

Immunohistochemical analysis of nestin, CD34 and TGFβ3 in facial tissue of children with complete unilateral and bilateral cleft lip and palate

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SUMMARY

Objective. This study aimed to evaluate levels of expression of nestin, CD34 and transforming growth factor β3 (TGFβ3) in facial tissue of children with complete unilateral (CU) and complete bilateral (CB) cleft lip and palate (CLP).

Materials and Methods. Twenty-nine CLP patients were enrolled in this study (18 boys and 11 girls). Tissue samples were collected during primary and repeated plastic surgery correction for CU (n=10) or CB (n=19) cleft palate (age range 3 months – 9 years, 6 months). Immunohistochemistry was used to assess levels of nestin, CD34 and TGFβ3. Positively stained cells were graded semi-quantitatively. Data were analyzed to compare cell counts between CUCLP and CBCLP, and CLP at an age before and during primary dentition and CLP in mixed dentition age patients using the Mann Whitney U-test (P<0.05).

Results. Moderate to abundant numbers of nestin immunoreactive structures were observed in the oral mucosa. CD34 antibodies labeled all microvessels in lamina propria of the CLP affected tissue, while low numbers of TGFβ3 positive cells were scattered in the connective tissue. There were no statistically significant differences between the study groups.

Conclusion. Expression of nestin in complete unilateral and bilateral cleft lip and palate affected soft tissue indicates a potential increase of tissue regeneration. CD34 positive oral mucosa cells suggest increased angiogenesis, while the sporadic expression of TGFβ3 indicates an insignificant role in the maintenance and growth of cleft affected oral mucosa stem or progenitor cells. Nevertheless, scarce expression of TGFβ3 suggests a role in cleft morphopathogenesis.

Keywords: cleft lip and palate, CD34, nestin, transforming growth factor β3, inflammation, wound healing.

INTRODUCTION

In humans, cleft lip and palate (CLP) is the fourth most common birth defect and the most common congenital malformation of the head and neck, with significant medical, psychological, and social ramifications. CLP is characterized by an incomplete formation of structures separating nasal and oral cavities including the lip, alveolus, hard and soft palate. It occurs in 1:600 children in European populations and correction involves prolonged treatment over many years (1, 2). Abnormal facial tissue development during gestation is caused by a complex interaction between multiple genetic and environmental factors

(3). In addition, the lack or excessive presence of a particular factor may cause irreversible changes resulting in the development of congenital abnormalities such as facial cleft (4, 5).

The oral mucosa is composed of stratified squamous epithelium and vascularized underlying connective tissue. Two different types of human adult stem cells have been identified in the oral mucosa: i) oral epithelial progenitor or stem cells that develop into epithelial cells and ii) other stem cells in the *lamina propria* of the gingiva (6). In general, stem cells can proliferate in response to injury and growth stimuli, resulting in one stem cell and one transient amplifying cell with limited proliferative potential (7). Egusa *et al.* (2012) reported that the oral and maxillofacial region is a rich source of adult stem cells (8).

CLP treatment is a complex procedure that may include multiple plastic surgical corrections, which may result in the formation of scar tissue, that ad-

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versely affects the growth and development of facial and oral cavity tissues.

Acute wound healing involves four phases including hemostasis, inflammation, proliferation, and remodeling (9). Various molecules contribute to the tissue regeneration associated with acute wound healing, including CD34, nestin and TGF β 3 molecules. Such molecules can be identified to allow analysis of wound healing, and can be useful to determine the rate or outcome of states of wound healing and therefore signal the success of surgical or pharmaceutical interventions.

CD34 is a commonly used lymphohematopoietic stem and progenitor stem marker, as well as endothelial cell marker, which identifies both newly formed blood vessels and pre-existing vessels. Nestin is a type VI intermediate filament protein initially identified as a marker of neural stem and neural progenitor cells. It is expressed in several human embryonal, fetal and adult tissues. It is transiently expressed in undifferentiated cells, including newly synthesized blood vessels (10, 11). TGF- β superfamily of cytokines contains more than 30 structurally related polypeptide growth factors, regulating a wide variety of cellular processes such as proliferation, differentiation, migration, epithelial mesenchymal transformation, and apoptosis, as well as physiological processes, including embryonic development, angiogenesis, and wound healing (12, 13).

