Oropharyngeal dysbiosis affects postoperative tissue reparative capacity in patients with congenital disorders of maxillofacial region

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Abstract: Background — One of the main causes of hyperergic postoperative tissue response could be a prolonged opening in the septum between normally isolated anatomical regions, e.g., of the nasal cavity and oropharynx in patients with congenital disorders of maxillofacial region, which leads to anomalous exchange of their microbiotas.

Objective — The objective of this study was to determine the composition of culturable facultative anaerobic microbiota of oronasopharyngeal mucosa, and to identify cytokine profiles in patients with congenital disorders of maxillofacial region in both preoperative and postoperative periods.

Methods and Results — Our study is based on the results of examining the children with unilateral congenital cleft of upper lip (CL) before and after surgery, as well as examining the children with simultaneous unilateral congenital cleft of upper lip and palate (CLP) before and after primary rhinocleoplasty. We used ELISA to determine the content of interleukin-10 (IL-10) and interleukin-10 (IL-10) in the samples of blood serum and mucosal surfaces. The study of culturable microflora was conducted in patients before the surgery and during a postoperative period, specifically at one, three, six, and twelve months. Isolation of pure bacterial cultures was performed via conventional bacteriological methods followed by identification using MALDI-TOF testing. Before the surgery, microbial colonization was observed at significantly higher levels in CLP children than in healthy children. After the surgery, microbiological indicators partially came to normal values solely in CL patients. Local IL-10 concentrations remained significantly higher than those found in healthy subjects. In terms of postoperative dynamics, blood plasma antioxidant activity declined below normal values in CLP patients.

Conclusion — Our study demonstrated the need for preoperative eradication of potential pathogens (e.g., Staphylococcus aureus, Klebsiella spp., Candida spp. and Streptococcus spp.), preferably, via using non-antibiotic approaches, such as probiotics and phage therapy, as well as supportive integrative therapy (e.g., using antioxidants).

Keywords: cleft lip, cleft palate, mucosa-associated microflora, dysbiosis, cytokines, serum antioxidant activity.

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Introduction

Orofacial clefts are among the most frequent congenital anomalies with a higher prevalence at birth than neural tube defects, but lower than cardiovascular malformations [1, 2, 3]. In Europe, the combined prevalence of cleft palate (CLP) and cleft lip (CL) at birth is approximately 1 in 700 live births, with certain ethnic and geographic variations [4]. It is believed that the pathogenesis of CL and/or CLP involves both genetic and environmental factors [5, 6]. Moreover, the disorders of maxillofacial region occupy a special place within a large group of congenital abnormalities due to severity of their clinical manifestations, as well as substantial socio-psychological impact on children [7, 8].

The outcomes of surgical interventions for congenital disorders of maxillofacial region greatly depend on the ability of postoperative tissue regeneration. To restore normal functions of the upper lip, it is not sufficient to just recreate its anatomical integrity; it is also necessary to restore the balance of muscle functioning in the region in order to ensure freedom and fullness of upper lip movements. However, postoperative scarring of the upper lip often suddenly reduces its functionality, thereby causing the dysfunction of the orbicularis oris muscle. Later in life, this disorder may interfere with normal articulation. Lip deformity may
lead to the social and psychological maladjustment of a child. Despite continuously improving cheiloplasty/cheilorhinoplasty techniques, along with pharmacological and other wound healing technologies, an incidence of long-term postoperative complications of this pathology remains quite high [9, 10]. As a result, an absolute majority of primary patients who underwent cheilorhinoplasty require repeated corrective interventions [11, 12].

The inefficiencies of surgical corrections of CL and/or CLP complications, as well their prevention, are largely due to insufficient knowledge of the mechanisms of their development. The causes of postoperative complications are likely to be different in different periods of a healing process, and may depend on the extent of surgical trauma and child’s age. Genetically predetermined features of tissue reaction to surgical trauma [13], infectious process in the wound, as well as activation of potential pathogens due to postoperative immunodeficiency, may also be involved [14, 15]. Besides, anomalous hyper- and hypoinflammatory reactions may affect wound healing process [16].

Even though research of various prospects for improving an outcome of regeneration processes was actively explored over the past decades (including consideration of individual polymorphism of genes potentially involved in a healing process [17]), our study did not provide results directly relevant to clinical practices.

