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Salivary peptide human neutrophil defensin 1–3 and its relationship with early childhood caries

Trophimus Gnanabagyan Jayakaran[1], C. Vishnu Rekha[2], Sankar Annamalai[3], Parisa Norouzi Baghkomeh[3]

[1]Department of Pediatric and Preventive Dentistry, SRM Dental College, [2]Department of Pediatric and Preventive Dentistry, Sathyabama Dental College and Hospital, [3]Department of Pediatric and Preventive Dentistry, Meenakshi Ammal Dental College and Hospital, Chennai, Tamil Nadu, India

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Address for correspondence:
Dr. Trophimus Gnanabagyan Jayakaran,
Department of Pediatric and Preventive Dentistry, SRM Dental College, Ramapuram, Chennai - 600 089,
Tamil Nadu, India.
E-mail: trophy.2000@gmail.com

ABSTRACT

Background: This study aimed to evaluate the relationship of the level of salivary peptides human neutrophil defensin (HNP) 1–3 in children with and without early childhood caries (ECC).

Materials and Methods: This *in vitro* study was conducted among 86 children of age 3–6 years who were divided into two groups: Group 1 – children with ECC (n = 43) and Group 2 – children without ECC (n = 43). Saliva samples were collected, and salivary peptide HNP1–3 levels were analyzed using enzyme-linked immunosorbent assay. The data collected were subjected to appropriate statistical analysis. Independent sample t-test was used to compare the mean salivary peptide levels of HNP1–3 in children with and without ECC. One-way ANOVA was used for intragroup comparison of the mean peptide levels between the ages. $P < 0.05$ was considered statistically significant.

Results: The mean age of the children in Group 1 and Group 2 was 5.12 ± 0.851 and 4.88 ± 0.879 years, respectively. A statistically significant decrease was seen in salivary peptide HNP1–3 levels in children with ECC (1.44 ng/ml) when compared to children without ECC (6.04 ng/ml) with $P < 0.001$. There were no statistically significant differences in the gender- and age-based comparisons.

Conclusion: A decrease in salivary peptide HNP1–3 levels might be a biological factor for predisposition to ECC and hence can be used as a predictive and a preventive tool in caries prevention.

Key Words: Defensin, early childhood caries, peptide, saliva

INTRODUCTION

Dental caries is a common disease that affects a large proportion of the world's population.[1] Extensive research indicates that dental caries is the result of bacterial infection and is also influenced by host and dietary factors. This dental caries, which is clinically manifested through the decalcification of dental tissues, and demonstrated in young children in an acute nature, is known as early childhood caries (ECC).[2] ECC is a serious public health problem in both developing and industrialized countries. It begins early in life, progresses rapidly in those who are at high risk, and often goes untreated.[3]

Human saliva possesses several functions involved in oral health and homeostasis, with an active

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protective role in maintaining oral health. It also contains appreciable quantities of free amino acids and low-molecular-weight peptides.[4,5] These molecules are exchangeable with plaque fluid, being not only potential metabolic substrates for the plaque microflora, but also products of microorganic metabolic pathways.[6] Antimicrobial peptides are natural antibiotics that provide a first line of defense against a wide spectrum of pathogens. These peptides may be particularly present in the oral cavity, where members of the microbial flora are present in high numbers at all times. Hence, levels of these molecules in saliva may express a higher or lower risk of developing this disease. The ability of peptides to kill or inhibit the proliferation of pathogenic microorganisms has been previously described.[7] In human adults, some peptides with antibacterial activity have been identified, including histatins, defensins, and the human cathelicidin LL37,[7,8] which demonstrates the potential importance of these molecules in oral infectious disease.

Recent research suggests that defensins and the cathelicidins are antibacterial agents in the oral cavity, whereas histatins are primarily antifungal agents.[9] The human β-defensins (hBDs) are widely expressed in oral tissues and the gingival epithelium. The hBD 1 and -2 have also been detected in salivary glands and ducts in the saliva.[10] The α-defensins human neutrophil defensin (HNP) 1–3 are expressed in neutrophils and participate in nonoxidative microbial death and have been identified in gingival crevicular fluid.[11] The human cathelicidin peptide LL37 is found in neutrophils and inflamed epithelia as well as in saliva.[7] This pattern of expression suggests that both the defensins and LL37 could have a role in protecting the tooth structure from caries as well as protecting oral mucosa, and there is scanty information in the literature regarding this.