Because nestin, CD34 and TGF β 3 molecules are essential for tissue regeneration, these signaling molecules were used as oral mucosa stem cells and angiogenesis markers in order to determine their expression in facial clefts from affected tissues obtained after primary and subsequent plastic surgeries.

MATERIALS AND METHODS

This study was independently reviewed and approved by the local Ethical Committee of the Riga Stradins University, Latvia (2003), and written informed consent was obtained from all parents after the nature of the study had been fully explained.

Samples for the study were retrieved from the Cleft Lip and Palate Centre of the Institute of Stomatology of the Riga Stradins University (Latvia). Twenty-nine CLP patients were enrolled in this study. Ten children had complete unilateral (CU) CLP, while 19 children had complete bilateral (CB) CLP. There were 18 boys and 11 girls among the affected patients. Samples of soft tissue were collected during surgical correction of the cleft between the ages of 3 months and 9 years, 6 months. Patient samples were obtained during various surgeries, such as labioplasty, veloplasty, uranoplasty and osteoplasty. Depending on the diagnosis and age, the patients were divided into four

groups: children with CUCLP (10 patients), children with CBCLP (19 patients), children with CLP at an age before and during primary dentition (18 patients, age range 3 months – 4 years, 8 months), and children with CLP in mixed dentition age (11 patients, age range 6 years, 4 months – 9 years, 6 months).

For conventional light microscopy and immunohistochemistry tissues were fixed for one day in a mixture of 2% formaldehyde and 0.2% picric acid in 0.1 M phosphate buffer (pH 7.2). Following this, they were rinsed in Tyrode's solution, containing 10% sucrose for 12 hours, and then embedded into paraffin. Four-micrometer thin sections were cut from each block, mounted on glass slides, deparaffinized, rehydrated through graded alcohol solutions and stained with hematoxylin and eosin.

Immunohistochemistry

Immunohistochemical method was applied for detection of nestin, CD34 and TGF β 3 (14).

Tissue sections were labeled with the following primary antibodies: mouse anti-nestin at 1:200 (ab22035; Abcam, Burlingame, CA, USA), mouse anti-CD34 at 1:200 (sc-19621; 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).

For the immunohistochemical staining protocol the following agents were used to visualize 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. Antibody Diluent (code-ab64211; Abcam, Burlingame, CA, USA);
4. HiDef DetectionTM reaction amplifier (code-954D-31; Sigma-Aldrich, Rocklin, CA, USA);
5. HiDef DetectionTM HRP polymer marker (code-954D-32; Sigma-Aldrich, Rocklin, CA, USA);
6. DAB Substrate Kit (code-957D-30; Cell Marque, Rocklin, CA, USA);
7. Hematoxylin (code-ab143166; Abcam, Burlingame, CA, USA);
8. Histological glue Pertex[®] (code-00811; HistoLab[®], Gothenburg, Sweden).

From each block 4 micrometer thin tissue cuts were got, which were put on slides covered with adhesive silan, deparaffinized in xylol and hydrated in alcohol solutions with decreasing concentration. Rinsing was done 2 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 Detection™ HRP polymer system was used for the mice or rabbit origin antibodies. In the case the current system was used after the primary antibody incubation and triple rinsing with TRIS buffer solution, HiDef Detection™ reaction amplifier was applied for 10 min at room temperature. After this processing, the preparations were rinsed three times for five minutes by TRIS buffer solution. After rinsing, using this polymer system, HRP chromogene was used for 5 min.

After chromogene, using HiDef Detection™ HRP polymer system, hematoxyline – cell nuclei dye was used. After staining with hematoxyline the micropreparations were rinsed in distilled water and dehydrated in increasing concentration alcohols, transparenting in xylol and covering with the glue Pertex®.

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

Images were captured by Leica DC 300F camera and image processing and analysis software was by Image-Pro Plus, Version 6.0 (Media Cybernetics, Inc., USA). The intensity of immunostaining was graded semi-quantitatively. A scale of 0 to ++++ was used as follows: 0 – no positive structures found in visual field, 0/+ – occasional positive structures seen in visual field, + – few immunoreactive structures seen in visual field, ++ – moderate number of immunoreactive structures seen in visual field, +++ – numerous immunoreactive structures seen in visual field, and ++++ – abundance of immunoreactive structures seen in visual field (15). The location of staining was observed and noted.

