Original Article

Neutrophil Count and Level of Interleukin-1β and Interleukin-8 in the Saliva of Three to Five Year Olds with and without Dental Caries

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Abstract

Objectives: Saliva plays an important role in prevention of dental caries. Neutrophils are the first defense mechanism of the immune system. Interleukins (ILs) can regulate the activity of neutrophils. This study aimed to assess the neutrophil count and level of IL-lβ and IL-8 in the saliva of children with and without dental caries.

Materials and Methods: This case-control study was performed on 90 preschool children between three to five years. Children were divided into three groups of caries-free, early childhood caries (ECC) and severe early childhood caries (S-ECC). Saliva was collected in tubes by the spitting method. Neutrophil count was assessed by Giemsa staining and the levels of IL-lβ and IL-8 in the saliva were assessed using ELISA. Data were analyzed by the Kruskal-Wallis and post hoc Games-Howell test.

Results: The mean levels of IL-1β and IL-8 and the mean neutrophil count in the caries free group were found to be 59.2± 59.15 pg/mL, 86.04± 96.12 pg/mL and 1342.66± 2222.412 pg/mL, respectively. These values were 36.78± 40.88 pg/mL, 76.12± 107.01 pg/mL and 2500± 3834.61 pg/mL in the ECC group and 48.75±47 pg/mL, 76.77± 70.63 pg/mL and 2353.1± 4583.81 pg/mL in the S-ECC group, respectively. There were no significant differences among the three groups in terms of the levels of IL-1β, IL-8 or the neutrophil count (P>0.05).

Conclusion: Since no significant difference was noted in the salivary levels of IL-1β and IL-8 or the neutrophil count among the groups, development of dental caries may be related to neutrophil chemotaxis defect.

Key works: Dental caries, Children, Saliva, neutrophil, Interleukin-8, Interleukin-1beta.

Journal of Dentistry, Tehran University of Medical Sciences, Tehran, Iran (2015; Vol. 12, No. 9)

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Received: 11 January 2015
Accepted: 27 May 2015

INTRODUCTION

Dental caries is a complex, multifactorial and dietary carbohydrate-modified infectious disease. The saliva is believed to be an important regulatory factor in the process of caries formation [1]. The flow rate and consistency of the saliva, its buffering capacity, remineralization and anti-microbial properties as well as the presence of immunological factors in the saliva affect the

process of caries formation [1,2]. Direct anti-bacterial effect is one of the protective mechanisms of the saliva, performed by different enzymes such as lysozymes. Since neutrophils are among the major sources of lysozyme secreted into the saliva, the important role of neutrophils cannot be overlooked in the protective actions of saliva [3].

In terms of the host immunological responses to caries, much attention has been paid to the role of neutrophils in the saliva as they are considered the first cells entering the gingival sulcus through the blood and are regarded as the first line of defense. In order to effectively control bacterial infections, neutrophils should serve their roles, including trans-endothelial migration, chemotaxis, opsonization, and phagocytosis, precisely and perfectly [4].

The American Academy of Pediatric Dentistry defined ECC as the presence of one or more decayed (cavitated and non-cavitated lesions), missing (due to caries) or filled tooth surfaces in any primary tooth in a child 71 months of age or younger. Any sign of smooth surface caries in children younger than three years of age is indicative of S-ECC. From three to five years of age, S-ECC is defined as one or more cavitated, missing (due to caries) or filled smooth surfaces in primary maxillary anterior teeth or decayed, missing or filled surfaces (dmfs) more than four at three years, more than five at four years or more than six at five years of age [1].

Interleukin-1 is an immune response-modifying cytokine, secreted by activated macrophages when encountered with microorganisms in the infection site; nevertheless, other cells such as neutrophils and endothelial cells are also able to secrete these cytokines. Interleukin-1 producing macrophages contain receptors for IL-1 and can be more activated under the effects of these cytokines which, per se, lead to increased metabolic activity of neutrophils as well as activation of endothelial cells to allow neutrophil migration through the lining of blood vessels. Interleukin-1β is the active and systemic form of IL-1, contributing to the production of IL-8 by macrophages and vascular endothelial cells. Interleukin-8 is secreted by macrophages and endothelial cells as the chemical attractant to draw neutrophils, leading to neutrophil infiltration. It has been established that in-vitro IL-8 results in neutrophil recruitment and increased release of lysosomal enzymes against bacteria [5-7].

