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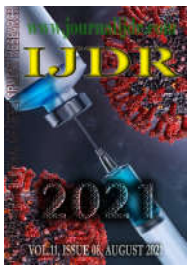

RESEARCH ARTICLE OPEN ACCESS

HISTOLOGICAL ANALYSIS OF RHBMP-2 ALVEOLAR BONE GRAFT IN INDIVIDUALS WITH CLEFT LIP AND PALATE

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ABSTRACT

This study histologically evaluated bone and soft tissues removed from the region of rhBMP-2grafted alveolar cleft in individuals with unilateral cleft lip with or withoutcleft palate (UCL+P), 6 months to 4 years after graft surgery.The experimental group consisted of 13 individuals with UCL+Pwith impacted canines in the rhBMP-2 (Infuse®) grafted alveolar region. The control group comprised six individuals with UCL+Pwith impacted canines in the noncleft side, near the ungrafted cleft or in the area grafted with autogenous iliac crest bone. At the time of surgical canine exposure, 6 to 46 months after secondary bone graft, biopsies of bone and soft tissues were obtained and submitted to histological analysis with hematoxylin and eosin.Microscopic sections of the experimental group showed fragments of viable bone tissue with normal osteocytes in lacunae, osteoblasts on the bone surface and medullary spaces filled by fibrous connective tissue and blood vessels. The bone tissue biopsies obtained 6 to 9 months after grafting showed more disorganized bone trabeculae, without lamellar formations and with greater osteocyte density per area, indicating lower degree of bone maturity. Biopsies taken 2 to 4 years after bone grafting were composed only of mature lamellar bone tissue containing incremental lines and osteocyte lacunae. Soft tissue sections revealed fragments of oral mucosa consisting of para-keratinized stratified squamous epithelium with underlying dense connective tissue.Fragments of bone and soft tissues removed from the rhBMP-2 grafted cleft area showed normal histological morphology, resembling the tissue characteristics of the control group.

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INTRODUCTION

The bone to be grafted in the alveolar cleft region can be autogenous, allogeneic or synthetic. The alveolar bone graft has been the favorite in the process of rehabilitation of cleft lip and palate and it is included in the therapeutic protocol of rehabilitation centers in worldwide, since its correct use enables form, function and aesthetics. When performed before the eruption of canine permanent, provides periodontal support for the eruption and preservation of the teeth adjacent to the cleft(SilvaFilho et al, 2013; Russell et al, 2016; Scalzone et al, 2019; Teng et al, 2019; Uribe et al, 2019; Osorio et al, 2020, Rosa et al, 2018). To this congenital craniofacial malformation, the autogenous secondary alveolar bone grafting has been the most widely used torepair the alveolar bone defect by anatomical joined to the adjacent bone and becoming indistinguishable in radiographic images after an average period of 3 months. Several radiographic researches have proved the success of the incorporation of the autogenous bone grafted in the subjacent bone tissue and the eruption of teeth by bone grafting (SilvaFilhoet al, 2013; Stasiak et al, 2019;

