Comparison of Neutrophil Apoptosis α-Defensins and Calprotectin in Children with and without Severe Early Childhood Caries

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ABSTRACT

Background: The infectious nature of severe early-childhood caries (S-ECC) points to the possible participation of immunologic host responses including neutrophils and their antimicrobial products. **Objectives:** The aim of this study was to determine the neutrophil apoptosis, α-defensins (HNP1-3) and calprotectin levels in the saliva of preschool children and the association with S-ECC. **Methods:** Oral examinations were performed on 87 children aged 3-5 years and non stimulated whole saliva samples were collected. Thirty of these subjects were considered S-ECC children, 30 with moderate caries (MC) and 27 were caries free (CF). To detect apoptosis, cell staining was done with Annexin-V-Fluos and propidium iodide, and they were analyzed by fluorescent microscopy. The concentration of α-defensins and calprotectin were assessed using ELISA. **Results:** There were no statistical differences between groups considering the HNP1-3 or calprotectin salivary levels (p=0.06 and p=0.23, respectively). The HNP1-3 and calprotectin levels were negatively correlated and the correlation was significant in MC group (p=0.03). Lower levels of apoptotic neutrophils were obtained from CF subjects as compared with S-ECC children (p=0.03). **Conclusions:** Our findings establish that apoptotic mechanisms could be implicated in the immunity responses associated with S-ECC. We cannot yet determine if the level of salivary α-defensins or calprotectin is predictive of S-ECC.

Keywords: Severe Early Childhood Caries, α-Defensins, Calprotectin, Neutrophil Apoptosis

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INTRODUCTION

Severe early-childhood caries (S-ECC) is an extremely destructive form of early-childhood caries involving multiple teeth, including the anterior maxillary teeth (1). The cause of S-ECC remains speculative. Although S-ECC is commonly assumed to be caused by prolonged use of a nursing bottle (2), but dietary intake alone may not account for the severe nature of this disease (3). As a consequence, current research aims at identifying risk factors for caries as well as identifying innate immunity that may protect against or prevent caries development (4).

Polymorphonuclear leukocytes (PMNs) are key components of the first line of defense against microorganisms (5). Pathogens have evolved diverse mechanisms to evade the innate immune response and escape clearance by immune cells. Modulation of PMN apoptosis is recognized as a key mode of immune evasion, altering the timing of PMN death (5). Shortened PMN survival may contribute to susceptibility to severe and recurrent infections in some pathological situations (6,7). Neutrophil apoptosis may be associated with a multitude of effector molecules which release within them. Density gradient separation of disrupted PMN reveals three major fractions with nonoxidative antimicrobial activity: primary (azurophil) granules, secondary (specific) granules, and cytoplasmic antimicrobial proteins. Antimicrobial substances known to reside in primary granules include defensins, elastase, collagenase, proteinase 3, myeloperoxidase, lysozyme, bacterial permeability factor, and cathepsin. Secondary granule proteins include lysozyme, collagenase and lactoferrin (8). The major cytoplasmic antimicrobial protein appears to be the zinc-binding protein, calprotectin (9).

The neutrophil α-defensins, HNP1-3, participate in nonoxidative microbial death (10) and were shown to opsonize several different bacterial strains, leading to enhanced phagocytosis (11). HNPs are found both in saliva (12) and in gingival crevicular fluid (GCF) (13), suggesting to play a role in the maintenance of microbial homeostasis.

Calprotectin is an S100 calcium- and zinc-binding protein, making up to 30% to 60% of the cytosolic protein of neutrophils (9). In addition to antimicrobial activities (14,15), calprotectin and its subunits appear to have regulatory functions in the inflammatory process and have been used as markers for disease activity (16,17). Calprotectin has been identified in various body fluids, including GCF (18-20) and saliva (21,22). In periodontitis, gingivitis, and other oral mucosal inflammatory diseases, the expression of calprotectin increases in the gingival epithelium (23,24).

The possible involvement of endogenous host-associated attributes in ECC process in children has so far received relatively little attention. The objective of this investigation is to determine the levels of α-defensins, calprotectin and neutrophil apoptosis in the saliva of preschool children and to study their association with S-ECC status.

