

Evaluation of the presence of MMP-2, TIMP-2, BMP2/4, and TGFβ3 in the facial tissue of children with cleft lip and palate

Liene Smane^{2*},

Mara Pilmane¹

¹ Institute of Anatomy and Anthropology,
Department of Morphology,
Riga Stradiņš University, Riga, Latvia

² Department of Pediatrics,
Children's Clinical University Hospital,
Riga, Latvia

Cleft lip and palate (CLP) is the most common defect affecting the face. The treatment consists of surgical reconstruction of the anatomical structures of the cleft. Part of the surgical treatment is reconstruction of the alveolar bone by means of autogenic bone grafting (osteoplasty). This study aimed to evaluate the levels of expression of extracellular matrix remodeling factors in the facial tissue of children with a complete unilateral (CU) and a complete bilateral (CB) CLP to assess whether the wound healing process is adequate. Twenty-two CLP patients were enrolled in this study. Tissue samples were collected during alveolar osteoplasty for unilateral ($n = 12$) or bilateral ($n = 10$) cleft palate, (age range from 6 years 8 months to 12 years 2 months). Control material was obtained in the case of tooth extraction (age range from 6 years 9 months to 14 years 5 months). Immunohistochemistry was used to assess the levels of matrix metalloproteinase-2 (MMP-2), tissue inhibitor of metalloproteinase-2 (TIMP-2), bone morphogenetic proteins 2 and 4 (BMP2/4), and transforming growth factor β3 (TGFβ3). Numbers of positively stained cells were graded semi-quantitatively. Data were analysed using the Kraskel-Wallis rank test and the Bonferroni correction. The total number of MMP2-positive cells was significantly lower in the CBCLP and in the control group than in the CUCLP ($p < 0.001$ after the Bonferroni correction). The total number of TIMP2-positive cells was significantly higher in the CUCLP than in the CBCLP and in the control group ($p < 0.001$; $p < 0.003$ after the Bonferroni correction). The overall number of BMP2/4, TGFβ3-positive cells was significantly higher in the CUCLP than in the CBCLP and in the control group ($p < 0.001$ after the Bonferroni correction). The decrease of the relative amount of statistically significant BMP2/4, TGFβ3, MMP-2, TIMP-2 containing bone cells in CBCLP patients identifies affected alveolar bone regeneration and remodeling process.

Keywords: cleft lip and palate, matrix metalloproteinase-2, tissue inhibitor of metalloproteinase-2, bone morphogenetic proteins 2 and 4, transforming growth factor β3

* Correspondence to: Liene Smane, Riga Stradiņš University, 16 Dzirciema Street, Riga, 1007, Latvia. Email: liene.smane@rsu.lv

INTRODUCTION

Orofacial clefts belong to the most common congenital anomalies affecting the face with a prevalence of complete unilateral (CU) and complete bilateral (CB) cleft lip and palate (CLP) of 1:1000 and 0.4:1000, respectively (1). Alveolar osteoplasty is an essential step in the overall management of patients with CLP. During osteoplasty, which is considered the gold standard, the bone is most often harvested from the iliac crest or tibia (2). As a result of surgery, the tissues may heal leaving a permanent defect that can negatively influence the growth of craniofacial tissues. The bone graft healing process and tissue remodeling are affected by the growth factors, matrix metalloproteinases (MMPs), tissue inhibitors of matrix metalloproteinases (TIMPs), and other signal molecules (3, 4).

The remodeling of extracellular matrix (ECM) in the alveolar bone can be regulated directly through the balance among the production of ECM molecules, the degradation of ECM by MMPs, and the inhibition of MMPs by TIMPs during bone healing (5). Nevertheless, the studies suggest that inhibiting the activation of MMP2 may result in bone defects and delayed remodeling of the ECM (6, 7).

The precise role of TIMP2 in bone healing is not known; however, it might be assumed that activation of TIMP2 results in locally increased concentrations of growth factors relevant for bone formation (8).

Growth factors have been investigated for the purpose of alveolar bone healing. Bone morphogenetic proteins (BMPs) such as BMP2, BMP4 that belong to the superfamily of the transforming growth factor β (TGF β) play a pivotal role in skeletal morphogenesis, repair and regeneration by inducing new bone formation (9). Loss of both BMP2 and BMP4 resulted in severe impairment of bone formation (10).

