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Research Article

Immunohistochemical Expression of Indian Hedgehog (IHH) during the Post-Natal Development of the Midpalatal Suture in Rats that Received Administration of Alendronate

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Abstract

Postnatal palatal growth arrest or deficiencies in the midpalatal area are common sequelae of physical injury. Since alendronate may induce a chondroprotection in several types of cartilage, the present study evaluated the immunoexpression of IHH in the area of the midpalate and compared results with the histomorphological aspects of this topography on 3rd and 7th day of life of newborn rats treated or alendronate-free.

Thirty-two newborn rats were included in this study: 16 in a control group, and 16 in a group that received intraperitoneal applications of 2.5 mg/kg/day of alendronate. The animals were euthanized on the 3rd or 7th day of administration of drug. The head of each rat was removed and prepared for histological and immunohistochemical analyses.

The group of animals that received alendronate had larger chondroid matrix deposition in areas where there was proliferation and isogenic formation of hypertrophic chondrocytes, and the analyses revealed immunoexpression of IHH. In addition, higher levels of IHH were detected in the mesenchymal tissue of the palatal raphe, forming an area of resting chondrocytes and a perichondral topography resulting from the differentiation of mesenchyme into chondrocytes.

In this pilot study may be indicated that Alendronate may be used as alternative in the treatment of orthopedic diseases, especially to promote the growth of the midpalatal area.

Keywords: *Alendronate; Midpalate; Chondroid Tissue; IHH*

Introduction

Postnatal craniofacial disorders associated to physical injury are a common concern in clinical practice, and may provokes activation of growth factors through inflammatory cytokine whose act in the suppression of the usual pathways in the formation of the midpalatal suture as well as pathways that are responsible for the differentiation of cells that originate in the neural crest and the mesenchyme [1]. As results, these pathological circumstances promote midpalatal growth disturbs conducting to serious consequences, such as craniosynostosis, midface hypoplasia or even precocious ossification of midpalate [2,3].

The usual pattern for the correction of these pathological conditions is usually through surgical approach. Although there are good results described in the literature about post-surgery success, their use is limited and may be contraindicated in conditions of other essentials diseases [4].

Based in this context, new strategies using substances or drugs that may stimulate differentiation of chondrocytes as well as osteogenesis sounds like a likely form treatment of disorders [5]. Herein, we hypothesized that the consecutive use of alendronate could be an option for chondrocyte proliferations in midpalatal areas, contributing for chondrocyte maturation and ossification of this anatomical site. This hypothesis is plausible since Nishitani., *et al.* [6] demonstrated that Alendronate accelerated bone formation without inhibiting its mineralization but thereafter inhibited bone remodeling in an osteochondral defect [5]. However, the real effect on midpalate growth and development remains unclear.

It is noteworthy that the control of development and osteochondral remodeling, as well as osteogenesis in midpalatal area seems to be regulate by the Hedgehog (HH) family [7,8]. Among these proteins, the Indian hedgehog (IHH) seems to work on induction of cell proliferation and differentiation, since this protein mainly on the osteogenic fronts of the calvarial, maxillary and mandibular bones. Besides that, IHH plays an important role in the site of chondroid plate growth and cartilage formation, as well as in early craniofacial development, including areas of growth of midpalatal derived from neural crest [9,10].

Thus, herein, we evaluated the immunoexpression of IHH in the midpalatal suture area in earlier stage of rat's midpalate development and compared results with histomorphometric findings in the same area, at 3 and 7 days.

Material and Methods

Animals and drug treatment

The Principles of Laboratory Animal Care (NIH Publication 85-23, revised in 1985) and national laws on animal use were observed for the present study with the approval of the institutional Ethical Committee for Animal Research (Protocol #266).

Thirty-two newborn rats were used in this study and they were randomly distributed into two distinct groups: a control group where the newborn rats received 1 mL of sterile 0.9% saline solution (n = 16) and a test group where the rats received 2.5 mg/kg/day of alendronate trihydrate (Biolife, Curitiba, Brazil, Lot number: 14042132C) (n = 16). Both saline and alendronate solutions were administered intraperitonially daily until euthanasia, which occurred on days 3 and 7 after birth (n = 8 for each time period in each treatment). Euthanasia was performed by brief exposure to isoflurane.