Statistical analysis

Data were entered onto a spreadsheet, and statistical analyses performed using SPSS software, version 20.0 (IBM Corp., Armonk, NY). A Mann-Whitney *U*-test for comparison of study groups was conducted. *P* values < 0.05 were considered statistically significant.

RESULTS

All results are summarized in Table. The present study consisted of 29 cases and results obtained for various markers are described.

Nestin

Nestin positive cells were identified in oral epithelium and the oral mucosa *lamina propria* (OMLP). We observed an abundant number of nestin positive cells

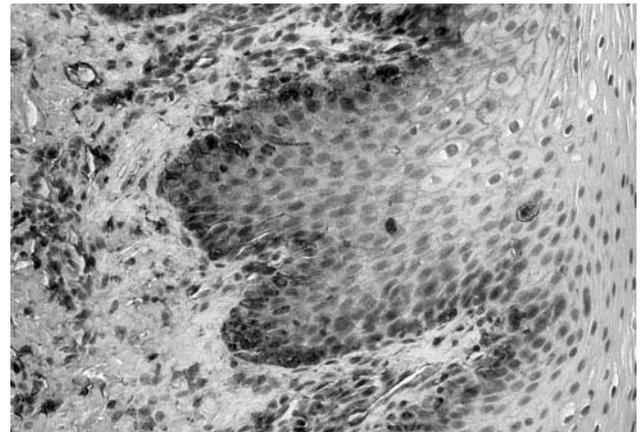


Fig. 1. Abundant nestin immunoreactive oral basal epithelial cells from cleft lip region of a 6-month-old child. Nestin immunohistochemistry, magnification $\times 200$.

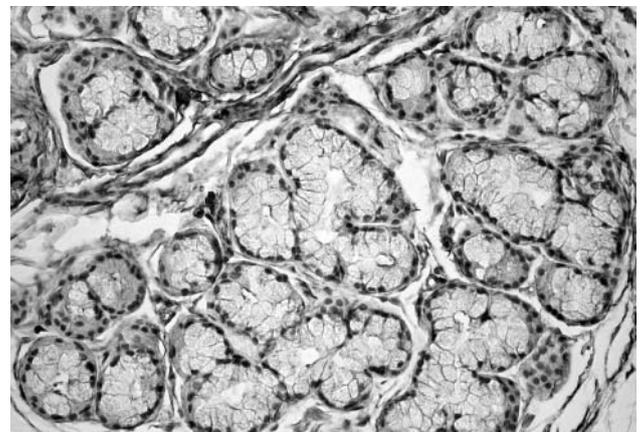


Fig. 2. Moderate positive staining for nestin salivary gland cells in cleft lip region of a 5-month-old child. Nestin immunohistochemistry, magnification $\times 200$.



Fig. 3. Numerous numbers of nestin-containing hair follicle outer root sheath cells. Nestin immunohistochemistry, magnification $\times 200$.