Hence, this *ex vivo* study was aimed at evaluating the relationship in the level of salivary peptide HNP1–3 in children with and without ECC.

MATERIALS AND METHODS

This *in vitro* study was conducted among 86 children of age 3–6 years who reported to the Pediatric Dental Outpatient Department of Meenakshi Ammal Dental College and Hospital, Chennai. The sample size was calculated based on previous studies[12] with a power of 80% using the Open Source Epidemiologic Statistics for Public Health, OpenEpi, Version 3.01 software 2013, California, USA. The study was approved by the Institutional Review Board of Meenakshi Ammal Dental College and Hospital, Meenakshi University, Chennai, with IRB number MADC/IRB/2015/80.

Parental consent was obtained prior to the study. Children from both genders of age 3–6 years were screened by a single qualified dentist for the presence or absence of ECC. Children with systemic or congenital diseases, or those who were using any medications during saliva collection period or earlier, were excluded from the study. Caries experience was determined through visual and tactile methods by using a dental explorer and a mouth mirror on a dental chair with dental light, and the mean decayed, missing, filled teeth (dmft) score was recorded.[12] The teeth were identified as being decayed when at least one surface presented with clinical signs of cavitated lesion. Eighty-six children from both genders were selected and separated into two groups with an equal distribution of 43 children in each group, based on the dmft score as, Group 1 children with ECC (dmft >1) and Group 2 children without ECC (dmft <1).

Two milliliters of unstimulated saliva was collected from both children with and without ECC into a wide-mouth 15-ml sterile plastic container as described by Fonteles *et al*.[13] Two milliliters of saliva was collected in the early hours of the day, and the participants were instructed to refrain from eating or drinking 1 h prior to the saliva sample collection to avoid contamination with nonsalivary components. One milliliter of the collected saliva samples was centrifuged at 3000 rpm for 10 min at 4°C in a cooling centrifuge (REMI Laboratory Instruments Pvt Ltd., Cama Industrial Estate, Mumbai, Maharashtra, India). The supernatant was transferred into individual sterile Eppendrof tubes and kept in a deep freezer for 20 days until all the samples were collected. The saliva samples were then kept in room temperature and were used to measure salivary HNP1–3 by enzyme-linked immunosorbent assay (ELISA) (Elabscience Biotechnology Co. Ltd, Houston, Texas, USA). The results were noted from the ELISA reader.

Independent sample *t*-test was used to compare the mean salivary peptide levels of HNP1–3 in children with and without ECC. Independent sample *t*-test was used for intergroup (between Groups 1 and 2) and intragroup (within one group) comparisons and

the intergroup comparisons of mean peptide levels between the ages separately. One-way ANOVA was used for intra-group comparison of mean peptide levels between the ages separately.

RESULTS

A total of 86 samples were collected for the study with an equal distribution of 43 samples in each group: Group 1 with ECC (mean age 5.12 ± 0.851) and Group 2 without ECC (mean age 4.88 ± 0.879). A comparison of the mean salivary peptide levels of HNP1–3 between the study groups showed a statistically significant lower salivary peptide level of HNP1–3 ($P < 0.001$) in Group 1, which was 1.44 ng/ml [Table 1].

Intragroup comparison between the mean salivary peptide levels of HNP1–3 based on the gender showed higher mean salivary peptide levels in males than in females in both groups, which was not statistically significant [Table 2]. Intergroup comparison between the mean salivary peptide levels of HNP1–3 based on the gender showed lower mean salivary peptide levels in the ECC group in both males and females when compared to children without ECC, which was statistically significant [Table 3].

The intragroup comparison of mean salivary peptide levels among different age groups did not show any statistically significant difference in both the groups [Table 4]. The intergroup comparison of mean salivary peptide levels among different age groups showed statistically significant difference in both the groups except for children aged 3 years, which was not statistically significant [Table 5].

