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Original paper

Pro-inflammatory cytokines and antioxidative enzymes as salivary biomarkers of dentofacial infections in children

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Abstract

Background. Dentofacial infection resulting from untreated dental caries or periodontal disease is a serious disease that can spread to deeper tissues of the face and neck.

Objectives. The present study aimed to analyze the salivary cytokine profile and oxidative stress parameters as potential biomarkers of acute odontogenic infections in children.

Material and methods. The prospective study group (DI) consisted of 28 children aged 3–17 years with acute dentofacial infections, and the control group (CG) comprised 52 children aged 4–17 years with uncomplicated dental caries. The cytokine profile was analyzed using the Bio-Plex Pro™ Human Cytokine 27-plex kit. In addition, oxidative stress parameters, such as catalase (CAT), glutathione reductase (GR), superoxide dismutase (SOD), manganese SOD (Mn-SOD), copper-zinc SOD (CuZn-SOD), total antioxidant capacity (TAC), total oxidant status (TOS), and malondialdehyde (MDA), in the saliva of children in both groups were compared.

Results. The levels of interleukin 6 (IL-6), macrophage inflammatory protein 1 alpha (MIP-1α) and tumor necrosis factor alpha (TNF-α) were significantly increased in children with dentofacial infections as compared to CG. In contrast, the levels of other pro-inflammatory cytokines, such as IL-1β, IL-1 receptor agonist (IL-Ra), IL-8, monocyte chemoattractant protein 1 (MCP-1), and MIP-1β, did not show statistically significant differences between the 2 groups. Among the measured oxidative stress and antioxidative parameters, only CAT and GR were elevated in children with dentofacial infections as compared to controls.

Conclusions. IL-6, MIP-1α, TNF-α, CAT, and GR can serve as selective biomarkers of oral cavity inflammation in children. These biomarkers can be useful in identifying and monitoring the progress and treatment of bacterial infections resulting in dentofacial inflammation.

Keywords: children, cytokines, saliva, antioxidative enzymes, dentofacial infections

Introduction

Dentofacial infections develop due to dental caries and periodontal diseases, such as gingivitis and periodontitis. They can spread to deeper tissues of the face and neck, constituting a life-threatening condition. The outcomes of dentofacial infections are related, at least in part, to the type of bacterial infection, host immunity, dietary factors, and the oral hygiene status.[1–5] Indeed, host immune responses, the quantity and virulence of bacteria, as well as the disease status all play critical roles in the development of bacterial infections, and determine prediction, prevention and intervention with regard to the infection. *Streptococcus mutans*, *Actinomyces* spp. and lactobacilli are considered the main bacteria responsible for the development of dentofacial infections,[6] as they produce a biofilm covering tooth surfaces and gum pockets.

While immune responses in cavities are triggered when odontoblasts in dental pulp become inflamed, the role of saliva proteins in this inflammation process is not fully understood. Therefore, understanding the innate markers underlying dentofacial infections is crucial to ensure oral health and effective protection against the development of cavities and their consequences, such as periodontal tissue inflammation. This focus is consistent with the recent strong emphasis on the natural defense system of the oral cavity and the role of saliva.[7,8]

Unlike blood, saliva is obtained noninvasively, which matters, especially in the case of children. Only a small amount of saliva is needed to analyze a full panel of cytokines and chemokines.[2,3,9,10] Saliva contains various proteins, such as cytokines, chemokines, proline-rich glycoproteins, mucins, immunoglobulins, agglutinins, lactoferrin, cystatins, and lysozyme,[11] which are important in the development of inflammatory processes and their prevention. The role of inflammatory processes in dentofacial infections in children and the contribution of saliva are not fully understood.