Histological processing

Immediately after euthanasia, the heads of 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 0.1M sodium phosphate. After decalcification for 3 weeks in 7% disodium ethylenediaminetetraacetic acid (EDTA) containing 0.5% formaldehyde in 0.1M 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 anatomic plane. Some sections were stained with hematoxylin and eosin to verify the histomorphology and histomorphometry of bone and chondroid matrix, whereas other sections were adhered to silanized slides (Sigma-Aldrich Chemie, Steinheim, Germany) for the immunohistochemical detection of IHH.

Immunohistochemistry processing

From each specimen, 3 slides containing fragments of 3-µm-thick sections were subjected to immunohistochemical detection of IHH protein. For antigen retrieval, deparaffinized sections were immersed in 10 mM sodium citrate buffer (pH 6.0) and subjected to microwave 3×5-minute cycles in a microwave. 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 4ºC with primary antibody anti-IHH (200 mg/mL, Santa Cruz Biotechnology, CA, USA, sc-

271101) at a dilution factor of 1:200. A labeled streptavidin/biotin antibody binding detection system (Universal HRP immunostaining kit-Diagnostic Biosystem, Foster City, CA, USA) was employed to detect the primary antibody. After washing in 0.05M 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, CA, USA). Sections were counterstained with Harris hematoxylin. For the negative control group, the primary antibodies were omitted, and the sections were incubated with nonimmune serum.

Image analysis

Images of microscopic 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 with 300 dpi resolution with an image size of 114.95 × 74.89 cm.

In each histological slice, it was verified amount of chondroid area, and areas composed by hypertrophic chondrocytes in midpalate region were accounted.

For histomorphometric measurements, the microscopic images were processed using Adobe Photoshop for Mac. Each micrograph obtained was transferred to the Image J program (https://imagej.nih.gov/ij) and areas of chondroid tissue, hyperplasic chondrocytes were carefully selected and measured, In each image the area of interesting occurred from midpoint of midpalate suture until the distal portion of evident bone fragment. An image of a 1-mm slide micrometer was used to calibrate all measurements. An average of 3 measurements for each parameter was then calculated for each specimen.

Yet, for histomorphological analysis, the data were evaluated within the monitoring period. A Shapiro-Wilk analysis was used to determine normality, followed by the Kruskal Wallis non-parametric test to verify significant differences among groups. A $p < 0.05$ was considered to be statistically significant.

An other hand, positive cells for IHH were accounted by semiquantitative score as follow: - (negative) from o to 1%; + from 1 to 25%, ++ from 25 to 50%, +++ from 50 to 75% and ++++ >75%, and the final score was performed such MODA. All accounted were performed both evident chondrocytes as well as connective tissue in midpalate area.

Results

All histomorphometric data for each parameter as well as immunohistochemical score are demonstrated in table 1, the fundamental aspects found among the specimens is described below.

Parameter analyzed	Day	Control	Alendronate	p
Area of chondroid tissue (mm^2)	3	1.34 ± 0.08	1.42 ± 0.11	0.523
	7	1.42 ± 1.01	1.73 ± 0.77	0.032
Area hyperplasic chondrocyte	3	1.12 ± 0.02	1.33 ± 0.21	0.437
	7	1.42 ± 1.01	1.69 ± 0.55	0.028
IHH in chondrocytes	3	+	++	
	7	+	++++	
IHH in mesenchymal tissue	3	+	+++	
	7	-	++++	

Table 1: *Histomorphometric and immunohistochemical data among the groups.*

For each same time period values p > 0.05 are statistically similar.

On the 3rd day, the histological aspects of midpalate of control group demonstrated a presence of fibrous connective tissue surrounded by hyaline cartilaginous tissue. The hyaline cartilage was composed by 2 to 3 layers of chondrocytes, which surrounded the hypertrophic chondrocytes area. In this time period, the IHH was expressed in scarce cells in the intercartilage mesenchyme, as well as some of the chondrocytes. On 7th days, we observed intense chondrocyte cells separated by dense connective tissue. Bone matrix was identified through hyperplastic chondrocytes. However, at 7 days the IHH was scarce.