Uribe et al, 2019; Osorio et al, 2020; Rosa et al, 2018). Properties such as biocompatible, osteoconductive and osteoinductive has been histologically proved in young Rhesus monkeys and seems to occur more rapidly in younger patients. However, has problems ofmorbidity of the donor site,postoperative pain, limitedavailability and additionally, there is a graft size limit that can require another surgery (SilvaFilho et al, 2013; Scalzone et al, 2019; Teng et al, 2019; Uribe et al, 2019; Osorio et al, 2020). Tissue bioengineering has been constantly searching for a synthetic bone substitute in order to avoid donor-site morbidity and discomfort.The bone morphogenetic proteins (BMPs) from the superfamily of growth factors are in the mature skeleton, participating in bone maintenance and repair of bone fractures. At higher than physiological doses BMP acts locally, concentrating the host mesenchymal cells and influencing their differentiation into osteoblasts. The BMP-2 presents in the osteoconductive allogeneic matrix is a glycoprotein of great influence on the osteoinductive process, since it effectively acts on the differentiation of pluripotent mesenchymal cells in osteogenic precursors(Szpalski et al., 2013; Carreira et al., 2014; Teng et al. 2019).The use of rhBMP synthetically by using recombinant technology would present similar performance relative to bone formation and regeneration in cleft lip and palate defects when compared with iliac crest bone graft (Rosa et al, 2018). In 2007, the *Food and Drugs Administration* (FDA) approved the commercial use of recombinant human bone morphogenetic protein (rhBMP-2) *INFUSE® Bone Graft (Medtronic Spinal and Biologics, Memphis, TN, USA)*for bone grafting for correction of alveolar defects in adult individuals (Herford et al., 2007; Woo, 2012). However, due to the off-label surgical utilization of rhBMP-2, the FDA's Manufacturer and User Facility Device Experience (MAUDE) reported its adverse effects, such as local inflammation, bone resorption and areas of excessive and heterotopic bone formation (Food and Drug Administration, 2010). Recent systematic reviews analyzing the use of BMP2 in the alveolar defects treatment and verified the use of BMP2 as the most favorable technique for bone formation but related factors such as variability in the doses of BMP2 used and the methods of protein release that can produce localized inflammation in the graft area (Osorio et al, 2020; Li et al, 2019).

The risk of adverse effects of utilization of rhBMP-2 in secondary alveolar bone graft (ABG) is unclear in the literature.Clinical trials included in a systematic review compared adverse events between the use of rhBMP-2 and illiac crest bone graft (ICBG)in patients in the age-range between 7 and 12 years old. These studies reported, after the utilization of rhBMP-2, adverse effects such as swelling and prolonged wound healing but yet not affecting tooth eruption. The majority of clinical trials evaluated presented small sample sizes; no power analysisand variability in time point measurements. These could be due to limited power of these studies. However, a retrospective largest clinical trial evaluated 501casesand showed a clinical graft success of 88.4% in the ICBG group and 90% in the rhBMP-2 group (Rosa et al, 2018). According to results of a recent systematic review and meta-analysis, further studies are necessary about the safety of rhBMP-2 for use in animals and humans (Uribe et al, 2019). Systematic reviews reported the lack of studies about clinical and volumetric analysis after alveolar cleft reconstruction using rhBMP-2 in humansidentifyingbone healing and enhanced mineralization in the graft area (URIBE et al; OSORIO et al, 2020; SCALAZONE et al). Despite the absence of osteogenic propertie, the histological process of incorporation of rhBMP-2 is similar to of autogenous graft? Four weeks after autogenous grafting,the formation of bone is originated from endosteal osteoblasts that delimit the trabecular bone surfaces. In this phase, this bone is immature and disorganized, with absence of Haversian systems. With the development of the grafting, a process of resorption and apposition, which will lead to formation of a mature bone with lamellar architecture and Harvesian systems, that is a complete structural integrity (Silva Filho et al, 2013). What about the rhBMP-2? By the data collected in literature, there are no histological reports of the use of bioactive materials containing proteins on reconstruction of alveolar cleft repair in humans. Only one previous study reported the histological characteristics after rhBMP-2 alveolar graft (total dose of 4.2mg) in sites rehabilitated with dental implants and showed the presence of thicker bone tissue rich in cells and blood vessels, (Freitas et al., 2016).Therefore, this study aimed at analyzing the histological characteristics of bone and soft tissue fragments harvested from the alveolar cleft area grafted with rhBMP-2, 6 months to 4 years after alveolar bone graft procedure, in patients with unilateral cleft lip with or without cleft palate.