Among the cytokines present in saliva, interleukin 1 beta (IL-1β), IL-2, IL-6, IL-8, tumor necrosis factor alpha (TNF-α), and interferon gamma (IFN-γ) play a key role in stimulating the immune system to fight off infection and inflammation. For example, IL-1β plays a role in inflammatory responses, cell death, apoptosis, and bone resorption. It is particularly involved in periodontal diseases, and is linked to TNF-α and IL-6.[12] Chemokines, such as IL-8, play a key role in the activation and migration of neutrophils, the first line of defense against bacteria that enter periodontal tissues from the circulatory blood.[13,14] When neutrophils reach the infected tissues through chemotaxis, they phagocytize and destroy the microorganisms by producing reactive oxygen species (ROS) and proteolytic enzymes. On the other hand, anti-inflammatory cytokines, such as IL-1 receptor antagonist (IL-1Ra) and IL-10, inhibit the production of pro-inflammatory cytokines and help to reduce inflammation.[15] In addition, cytokines such as IL-6 may have both pro-inflammatory and anti-inflammatory properties.

One key aspect related to saliva biology is the relationship between oral infection and inflammation and the role of oxidative stress in these processes. Oxidative stress refers to an imbalance between the production of ROS and the cellular antioxidant defense mechanisms. Reactive oxygen species are highly reactive molecules that can cause damage to cellular components and lead to lipid peroxidation, resulting in the generation of malondialdehyde (MDA).[16] If not countered by antioxidants, oxidative stress can lead to cellular damage and contribute to the development of various diseases. Among the markers of oxidative stress that can be measured in saliva, catalase (CAT) is an enzyme that catalyzes the breakdown of hydrogen peroxide (H_2O_2) to water (H_2O) and oxygen (O_2).[17–19] Glutathione reductase (GR) catalyzes the reduction of glutathione disulfide (GSSG) to its reduced form, glutathione (GSH), being an essential component of the glutathione antioxidant system.[20,21] Total antioxidant capacity (TAC) is a measure of the overall antioxidant capacity,[22] and total oxidative stress (TOS) is a measure of the overall oxidative stress level in biological samples.[19] Superoxide dismutase (SOD) and its isoforms, manganese SOD (Mn-SOD) and copper-zinc SOD (CuZn-SOD), catalyze the conversion of superoxide anions (O_2^-) to hydrogen peroxide (H_2O_2) and molecular oxygen (O_2).[23]

A relationship between dental infections and cellulitis and oxidative stress markers in saliva has recently been suggested.[24,25]

Hence, there is a pressing need for further research focusing on the role of saliva in inflammatory responses to dentofacial infections in children. By elucidating the molecular mechanisms underlying these processes, future studies have the potential to uncover novel diagnostic and therapeutic strategies for improving the management of these infections, and for promoting oral health in pediatric populations.[7,8,14,26]

Our study fills a critical gap in the literature by examining the role of salivary biomarkers in pediatric dentofacial infections. By offering novel insights into the pathogenesis and management of these conditions, we aim to advance both scientific understanding and clinical practice in pediatric oral healthcare.

Therefore, the present work aimed to evaluate whether salivary cytokines and oxidative stress parameters may serve as biomarkers of acute odontogenic oral and facial infections in children.

Material and methods

Study groups

The study was conducted in the years 2020–2022 in the Clinic of Pediatric Otolaryngology, Head and Neck

Surgery of the Department of Pediatric Surgery at the Medical University of Silesia (SUM), Katowice, Poland. It aimed to investigate the prevalence and potential biomarkers of acute dentofacial inflammation in children. The research embraced 2 groups of patients: a study group (DI) of 28 children (7 girls and 21 boys, aged 3–17 years; mean age: 8.67 ±4.64 years) with acute dentofacial infections; and a control group (CG) of 52 children (16 girls and 36 boys, aged 4–17 years; mean age: 8.38 ±3.67 years) with uncomplicated dental caries. The diagnosis of dental-related inflammatory conditions was determined according to the criteria established by the World Health Organization (WHO), which include clinical, radiographic and laboratory factors used to diagnose and classify different types of oral and dental diseases.[27] The WHO criteria provide a standardized approach for diagnosis, which can aid in developing treatment plans and tracking the disease over time. The study was approved by the Bioethical Committee of the Medical University of Silesia (SUM), with reference number PCN/0022/KB1/1/20.

