

The association between parameters of oral mucosal immunity and 25-hydroxyvitamin D in patients with rampant caries

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Aim. To determine the saliva level of immunoregulatory proteins in patients with rampant caries and 25-hydroxyvitamin D (25(OH)D) deficiency and evaluate the association of their concentration with 25(OH)D plasma level.

Materials and methods. The study was performed in two groups. The experimental group included 15 patients aged 20–22 years with rampant caries and the 25(OH)D plasma level of < 20 ng / ml. The control group encompassed 15 healthy age-matched volunteers with the 25(OH)D plasma level of 20–100 ng / ml. The concentrations of B7.2 (CD86), free active TGF- β 1, CTLA-4, PD-1, Tim-3, LAG-3, IGFBP-4, and ICAM-1 were assessed using flow cytometry. The levels of LL-37 and secretory immunoglobulin A (sIgA) were measured using ELISA. The Spearman's rank correlation coefficient was used to reveal a correlation between the indicated proteins and the 25(OH)D plasma level.

Results. A decrease in B7.2 (CD86), PD-1, Tim-3, sIgA, and LL-37 and elevation of IGFBP-4 and ICAM-1 saliva levels were detected in patients with rampant caries and 25-hydroxyvitamin D deficiency. A positive Spearman's rank correlation coefficient was revealed between plasma 25(OH)D and saliva levels of free active TGF- β 1, CTLA-4, B7.2 (CD86), LL-37, and sIgA. A negative correlation was revealed between 25(OH)D and ICAM-1.

Conclusion. 25(OH)D deficiency in patients with rampant caries is associated with decreased levels of B7.2 (CD86), PD-1, Tim-3, sIgA, and LL-37 and elevated levels of IGFBP-4 and ICAM-1 in the saliva.

Key words: rampant caries, 25-hydroxyvitamin D deficiency, mucosal immunity.

Conflict of interest. The authors declare the absence of obvious or potential conflict of interest related to the publication of this article.

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Conformity with the principles of ethics. All individuals signed an informed consent to participate in the study. The study was approved by the local Ethics Committee at Chita State Medical Academy (Protocol No. 9 of 24.06.2019).

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Взаимосвязь некоторых параметров мукозального иммунитета полости рта с уровнем витамина D у пациентов с множественным кариесом

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РЕЗЮМЕ

Цель – оценить содержание иммунорегуляторных молекул в слюне у лиц с множественным кариесом и дефицитом 25(OH)D₃ и определить взаимосвязи их величин с концентрацией 25(OH)D₃ в крови.

Материалы и методы. Обследованы две группы лиц в возрасте 20–22 лет. В одну включены 15 человек с кариесом и уровнем 25(OH)D₃ менее 20 нг/мл, в другую (контрольную) – 15 здоровых человек с содержанием 25(OH)D₃ 30–100 нг/мл. В ротовой жидкости определены концентрации растворимых форм молекул B7.2 (CD86), Free Active TGF- β 1, CTLA-4, PD-1, Tim-3, LAG-3, IGFBP-4, ICAM-1 методом проточной цитофлуориметрии, количество кателицидина LL-37, секреторного иммуноглобулина А (IgA) методом иммуноферментного анализа. Между определяемыми показателями рассчитан критерий корреляции Спирмена.

Результаты. У лиц с кариесом и дефицитом витамина D выявлено снижение значений Free Active TGF- β 1, B7.2 (CD86), PD-1, Tim-3, sIgA, кателицидина LL-37 и повышение уровня IGFBP-4 и ICAM-1 в слюне. Обнаружено наличие прямых корреляционных связей между количеством 25(OH)D₃ в крови, с одной стороны, и значениями Free Active TGF- β 1, CTLA-4, B7.2 (CD86), секреторного IgA, пептида LL-37 – с другой. Зафиксирована отрицательная взаимосвязь между величинами 25(OH)D₃ и ICAM-1.