MATERIALS AND METHODS

This study was approved by the Institutional Review Board of the University of Southeast of Bahia under the protocol #407.770. Parents/guardians signed the research consent. The study population included individuals with unilateral cleft lip with or without cleft palate (UCL+P)who underwent secondary alveolar bone graft surgery in the period between March of 2011 to December of2014.The eruption of maxillary canines was evaluated on panoramic and periapical radiographs obtained before onset of the comprehensive orthodontic treatment in all individuals assessed. The inclusion criteria were patients with impacted maxillary canines. Canine impaction was considered as the presence of unerupted maxillary canines with fully formed roots. The experimental group (n=13) comprised individuals diagnosed with impaction of permanent maxillary canines at the rhBMP-2 grafted cleft areawith indication of surgical exposure for orthodontic traction (9 males and 3 females). The mean age at the moment of graft surgery was 12.05 years (SD=0.78). Grafting was performed by two maxillofacial surgeons of a single center. Both surgeons used the same surgical standardized technique. Grafting was performed using1.5 mg/mLrhBMP-2 (Infuse®Bone Graft,Medtronic, Memphis. USA) with resorbable collagen membrane for alveolar bone graft surgery. Each kitwas used tooperate two individuals with unilateral cleft lip and palate. The mean age at surgery for orthodontic traction was 13.56 years (SD=0.64). The time elapsed between bone graft and biopsy ranged from 6 to 46 months. Surgical access was performed on the labial side of the alveolar ridge. During surgical exposure of impacted canines, bone tissue biopsies were harvested on the impacted crown region. Oral mucosa biopsies were taken from the border of the labial surgical flap. The control group (n=5) comprised individuals (2 males and 2 females) with maxillary impacted canine at the noncleft side (n=2) selected from the same initial population of the experimental group. Three individuals with impacted maxillary canine at the ungrafted cleft side were also included (n=3) in the control group. These ungrafted patients with unilateral cleft lip and palate were recruited from the orthodontic clinic at the same rehabilitation center. All individuals in the control group were referredfor surgical exposure of the impacted maxillary canines for orthodontic traction.Mean age at the surgery for canine exposure was 14.3 years (SD=1.46). During surgical exposure of impacted canines, bone tissue biopsies were harvested on the impacted crown region. Oral mucosa biopsies were taken from the border of the labial mucoperiosteal flap.

One patient from each group was lost due to insufficient amount of harvest tissue. *B*one and soft tissue fragments harvested during surgery were fixated in 10% formalin and sent to the Laboratory of Pathology of Dental School, University of São Paulo. The bone tissue fragments were placed in Morse's solution for 1 to 2 months. After decalcification, these fragments were submitted to routine laboratory processing and embedded in paraffin. Microscopic sections were obtained with 4-µm thickness. Two slides with several microscopic sections were obtained for each case analyzed, which were stained with Hematoxylin and Eosin (HE) according to the standard technique. The microscopic sections were analyzed by two examiners using a binocular light microscope (Axioskop 2 Plus, ZEISS). Following, images of the microscopic sections of each case were obtained on a machine Leica DM 500, using the software Leica Application Suite version 2.0.0. 2010. The periapical radiographs obtained before biopsy were associated to the photomicrographs of each case. The microscopic sections stained with HE were analyzed according to the morphological pattern of the bone tissue, describing the presence or absence of lamellar bone, osteoid matrix, basophilic

reversion lines, immature bone tissue, nutrient canals, osteoblasts, osteoclasts and osteocytes, besides observation of connective tissue with or without collagen fibers, as well as presence or absence of inflammation and blood vessels. The microscopic sections with soft tissue fragments described the present or not of atypical epithelium.

RESULTS

Experimental group: Macroscopically, the bone fragments exhibited irregular shape, white or light beige shade. Bone tissue6 to 12 months after ABG: three biopsies were available in this time interval revealing the presence of a denser and thick continuous bone, corresponding to alveolar cortical bone, covering a cancellous bone tissuewith large spaces between the trabeculae (Fig 1). The cortical bone was mostly composed of areas of dense lamellar bone surrounding nutrient canals, and the spaces between lamellae were filled with more immature bone tissuecontaining disorganized several osteocyte lacunae, entrapped in the bone matrix. Adjacent to the cortical bone, the cancellous bone was composed of a mixture of more organized bone tissuecontaining osteocyte lacunae concentrically dispersed in the bone matrix, basophilic reversion lines and an extensive layer of immature bone followed by a thin layer of osteoid matrix weakly stained by eosin, indicating continuous deposition of bone tissue.