Regarding the high prevalence of caries and the associated complications, the present study aimed to assess the neutrophil count (one of the most important anti-caries factors) and levels of IL-1β and IL-8 in the saliva as the most significant factors affecting both the count and function of neutrophils.

MATERIALS AND METHODS

This case-control study was conducted on three to five year-old preschool children in all preschools of the city of Babol who were selected by convenience sampling.

The inclusion criteria consisted of no history of drug consumption (chemotaxis-inducing drugs such as levamisole, H2 antagonists and interferon, anti-inflammatory steroidal and non-steroidal drugs and antibiotics) in the past two months, no history of systemic disease, no permanent tooth erupting, no fluoride therapy during the past six months and no history of inflammatory or infectious dental lesions.

The study protocol was approved by the Ethics Committee of Babol University of Medical Sciences (code: 2865). Parents signed written informed consent forms and filled out a form asking for their child's demographics. Children were enrolled after their parents gave consent for their participation in the study. Intraoral examination was then performed by a pediatric dentist. A total of 90 three to five year-old children selected by convenience sampling were divided into three groups of 30 children each namely the caries-free, ECC and S-ECC groups.

Those who did not have a carious tooth in clinical examination by a specialist were assigned to the caries-free group. Those with dmfs (decayed, missing or filled surfaces) ≤4 at three years, dmfs ≤5 at four years, and ≤6 at five years of age, according to Pinkham's definition, were assigned to the ECC group, and those with one or more cavitated, missing (due to caries) or filled surfaces in primary maxillary anterior teeth and/or dmfs more than the ECC group with respect to age were assigned to the S-ECC group [1].

Unstimulated saliva was collected using the spitting method. Children were asked to avoid eating, drinking, brushing and using dental floss for 90 minutes before sampling. Saliva sampling was done at 10 AM. Then, 3-5 mL of the saliva was transferred into 50 mL capped tubes. Samples collected each day were transferred to a laboratory in a dry ice container. For cellular analysis, 50 µL of the saliva samples was stirred using LS-100 shaker (Labtron Equipment Ltd, Hampshire, UK), and smear was prepared from the fluid obtained. Samples were fixed with Pathofix (Padtan Teb, Tehran, Iran) after being dried, and Giemsa staining was performed afterwards.

The samples were then coded so that the counter was blinded to the group allocation of specimens. All the slides were observed by one person using Olympus CX31 optical microscope (Olympus, Tokyo, Japan) and were examined from left to right in consecutive fields.

The number of neutrophils was counted and multiplied by 20 to be expressed in millimeters. The remaining saliva samples were centrifuged for 10 minutes at 5000 rpm (Spectrafuge^{TM} 24D, Labnet International Inc., NJ, USA), and the supernatant was collected by a sampler and divided into two capped microtubes which were kept at -80 °C until the day of the experiment. On the day of the experiment, all samples were evaluated by ELISA diagnostic kits manufactured by eBioscience (Platinum ELISA, eBioscience, San Diego, USA) according to the manufacturer's instructions using ELISA Reader RT-2100C (RT-2100C microplate reader, CHINCAN, Shanghai, China), and levels of ILs were reported in pg/mL of saliva. Data were analyzed by SPSS version 18 and the Kruskal Wallis test was used to examine the difference in the amount of IL-1β, IL-8 and neutrophil counts among the three groups. For further statistical analysis and intergroup comparisons, Games-Howell test was applied.

RESULTS

Ninety children between three to five years were enrolled. The mean levels of IL-1β and IL-8 and neutrophil counts (mL of saliva) in the three study groups are shown in Table 1. The mean levels of IL-1β and IL-8 are shown in Figure 1 in the three groups.

Using the Kruskal Wallis test, no significant difference was found in the levels of IL-1β, IL-8 and the neutrophil counts among the three groups (Table 1).

