *Cytokine Growth Factor Rev* 2009;**20**:475–80.
30. Ko FC, Karim L, Brooks DJ, Bouxsein ML, Demay MB. Bisphosphonate withdrawal: effects on bone formation and bone resorption in maturing male mice. *J Bone Miner Res* 2017;**32**:814–20.
31. Qin A, Cheng TS, Pavlos NJ, Lin Z, Dai KR, Zheng MH. V-ATPases in osteoclasts: structure, function and potential inhibitors of bone resorption. *Int J Biochem Cell Biol* 2012;**44**:1422–35.

32. Dangelo M, Sarment DP, Billings PC, Pacifici M. Activation of transforming growth factor beta in chondrocytes undergoing endochondral ossification. *J Bone Miner Res* 2001;**16**:2339–47.
33. van der Kraan PM, Blaney Davidson EN, Blom A, van den Berg WB. TGF-beta signaling in chondrocyte terminal differentiation and osteoarthritis: modulation and integration of signaling pathways through receptor-Smads. *Osteoarthritis Cartilage* 2009;**17**:1539–45.
34. Wang W, Rigueur D, Lyons KM. TGFbeta signaling in cartilage development and maintenance. *Birth Defects Res C Embryo Today* 2014;**102**:37–51.
35. Tekari A, Luginbuehl R, Hofstetter W, Egli RJ. Transforming growth factor beta signaling is essential for the autonomous formation of cartilage-like tissue by expanded chondrocytes. *PLoS One* 2015;**10**:e0120857.
36. Ruggeri A, Dell'orbo C, Quacci D. Electron microscopic visualization of proteoglycans with Alcian Blue. *Histochem J* 1975;**7**:187–97.

Address:
Allan Fernando Giovanini
Universidade Positivo
R Pedro Viriato Parigot de Souza 5300
Campo Comprido
Curitiba
PR 81280-330
Brazil
Tel.: +55 41 3317 3456. Fax: +55 41 3336 5962
E-mail: afgiovanini@gmail.com