It is also known that tissues, injured after any surgical intervention, cause the release of cytokines, prostaglandins and other mediators in charge of activating nonspecific immune response with an involvement of leukocytes, platelets, endothelial cells, and extracellular matrix [18]. Such kind of response inevitably results in oxidative processes, physiologically aimed at cleansing necrotic tissues while stimulating granulation and epithelialization. Such reaction includes activation of leukocytes and may lead to excessive release of free radicals causing an oxidative tissue damage [19, 20]. On the other hand, a sharp drop in radical formation was presumed to contribute to the development of infectious processes on wound surfaces [16, 21].

It has also been demonstrated that changes in the mediator system, which regulates the course of inflammatory and regenerative reactions, are primarily associated with the extensive production of proinflammatory cytokines, such as interleukin-1β (IL-1β) and Tumor Necrosis Factor-α (TNFα), causing the disbalance of a normal ratio between proinflammatory and anti-inflammatory mediators, as well as altered concentrations of fibrogenic growth factors, such as Transforming Growth Factor-β (TGF-β) [18, 22]. An augmented physical pressure during the postoperative wound closure may lead to the damage of intracellular cytoskeleton of fibroblasts, thereby causing an overexpression of TGF-β and other cytokine genes, overdeveloped matrix formation, and hypertrophic scar development [23, 24].

The present-day concept of scar formation also considers an involvement of mast cell activation, which are present in large numbers in hypertrophic scars. These cells contribute to the scarring process by initiating excessive collagen formation due to secretion of histamine, which promotes vasodilation and the exit of plasma proteins into the extracellular space [25].

Anatomical anomalies of nasopharyngeal area in patients with congenital disorders of maxillofacial region result in imbalanced microbiota there [26]. This leads, in particular, to developing inflammatory diseases of oronasopharyngeal area [27]. The chronic inflammation, in turn, affects the condition of mucosal surfaces [28]. The continuous persistence of potential pathogens, such as *Staphylococcus aureus*, at mucosal surfaces promotes formation of pathogenic biofilm, which affects local immunity processes and may, therefore, be considered a leading pathogenic factor of inflammatory complications [29].

In regard to that, currently available data indicate that one of the main causes of hyperergic postoperative tissue response is a prolonged opening in the septum between normally separated anatomical regions, e.g., the nasal cavity and oropharynx. This would lead to the development of anomalous microbiota, i.e., non-characteristic for both sites.

Hence, the objective of our study was to determine possible interrelations between the composition of culturable facultative anaerobic microbiota of oronasopharyngeal mucosae and cytokine profiles in patients with congenital disorders of maxillofacial region in both pre- and postoperative periods.

**Material and Methods**

**Study subjects**

Our study was based on the results of examining 15 children (8 girls, 7 boys, 9-12 months of age) with unilateral congenital CL before surgical operation and 17 children (11 girls, 6 boys, 1-3 years of age) with unilateral congenital CL; 16 children (7 girls, 9 boys, 8-12 months of age) with unilateral congenital CLP before surgical operation and 22 children 1.5-3 years old who underwent primary cheilorhinoplasty. The surgery involved either the reconstruction of upper lip (CL patients), or the reconstruction of upper lip and elimination of unilateral flattening of the nose ala on the side of the cleft (CLP patients). The control groups for examining normal blood levels of cytokines consisted of healthy donors of matching ages, 15 children in each.

Signed informed consent was obtained from the parents. The study design was approved by the Ethics Committee at Kabardino-Balkarian State University. Patient data entry for subsequent analysis was carried out in anonymous mode, with each patient assigned an individual number. The study was conducted in compliance with the Russian Federation Law of December 30, 2017, based on Declaration of Helsinki and its later amendments.

**Immunological and biophysical methods**

To isolate leukocytes, blood (2-5 mL) was taken on an empty stomach from ulnar vein into siliconized polypropylene tubes with EDTA as anticoagulant. Blood was layered upon an equal volume of ficoll-verografin (with a specific gravity of 1.199 g/cm³) and centrifuged at 400 g for 40 minutes to collect leukocytes in interphase. The cells were transferred into the tube, washed twice by centrifugation (400 g, 10 minutes), counted, and the concentration of cells was adjusted to 10⁶ cells/mL.