Table 1. The mean levels of IL-1β and IL-8 (in pg/mL) and the neutrophil counts (mL of the saliva) in the three study groups

Group	Number	Mean IL-1β level (±SD)	Mean IL-8 level (±SD)	Mean neutrophil count (±SD)
Caries-free	30	59.2 (±59.15)	86.04 (±59.15)	1342.66 (±2222.412)
ECC	30	36.78 (±40.88)	76.12 (±107.01)	2500 (±3834.67)
S-ECC	30	48.75 (±47)	76.77 (±70.63)	2353.1 (±4583.81)
P-value		P=0.382	P=0.862	P=0.467

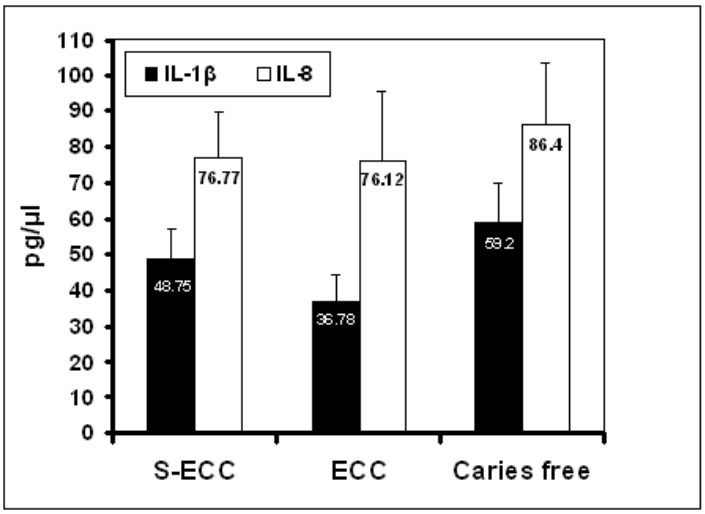

Fig. 1. The mean (±SE) level of IL-1β and IL-8 in the three groups (pg/mL).

Intergroup comparisons also showed no significant difference among the three groups in terms of the levels of IL-1β, IL-8 or the neutrophil count (Table 2).

DISCUSSION

The present study showed no significant difference in the levels of IL-1β and IL-8 or the neutrophil counts among the caries-free, ECC and S-ECC groups.

According to a study by Toomarian and Saberi in 2006, more neutrophils are recruited into the oral cavity in presence of nursing caries in three to five year-old children, which can be due to high levels of chemotactic factors released by Streptococcus mutans i.e. the cause of nursing caries [8]; in other words, in presence of Streptococcus mutans, there is an increase in neutrophil migration from the blood to the saliva as reported in studies by Scully (1982)[9] and Tempel et al, (1970)[10] on the role of polymorphonuclear leukocytes in periodontal disease; however, such finding was not obtained in the current study.

In a study by Izumi et al, in 1995, it was stated that the number of neutrophils increased with development of caries and they appear when the carious lesion is approximately 2 mm away from the pulp chamber [11].

Although the neutrophil counts were lower in caries-free group compared to the ECC and S-ECC groups, this difference was not statistically significant. Such controversy in results may be related to how the neutrophils were evaluated since in the study by Izumi et al, the number of existing neutrophils had been examined in decayed tooth pulp, while in our study, the neutrophil count was evaluated in the saliva [11].

Van Dyke et al, in 1982 demonstrated that products released from Gram-positive bacteria inhibit neutrophil chemotaxis. In fact, these products compete with the chemotactic agents for their receptors on neutrophils; binding of these products to the receptors inhibits chemotaxis. It seems that no difference in the number of neutrophils among the three groups in our study may be attributed to their chemotaxis defect due to the function of Gram-positive bacteria such as streptococci [12]. Moore and Gregory in 1998 also investigated the activity of neutrophils against Streptococcus mutans isolated from the saliva of patients with and without caries as well as those with root surface caries and found that the mean neutrophil activity in caries-free subjects was 25-30% higher than that in subjects with caries [13].

On the contrary, the mean neutrophil activity in Streptococcus mutans-induced root surface caries was 45-50% lower than that in other groups. They suggested that biological alterations in Streptococcus mutans lead to difficulty in being detected by neutrophils, and the colonization and survival of Streptococcus mutans are, therefore, higher on the tooth surfaces [13]. However, Steinberg et al, in 1999 showed that Streptococci with extracellular polysaccharides contributed to reduced production of toxic oxygen products by neutrophils, and no similar effect was observed by Streptococci without extracellular polysaccharides [14]. Kowolick et al, also revealed elevated number of white blood cells and neutrophils with increasing level of plaque accumulation [15]. In studies by Toomarian and Saberi in 2006 [8], Scully in 1982 [9] and Kowolick et al, in 2001 [15], it was concluded that elevated number of Streptococcus mutans was associated with increased number of saliva neutrophils; but, such an evaluation was not made in the current study and only the presence and the severity of dental caries were addressed.