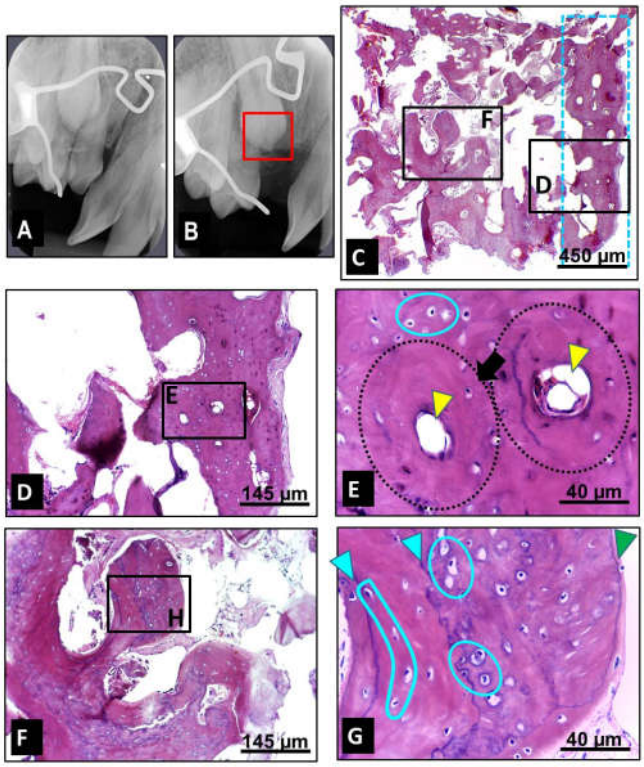

Figure 1. Male individual with a right complete cleft lip and palatesubmitted to ABGwith rhBMP-2 at 11.1 years of age: A-B). Periapical radiographs obtained beforeABG surgery (A) and 6 months after bone graft procedure (B) demonstrating impaction of the permanent canine and the biopsy area (red square). Histological images ofbone tissuestained with HE show: C) Panoramic aspect of greater fragment of biopsy (≅ 2.5 mm^2) formed by a thicker and continuous bone (highlighted in blue dashed line) and the remaining by cancellous bone tissuecontaining spaces between trabeculae. 4X magnification. D-E) Detail of lamellar bone tissue (black arrow) whose lamellae and osteocyte lacunae are arranged parallel to the nutrient canal (yellow arrowhead), and the space between them with more immature/disorganized bone tissuewith randomly dispersed osteocytes. 10X and 40X magnifications. F-G). Region of fragment composed of a mixture of more organized bone tissuecontaining small osteocyte lacunae (area surrounded in blue)dispersed in the bone matrix, basophilic reversion lines (blue arrowhead)and extensive layer of immature bone followed by a thin layer of osteoid matrix slightly stained by eosin (green arrowhead), indicating continuous bone tissue deposition. 10X and 40X magnifications.

Bone tissue13 to 24 months after ABG: six biopsies were obtained within this interval. Some regions of the biopsy exhibited recently formed bone tissue associated with connective tissue, containing recently entrapped osteocytes and active osteoblasts. Despite the higher degree of bone maturity, there were areas of immature bone tissuewith active osteoblasts on the bone surface covered by fibrous connective tissue. Slight reversion/incremental lines and osteocyte lacunae dispersed into the bone matrix were observed (Fig. 2).