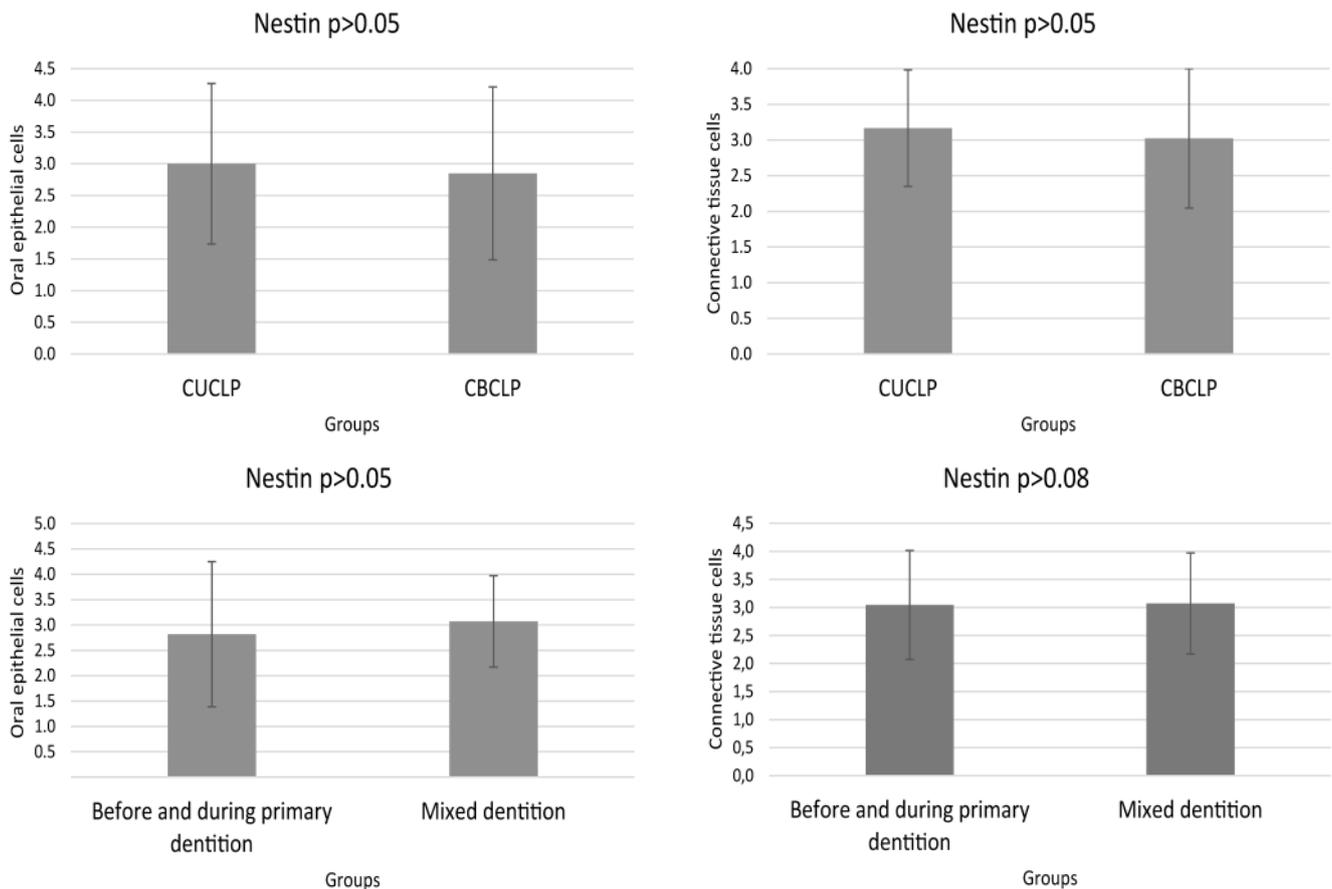


Fig. 4. Comparison of nestin expression in complete unilateral (CU) and bilateral (CB) cleft lip and palate (CLP) affected oral mucosa before and during primary and mixed dentition. No statistically significant differences were observed.

in oral epithelium of four CLP patients, but moderate numbers in seventeen patients. Nestin protein expression was mainly observed in the basal layer of the oral epithelium (Fig. 1). Moderate to abundant numbers of immunoreactive structures were also observed in the connective tissue, and endothelial and salivary gland cells (Fig. 2). Moreover, nestin-positive cells were detected in the outer root sheath basal layer of hair follicles but were not observed in the inner root sheath (Fig. 3). There was no significant difference in mean nestin-positive cell numbers between the study groups (Fig. 4).

CD34

CD34 antibodies labeled all microvessels in the papillary and reticular layers of the lamina propria

from CLP affected tissue. The number of CD34-positive endothelial cells varied from moderate to abundant in all specimens from the study groups (Fig. 5). There was no significant difference in the mean numbers of CD34-positive endothelial cells between the groups (Fig. 6). We observed CD34-positive spindle shaped stromal cells in all CLP cases (Fig. 7) and although few to moderate numbers were observed in the visual field there was no significant difference in mean numbers between the study groups (Fig. 8).