MATERIALS AND METHODS

One hundred and twenty 3-5 year-old children from six preschool centers in north Tehran were included in this study. The study protocol was approved by the Ethics Review Committee of Shahid Beheshti University of Medical Sciences. Informed consent was obtained from all children's parents or guardians prior to the study. A brief health history survey was completed by parents and only children with healthy

background, no periodontal or infectious disease, dental abscess, and also without history of taking any kind of medication or congenital syndromes were entered to the study. Information about the child's duration and type of nocturnal nursing, birth type and order was obtained. The educational level of parents was assessed separately for the fathers and the mothers and categorized into four: illiterate, primary or middle school, high school, and university education.

Oral examinations were performed by a single calibrated investigator (N.H.) and the subjects were allocated according to the range of caries experience as S-ECC, moderate caries (MC) and caries free (CF). The criterion for caries diagnosis was according to the WHO recommendations (25). Decayed teeth included the teeth with visually diagnosed cavitated lesions. If doubt existed, the surface was investigated with a CPI probe. When the tip failed to enter the lesion, the tooth was recorded as sound. The child was considered to have S-ECC if he/she had dmf score of more than four, five and six at age of 3, 4 and 5 years, respectively (26). Children were considered CF when they did not show any clinical signs of dental caries.

Nonstimulated whole saliva (1 ml) was collected by means of a sterile disposable polyethylene Pasteur pipette. Sample collection was conducted between 9 and 11 a.m. The salivary samples were placed immediately into transport fluid micro tubes, codified, and transferred on ice to the laboratory. The tubes were stored at -70ºC. The concentrations of α-defensin HNP1-3 and calprotectin were determined by commercially available enzyme-linked immunosorbent assay (ELISA) kits (α-defensin: HK 317, HyCult Biotechnology, Uden, Netherlands; Calprotectin: HK 325, HyCult Biotechnology, Uden, Netherlands). Data were presented in ng/ml for HNP1–3 and in μg/ml for calprotectin concentration.

Apoptosis was detected using Annexin-V-FLUOS Staining Kit (Roche Applied Science, Mannheim, Germany) as recommended by the manufacturer. This assay includes Annexin-V-Fluorescein for the detection of apoptosis and Proidium iodide (PI) as an indicator of necrosis. Annexin-V binds phosphatidylserine (PS) and is translocated to the external membrane surface in apoptotic cells (27), and PI, a DNA stain, permeates necrotic cells. The cells were analyzed by fluorescent microscopy and the percentage of apoptotic cells was calculated from the proportion of Annexin V positively stained neutrophils in relation to total neutrophils.

Statistical analysis was performed using SPSS 15 software (SPSS Inc., Chicago, IL). Chi-square test was used to assess distribution equivalence of age and gender in 3 groups. Comparison of apoptotic neutrophil percentages and calprotectin levels was made using one-way ANOVA. Comparison of the HNP1-3 levels was made using Kruskal-Wallis test. Correlations between HNP1-3 levels, calprotectin levels, and neutrophil apoptosis percentages were analyzed using Pearson and Spearman's correlation coefficients. The level of significance was regarded as $p<0.05$.

RESULTS

Thirty three samples were eliminated either because of incomplete questionnaire or insufficient sample; so that 48 females and 39 males finally participated in the study. All children were Iranian, between 3 and 5 years of age and in the primary dentition period. Thirty children were considered S-ECC, 30 with MC, and 27 were CF (Table 1).

There were no statistical differences between groups considering the demographic characteristics of the children (p>0.05).

Table 1. Demographic characteristics of the children.

Group		S-ECC	MC	CF	Total
number		30	30	27	
Gender (%)	Male	53.3	43.3	37	44.8
	Female	46.7	56.7	63	55.2
Age in years (%)	3	33.3	30	40.7	34.5
	4	33.3	26.7	29.6	29.9
	5	33.3	43.3	29.6	35.6
Nocturnal nursing	Yes	83.3	73.3	74.1	77
	No	16.7	26.7	25.9	23
Type of nocturnal nursing	Brest feeding	43.3	46.7	44.4	44.8
	Bottle feeding	30	10	22.2	20.7
	Both	26.7	43.3	33.3	34.5
Duration of nocturnal nursing (month)	Mean	21.54	21.55	16.3	20
	SD	16.03	10.92	13.19	13.73
Type of birth	Cesarean	73.3	83.3	77.8	78.2
	Normal	26.7	16.7	22.2	21.8
Order of birth	First	73.3	73.3	59.3	69
	Second	26.7	26.7	40.7	31
Mother's educational level	Illiterate	3.3	0	0	1.1
	Primary or middle school	6.7	6.7	3.7	5.7
	High school	36.7	23.3	25.9	28.7
	University education	53.3	70	70.45	64.4
Father's educational level	Illiterate	0	0	0	0
	Primary or middle school	10	3.3	0	4.6
	High school	30	36.7	22.2	29.9
	University education	60	60	77.8	65.5

The mean percentage of apoptotic neutrophils are summarized in Table 2. Significantly lower numbers of apoptotic neutrophils were obtained from CF subjects as compared with S-ECC children (p=0.03).