Заключение. На фоне дефицита витамина D при множественном кариесе в ротовой жидкости регистрируются низкие концентрации Free Active TGF- β 1, B7.2 (CD86), PD-1, Tim-3, секреторного IgA, кателицидина LL-37 по сравнению с контролем, но увеличены значения IGFBP-4 и ICAM-1.

Ключевые слова: множественный кариес, гиповитаминоз D, мукозальный иммунитет.

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Источник финансирования. Авторы заявляют об отсутствии финансирования при проведении исследования.

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INTRODUCTION

Dental caries is a multifaceted, dynamic pathological process accompanied by demineralization and proteolysis of dental hard tissues [1]. This chronic disease is widespread among both adults and children [2]. Around the world, there are approximately 2.4 billion people with untreated dental caries [3], which in turn determines the importance of developing prognostic methods and finding ways to eliminate its causes.

Dental caries is formed due to complex internal and external causes, such as dietary habits (frequency of sugar consumption), acidogenic bacteria, and impaired local immunity [2]. Numerous salivary proteins are involved in innate and adaptive immune responses in the oral mucosa. Resistance or susceptibility to dental caries significantly correlates with changes in the salivary protein level that modulate the oral microflora. Therefore, the saliva protein profile is a sensitive indicator of dental health and a non-invasive prognostic biomarker [4].

A number of studies showed that rampant caries develops in patients with a lack of vitamin D [5]. The active form of vitamin D, calcitriol, is one of the main hormones that regulate calcium and phosphorus metabolism and provide mineralization of dental hard tissues [6]. Additionally, this biologically active substance can modulate activity of immune cells and functioning of the immune system and contribute to synthesis and release of antimicrobial peptides, in particular, cathelicidin LL-37 [7–9].

Our earlier study revealed the association between low level of 25(OH)D in the blood serum of patients with moderate to severe caries. In our opinion, this confirms the literature data that a lack of bioactive vitamin D plays an important role in the development of dental caries.

The aim of this study was to assess the level of some immunoregulatory salivary proteins in patients with rampant caries and vitamin D deficiency and to determine the relationship between these values and the concentration of 25(OH)D in the blood.

MATERIALS AND METHODS

The study involved 30 male ChSMA students aged 20–22 years who were divided into two groups. The first group (control) included 15 healthy people (the Decayed, Missing, and Filled Teeth (DMFT) index was 0.00 (0.00; 0.00)) with a normal vitamin D level (30–100 ng / ml). The second group included 15 people with rampant caries (DMFT index was 10.3 (9.5; 11.5)) and vitamin D deficiency (25(OH)D was less than 20 ng / ml). The groups were formed taking into account the “Clinical guidelines of the Russian Association of Endocrinologists on diagnosis, and prevention of vitamin D deficiency in adults (2016)”. The serum level of 25(OH)D was determined using the chemiluminescent immunoassay (Immunoassay Analyzer Access 2, Beckman Coulter, USA).

All participants signed an informed consent to take part in the study. The study adhered to the ethical principles of The Declaration of Helsinki of the World Medical Association (as amended in 2013). The levels of soluble membrane proteins (membrane protein of the immunoglobulin superfamily, the product of the *CD86* gene, B7.2 (CD86), free active TGF- β 1, co-inhibitory receptors (cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4), programmed cell death-1 (PD-1), T-cell immunoglobulin and mucin domain-3 (Tim-3), lymphocyte-activation gene-3 (LAG-3), insulin-like growth factor binding protein-4 (IGFBP-4),

and intercellular adhesion molecule-1 (ICAM-1)) were determined in the oral fluid collected from all the participants using the Human Immuno-Oncology Checkpoint Protein Panel 1 bead-based multiplex panel (Biolegend, USA).

The analysis of the oral fluid was performed without dilution, all stages of the study were conducted according to the kit instructions (https://www.biolegend.com/Files/Images/media_assets/pro_detail/datasheets/750000504_HU_Immune_Checkpoint_Panel_1_Manual_R01.pdf). The results were evaluated using a flow cytometer Cytoflex LX (Beckman Coulter, USA).