The inclusion criteria were children with dentofacial infections who were free of any systemic diseases and had not taken any medications in the past month. The exclusion criteria comprised the occurrence of systemic conditions that prevented the continuation of the study, the lack of cooperation of a child and the refusal of a parent to participate in the study. All legal guardians and children over the age of 16 signed an informed consent form for the study (Fig. 1).

The examinations were conducted by a single doctor (B.E.O.W.), visually and by touch, and then intraorally using a probe and a mirror. In the DI group, the number of teeth with caries and teeth causing inflammation was determined. In CG, the number of teeth with uncomplicated caries was determined. Saliva was collected in the morning between 8 a.m. and 11 a.m. on an empty stomach, after rinsing the mouth with water and waiting for 10 min. The saliva was then centrifuged at 3,000 rpm for 10 min at 4°C in a Centurion centrifuge (Centurion Scientific Ltd., Chichester, UK) and stored at –80°C for further studies. The importance of the procedure was explained to parents and older children.

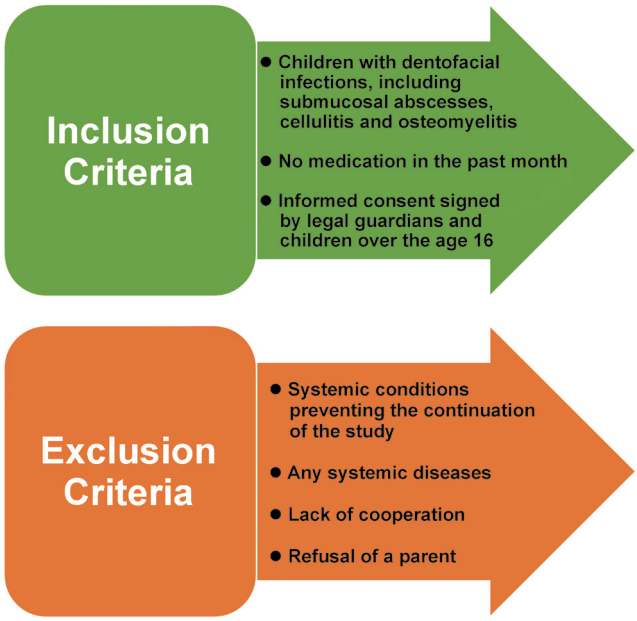

Fig. 1. Inclusion and exclusion criteria

Assessment of inflammatory mediators

The cytokine and chemokine levels were assessed using the Bio-Plex® 200 System and the Bio-Plex Pro™ Human Cytokine 27-plex kit (Bio-Rad Laboratories, Hercules, USA), according to the manufacturer's instructions (Fig. 2A).[28–30] The analyses were performed in the Department of Microbiology and Immunology, Faculty of Medical Sciences in Zabrze, Medical University of Silesia (SUM), Katowice, Poland. All procedures followed the Good Laboratory Practice (GLP) standards. To avoid bias, all samples were anonymized and numbered. All analytical methods were under continuous interlaboratory quality control, and met the criteria of the Central Center for Quality Testing in Laboratory Diagnostics (Lodz, Poland) and Labquality (Helsinki, Finland).

Assessment of oxidative stress and antioxidative potential

The activity of SOD and its isoenzymes (Mn-SOD and CuZn-SOD) was measured as described by Oyanagui.[31] In this method, xanthine oxidase produces superoxide anions that react with hydroxylamine, forming nitric ions. These ions react with naphthalene diamine and sulfanilic acid, generating a colored product, which is proportional to the amount of superoxide anions produced and negatively proportional to the activity of SOD. Absorbance was measured at a wavelength of 550 nm. The enzymatic activity of SOD was expressed in nitric units (NUs). The assessment of Mn-SOD and CuZn-SOD activity employed similar approaches, using potassium cyanide (KCN) as the inhibitor of CuZn-SOD activity. The activity of SOD is equal to 1 NU when it inhibits nitric ion production by 50%. The activity of SOD, Mn-SOD and CuZn-SOD was expressed in NU/mg of protein.