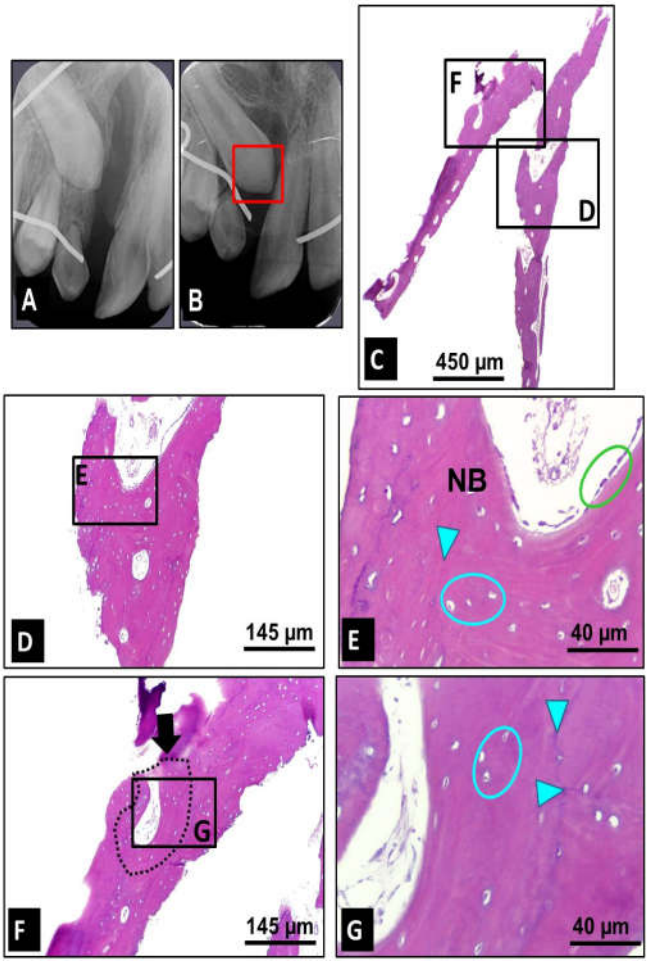

Figure 2. Male individual with a right complete cleft lip and palatesubmitted to ABG with rhBMP-2 at 12.6 years of age: A-B). Periapical radiographs before ABG (A) and 21 months after the bone graft surgery (B) demonstrating impaction of the permanent canine and biopsy area (red square). Histological images of bone tissue stained with HE show:C) Panoramic aspect of biopsy fragments (one fragment ≅ 4.5 x 0.5 mm^2 and one fragment ≅ 2.5 x0.5 mm^2). 4X magnification. D-G) Details of fragments formed by mature lamellar bone tissue (black arrow) containing slight reversion/incremental lines (blue arrowhead) and osteocyte lacunae dispersed in the bone matrix externally lined by osteoblasts (area surrounded in green). 10X and 40X magnifications

Bone tissue after24 months or more after ABG: three biopsies were obtained at this period. Bone tissueshowed dense bone trabeculae externally surrounded by fibrous connective tissue and others composed of bone marrow. Several reversion lines with signs of resorption and new bone formation indicated intensive bone remodeling (Fig. 3). Only three biopsies of soft tissue were adequate for analysis, taken 9, 13 and 46 months after rhBMP-2 bone graft surgery.

Thick parakeratinizedstratified epithelium containing long and narrow epithelial papillae were observed in the mucosal tissue. In all biopsies it was possible to identify the basal, spinousand granular layers of epithelium with normal aspect. At the interface between epithelium and connective tissue, upward projections of the fibrous connective tissue, such as connective papillae, interdigitated with epithelial crests (Fig 4). The lamina propria was composed of fibrous connective tissue containing fibroblasts and blood vessels (Fig. 4).