On 3rd day, the specimens that received alendronate reveled similar pattern when compared to control group. However, several cells that compounded the mesenchyme connective tissue revealed intense immunopresence for IHH. On other hand, few isogenic and proliferative groups of hypertrophic chondrocytes, IHH+ shape identified. In the 7th day, an intense area of hypertrophic chondrocytes was observed, forming well-characterized isogenic IHH+ areas, in relation to chondroid area. Surrounding the majority of chondrocytes, we identified 4 to 6 layers of hyaline cartilage and one IHH + mesenchyme throughout the length of the midpalate. In this period, the presence of bone deposition in these specimens was not identified.

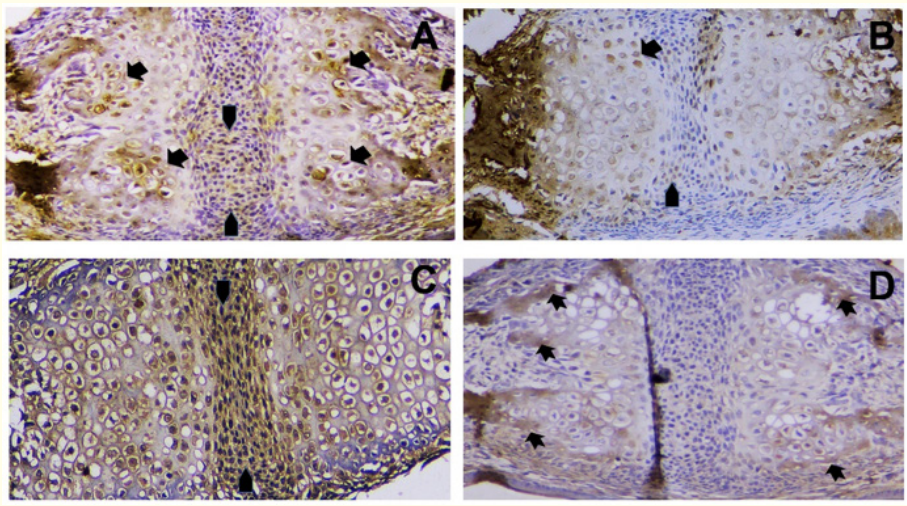

Figure 1: *Micrograph A demonstrates aspects in specimens that received alendronate on 3rd day post application. Verify the intense presence of IHH among the cartilage, especially in hypertrophic chondrocyte (arrows), as well as in isolated mesenchymal cells in intercartilage area (head of arrows). Figure (B) demonstrates scarce presence of IHH in control group. Note the restrict expression of IHH both in scarce cartilage (Arrow) and mesenchymal cells (Head of arrows). C shows the intense presence of IHH in specimens that received alendronate. Note strong presence of IHH either proliferative hypertrophic cartilage (Arrow), or intense presence of mesenchymal cells (head of arrow), that is a distinct condition of control (D) that demonstrates lack of IHH in cartilage, but discrete IHH in areas of bone tissue (arrow). Original magnification 100×.*

Discussion and Conclusion

In this study, we verified that the use of alendronate altered the histological aspect in formation of the midpalatal area. In specimens that received alendronate we verified formation of a net of isogenic groups of hyperplasic chondrocytes, a condition that culminated with a lower area of mineralization and an increase in the cellular contingent of hypertrophic/proliferative chondrocytes. These results coincided to increased in the immunoexpression of IHH. Besides that, the mesenchymal connective fibrous tissue that formed the midpalate, also exhibited IHH+, in contrast to control group.

Together, these results suggested that alendronate induces a sequence of events that mimics the endochondral metaphyseal area, at the same time that promotes a transdifferentiation of mesenchyma cells into a rest-like zone.

In fact, it is previously showed by our group that the alendronate works in order to the chondroprotection in the midpalate suture area, promoting an expansion of the chondroid area at the same time that delay the mineralization in this topographic area [5]. Herein we have indicated that all of these sequences of events seem to be associated to increase in IHH presence.