CAT activity was evaluated according to the method described by Johansson and Borg.[17] The method is based on the reaction of the enzyme with methanol in the presence of optimal concentrations of H_2O_2. Formaldehyde produced in the reaction is measured spectrophotometrically at 550 nm as the Purpald® dye (Avantor Performance Materials Poland, Gliwice, Poland). GR activity was measured as described by Richterich and Colombo.[32] The activity was expressed in IU (international unit)/g of protein.

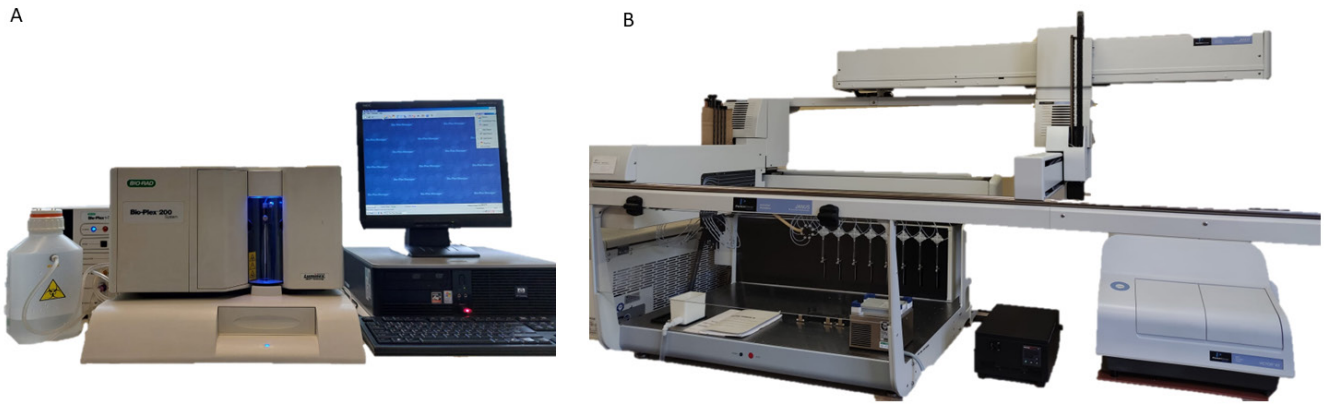

Fig. 2. Bio-Plex 200 System (A) and the JANUS automated analyzer (B)

TAC and TOS were measured according to Erel's protocols.[19] When assessing TAC, a colored 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) radical cation ($ABTS^{*+}$) solution is decolorized by the antioxidants present in the analyzed sample. The reaction efficiency depends on the level of antioxidant compounds. The color change was measured as a change in absorbance at 660 nm, using an automated analyzer (JANUS™; PerkinElmer Inc., Waltham, USA) calibrated with Trolox® (Sigma Aldrich Chemie, Steinheim, Germany) (Fig. 2B). The data is shown in mmol/g. The TOS assay is based on the oxidation of ferrous ions to ferric ions by the oxidant species present in an acidic medium. The measurement of ferric ions with xylenol orange was analyzed as a change in absorbance at 560 nm, using the same automated analyzer calibrated with H_2O_2. The data is expressed in µmol/g.

The MDA levels as a marker of lipid peroxidation were measured fluorometrically as 2-thiobarbituric acid-reactive substances (TBARS), as described by Ohkawa et al.,[33] at 515 nm and 522 nm excitation wavelengths, using the automated analyzer. The TBARS values are expressed as MDA equivalents. Tetraethoxypropane was used as the standard. The concentrations are given in µmol/g.

Statistical analysis

Statistical analysis was performed using Statistica 13 (TIBCO Software Inc., Palo Alto, USA). Student's *t* test and the Wilcoxon signed-rank test were used for the statistical analysis of parametric and nonparametric samples, respectively.