There are three TGF β isoforms: TGF β 1, TGF β 2, and TGF β 3 in mammals that control adhesion, proliferation, differentiation and transformation of many cell types (11). Nonetheless, excess expression of those markers increases bone formation (12). Inactivation of TGF β 3 results in developmental defects that include craniofacial malformations (13).

This study investigated the expression of MMP2, TIMP2, BMP2/4, and TGF β 3 in CUCLP and CBCLP to identify possible changes in signaling pathways that could lead to the aberrant alveolar bone remodeling after osteoplasty observed in CLP.

MATERIALS AND METHODS

Study population

Samples for the study were retrieved from the Cleft Lip and Palate Centre of the Institute of Stomatology of Riga Stradiņš University (RSU). Twenty-two patients – 16 boys and six girls were available for this study. Ten children had CBCLP, and 12 children had CUCLP. The age at the time of bone grafting ranged from 6 years 8 months to 12 years 2 months. In all 22 cases, bone from the iliac crest was transplanted. This study was independently reviewed and approved by the local Ethical Committee of the Riga Stradiņš University. Written informed consent was obtained from all parents after the nature of the study had been fully explained.

Fixation of studied tissue material

For conventional light microscopy and immunohistochemistry, tissues were fixed for one day in Stefanini/Zamboni solution, which was first used by Stefanini et al. in 1967 for the fixation of spermatozooids (14). After fixation, the study material was taken to the Laboratory of Morphology (Institute of Anatomy and Anthropology, Riga Stradiņš University) for rinsing with Tyrode solution for 24 hours, for decalcification with BIODER R solution for 24 hours, for dehydration in increasing concentration alcohol solutions, for degreasing in xylol for 30 min. Then it was immersed in paraffin I for one hour and in paraffin II for two hours. After that, it was immersed in melted paraffin for solidification and further processing to do the routine (hematoxylin and eosin) and immunohistochemical method.

Routine histological staining method

Four micrometre-thin sections were cut from each block, mounted on glass slides, deparaffinised, rehydrated through graded alcohol solutions, and stained with hematoxylin and eosin (15).

Immunohistochemistry

Tissue sections were labelled with the following primary antibodies: mouse anti-MMP-2 at 1:100

(sc-53630; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), mouse anti-TIMP-2 at 1:100 (sc-21735; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), rabbit anti-TGF β 3 at 1:200 (sc-82; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), goat anti-BMB2/4 at 1:100 (AF355; R&D Systems, Inc., Minneapolis, USA).

For the immunohistochemical staining protocol, the following agents were used to visualize the antigen-antibody reaction:

1. EDTA (pH 9.0) buffer (code-T0103; Diapath, Martinengo BG, Italy);
2. TRIS buffer (code-15-M106; Bio-Optica, Milano, Italy);
3. BIODÉC R decalcification solution (code-05-M03009; Bio-Optica, Milano, Italy);
4. Antibody Diluent (code-ab64211; Abcam, Burlingame, CA, USA);
5. HiDef DetectionTM reaction amplifier (code-954D-31; Sigma-Aldrich, Rocklin, CA, USA);
6. HiDef DetectionTM HRP polymer marker (code-954D-32; Sigma-Aldrich, Rocklin, CA, USA);
7. Goat ImmunoCruzTM ABC staining system (code-sc-2023; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), which includes biotin-containing secondary and tertiary antibodies, containing avidin and biotin-horseradish peroxidase solution;
8. DAB Substrate Kit (code-957D-30; Cell Marque, Rocklin, CA, USA);
9. Hematoxylin (code-ab143166; Abcam, Burlingame, CA, USA);
10. Histological glue Pertex[®] (code-00811; HistoLab[®], Gothenburg, Sweden).

From each block, 3- μ m tissue cuts were obtained and placed on slides covered with adhesive silan deparaffinised in xylol and hydrated in alcohol solutions of decreasing concentration. Rinsing was done two times every five minutes with TRIS buffer solution, boiling in EDTA buffer for 5 min in microwave. Endogenous peroxidase activity with 3% peroxidase was blocked for 10 min. All antibodies used in research were diluted with Antibody Diluent.