Table 2. Percentage of salivary apoptotic neutrophils.

Group	No.	Mean	SD	95% CI[#] for mean	Minimum	Maximum
S-ECC	30	30.77	14.80	25.24-36.30	7	60
MC	30	26.33	13.39	21.33-31.33	8	52
CF	27	23.00	11.95	18.27-27.73	8	50
Total	87	26.83	13.70	23.91-29.75	7	60

[#]CI: Confidence Interval

The mean levels of HNP1-3 and calprotectin are summarized in Tables 3 and 4, respectively.

Table 3. The level of salivary HNP1-3 in different groups of patients.

Group	No.	Mean (ng/ml)	SD	95% CI# for mean	Minimum	Maximum	Median	Mean rank
S-ECC	30	87.07	7.79	14.16-89.98	40.43	105.51	87.56	42.15
MC	30	86.91	13.61	81.82-91.99	28.89	101.19	92.29	52.37
CF	27	84.13	17.34	17.26-90.99	2.35	100.69	86.22	36.76
Total	87	86.10	13.25	83.27-88.93	2.35	105.51	-	-

*NA: Not applicable
#Confidence Interval

Table 4. The level of salivary Calprotectin in different groups of patients.

Group	No.	Mean (μg/m)	SD	95% CI# for mean	Minimum	Maximum
S-ECC	30	2.84	1.06	2.44-3.24	1.49	5.64
MC	30	2.37	1.48	1.81-2.92	0.95	6.98
CF	27	2.92	1.43	2.36-3.49	1.42	8.67
Total	87	2.70	1.34	2.42-2.99	0.95	8.67

#Confidence interval

There were no significant differences between children's caries severity and HNP1-3 salivary levels (p=0.06). Moreover, Calprotectin level was not significantly different among the groups (p=0.23). Additional analysis showed that there was a significant negative correlation between HNP1-3 and calprotectin levels in MC group (p=0.03) (Table 5).

Table 5. Correlation between neutrophil apoptosis, HNP1-3 and calprotectin salivary levels within groups.

	Correlation coefficient (p value)		
	S-ECC (n=30)	MC (n=30)	CF (n=27)
Neutrophil apoptosis and HNP1-3	0.095 (0.618)	-0.033 (0.861)	0.074 (0.715)
Neutrophil apoptosis and calprotectin	0.313 (0.093)	-0.232 (0.217)	-0.236 (0.235)
HNP1-3 and calprotectin	-0.05 (0.980)	-0.401(0.028)*	-0.362(0.064)

*Statistically significant correlation at p< 0.05

DISSCUSION

Severity of dental caries varies widely among individuals, and this variability is probably due to differences in microflora as well as to differences in the immune responses against oral microflora. In general, the oral immune network prevents the invasion of the host by the oral microflora (28). PMNs are important effector cells in first-line defense against bacterial pathogens (28). Indeed, induction of death in PMNs by cariogenic bacteria might play an important role in the initiation and progression of dental caries. In this report, we provide the first hand evidence that the percentage of the apoptotic neutrophils obtained from the saliva of S-ECC children is significantly higher than the number found in CF children.

Apoptosis is an essential process in which cells are deleted in a controlled manner without perturbing surrounding cells and tissues and is observed in many biological phenomena, such as involution of the thymus, turnover of enteric crypt epithelial cells, and remodeling of embryonic tissues (29). This is an intrinsic cellular process that can also be induced by a variety of environmental factors, such as ionizing radiation, stress-related hormones, oncogenes and viral infection (30-34). Apparently in these cases apoptosis serves in favor of microorganism as the defense mechanism is impaired. By this logic, apoptosis may also occur in cells infected by pathogenic bacteria (28). In fact, there are a number of reports of the death of immune cells induced by bacterial pathogens (35-38). In this respect, we propose that an increase in neutrophil apoptosis might serve to decrease the ability of the host to regulate the development of an immune response to cariogenic pathogens, thereby impairing the development of innate immune system cross-talk needed to clear the infection. Furthermore, we have tried to correlate the percentage of these apoptotic cells with the levels of α-defensins and calprotectin in saliva.