The study sample size was calculated based on mean and standard deviation ($M \pm SD$), as exhibited in the paper by Menon et al.[34] The accepted level of significance was set at $p \leq 0.05$, with a wanted power of 90%. Using a sample size of 24 patients per group, the study would have had a power of 90.9% to yield statistically significant results under the abovementioned conditions.

Results

Impact of dentofacial infection on the levels of pro-inflammatory and anti-inflammatory cytokines in saliva

Table 1 shows the levels [pg/mL] of all cytokines detected in the saliva of all the patients examined. The table includes the $M \pm SD$, minimum (min) and maximum (max) values for each cytokine in each group, as well as the *p*-values indicating the level of statistical significance of the differences between the 2 groups. The results indicate that among the measured pro-inflammatory cytokines, the levels of IL-6 (Fig. 3A), macrophage inflammatory protein 1 alpha (MIP-1α) (Fig. 3B) and TNF-α (Fig. 3C) were significantly higher in children with dentofacial infections as compared to controls with uncomplicated dental caries. In contrast, the levels of IL-1β, IL-1 receptor agonist (IL-Ra), IL-8, monocyte chemoattractant protein 1 (MCP-1), and MIP-1β did not show statistically significant differences between the 2 groups.

Impact of dentofacial infection on oxidative stress and antioxidative parameters in saliva

Table 2 presents the results for oxidative stress and antioxidative factors in the saliva of children with dentofacial infections as compared to controls. The table includes the $M \pm SD$, min and max values, and the *p*-values for each parameter in both groups. Among the studied oxidative stress and antioxidative indicators, the activity of CAT and GR was higher in the DI group as compared to CG, indicating greater antioxidative protection. Specifically, the mean CAT level was 25.52 ±14.50 IU/g, with a minimum of 6.04 IU/g and a maximum of 77.05 IU/g in controls. In children with dentofacial infections, the mean CAT level was higher by 68%, at 42.99 ±19.97 IU/g, with a minimum of 15.63 IU/g and a maximum of 95.42 IU/g (Fig. 4A).

Table 1. Levels of pro-inflammatory and anti-inflammatory cytokines and chemokines [pg/mL] in the saliva of children with dentofacial infections as compared to controls

Variable	Groups						*p*-value
	DI			CG			
	M ±*SD*	min	max	*M* ±*SD*	min	max	
IL-1β	303.18 ±304.54	0.07	1,141.27	167.31 ±181.08	16.22	931.73	0.060
IL-Ra	13,023.07 ±14,951.81	351.39	55,289.18	21,925.36 ±35,614.43	785.40	151,235.55	0.710
IL-6	132.42 ±220.74	0.56	916.60	24.54 ±49.00	0.74	328.94	0.000*
IL-8	956.69 ±1,582.35	0.27	8,168.46	691.86 ±884.51	13.85	3,888.11	0.720
MCP-1 (MCAF)	38.45 ±30.29	0.11	124.16	41.83 ±48.46	2.89	259.23	0.450
MIP-1α	14.86 ±22.02	0.56	93.73	4.61 ±4.36	1.13	26.38	0.020*
MIP-1β	35.29 ±50.36	0.49	180.63	16.21 ±17.76	2.63	103.18	0.100
TNF-α	39.61 ±33.14	3.23	121.91	24.93 ±18.84	5.61	93.38	0.040*

Groups: DI – children with acute dentofacial infections; CG – control group (children with uncomplicated dental caries).
IL – interleukin; IL-Ra – IL-1 receptor agonist; MCP-1 (MCAF) – monocyte chemoattractant protein 1 (monocyte chemotactic and activating factor); MIP – macrophage inflammatory protein; TNF-α – tumor necrosis factor alpha; *M* – mean; *SD* – standard deviation; min – minimum; max – maximum; * statistically significant.