In a study by Toomarian et al, in 2011 on the role of neutrophils in dental caries, it was demonstrated that the number of apoptotic neutrophils was higher in patients with ECC than caries-free individuals and it can be justified by noting that even though neutrophils pass through the gingival sulcus in presence of dental caries, they will die of bacterial agents and the number of active neutrophils will be similar to that of caries-free individuals [16]. Although neutrophil counts were lower in caries-free group compared to other groups in the current study, this difference was not statistically significant. Moreover, in studies conducted so far, no attention has been paid to the role of IL-1β and IL-8 with regard to the degree of dental caries.

In a study by McLachlan et al, in 2004, increased levels of IL-1β and IL-8 were noted in carious dental pulp. Normally, IL-1β plays an important role in neutrophil migration through the lining of blood vessels via affecting the endothelial cell lining of blood vessels. In addition, as a chemokine, IL-8 causes neutrophils to migrate towards the site of chemotaxis [17].

Table 2. Intergroup comparison of the mean variables using the Games-Howell test

Dependent variable	Group	Group	Difference between the means	P-value
Neutrophil count	Caries-free	ECC	- 1157.33	0.353
		S-ECC	-1010.43	0.537
	ECC	Caries-free	- 1157.33	0.353
		S-ECC	146.89	0.991
	S-ECC	Caries-free	1010.43	0.537
		ECC	- 146.89	0.991
IL-8 level	Caries-free	ECC	9.91	0.927
		S-ECC	9.26	0.919
	ECC	Caries-free	- 9.91	0.927
		S-ECC	- 0.65	1
	S-ECC	Caries-free	- 9.26	0.910
		ECC	0.65	1
IL-1β level	Caries-free	ECC	22.42	0.228
		S-ECC	10.44	0.740
	ECC	Caries-free	- 22.42	0.228
		S-ECC	- 11.97	0.547
	S-ECC	Caries-free	- 10.44	0.740
		ECC	11.97	0.547

However, in our study, the presence of neutrophils was not dependent upon increase in IL-8 levels and, perhaps, the role of other chemokines such as bacterial lipopoly-saccharides and C5a component of the complement system should be taken into account considering the increased number of neutrophils in groups with dental caries compared to the caries-free group; hereof, higher degree of active caries leads to greater neutrophil recruitment to the site due to presence of higher levels of chemotactic factors derived from cariogenic Streptococci such as Formyl Methionine-Leucine-Phenylalanine (fMLP), as well as the factors resulting from the effects of bacteria on the complement system such as C5a. The difference between the results obtained in this study and those of other investigations may also be due to the number of samples studied. Variables such as level of education of parents, daily intake of sugar and nutrition during infancy as factors playing a role in the formation of dental caries, were not evaluated in our study. Further studies are required to assess the activity of salivary neutrophils and their count with more accurate quantitative techniques such as flow cytometry. Also, the relationship of cariogenic bacteria with the salivary neutrophil count can be an interesting research topic for future studies.

CONCLUSION

Lack of a significant difference in the neutrophil count among the three study groups was in line with the lack of a significant difference in IL levels among the three groups; because IL-1 β and IL-8 are chemotactic for neutrophils. This finding may be due to the neutrophil chemotaxis defect because of the increased levels of the afore-mentioned ILs in presence of dental caries. Therefore, further studies are required to evaluate the reasons behind the chemotaxis defects at various levels.

ACKNOWLEDGMENTS

The authors would like to thank the Deputy of Research and Technology of Babol University of Medical Sciences for their financial support and also the staff of Cellular and Molecular Biology Research Center of Babol University of Medical Sciences, especially Mr. Aghajanpour for technical assistance with ELISA. This paper is part of a research project (No. 8929313), approved by the Research Council, and the thesis project of Dr. Mohammad Ali Zaghian in the Dental Faculty of Babol University of Medical Sciences.

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