DISCUSSION

Salivary defense plays a significant role as a part of innate immune system in maintaining the health of the oral cavity by its antimicrobial activities including microorganism aggregation and clearance from the oral cavity and immune surveillance.[14,15] Antimicrobial peptides which are locally expressed or systemically recruited as mediators, are endogenous and gene encoded and provide a rapid first line of defense against invading microorganisms.[15-18]

Defensins are small, cationic antimicrobial peptides that can kill a wide variety of Gram-positive and Gram-negative bacteria, fungi, as well as enveloped viruses such as herpes simplex. Defensins are grouped into two subfamilies referred to as α- and β-defensins. Till date, six α-defensins have been identified in humans; four have been found in neutrophils and are called HNPs1–4. α-defensins are synthesized as precursors which are stored as granules and are proteolytically cleaved and activated before release.[19,20]

HNP1–3 in saliva may contribute to resistance of caries by direct antimicrobial properties, either alone or in combination with other saliva components or by preventing biofilm formation on the surface of the tooth through its ability to bind to bacterial outer membranes.[12]

Proteins that originate peptides in whole saliva are derived mainly from the secretions of the parotid, submandibular, sublingual, and minor salivary glands. Nevertheless, a small amount of protein present in whole saliva originates from oral microorganisms, crevicular fluid, epithelial cells, polymorphonuclear leukocytes, and dietary compounds.[12] As salivary compounds can undergo changes in the presence of oral disease, such as dental caries, this study aimed to find the possible association between salivary peptide HNP1–3 in children with and without ECC using ELISA.

Although many studies have identified peptides in saliva,[6-8,12,14,21] very few studies have associated salivary peptides and caries experience,[6,12,14,21] and of these, only two studies have investigated its relationship with ECC.[12,21]

Previous studies have reported the collection of unstimulated saliva for peptide analysis and stimulated saliva for microbial analysis, as stimulated saliva removes even the plaque present on the tooth surface which will help in microbial analysis.[12] In the present

Table 1: Comparison of mean salivary peptide levels of human neutrophil defensin 1-3 in children with and without early childhood caries

Variable	Group	*n*	Mean (ng/ml)±SD	*t*	*P*
Salivary peptide HNP1-3	Group 1 (with ECC)	43	1.44±0.87	6.920	<0.001
	Group 2 (without ECC)	43	6.04±4.24		

HNP: Human neutrophil defensin; ECC: Early childhood caries; SD: Standard deviation

Table 2: Intragroup comparison of mean salivary peptide levels of human neutrophil defensin 1-3 based on gender in children with and without early childhood caries

Group	Gender	*n*	Mean peptide (ng/ml)±SD	*t*	*P*
Group 1 (with ECC)	Male	22	1.517±1.010	0.374	0.710
	Female	21	1.416±0.728		
Group 2 (without ECC)	Male	22	6.632±4.209	0.939	0.353
	Female	21	5.415±4.288		

ECC: Early childhood caries; SD: Standard deviation

Table 3: Intergroup comparison of mean salivary peptide levels of human neutrophil defensin 1-3 based on gender in children with and without early childhood caries

Group	Gender	*n*	Mean peptide (ng/ml)±SD	*t*	*P*
Group 1	Male	22	1.517±1.010	5.54	<0.001
Group 2		22	6.632±4.209		
Group 1	Female	21	1.416±0.728	4.21	<0.001
Group 2		21	5.415±4.288		

SD: Standard deviation

Table 4: Intragroup comparison of mean salivary peptide level of human neutrophil defensin 1-3 based on the age of children with and without early childhood caries

Group	Age	*n*	Mean peptide (ng/ml)	SD	*F*	*P*
Group 1 (with ECC)	3	2	1.310	0.179	0.502	0.683
	4	7	1.835	1.621		
	5	18	1.437	0.820		
	6	16	1.361	0.495		
	Total	43	1.467	0.874		
Group 2 (without ECC)	3	4	6.046	4.857	0.086	0.968
	4	7	5.520	3.745		
	5	22	6.344	4.500		
	6	10	5.722	0.495		
	Total	43	6.038	0.874		

ECC: Early childhood caries; SD: Standard deviation

study, unstimulated saliva was collected for the assay, which is in accordance with Ribeiro *et al*. who stated that children displayed uncooperative behavior on collecting stimulated saliva.[12]

The results of the present study suggest that the mean salivary peptide level of HNP1–3 was significantly less in children with ECC (1.44 ng/ml) when compared to those without ECC (6.04 ng/ml). This is in accordance with studies done by Tao *et al*.[14] and Ribeiro *et al*.,[12] where the levels of HNP1-3 were significantly high in caries-free individuals when compared to children with ECC.