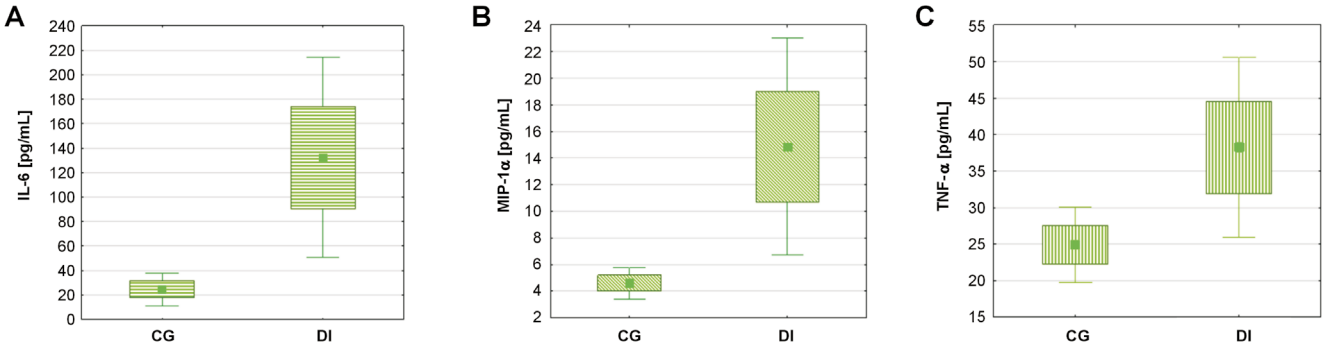

Fig. 3. Box–whisker plot showing the values for the interleukin 6 (IL-6) (A), macrophage inflammatory protein 1 alpha (MIP-1α) (B) and tumor necrosis factor alpha (TNF-α) (C) levels in the dentofacial infections (DI) group and the control group (CG)

The box represents interquartile range (*IQR*), the square inside the box is median (*Me*), and the whiskers represent the min and max values. The dots outside the whiskers represent outliers ($p < 0.01$).

Table 2. Levels of various oxidative stress parameters and antioxidative factors in children with dentofacial infections as compared to controls

Variable	Groups						*p*-value
	DI			CG			
	M ±*SD*	min	max	*M* ±*SD*	min	max	
CAT [IU/g]	42.99 ±19.97	15.63	95.42	25.52 ±14.50	6.04	77.05	<0.010*
GR [IU/g]	6.94 ±5.99	0.43	26.36	3.16 ±2.73	–0.02	15.32	<0.010*
TAC [mmol/g]]	0.05 ±0.11	0.00	0.49	0.08 ±0.20	0.00	1.24	0.850
TOS [μmol/g]	1.98 ±2.42	0.04	10.13	2.00 ±1.89	0.20	8.15	0.600
SOD [NU/mg]	5.59 ±3.13	1.54	10.56	5.92 ±4.59	0.62	22.36	0.960
Mn-SOD [NU/mg]	13.49 ±32.24	0.37	105.00	3.45 ±3.30	0.15	13.86	0.450
CuZn-SOD [NU/mg]	1.68 ±1.75	0.00	6.00	2.53 ±2.42	0.00	9.57	0.450
MDA [μmol/g]	0.26 ±0.29	0.03	0.98	0.23 ±1.17	0.01	1.17	0.800

Groups: DI – children with acute dentofacial infections; CG – control group (children with uncomplicated dental caries).
CAT – catalase; GR – glutathione reductase; TAC – total antioxidant capacity; TOS – total oxidative stress; SOD – superoxide dismutase; Mn-SOD – manganese SOD; CuZn-SOD – copper-zinc SOD; MDA – malondialdehyde; * statistically significant.