TGFβ3

TGFβ3-positive cells were present in fifteen tissue samples, and immunoreactivity was detected in fibroblasts around blood vessels and macrophages

Table. Semiquantitative distribution of immunoreactive structures in patients with unilateral and bilateral cleft lip and palate

Study groups	CUCLP			CBCLP			Before and during primary dentition age			Mixed dentition age		
	Nestin	CD34	TGFβ3	Nestin	CD34	TGFβ3	Nestin	CD34	TGFβ3	Nestin	CD34	TGFβ3
Epitheliocytes	+++	0	0	+++	0	0	+++	0	0	+++	0	0
Connective tissue cells	+++	+	0/+	+++	++	0/+	+++	++	0/+	+++	+	0/+
Endotheliocytes	++	+++ /++++	0	++	++++	0/+	++	++++	0/+	+++	+++	0/+

TGF – transforming growth factor; CUCLP – complete unilateral cleft lip and palate; CBCLP – complete bilateral cleft lip and palate

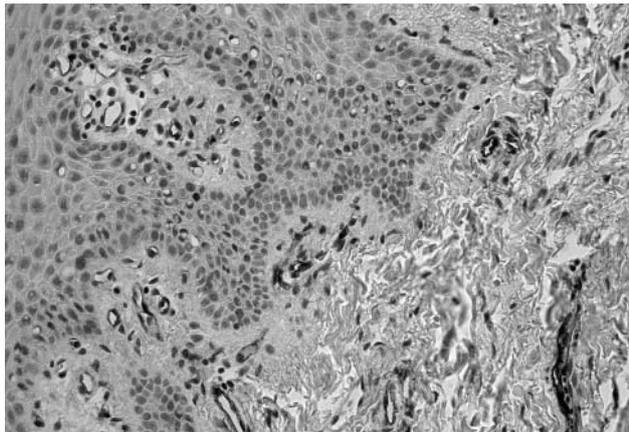


Fig. 5. Oral mucosa of a patient with complete bilateral cleft lip and palate. CD34-positive microvessels build a network of papillary and reticular layers. CD34-positive microvessels display cells with filopodial projections. CD34 immunohistochemistry, magnification $\times 200$.

(Fig. 9). The number of TGF β 3-positive cells varied from occasional to few in CBCLP cases. In the tissue samples, obtained from CUCLP patients, TGF β 3-positive connective tissue cells varied from separate (0/+) to few (+) in the visual field. TGF β 3-positive cells were observed in all groups; however, there was no significant difference between them (Fig. 10). The appearance of TGF β 3-positive connective tissue cells in the current study varied from occasional to few in the visual field of samples from children with clefts before and during primary dentition, as well as mixed dentition age. No immunohistochemical staining for TGF β 3 was observed in other structures of the oral mucosa, such as the oral epithelium.

DISCUSSION

Identification of either stem cells or transient amplifying cell locations will provide an understanding of normal and abnormal epithelial growth and tissue regeneration of the oral cavity. Cells of the oral mucosa lamina propria (OMLP) originate from the embryonic neural crest (16). Wounds in the oral mucosa heal by regeneration and without scar formation indicating the existence of a primitive neural crest stem cell-derived population in the OMLP (17).

The results of this study showed that nestin expression was present in the tissues of all patients. Therefore, we suggest that nestin expression in oral mucosa from clefts might be related to tissue regeneration. Wiese *et al.* (2004) reported that nestin-expressing cells played a central role in cellular proliferation, differentiation and migration after re-activation. Of note, adult tissue cells upregulate nestin after injury suggesting a potential role of nestin in tissue regeneration. Nestin upregulation after injury may be controlled by extracellular