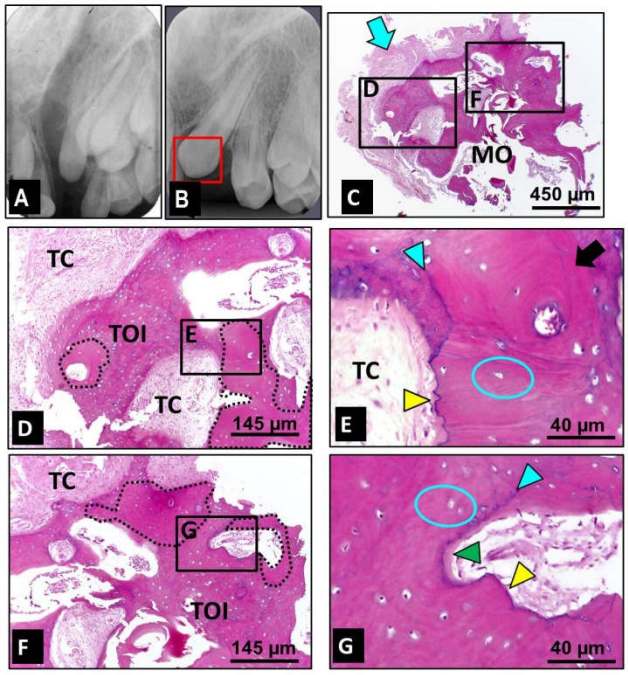

Figure 3. Male individual with complete left cleft lip and palate, submitted to ABG with rhBMP-2 at 12.7 years of age: A-B). Periapical radiographs obtained at the day of ABG (A) and 46 months after bone graft (B) demonstrating impaction of the permanent canine and area of biopsy (marked in red). Histological images of bone tissue stained with HE: C) Panoramic aspect of biopsy fragments (≅ 2mm^2) formed by dense bone trabeculae externally surrounded by fibrous connective tissue (TC)(blue arrow) and others compose of bone marrow. 4X magnification. D-G) Details of fragments formed by mature lamellar bone tissue (black arrow) containing reversion lines (blue arrowhead) with signs of resorption (yellow arrowhead) and immature new bone formation (TOI)(green arrowhead) and osteocyte lacunae (area surrounded in blue) dispersed in the bone matrix (MO). 10X and 40X magnifications.

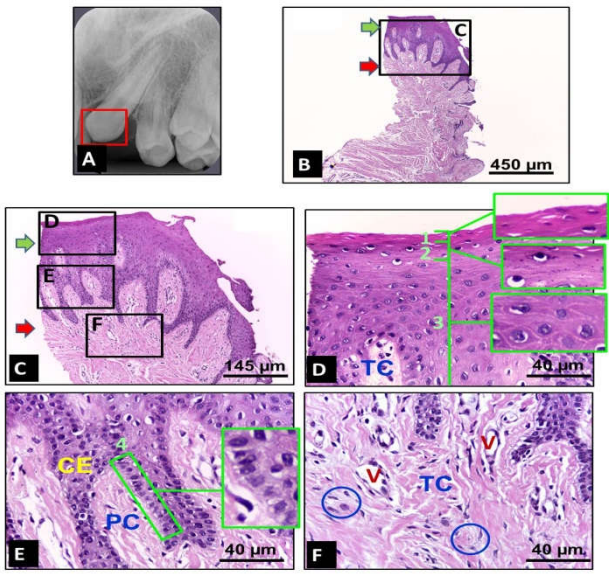

Figure 4. Male patient with complete left cleft lip and palate submitted to ABG with rhBMP-2 at 12.7 years of age. A) Periapical radiograph obtained 46 months after grafting, demonstrating the area of biopsy removal (area contoured in red). Histological images of oral mucosa tissue stained with HE: BC) Panoramic image of biopsy fragment exhibiting the lining epithelium (green arrow) and adjacent lamina propria(red arrow) composed of dense connective tissue. 4x and 10x magnification. D) Detail of epithelium exhibiting the parakeratinized/queratinized surface(1) containing pyknotic nuclei, granular layer (2) containing keratohyalin granules, and spindle layer (3) with keratinocytes exhibiting in detail the "intercellular bridges" or spinous between cells. 40x and 100x magnification; E) Detail of epithelialcrests (CE) demonstrating the basal layer (4) composed of columnar cells and connective papillae (PC). 40x and 100x magnifications. F) Detail of lamina propria composed of dense connective tissue (TC) containing fibroblasts (blue circle) and blood vessels (V). 40x magnification.

Control group: Control group bone tissue demonstrated lamellar bone formation surrounding new nutrient canals. Spaces between lamellae were filled with more immature bone tissue containing large osteocyte lacunae or a more organized lamellar bone tissue containing small nutrient canals and osteocyte lacunae following the lamellar arrangement. Reversion lines and nutrient canals were observed suggesting bone remodeling (Fig. 5). Soft tissue showed normal aspects with a slight inflammatory infiltrate at the mucous membrane layer (Fig. 6).