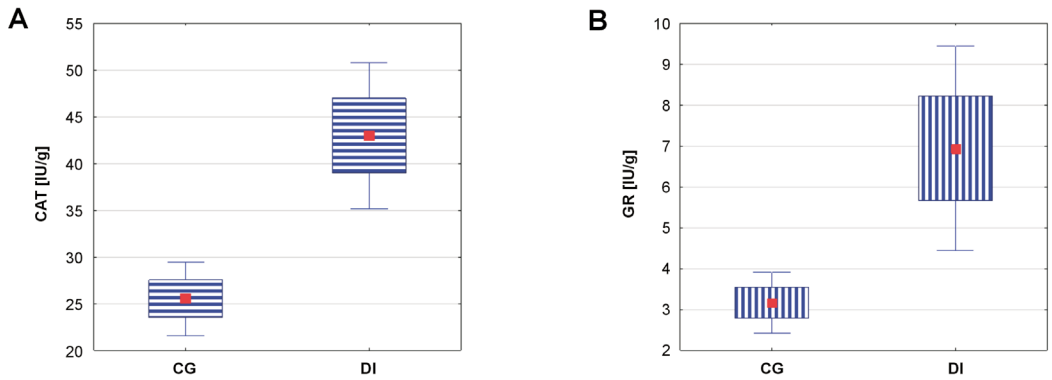

Fig. 4. Box–whisker plot showing the values for the catalase (CAT) (A) and glutathione reductase (GR) (B) levels in the dentofacial infections (DI) group and the control group (CG)

The box represents *IQR*, the square inside the box is *Me*, and the whiskers represent the min and max values. The dots outside the whiskers represent outliers ($p < 0.01$).

Regarding GR, the mean activity in CG was 3.16 ±2.73 IU/g, with a minimum of –0.02 IU/g and a maximum of 15.32 IU/g. In the DI group, the mean activity was higher by more than 100%, at 6.94 ±5.99 IU/g, with a minimum of 0.43 IU/g and a maximum of 26.36 IU/g (Fig. 4B). The differences between the 2 groups in terms of all other oxidative stress parameters and antioxidative factors did not reach statistical significance.

Discussion

This study aimed to determine the levels of interleukins and oxidative stress parameters in the saliva of children with dentofacial infections. This is an important clinical problem, as inflammation in the oral cavity and face region can be caused by several common dental conditions, such as tooth infections, abscesses and periodontal diseases. To date, there have only been a limited number of studies on this topic in the literature, making this research a pioneering effort in the field. To address inflammation in dentistry, strategies such as utilizing chitosan coatings, exploring other natural polymers and embracing the principles of green dentistry can aid in mitigating inflammatory responses and promoting oral health.[35–37] Caries was excluded from this study to focus specifically on dentofacial infections and their associated biomarkers. Nevertheless, there is a need to establish certain biomarkers of dentofacial infection as important prognostic and preventive factors for caries and the threatening complications of this disease.[27] The results of the present study indicate that saliva can be used to study biomarkers that may impact the development of acute deep carious infections in children.

The results of our study indicate that the detection of IL-6 and TNF-α in saliva samples can be used as an indicator of acute inflammation within the oral cavity in children. Indeed, both cytokines were significantly elevated in children with dentofacial infections as compared to controls. They are key mediators of acute inflammation and are responsible for specific immune responses during inflammation. Our results are in line with the reported literature. For example, Gornowicz et al. in their study investigated the levels of TNF-α, IL-6 and IL-8 in patients with and without dental caries, and found statistically significantly elevated levels of these cytokines in the saliva of patients with caries.[13] Moreover, Menon et al. showed that IL-6 significantly correlated with early enamel caries (EEC), and its levels decreased after caries treatment in children.[34] Sharma et al. studied the same cytokines in children with EEC and came to similar conclusions.[38] Zielińska et al. showed that higher levels of TNF-α correlated with high levels of aerobic bacteria, indicating an early immune response.[10] Rinderknecht et al. showed an elevated level of pro-inflammatory interleukins IL-6 and IL-8 in children with periodontitis, and proposed that they might serve as a prognostic or confirming factor for oral inflammation.[11] In contrast, Yoshida et al. showed a significant decrease in the levels of TNF-α, IL-1β, IL-6, and IL-8 under the influence of periodontitis treatment in children with gum inflammation and cerebral palsy.[15] Overall, the results of the present study and literature data suggest that TNF-α and IL-6 may serve as reliable biomarkers of caries and oral inflammation in children. TNF-α, a pivotal cytokine in immune responses, may play a role in dentofacial anomalies, particularly in periodontal diseases, although the exact impact remains under investigation due to inconsistent findings across studies.[39]

Our study indicates that MIP-1α can also serve as a biomarker of dentofacial infection.