To obtain blood plasma, peripheral venous blood was collected on an empty stomach from cubital vein into sterile siliconized polypropylene tubes with EDTA as anticoagulant. Stabilized blood was centrifuged at 400g for 5 minutes, and resulting supernatant was collected.

To study cytokines of the mucosae of mouth, nose and pharynx, sterile dental pins were used as previously described [30]. The pins were applied to the surfaces for 30 s then placed into Eppendorf tubes with 1 mL of sterile saline for 40 min. After that,
pins were removed and solutions were evaluated for cytokine activity.

Antioxidant activity of blood plasma was determined sensu Vasiljeva et al. [31].

To investigate the content of IL-1β and interleukin 10 (IL-10) in blood serum and mucosal samples, ELISA kits were used (Vector Best, Russia). The test sample, 30 μL, was added to same amount of incubation buffer and kept at room temperature for 45 minutes. Then, the content of wells was removed, and the wells were rinsed four times. Biotinylated conjugate (100 μL) was added, and the mixture was kept at room temperature for two hours. Following the incubation, the sample was rinsed four times, followed by the additions of a chromogenic solution; and after a 30-minute incubation, 100 μL of stop solution was added, and the optical density was read at 450 nm.

Microbiological methods

The study of culturable microbiota was conducted on patients before the surgery and then at one, three, six, and twelve months after it. The isolation of pure bacterial cultures was performed by conventional bacteriological methods, followed by identification via MALDI-TOF Biotyper (Bruker, Germany).

Statistical analysis

The normality of data was tested using the online tool, AI-Therapy Statistics, at https://www.ai-therapy.com/psychology-statistics/distributions/normal, which generated histograms, a Q-Q plots, calculated skewness, kurtosis and performed Kolmogorov-Smirnov and Shapiro–Wilk tests for normality. Then, Student’s t-test was employed in MS Excel. P-value of less or equal 0.05 implied statistical significance. The microbial counts were converted to log_{10} of the number of viable bacterial colony forming units (CFU) per mL. The results were presented as means ± their standard deviations.

Results

Microbiological features of oronasopharyngeal mucosa in patients with congenital disorders of maxillofacial region

We discovered that the level of total microbial colonization of oronasopharyngeal mucosa of CL patients in preoperative period was 3.5±0.5 log CFU/mL, but it was not significantly different from microbial counts in healthy children (p=0.074). However, in children with CLP, total microbial counts of mucosae of the mouth, pharynx, and nose were significantly higher (4.5±0.5 log CFU/mL) than in healthy children of age-matching group (2.5±0.5 log CFU/mL, p<0.05) (Figure 1).

To assess the presence and severity of dysbiotic changes, we determined the frequency of potentially pathogenic and pathogenic microorganisms in nasal, pharyngeal and oral microbiotas. Among the strains of potentially pathogenic species, Klebsiella pneumoniae and Candida albicans were the most frequent isolates, while top pathogenic representative of mucosal microbiotas in nasal, pharyngeal and oral cavities was S. aureus. In some cases, α-hemolytic streptococci were isolated (Streptococcus pneumoniae). None of these microorganisms were isolated from healthy children (Table 1). The possible explanation for this finding was the presence of abnormal opening between oral and nasal cavities, creating specific microenvironmental conditions at both biotopes, thereby causing the development of deteriorated microbiota (i.e., dysbiosis). On the other hand, we established an increased frequency (5%) of yeast-like fungi in healthy children without clinical signs of dysbiosis, compared with 0% in children of comparable age with CL; even though, before the surgery, children with CLP exhibited elevated levels of Candida spp. These findings imply an urgent need for further research to determine the cause of increased Candida spp. frequency.

In postoperative period, total viable counts of microorganisms normalized, except for the microbiota of nasal cavity, but only in CL patients, whereas in the group of operated CLP children, the counts remained significantly higher (p<0.05, compared with the group of healthy children). The percentage of S. aureus remained high in both groups of operated children.