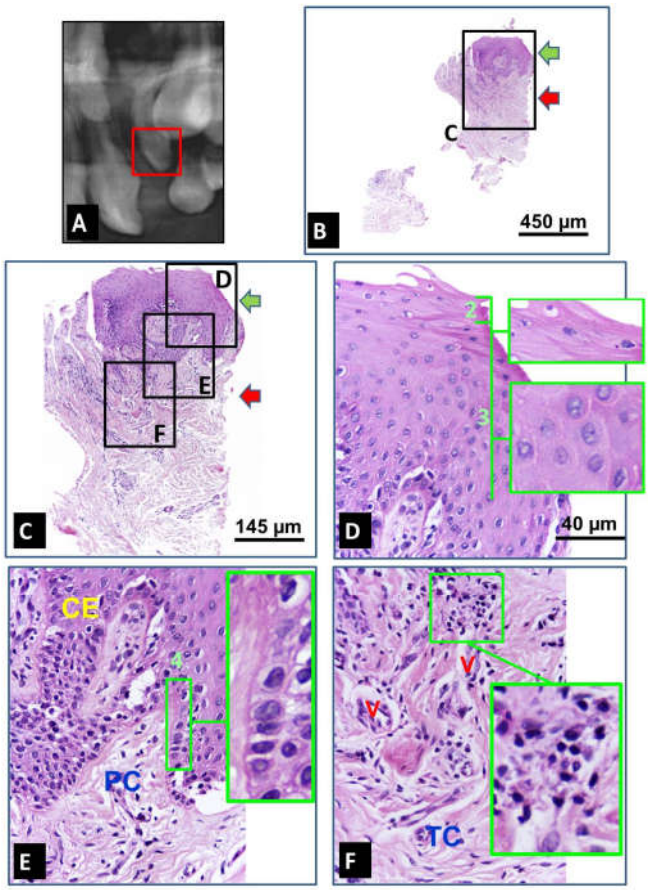

Figura 6. Male patient with complete left cleft lip and palate left side without bone graft at 12.5 years of age A) Periapical radiograph obtained in the date of surgery and area of biopsy (area contoured in red). B) Panoramic image of biopsy fragments. Histological images of oral mucosa tissue stained with HE: BC) Panoramic image of biopsy fragment (≅3,6mm^2) exhibiting the lining epithelium (green arrow) and adjacent lamina propria (red arrow) composed of dense connective tissue. 4x and 10x magnification. D) Detail of epithelium exhibiting granular layer (2) containing few keratohyalin granules and spindle layer (3) with keratinocytes exhibiting in detail the "intercellular bridges" between cells. 40x and 100x magnification.E) Detail of epithelial crests (CE) demonstrating the basal layer (4) composed of columnar cells and connective papillae (PC). 40x and 100x magnification. F) Detail of lamina propria composed of dense connective tissue (TC) containing fibroblasts, blood vessels (V) and slight inflammatory infiltrate (area surrounded in green). 40x magnification.

DISCUSSION

In this study,periapical radiographies and histological images results showed bone formation. Periapical radiographies findings obtained before bone graft surgery and 6 months to 4 years after bone graft procedure showed radiopaces areas through the graft suggestive of newly formed bone.Radiographic follow-up demonstrated the adaptation of the rhBMP-2 to the host area, making it impossible to distinguish the mesial and distal limits of the cleft. In addition, radiographical images revealed canines migrate toward the occlusal plane through the grafted bone with periodontal condition. Theseresults are similar with other studies in the literature in which teeth erupted through the grafted bone of cancellous bone of the iliac

crest (Uribe et al, 2019; Scalzone et al, 2019). The graft with rhBMP-2 is quickly incorporated and vascularized and, most importantly, does not interfere in the formation of the teeth adjacent to the cleft. This fact is shown in the radiographs of patients in Figures1-4A and has been also verifiedin histological findings of this study. The presence of the tooth contributes to the preservation of the grafted area and to the differentiation of the periodontal support. To our knowledge, therewas no studythat investigatemicroscopically analyzed human bone tissue fragments harvested from the alveolar cleft area graftedwith rhBMP-2. In the present study,the tissue biopsy was obtained during oral surgery for surgical access and orthodontic traction of impacted canines at the cleft area 6 months to 4 years after secondary alveolar bone graft procedure. The histologicalanalyses of soft and bone tissue samples harvested from the cleft area graftedwith rhBMP-2 were compatible with the morphological pattern of normality described by Ten Cate (2013) and with the control group. These results corroborate the findings from a previous clinical study (Freitas et al. 2016) who observed similar outcomes in fragments removed from the edentulous alveolar ridge grafted with rhBMP-2 before placement of endosseous implants. The microscopic sections of bone fragments obtained 6 months after graft with rhBMP-2 revealed bone tissue rich in blood vessels and cells, composed of immature bone trabeculae suggesting bone remodeling and stromal tissue without inflammation.