Several oxidative stress parameters have been proposed in the literature as potential biomarkers of acute oral and facial inflammation. One of them is the MDA level as a marker of lipid peroxidation, since inflammation can lead to enhanced oxidation of lipids in cell membranes. In our study, the MDA levels remained unchanged in children with dentofacial infections, suggesting that the levels of oxidative stress did not reach the threshold required for an increase in this parameter. The lack of changes in TOS in the DI group as compared to controls confirms this notion.

It is noteworthy that matrix metalloproteinase 8 (MMP-8) and MMP-20 may serve as additional potential biomarkers for assessing the severity of early childhood caries (ECC) and for monitoring treatment outcomes in pediatric patients.[26]

The novel results of the present study indicate increased activity of CAT and GR in the saliva of children with dentofacial infections as compared to CG. These effects may be responsible for the lack of changes in the MDA levels in these children, as both enzymes exert potent antioxidative protection. Indeed, changes in GR activity and/or expression levels have been reported in various diseases, including inflammatory conditions. Glutathione is an important antioxidant that helps protect cells from oxidative damage. Changes in the glutathione levels have been frequently assessed as an indicator of oxidative stress and inflammation in oral and facial tissues. It is important to note that these parameters may be influenced by other factors, such as diet, lifestyle and the disease state.[40,41] Surprisingly, we did not observe any alterations in the activity of SOD and its isoenzymes, Mn-SOD and CuZn-SOD, which have also been used as indicators of oxidative stress in oral and facial tissues.[17,31]

While the results of the present study on saliva-related inflammatory biomarkers are novel and highly promising, it should be noted that collecting saliva samples from young children can be challenging. Some children are uncooperative and do not want to spit into the container, making it difficult to obtain a sufficient sample. Additionally, children who are being prepared for general anesthesia are usually fasting and poorly hydrated, which results in a very poor saliva flow and makes it difficult to collect even a small amount of saliva. Thus, our study also highlights the importance of developing more convenient and noninvasive methods for collecting saliva samples from children.

Limitations

In addition to its numerous advantages, it is important to acknowledge the limitations of saliva-based diagnostics. While saliva offers a noninvasive means of sample collection, its composition can be influenced by various factors, such as diet, the hydration status, the circadian rhythm, and medications, which may introduce variability in the biomarker levels and affect the accuracy of diagnostic tests. Furthermore, the sensitivity and specificity of saliva-based assays may vary depending on the target biomarker and the detection method employed, necessitating validation studies to ensure reliability and reproducibility. Additionally, the current understanding of salivary biomarkers and their diagnostic utility for specific oral healthcare problems is still evolving, requiring further research to establish standardized protocols and reference ranges.

Conclusions

This study suggests that the levels of selected pro-inflammatory cytokines, such as IL-6, MIP-1α and TNF-α, and the activity of antioxidative enzymes, such as CAT and GR, can be used as biomarkers of inflammatory states of the oral cavity and face in children. These biomarkers can provide an insight into inflammatory and oxidative stress responses in children, and may aid in understanding the underlying mechanisms of the disease and in developing potential therapeutic strategies.

Ethics approval and consent to participate

The study was approved by the Bioethical Committee of the Medical University of Silesia (SUM), Katowice, Poland, with reference number PCN/0022/KB1/1/20. All legal guardians and children over the age of 16 signed an informed consent form for the study.

Data availability

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Consent for publication

Not applicable.

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