Additionally, the levels of antimicrobial peptide cathelicidin LL-37 and secretory immunoglobulin A (sIgA) were assessed by enzyme-linked immunosorbent assay (ELISA) using Hycult Biotechnology (Denmark) and IFA-BEST (Russian Federation) reagent kits, respectively. The data are presented as the median and the interquartile range $Me(Q_{25}; Q_{75})$; the Mann – Whitney test was used to compare two independent samples. The non-parametric Spearman's rank-order correlation coefficient was calculated for the correlation analysis. A *p* value of < 0.05 was considered statistically significant. The Statistica 10 software packages were used for statistical processing of the data.

RESULTS AND DISCUSSION

The analysis of the research results showed that with rampant caries, free active TGF- β 1 was significantly reduced in the saliva (by 67.82%) compared with the control group (Figure).

Tooth pulp is known to contain a number of TGF isoforms that are either expressed by odontoblasts, macrophages, and T- and B-lymphocytes in the tooth pulp at the plasma membrane and/or are bound to the extracellular matrix [10]. It was revealed that TGF- β 1 induces synthesis of type III collagen in odontoblasts, regulates transcription of non-collagenous proteins (dentin sialophosphoprotein (DSPP), dentin matrix protein 1 (DMP1)) [10], and is crucial for the reparative process and development of teeth by regulating proliferation and differentiation of cells [10–12]. It is assumed that the physiological function of TGF- β in mature odontoblasts contributes to formation of secondary dentin, mineralization of intact and healthy teeth, as well as to degradation of the matrix in case of injury [11]. Probably, TGF- β 1 is also involved in antimicrobial protection of the tooth, although this mechanism is not fully understood.

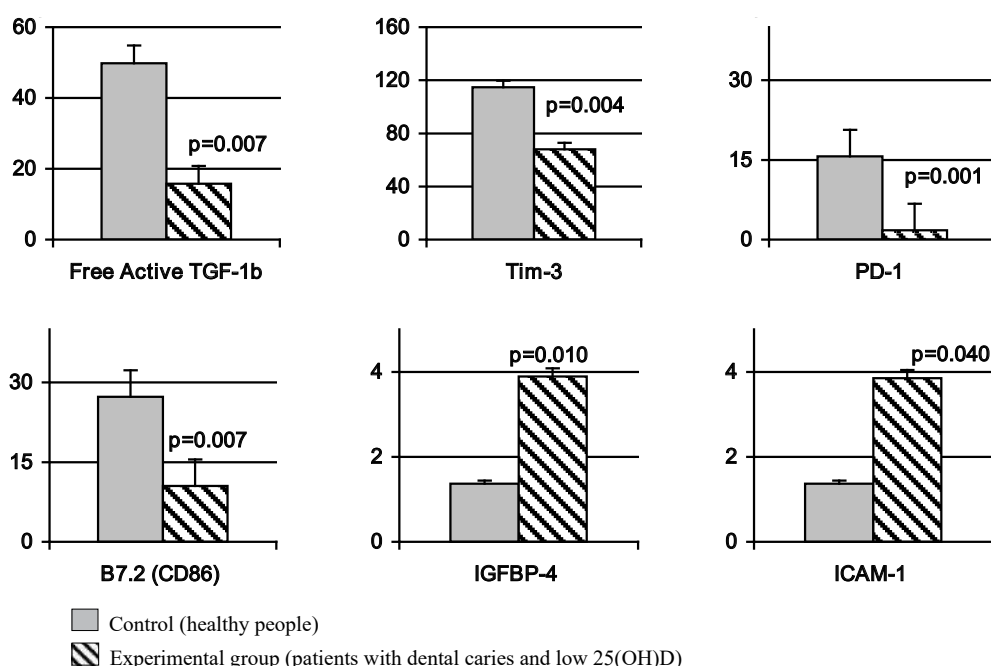


Figure. The saliva level of soluble membrane proteins regulating the immune response, pg / ml

We found a direct moderate relationship ($r = 0.67$; $p < 0.001$) between the levels of free active TGF- β 1 in the saliva and 25(OH)D in the blood in individuals with rampant caries and vitamin D deficiency. No such correlation was found in the control group. There are also data in the literature on the association between TGF- β 1 and vitamin D in patients with some diseases [13] and on the role of vitamin D in TGF- β 1 metabolism [14].