Our results are also in accordance with histological evaluations of bone fragments obtained from areas grafted with BMP-2 in clefts created in animal models (Sawada et al., 2009; Aghaloo et al., 2010; Deng et al., 2015; Mostafa et al., 2015). These studiesrevealed immature trabecular bone tissue containing several osteocyte lacunae, rimming of osteoblasts on the surfaceand fibrous connective tissue rich in blood vessels, with mild foci of mononuclear inflammatory infiltrate.The microscopic sections of the present study, at least 24 months after graft with rhBMP-2, presented similar histologicalaspects as those reported by Francis et al (2013) in sections obtained from an individual with cleft lip and palate, 42 months after secondary bone graftwith rhBMP-2, evidencing mature lamellar bone tissue with osteocyte lacunae dispersed in the bone matrix filled with fibrous connectivetissue. It showed histological normal aspect combined to viable bone tissue. In the present study, the histological findings of bone tissue fragments obtained from areas grafted with with 4.2 mg rhBMP-2 *Infuse*® revealed viable bone tissue without ectopic ossification, absence of poor bone structure and/or bone resorption. Despite the clinical utilization of rhBMP-2*Infuse and AMPLIFY*™, DeVine et al (2012) highlighted that examiners of the FDA cautioned about the clinical use of rhBMP-2with total dose of40 mg (commercial brand *AMPLIFY*™), since the high concentration of this protein may provide successful promotion of bone formation, but also adverse effects in the long term. Who 2013 reported e databank of the *FDA's Manufacturer and User Facility Device Experience* evidenced that, among the twelve reports of ectopic ossification after utilization of rhBMP-2 *AMPLIFY*™ in orthopedic surgeries, five reported that the individuals required another surgery to remove the extra bone. Although previous data indicated an association between high dosage of rhBMP-2 and occurrence of potentially malignant pathologies (Carragee et al, 2013), no individual in the sample demonstrated atypical ossification or excess of tissue volume at the grafted alveolar ridge.

Silva filho et al (2013) related that physiological principle of integration of any bone transplant is the osteoclastic resorption, considered bioestimulation to bone neoformation and primary factor on the process of graft remodeling, influencing its mineralization proportion. The process of graft incorporation with initial formation of immature primary bone and its maturation in secondary lamellar bone is also related to the graft vascularization, associated with the graft characteristics and the receptor site. This physiological principle can explain the histological findings in our study of bone fragments obtained from areas after 12 and 46 months grafted with rhBMP-2 (*Infuse)*that revealed viable bone tissue with mature lamellar bone tissue containing reversion lines with signs of resorption and immature new bone formation, suggesting a normal cycle of resorption/remodeling. In the control group bone tissue demonstrated lamellar bone formation surrounding new nutrient canals without resorption. The mean age at the surgery for canine exposure was 14.3 years (SD=1). With respect to oral mucosatissue of the experimental group, it showedhistological normal aspect combined to viable bone tissue. To our knowledge no previous studies on histological analysis of soft tissue adjacent to the rhBMP-2 graft was found. The microscopic sections with soft tissue fragments did not present atypical epithelium that might indicate some potentially malignant alteration by the World Health Organization (Gale et al., 2005). Further studies with longer follow-up should be conducted.

CONCLUSION

The histological aspects of bone and soft tissue at the cleft area grafted with 4.2mg rhBMP-2 revealed bone remodeling and absence of malignant alterations in the period of 6 to 46 months after surgery. In addition, further studies with a higher number of patients should be performed to confirm or not these results.

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