HiDef DetectionTM HRP polymer system was used for the antibodies of mice or rabbit origin. In the case the current system was used after primary antibody incubation and triple rinsing with TRIS

buffer solution, HiDef DetectionTM reaction amplifier was applied for 10 min at room temperature. After this processing, the preparations were rinsed three times for five minutes in TRIS buffer solution. After rinsing, using this polymer system, HRP chromogene was used for 5 min.

ImmunoCruzTM ABC staining system was used for antigens of goat origin. In the case ABC staining system was used, micropreparations were incubated in 1.5% blocking serum TRIS buffer solution up to one hour at room temperature. Then preparations with primary antibodies were incubated for one hour at room temperature. After rinsing three times for five minutes in TRIS buffer solution, the cuts were incubated for 30 min with biotin-containing secondary antibody (biotinylated goat Ig). The samples were then rinsed three times for five minutes in TRIS buffer solution and incubated with tertiary antibody for 30 min at room temperature, after which they were rinsed with TRIS buffer solution. Next, the DAB Substrate Kit was used for 10 min.

After chromogene, using both goat ImmunoCruzTM ABC staining system and HiDef DetectionTM HRP polymer system, the cell nuclei dye hematoxyline was used. After staining with hematoxyline, the micropreparations were rinsed in distilled water and dehydrated in alcohols of increasing concentration, transparenced in xylol and covered with glue Pertex[®] glue.

In parallel cuts of the mentioned preparation, the primary antibody was substituted by Antibody Diluent. These cuts were used as the negative control. Positive controls (in tissues which always have a positive reaction) were prepared for each preparation series as well.

Semi-quantitative method

The intensity of immunostaining was graded semi-quantitatively on a scale of 0 to +++++, as follows: 0, no positive structures; 0/+, occasional positive structures; +, few immunoreactive structures; ++, a moderate number of immunoreactive structures; +++, numerous immunoreactive structures; +++++, an abundance of immunoreactive structures in the visual field (16).

Statistical analysis

Statistical analyses were performed using SPSS v.20.0 (IBM Corp., Armonk, NY, USA). For the assessment of the research data, nonparametric

statistical methods were applied. For the comparison of several unconnected groups, Kruskal–Wallis range dispersion analysis was used. For the comparison of five groups in pairs, the Bonferroni correction for the detection of p value significance level was used.

RESULTS

MMP2

MMP2-positive cells were detected in all CUCLP cases and the presence of MMP2-positive osteocytes was occasional to abundant (Fig. 1). MMP2-positive cells were present in all CBCLP bone tissue samples. The number of MMP2-positive osteocytes varied from occasional to numerous (Fig. 2). In all control

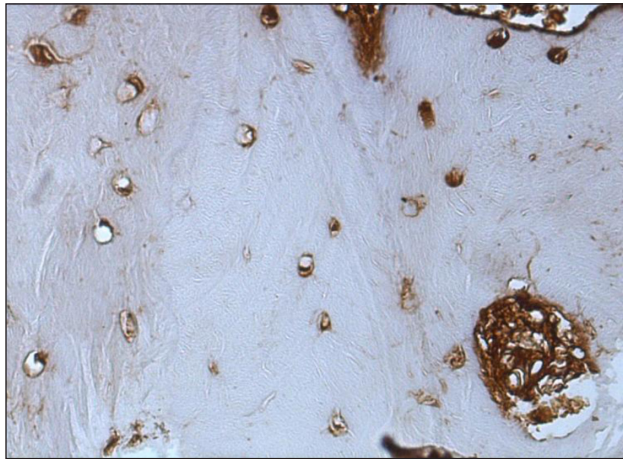


Fig. 1. Moderate MMP2-positive osteocytes in a child aged 7 years and 1 month with CUCLP osteoplasty, $\times 200$