Co-stimulatory and co-inhibitory molecules, also referred to as immune checkpoints, play an important role in the development of an adequate cellular immune response. There are several other pathways of co-stimulation and co-inhibition of T-cells. Co-stimulation is provided by binding B7.2 (CD86) protein to CD28 localized at the T-cell membrane. The co-inhibitory signal, which limits the cellular immune response, is induced by receptors that include molecules of the CTLA-4 family (Tim-3, LAG-3, and TIG-IT). The expression of these molecules begins at the membrane of T-cells after activation of the latter, by binding to B7.2 (CD86) protein, they inhibit the formation of effector T-cells.

The second component of the immune checkpoint is a co-inhibitory receptor PD-1, whose function is somewhat different from that of CTLA-4. Both receptors suppress proliferation of T-cells, their survival, and cytokine synthesis. However, CTLA-4 suppresses the cellular immune response in the early phase pri-

marily in lymphoid tissues, while PD-1 does the same in the late phase in peripheral tissues [15]. Co-inhibitory receptors are crucial for maintaining immune homeostasis and preventing the development of autoimmunity, while providing effective immune responses to destroy pathogenic microorganisms [15].

In our study, the values of the following parameters were significantly reduced in the group of people with rampant caries and vitamin D deficiency (compared with the control group): PD-1 – by 83.82% ($p = 0.001$), Tim-3 – by 40.75% ($p = 0.004$), and B7.2(CD86) – by 61.47% ($p = 0.007$) (Figure). The LAG-3 levels only showed a downward trend and were 50.11% lower than in the control group.

A correlation analysis revealed a direct correlation between the values of CTLA-4 in the saliva and 25(OH)D ($r = 0.63$; $p = 0.010$) in the blood serum, as well as between the values of B7.2(CD86) and 25(OH)D ($r = 0.70$; $p < 0.001$) in patients with dental caries and vitamin D deficiency. Moreover, in this group, a direct relationship was found between the values of CTLA-4 and B7.2(CD86) in the saliva ($r = 0.77$; $p < 0.001$). In the control group, correlations were found only between the values of CTLA-4 and B7.2(CD86) ($r = 0.56$; $p = 0.019$).

Our study also identified an increased level of IGFBP-4 by 187.39% ($p = 0.01$) in the saliva in patients with rampant caries compared with the control (Figure). IGFBP-4 is a protein that modulates the

effect of insulin-like growth factor-1 (IGF-1). Insulin-like growth factor, its receptors, and binding proteins (IGFBPs) are critical for adequate development, tissue growth, metabolism, and homeostasis [16]. Additionally, IGF-1 is the most abundant growth factor in the bone matrix [16], with its molecular mechanisms being involved in osteogenic differentiation. However, there is a lack of information about the functions of six types of its high-affinity IGF-binding proteins (IGFBP 1–6). Available studies are mainly focused on the role of IGFBP-4 and IGFBP-5 in bone tissue formation [17].

We also found that the saliva ICAM-1 values in patients with caries increased by 181.02% ($p = 0.04$) compared with the control group (Figure). Interestingly, this group showed a negative relationship ($r = -0.56$; $p = 0.024$) between the values of the vitamin D metabolite and the values of ICAM-1; in the control group, the relationship was weaker ($r = -0.44$; $p = 0.047$). ICAM-1 is a protein regulating interactions between immune cells and vascular endothelium [18], thus providing strong adhesion of white blood cells to the vessel wall and facilitating penetration of these cells into the intima. Inflammation increases the expression of ICAM-1 [18]. There are studies showing that the level of sICAM-1 in the blood correlated with the severity of periodontitis [<https://www.ncbi.nlm.nih.gov/nlmcatalog?term=%22J+Periodontol%22%5bTitle+Abbreviation%5dhttps://pubmed.ncbi.nlm.nih.gov/23688098/19>]. C.L. Greillera et al. demonstrated that vitamin D metabolites attenuate RV-induced expression of ICAM-1 [20].