Local and serum cytokine profiles and antioxidant blood plasma activity in patients with maxillofacial congenital disorders

The analysis of blood cytokines indicated that in the group of patients with CLP, the levels of IL-1β remained above the norm after an initial postoperative increase for a longer time, compared with CL children, and normalized only by day 10 after the surgery vs. day 5 in CL children (Table 2). The difference was most likely due to a larger volume of the surgical intervention in lip and palate plastic surgery.

The levels of anti-inflammatory cytokine interleukin-10 (IL-10), which is a major regulator of innate immunity cell activity, exhibited a different trend: after an initial drop in both groups on days 1 and 3 in postoperative period, its levels grew back to initial (i.e., preoperative) values on days 5 and 10 (Table 3).

The local concentrations of proinflammatory cytokine IL-1β increased significantly in the early postoperative period (Figure 2). However, before surgery, local levels of IL-1β were significantly higher than those in healthy patients (p<0.05).

Blood plasma antioxidant activity decreased below normal values in the group of patients on days 5-10 after the surgical intervention of cheilorrhinoplasty (p<0.05) (Table 4).
Table 1. Potentially pathogenic microorganisms in oronasopharyngeal region of children with congenital disorders of maxillofacial region before and after surgery

| Patient groups                        | Isolation frequency, % |
|---------------------------------------|------------------------|
|                                       | S. aureus | K. pneumoniae | S. pneumoniae | C. albicans |
| Children with CL before surgery       | 36.4      | 9.1           | 0             | 0           |
| Children with CL after surgery        | 33.3      | 0             | 0             | 0           |
| Children with CLP before surgery      | 63.3      | 12.2          | 12.2          | 16.3        |
| Children with CLP after surgery       | 16.3      | 0             | 0             | 0           |
| Healthy children (control)            | 0         | 0             | 0             | 5.0         |

CL, children with unilateral congenital cleft of upper lip; CLP, children with unilateral congenital cleft of upper lip and palate.

Table 2. Serum levels of IL-1β in children with maxillofacial congenital disorders before and after surgery, pg/mL±SD

| Patient groups                        | Observation period, days |
|---------------------------------------|--------------------------|
|                                       | Before surgery | 3 | 5 | 10 |
| Children with CL                      | 13.0±3.0          | 18.3±5.0² | 17.5±5.0¹ | 14.0±3.0 | 14.0±3.0 |
| Children with CLP                     | 13.0±3.0          | 23.0±3.0² | 23.5±1.5¹ | 20.5±2.5¹ | 18.5±1.5² |
| Healthy children (control)            | 15.0±5.0          | NA          | NA          | NA          | NA          |

1 p<0.05 as compared with control group (healthy children); 2 p<0.05 CL vs. CLP groups; 3 p<0.05 as compared with previous time point (day). NA, not applicable; CL, children with unilateral congenital cleft of upper lip; CLP, children with unilateral congenital cleft of upper lip and palate.

Table 3. Serum levels of IL-10 in children with maxillofacial congenital disorders in dynamics, before and after surgery, pg/mL±SD

| Patient groups                        | Observation period, days |
|---------------------------------------|--------------------------|
|                                       | Before surgery | 3 | 5 | 10 |
| Children with CL                      | 10.5±2.5          | 8.5±2.5¹ | 10.5±2.5¹ | 10.0±3.0 | 10.0±2.0 |
| Children with CLP                     | 10.0±1.0          | 5.0±3.0¹ | 7.5±1.5² | 9.5±2.5 | 9.5±1.5 |
| Healthy children (control)            | 10.0±2.0          | NA          | NA          | NA          | NA          |

1 p<0.05 as compared with control group (healthy children); 2 p<0.05 CL vs. CLP groups; 3 p<0.05 as compared with previous time point (day). NA, not applicable; CL, children with unilateral congenital cleft of upper lip; CLP, children with unilateral congenital cleft of upper lip and palate.

Table 4. Levels of blood plasma antioxidant activity in children with congenital disorders of maxillofacial region before and after surgery, %

| Patient groups                        | Observation period, days |
|---------------------------------------|--------------------------|
|                                       | Before surgery | 3 | 5 | 10 |
| Children with CL                      | 50.0±6.0          | 62.0±3.0² | 59.0±6.0 | 55.0±6.0 | 51.0±6.0 |
| Children with CLP                     | 53.0±5.0          | 65.0±2.0² | 62.0±6.0¹ | 48.0±4.0¹² | 47.0±5.0² |
| Healthy children (control)            | 55.0±2.0          | NA          | NA          | NA          | NA          |

1 p<0.05 as compared with control group (healthy children); 2 p<0.05 CL vs. CLP groups; 3 p<0.05 as compared with previous time point (day). NA, not applicable; CL, children with unilateral congenital cleft of upper lip; CLP, children with unilateral congenital cleft of upper lip and palate.