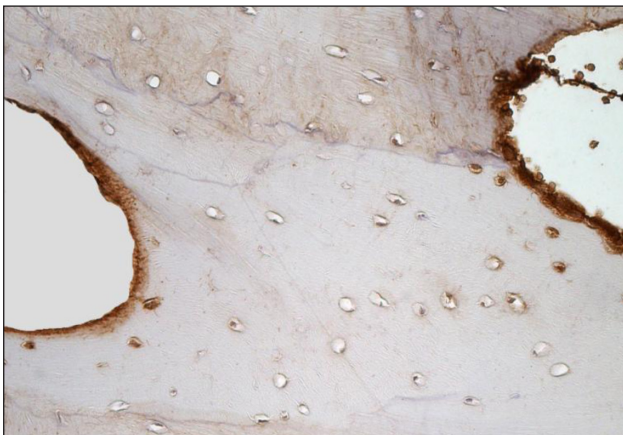


Fig. 2. Occasional MMP2-positive osteocytes in a child aged 9 years and 6 months with CBCLP osteoplasty, $\times 200$

specimens MMP2-positive cells were present from occasional to moderate (Fig. 3). The total number of MMP2-positive cells was significantly lower in the CBCLP and control group than in the CUCLP ($p < 0.001$ after the Bonferroni correction).

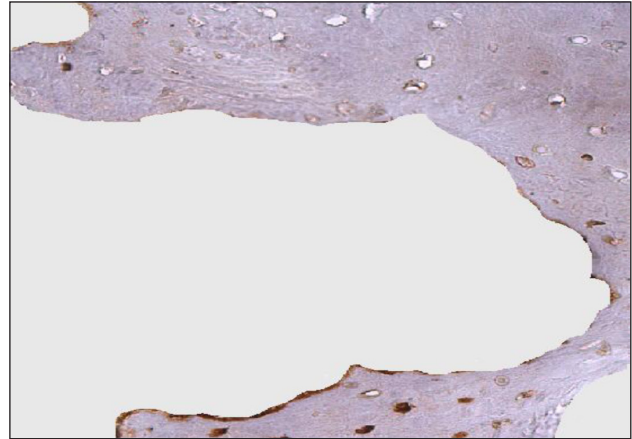


Fig. 3. Occasional MMP2-positive osteocytes in bone tissue from an unaffected child aged 10 years and 2 months, $\times 200$

TIMP2

TIMP2-positive cells were observed in all groups; however, there was a significant difference between them. The number of TIMP2-positive osteocytes varied from few to abundant in all CUCLP cases. In CBCLP specimens, TIMP2-positive cells in the bone tissue were occasional to numerous (Fig. 4). In controls, the number of TIMP2-positive bone cells varied from occasional to



Fig. 4. Numerous TIMP-2-containing osteocytes in a child aged 7 years and 8 months with CBCLP osteoplasty, 1×100

moderate (Fig. 5). The total number of TIMP2-positive cells was significantly higher in the CUCLP than in the CBCLP and in the control group ($p < 0.001$; $p < 0.003$ after the Bonferroni correction).

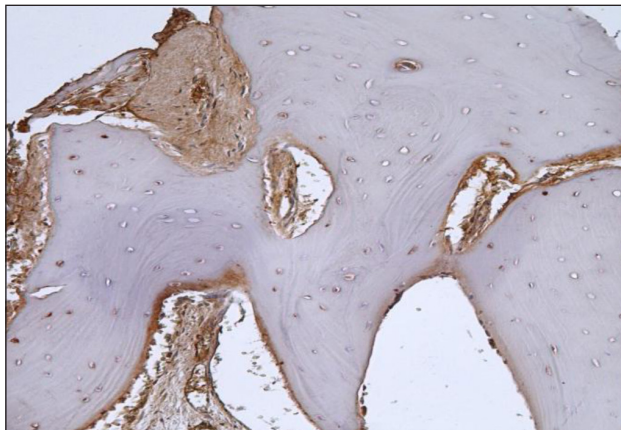


Fig. 5. Occasional MMP2-positive osteocytes in bone tissue from an unaffected child aged 10 years and 2 months, $\times 200$

BMP2/4

BMP2/4-positive bone cells were present in all hard tissue samples, and immunoreactivity was detected in osteoblasts and osteocytes. The number of BMP2/4-positive bone cells varied from moderate to abundant in all CUCLP cases (Fig. 6), but positive osteocytes in the CBCLP group were occasional to numerous (Fig. 7). Few to numerous BMP2/4-positive osteocytes were present in the control group specimens (Fig. 8). The total number of BMP2/4-positive bone-forming cells