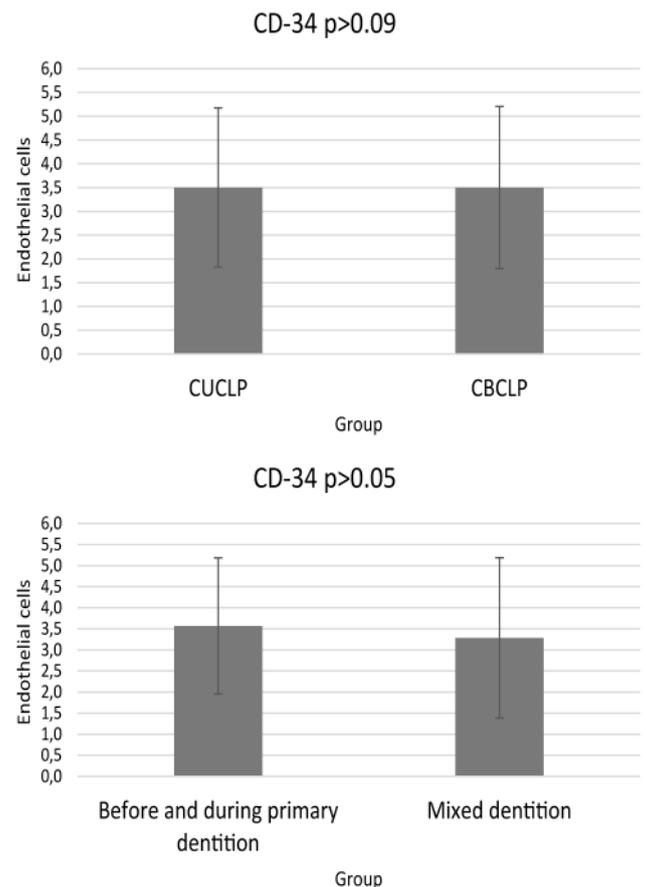


Fig. 6. Comparison of CD34 expression in CUCLP and CBCLP affected endothelial cells before and during primary and mixed dentition age. No significant differences between groups were observed.

factors, such as growth factors, cell-cell interactions, transcriptional regulation and intermediate filament remodeling (18). Nestin expression has been reported in human and rat oral mucosal lamina propria, nasal mucosa, hair follicle, skin progenitor cells and in newly synthesized blood vessels (19-24).

CD34 is a 110-kDa transmembrane surface glycoprotein. The biological function and regulation of CD34 is not well understood. Normally it is expressed on hematopoietic and progenitor stem cells, endothelium, embryonic fibroblasts, the interstitial cells of Cajal, dendritic and mast cells. Barth *et al.* (2004) hypothesized that CD34-positive connective tissue cells may act as multipotent mesenchymal cells (25). CD34 has been used as a marker for epidermal stem cells in mouse hair follicles, and is expressed in the outer root sheath cells of human hair follicles (26, 27). However, CD34 expression is not specific for epithelial stem and progenitor cells in the oral mucosa. However, the present study demonstrated abundant CD34-positive microvessels in the lamina propria. CD34 is an important marker for angiogenesis and represents microvascular density in normal and pathological conditions of

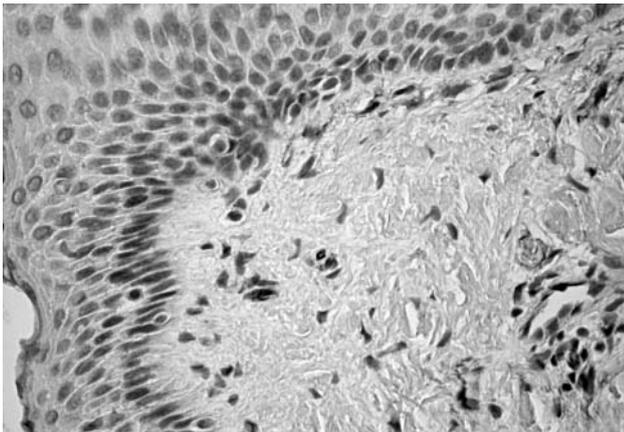


Fig. 7. CD34-positive spindle shaped stromal cells in all CLP cases. CD34 immunohistochemistry, magnification $\times 400$.