On the contrary, Fonteles *et al*.[13] had reported the presence of free proline, a free amino acid, which increased the risk of ECC. Similarly, Dunsche *et al*.[10] observed the higher salivary concentration of HNP-1 in patients with oral diseases such as lichen planus, leukoplakia, and squamous cell carcinoma in contrast to healthy individuals. Mumcu *et al*.[15] identified the increase in salivary HNP1–3 in children with Behcets disease. The large variation in the concentration of α-defensins in saliva could be due to polymorphism in sequence and copy numbers in the gene encoding these peptides.

In the present study, even though there is an increase in the mean salivary peptide HNP1–3 level in males than in females, it was not statistically significant.

In general, a sexual dimorphism has been reported in immune function, with females being more "immunocompetent" than males.[22] Srivastava *et al*. also reported higher gene expression in females related to age in human parotid gland.[23] In the literature, no data exist on differences in salivary peptide HNP1–3 levels between males and females.

There are no other studies in literature comparing the salivary HNP1–3 levels in children with and without ECC based on the age. However, few studies have associated salivary peptides and caries experience in adolescents and young adults. Gornowicz *et al*.[24] reported a decrease in salivary peptide in young adults with caries when compared to those who were caries free. However, in the present study, there is no significant difference in mean salivary peptide HNP1–3 level in both the groups based on age.

The prediction of caries risk has been of long-standing interest and is very important for the development of new preventive strategies for caries. This is especially significant for young children and for children with special health-care needs.[21] Saliva is an easily available fluid which can be collected noninvasively and used to measure and monitor the risk for caries. The assay for HNP1–3 is easy to perform and can be done in <1 ml whole saliva. Based on the present study, the inverse correlation of HNP1–3 with caries experience suggests its possible protective effect. Conversely, low levels of HNP1–3 may result in increased susceptibility to caries.

However, further studies with large sample size are required to confirm the correlation of salivary peptide level and the caries susceptibility and to consider it as a valuable biomarker for predicting and preventing ECC.

Table 5: Intergroup comparison of mean salivary peptide level of human neutrophil defensin 1-3 based on the age of children with and without early childhood caries

Group	Age	*n*	Mean peptide (ng/ml)±SD	*t*	*P*
Group 1	3	2	1.310±0.179	1.30	0.263
Group 2		4	6.046±4.857		
Group 1	4	7	1.835±1.621	2.389	0.034
Group 2		7	5.520±3.745		
Group 1	5	18	1.437±0.820	4.55	<0.001
Group 2		22	6.344±4.500		
Group 1	6	16	1.361±0.495	4.016	<0.001
Group 2		10	5.722±0.495		

SD: Standard deviation

In addition, future studies could be done by synthesizing specific peptides with protective properties and adding to dental materials, toothpastes, topical fluorides, chewing gums, and other preventive products or may be used independently providing anticariogenic effect.

CONCLUSION

Within the limitations of this study, the following conclusions can be drawn:

- The mean salivary peptide HNP1–3 level was significantly lower in children with ECC when compared to children without ECC
- The mean salivary peptide HNP1–3 level was slightly high in males when compared to females within each group, which was not statistically significant. The mean salivary peptide HNP1–3 level was less in children with ECC for both the genders when compared to children without ECC, which was statistically significant
- The mean salivary peptide HNP1–3 level did not show any significant difference based on their age within the group. The mean salivary peptide HNP1–3 level was statistically lower in children with ECC for all age groups except for 3-year-old children, which was statistically significant.

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Conflicts of interest

The authors of this manuscript declare that they have no conflicts of interest, real or perceived, financial or non-financial in this article.