The real condition of how Alendronate encourages the immunoexpression of HHI remains an inference. However, a reason that should be taken into consideration in order to explain this result is the probable induction of TGF-beta through alendronate [11,12].

Corroborating with this premise, a study performed by Erlebacher and Derynck (1996) [13] demonstrated that enhancing effects of TGF-beta was responsible on specific enhancement of IHH expression in the cell of chondro-osteoblastic lineage revealed a novel pathway of the action of this pluripotent growth factor in endochondral metabolism.

These results also seem to be supported by Tekari., *et al.* [14] who demonstrated that the expansion of chondrocytes seems to be strictly associated with functional endogenous TGF-β signaling. The authors demonstrated that TGF-β receptor inhibited the cytokine-dependent pathway, a condition that coincided with arrest of cartilaginous growth and chondroid differentiation. Here we added that these effects may occurs via IHH.

As, our results demonstrate that alendronate inhibited precoces mineralization, and promotes an increased on expression of IHH. These changes enhanced endochondral ossification through chondrocyte expansion induced by alendronate. Under these conditions alendronate contributes to orthopedic growth of the maxilla, providing new evidence that alendronate may be a likely alternative for treatment of diseases affecting midpalate.

Bibliography

1. Li J., *et al*. "Disrupting the intrinsic growth potential of a suture contributes to midfacial hypoplasia". *Bone* 81.6 (2015): 186-195.

2. Weng M., *et al*. "A review of FGF signaling in palate development". *Biomedicine and Pharmacotherapy* 103 (2018): 240-247.

3. Wurdak H., *et al*. "Inactivation of TGF beta signaling in neural crest stem cells leads to multiple defects reminiscent of DiGeorge syndrome". *Genes and Development* 19.5 (2005): 530-535.

4. Murthy PS., *et al*. "Pre surgical nasoalveolar molding: changing paradigms in early cleft lip and palate rehabilitation". *Journal of International Oral Health* 5.2 (2013): 70-80.

5. Vieira JS., *et al*. "Alendronate induces postnatal maxillary bone growth by stimulating intramembranous ossification and preventing premature cartilage mineralization in the midpalatal suture of newborn rats". *International Journal of Oral and Maxillofacial Surgery* (2019).

6. Nishitani K., *et al*. "Positive effect of alendronate on subchondral bone healing and subsequent cartilage repair in a rabbit osteochondral defect model". *American Journal of Sports Medicine* 37.1 (2009): 139S-147S.

7. St-Jacques B., *et al*. "Indian hedgehog signaling regulates proliferation and differentiation of chondrocytes and is essential for bone formation". *Genes and Development* 13.16 (1999): 2072-2086.

8. McMahon AP., *et al*. "Developmental roles and clinical significance of hedgehog signaling". *Current Topics in Developmental Biology* 53 (2003): 1-114.

9. Long F., *et al*. "IHH signaling is directly required for the osteoblast lineage in the endochondral skeleton". *Development* 131.6 (2004): 1309-1318.

10. Bitgood MJ and McMahon AP. "Hedgehog and Bmp genes are coexpressed at many diverse sites of cell-cell interaction in the mouse embryo". *Developmental Biology* 172.1 (1995): 126-138.

11. Jia J., *et al*. "Prolonged Alendronate Treatment Prevents The Decline In Serum TGF-β1 Levels And Reduces Cortical Bone Strength In Long-Term Estrogen Deficiency Rat Model". *Bone* 52.1 (2013): 424-342.

12. Giovanini AF., *et al*. "Prolonged use of alendronate alters the biology of cranial repair in estrogen-deficient rats' associated simultaneous immunohistochemical expression of TGF-β1+, α-ER+, and BMPR1B". *Clinical Oral Investigations* 22.5 (2018): 1959-1971.

13. Erlebacher A and, Derynck R. "Increased expression of TGF-beta 2 in osteoblasts results in an osteoporosis-like phenotype". *Journal of Cell Biology* 132.1-2 (1996): 195-210.

14. Tekari A., *et al*. "Transforming growth factor beta signaling is essential for the autonomous formation of cartilage-like tissue by expanded chondrocytes". *PLoS One* 10.3 (2015): e0120857.

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