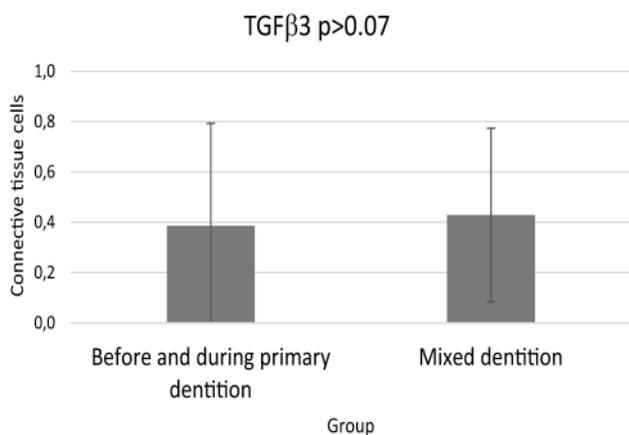


Fig. 9. Occasional TGFβ3-positive macrophages and fibroblasts in the OMLP. TGFβ3 immunohistochemistry, magnification $\times 400$.

oral mucosa. Angiogenesis describes the growth of blood vessels from pre-existing blood vessels and is essential in development, reproduction, and tissue repair. This process initially involves proliferation, sprouting, migration of endothelial cells and is controlled by several signaling molecules, including TGFβ (28, 29).

In this study, ten cases showed occasional TGFβ3 positive structures around blood vessels and five cases contained few TGFβ3 positive macrophages in the visual field. The TGFβ superfamily of cytokines contains more than 30 structurally related polypeptide growth factors, regulating a wide variety of cellular processes such as proliferation, differentiation, migration, epithelial mesenchymal transformation, and apoptosis, as well as physiological processes, including embryonic development, angiogenesis, and wound healing (30). Furthermore, TGFβ signals play important roles in the maintenance and growth of stem cells (31). TGFβ3 is a critical molecule for palatal fusion and mutation of the TGFβ3 gene, as well as inhibition of its expression in humans may result in cleft palate (32, 33).

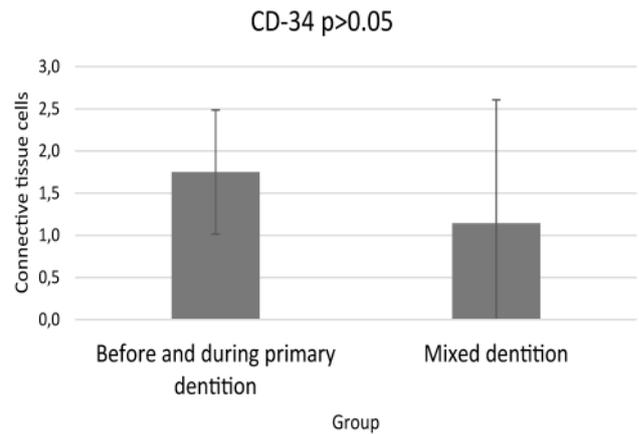


Fig. 8. CD34 expression in CLP patients before and during primary and mixed dentition age. No statistically significant differences were observed.

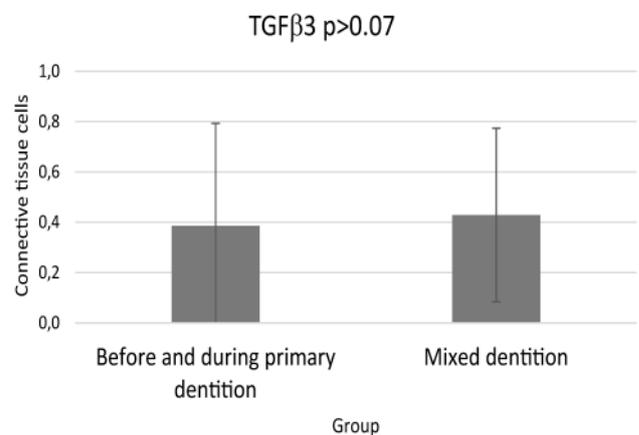


Fig. 10. Comparison of TGFβ3 expression between study groups. No statistically significant differences were observed.

CONCLUSIONS

The prominent expression of nestin in complete unilateral and complete bilateral cleft lip and palate affected soft tissue indicates a possible increase of tissue regeneration. CD34 positive oral mucosa cells indicate increased angiogenesis in complete unilateral and bilateral cleft lip and palate patients, while the sporadic expression of TGFβ3 indicates its insignificant role in the maintenance and growth of cleft affected oral mucosa progenitor cells. Nevertheless, scarce expression of TGFβ3 suggests a role in cleft morphopathogenesis.

STATEMENT OF CONFLICTS OF INTEREST

The authors declare that they have no conflict of interest.

ACKNOWLEDGMENT

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