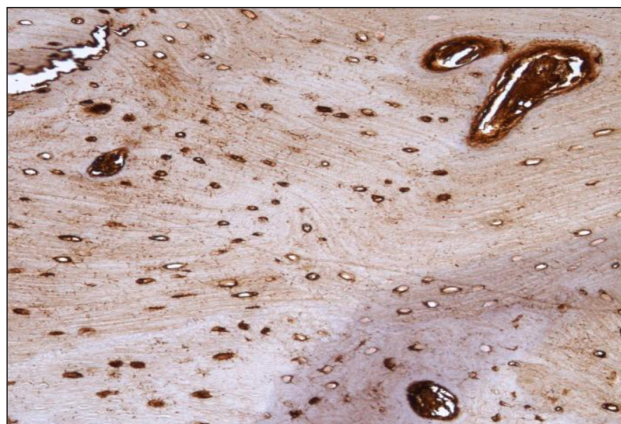


Fig. 6. Numerous BMP2/4-containing osteocytes in a child aged 6 years and 8 months with CUCLP osteoplasty, $\times 200$

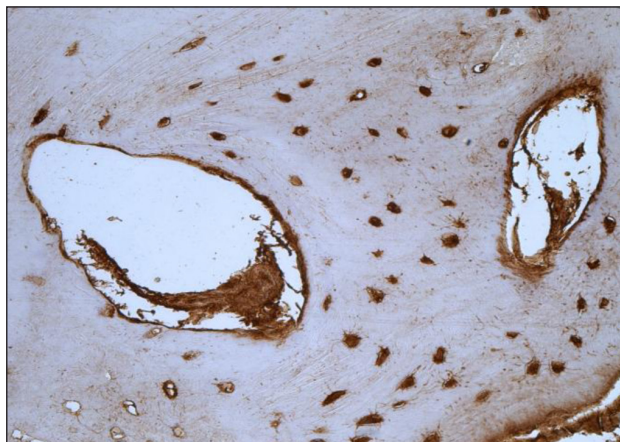


Fig. 7. Numerous BMP2/4-positive osteocytes in a child aged 8 years and 6 months with CBCLP osteoplasty, $\times 200$

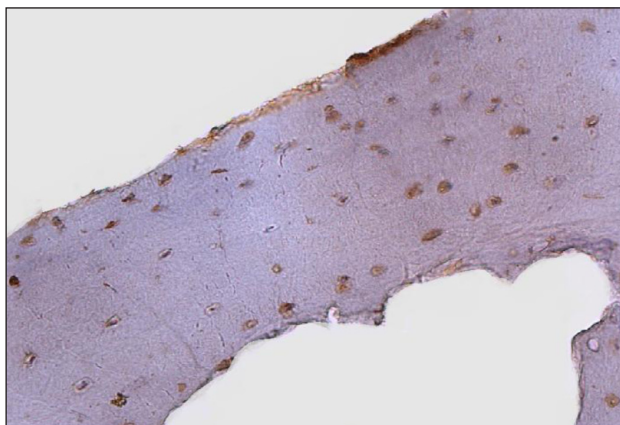


Fig. 8. Numerous BMP2/4-positive osteocytes in bone tissue from an unaffected child aged 11 years and 7 months, $\times 200$

was significantly lower in the CBCLP than in the CUCLP ($p < 0.001$ after the Bonferroni correction).

TGF β 3

Of the 12 CUCLP specimens, all were positive for TGF β 3, although the signal was detected in moderate to abundant cells (Fig. 9). TGF β 3-positive cells were detected in all CBCLP cases, and the presence of TGF β 3-positive osteocytes was moderate to numerous (Fig. 10). In all control specimens, few to numerous TGF β 3-positive cells were observed (Fig. 11). The total number of TGF β 3-positive cells was significantly higher in the CUCLP than in the CBCLP and in the control group ($p < 0.001$ after the Bonferroni correction).

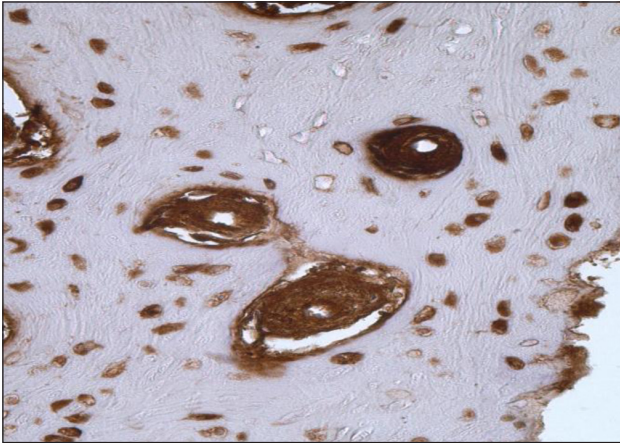


Fig. 9. Moderate TGFβ3-positive osteocytes in a child aged 8 years and 5 months with CUCLP osteoplasty, ×100