The concentration of HNP1-3 in non stimulated saliva of preschool children has not been previously reported, although healthy adults and middle school children had a mean value of 0.8 μg/ml (39) and 0.94 μg/ml HNP1-3 (4), respectively. The large variation found in the concentration of α-defensins in saliva of preschool children in this study could be attributed to previously demonstrated polymorphisms in sequence and copy number of the genes encoding these peptides (40,41), as well as to variations in neutrophil efflux via the gingival fluid (4). Neutrophils, that migrate into the oral cavity via GCF, and also salivary duct cells are the sources of HNP1-3 (4). In clinically healthy gingiva, approximately 30,000 neutrophils per minute migrate through the junctional epithelia of all human teeth into the oral cavity (42).

Due to the infectious nature of ECC and the potential for the participation of HNP1-3 in its pathogenesis, we hypothesized that HNP1-3 in CF children may present higher concentrations compared to children with carious lesions. Tao et al. already claimed that individuals with higher levels of HNP1-3 would be better protected from dental caries (4). However, our results did not prove this hypothesis, and in fact we did not find any significant differences regarding HNP1-3 levels among the groups. Nevertheless, the difference of age in two studies should be kept in mind. The HNP1-3 may not be expressed in preschool children as much as in older children, considering that the host defense mechanisms are not completely established in younger children (43). It should also be noted that the presence of loose teeth and mucosal inflammation could deteriorate the levels of α-defensins in saliva of middle school children.

The results of the present study may partly be interpreted by the simultaneous presence of gingival inflammation in patients with caries. It should be noted that parents of children with ECC usually show a poor quality in the performance of oral hygiene procedures, which can favor the development of pathogenic dental plaque, especially in the cervical third of the child's teeth, leading to the establishment of gingivitis (43,44). Because the gingival fluid increases during gingival inflammation, it is most likely that patients with gingivitis will show more neutrophil efflux, resulting in higher levels of HNP1-3 in the saliva. The results could also be due to the higher percentage of apoptotic neutrophils in the S-ECC group compared to CF group and thereby their degranulation may result in the higher production of HNP1-3.

A novel aspect of the present study was the detection of calprotectin in saliva of preschool children. The primary source of calprotectin in tissues and body fluids has

been suggested to be the cytosol of dead or lysed neutrophils (45). Although many studies have shown a correlation between salivary proteins and glycoproteins with caries experience (46,47), but no study has investigated the association between salivary calprotectin level and caries experience. Considering broad-spectrum antimicrobial effects of calprotectin (14,15), we hypothesized that its expression may kill or inhibit the growth of cariogenic bacteria within oral cavity. It has been suggested that an important antimicrobial mechanism of calprotectin is through competition for zinc, which deprives organisms of a required nutrient (48,49).

In periodontitis, calprotectin levels in GCF from diseased sites are significantly higher than those in GCF from healthy sites and show positive correlations with several clinical and biochemical markers, including probing depth, interleukin 1b, prostaglandin E2, collagenase, and aspartate aminotransferase activity (50,51). It has been demonstrated that expression of calprotectin protects epithelial cells in culture against binding and invasion by *Porphyromonas gingivalis*. In periodontal disease, calprotectin may augment both the barrier protection and innate immune functions of the gingival epithelium to promote resistance to *Porphyromonas gingivalis* infection (52). However, our results show that maybe this is not true for dental caries. There was not necessarily a negative relationship between the calprotectin level and the severity of caries in children. Although the presence of calprotectin evokes soft tissue response, hard tissue seems not to be affected much.

Although no significant correlation was found between HNP1-3 and calprotectin levels and caries severity in our subjects, this may have been due to the relatively small sample number. A study of a larger sample of preschool children at various caries levels would be required to explore the role of salivary HNP1-3 and calprotectin in this population. The sample number in our study was significantly limited due to the problems in recruiting the subjects who were able to participate in the process of saliva collection. The controversial findings published in the literature and the results obtained in this report suggest that, in order to prevent diseases such as the S-ECC, it could be advantageous to study the role of salivary components such as defensins and calprotectin in young children.

Overall, PMN apoptosis or survival during caries infections can have important consequences in promoting or impairing the ability of the host to clear infection. Future studies will hopefully provide a clearer picture of the nature of these pathological interactions so as to provide novel, therapeutic targets for the treatment of dental caries. Indeed, it remains to provide proof for the concept of inhibiting PMN death early after infection as a mechanism in preventing further dental disease evolution to ECC.

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