An increased antioxidant capacity was found in blood plasma of CL and CLP patients, as compared with healthy children, on day 1 on the postoperative period (Table 4). However, on days 5-7 after the surgery, antioxidant capacity of plasma in the group of patients with more severe defect (CLP) was below normal, which may imply the lack of a compensatory increase of anti-radical enzymes.

Discussion

Preoperative condition of oral, nasal and pharyngeal mucosae directly affects the surgery outcome [9, 12, 33]. Therefore, the quantitative and qualitative composition of microbiota can be considered as one of the major factors influencing a wound healing process. In regard to this, we examined culturable aerobic and facultative microbiota of mucosal surfaces of oronasopharyngeal region, as well as the parameters of pro- and anti-inflammatory immunity and indices of antioxidant activity in CL and CLP patients before and after the surgery. Our findings indicated that children with CLP, but not with CL, had higher mucosal microbial colonization of oropharyngeal region, compared with healthy children. This finding was consistent with the literature data [24, 26, 32]. Hence, we can confirm the severity of
congenital disorder of the maxillofacial region that directly affects the state of local microbiota [26, 34].

Continuous postoperative presence of microorganisms with high persistent abilities, such as S. aureus, in our patients may indicate their contamination from parents and/or because of inadequate hygiene [29]. High prevalence of S. aureus and significantly higher levels of microbial colonization of mucosa in children, who underwent the surgery for CLP, suggest an involvement of other systemic and local factors, such as mucosal dryness and reduction in local immunological reactivity, which could make the eradication of a pathogen (e.g., S. aureus), rather difficult [29, 34].

As far as we know, ours is the first study that combined microbiological, immunological and biophysical methods to establish the causes for postoperative complications in children with CL and CLP. Our results indicate that during postoperative period, microbiological parameters partially normalize in CL, but not in CLP patients. However, S. aureus occurrence remained high. Moreover, C. albicans were isolated from three patients implying the condition of local dysbiosis.

The markers of the body’s response to operative trauma include blood and tissue levels of free radicals, specifically, production of free oxygen and nitrogen species by phagocytic cells, as well as the antioxidant capacity of tissues and blood plasma [35]. Reactive oxygen species and nitric oxide are potent bactericidal agents protecting a host from infectious agents, including the situation when normal physical barriers are damaged during the surgery [36].

The results of our study indicated that reactive oxygen species were released during the active phase of wound healing process. However, on days 5-7, the antioxidant capacity of plasma in CLP patients was below normal, which presumes the deficiency of compensatory increase in the production of anti-radical enzymes. It is known that the elevated concentrations of free oxygen and nitrogen radicals can interfere with wound healing due to the inhibition of collagenase activity [37, 38]. The expression of collagenase in fibroblasts of hypertrophic scars is downregulated, which results in a reduced collagen degradation and, therefore, in connective tissue remodeling, along with postoperative complications [39].

Conclusion
In children with CLP, the total microbial counts and the percentage of S. aureus on mucous membranes of the mouth, pharynx and nose were significantly higher than those in healthy children of the age-matching group. Prior to the surgery, the local levels of IL-1β were significantly higher than those found in healthy individuals, which was the sign of proinflammatory nature of tissues. Blood plasma antioxidant activity after surgery declined below normal values in the group of patients after cheliorhinoplasty, which indicated the deficiency of compensatory increase in the production of anti-radical enzymes. The study showed the need for preoperative eradication of such potential pathogens as S. aureus, Klebsiella spp., Candida spp., and Streptococcus spp., preferably, via using non-antibiotic approaches, such as probiotics and phage therapy, as well as supportive integrative therapy (e.g., using antioxidants).

Ethical Approval
All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with 1964 Declaration of Helsinki and its later amendments, or comparable ethical standards.

Conflict of Interest
The authors declare no conflicts of interest.

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