Fig. 10. Moderate (++) TGFβ3-positive osteocytes in a child aged 8 years and 6 months with CBCLP osteoplasty, ×100

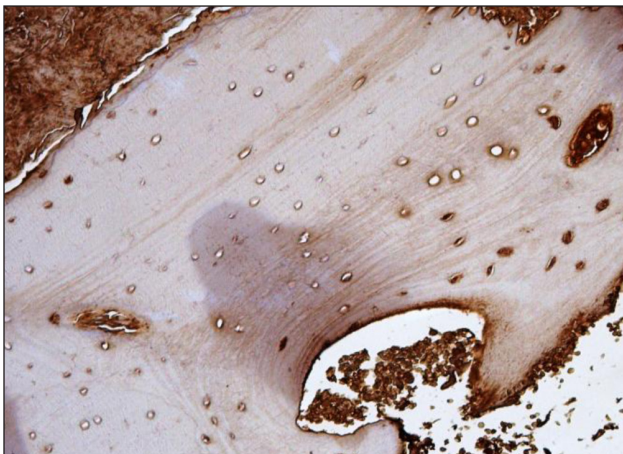


Fig. 11. Moderate TGFβ3-positive osteocytes in bone tissue from an unaffected child aged 10 years and 2 months, ×100

DISCUSSION

Expression analysis in human alveolar bone tissue from patients with CB and CUCLP has not been performed before. Hence, we conducted the present study in order to obtain more information on bone remodeling and regeneration of CLP in general.

Considering the fact that alveolar bone healing and remodeling are a complex process coordinated by cells, bioactive factors, and extracellular matrix, stimulating preosteoblast proliferation, differentiation and migration, different factors that affect the bone regeneration have been investigated. Molecular mechanisms of alveolar bone remodeling in relation to facial clefts and osteoplasty used for their treatment have not been widely studied. In a recent study, MMP-2 expression and its role in the alveolar bone regeneration in rats after a tooth extraction were proved (17). Scientists from Brasil reported that by stimulating MMP-2 expression, bone remodeling and successful wound healing were stimulated too (18). It also was found that a decreased MMP-2 functional activity was negatively influencing the bone quality (19). Kramer et al. found out that injection of TIMP-2 into the cranial bone defect areas stimulated the healing process (20).

Assessing MMP-2 and TIMP-2 presence in the material acquired during osteoplasty, decreased bone cells containing this protein were observed in children with the most severe cleft type, i.e., CBCLP. This finding can perhaps be explained by the fact that the alveolar bone remodeling potential in patients with CBCLP is less marked than in patients with CUCLP.

In recent studies with animal models it has been proved that BMP-4 can also induce hard tissue regeneration (21, 22). In his study Torrecillas-Martínez emphasizes that BMP-4 plays an essential role in bone homeostasis and regeneration after surgical expansion of the maxillary sinus (23). One can also find some recent studies pointing to a positive association of BMP-4 gene with CLP in different populations (24–26). Using the immunohistochemical method, Suzuki et al. identified BMP-2 expression in dental germs and mandibular tissues in human embryos, which confirmed the importance of this protein in the morphogenesis of facial and oral tissues (27).

In our study, we observed a significantly decreased BMP2/4 presence in children with CBCLP ($p < 0.001$). In children with CUCLP, rich BMP2/4 presence in osteocytes was seen. Thus, even after the bone trauma due to the operation, bone healing and regeneration capacities were more pronounced in patients with CUCLP than in patients with the most severe cleft type, and in more severe cleft cases the bone healing and growth potential were essentially reduced. The finding in the controls material gives evidence to the importance of BMP2/4 in the regulation of a normal tissue function. The findings of this study show that children with CBCLP have a reduced bone regeneration potential, therefore there may be a greater probability that the wound in these children would heal with a permanent defect. Similar results were reported by Alarmo and Torrecillas-Martínez et al. (28).