REFERENCES

1. Loesche WJ. Role of *Streptococcus mutans* in human dental decay. Microbiol Rev 1986;50:353-80.
2. Hicks J, Garcia-Godoy F, Flaitz C. Biological factors in dental caries: Role of saliva and dental plaque in the dynamic process of demineralization and remineralization (part 1). J Clin Pediatr Dent 2003;28:47-52.
3. Weinstein P, Domoto P, Koday M, Leroux B. Results of a promising open trial to prevent baby bottle tooth decay: A fluoride varnish study. ASDC J Dent Child 1994;61:338-41.
4. Pobozy E, Czarkowska W, Trojanowicz M. Determination of amino acids in saliva using capillary electrophoresis with fluorimetric detection. J Biochem Biophys Methods 2006;67:37-47.
5. Schipper RG, Silletti E, Vingerhoeds MH. Saliva as research material: Biochemical, physicochemical and practical aspects. Arch Oral Biol 2007;52:1114-35.
6. Perinpanayagam HE, Van Wuyckhuyse BC, Ji ZS, Tabak LA. Characterization of low-molecular-weight peptides in human parotid saliva. J Dent Res 1995;74:345-50.
7. Murakami M, Ohtake T, Dorschner RA, Gallo RL. Cathelicidin antimicrobial peptides are expressed in salivary glands and saliva. J Dent Res 2002;81:845-50.
8. Ayad M, Van Wuyckhuyse BC, Minaguchi K, Raubertas RF, Bedi GS, Billings RJ, *et al*. The association of basic proline-rich peptides from human parotid gland secretions with caries experience. J Dent Res 2000;79:976-82.
9. Oppenheim FG, Xu T, McMillian FM, Levitz SM, Diamond RD, Offner GD, *et al*. Histatins, a novel family of histidine-rich proteins in human parotid secretion. Isolation, characterization, primary structure, and fungistatic effects on *Candida albicans*. J Biol Chem 1988;263:7472-7.
10. Dunsche A, Açil Y, Dommisch H, Siebert R, Schröder JM, Jepsen S. The novel human beta-defensin-3 is widely expressed in oral tissues. Eur J Oral Sci 2002;110:121-4.
11. McKay MS, Olson E, Hesla MA, Panyutich A, Ganz T, Perkins S, *et al*. Immunomagnetic recovery of human neutrophil defensins from the human gingival crevice. Oral Microbiol Immunol 1999;14:190-3.
12. Ribeiro TR, Dria KJ, de Carvalho CB, Monteiro AJ, Fonteles MC, de Moraes Carvalho K, *et al*. Salivary peptide profile and its association with early childhood caries. Int J Paediatr Dent 2013;23:225-34.
13. Fonteles CS, Guerra MH, Ribeiro TR, Mendonça DN, de Carvalho CB, Monteiro AJ, *et al*. Association of free amino acids with caries experience and mutans streptococci levels in whole saliva of children with early childhood caries. Arch Oral Biol 2009;54:80-5.
14. Tao R, Jurevic RJ, Coulton KK, Tsutsui MT, Roberts MC, Kimball JR, *et al*. Salivary antimicrobial peptide expression and dental caries experience in children. Antimicrob Agents Chemother 2005;49:3883-8.
15. Mumcu G, Cimilli H, Karacayli U, Inanc N, Ture-Ozdemir F, Eksioglu-Demiralp E, *et al*. Salivary levels of antimicrobial peptides Hnp 1-3, Ll-37 and S100 in Behcet's disease. Arch Oral Biol 2012;57:642-6.
16. Soehnlein O. Direct and alternative antimicrobial mechanisms of neutrophil-derived granule proteins. J Mol Med (Berl) 2009;87:1157-64.
17. Kanmura S, Uto H, Numata M, Hashimoto S, Moriuchi A,

Fujita H, *et al*. Human neutrophil peptides 1-3 are useful biomarkers in patients with active ulcerative colitis. Inflamm Bowel Dis 2009;15:909-17.
18. Gomes Pde S, Fernandes MH. Defensins in the oral cavity: Distribution and biological role. J Oral Pathol Med 2010;39:1-9.
19. Abiko Y, Nishimura M, Kaku T. Defensins in saliva and the salivary glands. Med Electron Microsc 2003;36:247-52.
20. Dale BA, Tao R, Kimball JR, Jurevic RJ. Oral antimicrobial peptides and biological control of caries. BMC Oral Health 2006;6 Suppl 1:S13.
21. Colombo NH, Ribas LF, Pereira JA, Kreling PF, Kressirer CA, Tanner AC, *et al*. Antimicrobial peptides in saliva of children with severe early childhood caries. Arch Oral Biol 2016;69:40-6.
22. Nunn CL, Lindenfors P, Pursall ER, Rolff J. On sexual dimorphism in immune function. Philos Trans R Soc Lond B Biol Sci 2009;364:61-9.
23. Srivastava A, Wang J, Zhou H, Melvin JE, Wong DT. Age and gender related differences in human parotid gland gene expression. Arch Oral Biol 2008;53:1058-70.
24. Gornowicz A, Tokajuk G, Bielawska A, Maciorkowska E, Jabłoński R, Wójcicka A, *et al*. The assessment of sIgA, histatin-5, and lactoperoxidase levels in saliva of adolescents with dental caries. Med Sci Monit 2014;20:1095-100.