In addition to other protective mechanisms, human saliva contains several immunoglobulins, which account for about 5–15% of all salivary proteins [21]. The main subclass of immunoglobulins found in the saliva is IgA (50–60%), which acts as the first line of defense [4]. Several studies examined the relationship between salivary immunoglobulins and caries formation. A. Bagherian et al. [22] and T.K. Fidalgo et al. [23] found higher IgA concentrations in this disease, but another study reported a negative relationship between the level of this immunoglobulin in the saliva and the intensity of caries in children aged 3–6 years [24]. At the same time, no correlations were found between the concentration of this immunoglobulin and the intensity of the pathological process [22].

In our study, we registered that in patients with rampant caries the IgA level in the saliva decreased by 77.62% ($p < 0.001$) compared with the control group, and the concentration of LL-37 decreased by

62.5% ($p = 0.045$) (Table). The correlation analysis revealed a positive relationship between the values of the vitamin D metabolite and the sIgA ($r = 0.88$; $p = 0.001$) and cathelicidin LL-37 ($r = 0.52$; $p = 0.037$) levels in both experimental and control groups.

Table

The saliva concentration of antimicrobial peptides in the oral cavity of patients with rampant caries and 25(OH)D deficiency, $Me (Q_{25}; Q_{75})$		
Parameter	Control group, $n = 15$	Experimental group, $n = 15$
Secretory IgA, g/l	55.00 (50.00; 61.46)	12.31 (9.11; 15.53) $p < 0.001$
Cathelicidin LL-37, ng/ml	0.56 (0.37; 0.72)	0.21 (0.13; 0.38) $p = 0.045$

Note: p – the significance level compared with the control group.

Cathelicidins are antimicrobial peptides essential for innate immunity in the oral cavity [25, 26]. S. Davidopoulou et al. found that the concentration of LL-37 in the saliva was lower in children with highly active dental caries compared with children without this pathology [27]. A bioactive form of vitamin D affects a Th-2 immune response, diminishing the expression of Th-1 cytokines and stimulating release of Th-2 cytokines, in particular, IL-4 [9], which might influence the production of immunoglobulins, including IgA.

Therefore, literature data and the results of this study indicate an imbalance of mucosal immunity as an important factor in the development of dental caries. Additionally, in our opinion, vitamin D deficiency in the body can contribute to the development of this pathology along with decreased levels of free active TGF- β 1, B7.2(CD86), PD-1, Tim-3, sIgA, and cathelicidin LL-37 in the saliva and increased levels of IGFBP-4 and ICAM-1. Prescription of vitamin D may alleviate the detected disorders in the oral mucosal immunity.

CONCLUSION

In vitamin D deficiency and rampant caries, low levels of immunoregulatory free active TGF- β 1, B7.2 (CD86), PD-1, Tim-3, sIgA, and cathelicidin LL-37 and high concentrations of IGFBP-4 and ICAM-1 are found in the oral fluid compared with the control group.

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Authors contribution

Putneva A.S., Karavaeva T.M., Maksimenya M.V., Tereshkov P.P., Tsybikov N.N. – conception and design of the study. Putneva A.S., Tereshkov P.P., Mishchenko M.N., Fefelova E.V. – collection and processing of clinical and biological material. Tereshkov P.P., Putneva A.S., Parshina A.A. – statistical processing, graphic design. Putneva A.S., Karavaeva T.M., Maksimenya M.V. – drafting of the article. Tsybikov N.N., Fefelova E.V., Tereshkov P.P., Mishchenko M.N., Parshina A.A. – editing of the manuscript. Putneva A.S., Karavaeva T.M., Maksimenya M.V., Tereshkov P.P., Mishchenko M.N., Fefelova E.V., Tsybikov N.N., Parshina A.A. – final approval of the manuscript for publication.

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