TGF β 3 has been identified to regulate the osteoblast differentiation, thus TGF β 3 gets involved in bone remodeling (29). During bone resorption, osteoclasts activate latent TGF β 3, which stimulates bone formation. These findings shows that TGF β 3 is the leading factor in the bone remodeling phase of the healing process (30).

Comparing the tissue samples obtained from osteoplasty, we observed a moderately decreased relative amount of TGF β 3 osteoblasts and osteocytes in patients with CBCLP and in the control group. This fact could be explained by insufficient cell proliferation, differentiation and apoptosis in the most severe cleft type, i.e., CBCLP.

CONCLUSIONS

The decrease of the relative amount of statistically significant BMP2/4, TGF β 3, MMP-2, and TIMP-2 containing bone cells in CBCLP patients shows a decrease in the alveolar bone regeneration and remodeling process.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest regarding the publication of this article.

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Liene Smāne, Mara Pilmane

VAIKŲ, TURINČIŲ NESUAUGUSIUS LŪPĄ IR GOMURĮ, MMP-2, TIMP-2, BMP2 / 4 IR TGFβ3 NUSTATYMAS VEIDO AUDINYJE

Santrauka

Įvadas. Nesuaugusi lūpa ir gomurys yra labiausiai paplitęs veido pažeidimas. Gydytas susideda iš anatomiinių struktūrų chirurginės rekonstrukcijos. Chirurginio gydymo dalis yra alveolinio kaulo rekonstrukcija, pasitelkiant autogeninį kaulų persodinimą (osteoplastiją). Šio tyrimo tikslas – nustatyti, ar ekstraląstelinio matriksio remodeliacijos veiksnių ekspozicija yra pakankama vaikų, turinčių visišką vienpusį ir abipusį lūpos ir gomurio nesuaugimą veido audinyje; siekta įvertinti, ar žaizdų gijimo procesas yra pakankamas. Tyrime dalyvavo dvidešimt du pacientai.

Medžiaga ir metodai. Audinių mėginiai buvo paimti atliekant alveolinę osteoplastiką esant vienpusiam ($n = 12$) arba abipusiam ($n = 10$) gomurio nesuaugimui (6 metai, 8 mėnesiai – 12 metų, 2 mėnesiai). Kontrolinė me-

džiaga gauta danties ekstrahavimo atveju (6 metai, 9 mėnesiai – 14 metų, 5 mėnesiai). Imunohistochemija buvo naudojama matriksio metaloproteinazės-2 (MMP-2), metaloproteinazės-2 (TIMP-2) audinių inhibitoriaus, 2 ir 4 kaulų morfogenetinių baltymų (BMP2 / 4) ir transformuojančio augimo veiksnio β3 (TGFβ3) matavimų lygiui įvertinti. Teigiamai nudažytų ląstelių skaičius buvo suskirstytas pusiau kiekybiškai. Duomenys išanalizuoti taikant Kraskel-Wallis'o testą ir Bonferroni pataisą.

Rezultatai ir išvados. Bendras MMP2 teigiamų ląstelių kiekis visiško abipusio nesuaugimo ir kontrolinėje grupėje buvo daug mažesnis nei visiško vienpusio nesuaugimo grupėje ($p < 0,001$ po Bonferroni korekcijos). Bendras TIMP2 teigiamų ląstelių skaičius buvo didesnis nei visiško vienpusio bei abipusio nesuaugimo ir kontrolinėje grupėse ($p < 0,001$; $p < 0,003$ po Bonferroni korekcijos). Bendras BMP2 / 4, TGFβ3 teigiamų ląstelių skaičius buvo didesnis visiško vienpusio nesuaugimo grupėje nei visiško abipusio nesuaugimo ir kontrolinėje grupėse ($p < 0,001$ po Bonferroni korekcijos). Visiško abipusio nesuaugimo grupės pacientams santykinai sumažinus kaulų ląstelių, turinčių statistiškai reikšmingus BMP2/4, TGFβ3, MMP-2, sumažėja alveolių kaulų regeneracijos ir remodeliavimo procesas.

Raktažodžiai: nesuaugusi lūpa ir gomurys, matriksio metaloproteinazė-2, metaloproteinazės-2 audinių inhibitorius, 2 ir 4 kaulų morfogenetiniai baltymai, transformuojantis augimo veiksnys β3