Evaluation of Salivary HNP1-3 profile in children with and without early childhoodcaries using Liquid Chromatography Mass Spectrometry (LC-MS)

Section A-Research paper

EVALUATION OFSALIVARY HNP1-3PROFILEIN CHILDREN WITH AND WITHOUT EARLY CHILDHOODCARIES USING LIQUID CHROMATOGRAPHY MASS SPECTROMETRY (LC-MS)

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Abstract

Aim: To evaluate the salivary HNP1-3profile in children with and without Early Childhood Caries.
Materials and Methods:A pilot study with a convenience sample (n=10) were included in the study and were divided into two groups. Group 1 – children with early childhood caries (n=5) and Group 2 – children without early childhood caries (n=5). Saliva samples were collected and salivary HNP1-3 levels were analysedusing Liquid chromatography mass spectrometry.
Results: Pool chromatograms showed qualitative differences in HNP1-3 (1744 m/z) profile, noted in peaks 14 and 21, were present in the caries free pool, but absent in the ECC pool.
Conclusion:A decrease in the salivary peptides HNP1-3 profile in children with ECCcan help establish specific preventive and treatment protocols.

Keywords: Saliva, defensin, Early childhood caries, Liquid Chromatography Mass spectrometry

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1. Introduction:

Dental cariesis an infectious process which leads to the breakdown of enamel. It is a multifactorial disease, formed through a complex interaction between cariogenic acid-producing bacteria in combination with fermentable carbohydrates and other dietary, genetic, behavioural, social, and cultural factors.[1,2]Children are susceptible to caries as soon as the first teeth erupt, which usually occurs around 6 months of age.This dental caries, which clinically manifests as decalcification of dental tissues, and demonstrated in young children in an acute nature is known as Early Childhood Caries (ECC).[3]Over the past several decades, dentalcaries experience in children has focused predominantly on early childhood caries(ECC) which is also considered as a global public health problem that continues to be a major threat in a child'soral health. [4,5,6]ECC is defined as the presence of one or more decayed (non-cavitated or cavitated lesions), missing (due to caries), or filled tooth surfaces in any primary tooth in a child under the age of six. [7]ECC is a serious oral health problem, especially in the under privileged communities in both developing and industrialized countries. [8,9]

Saliva plays an important role in the caries risk prediction because of its composition, which, because of its composition, can be used as a medium for monitoring the primary factors in the etiology of dental caries. [10]Salivary defence systems play a major role in maintaining the health of the oral cavity and preventing caries.Salivary defences comprise factors which reverse or inhibit demineralization of exposed tooth surfaces, which include simple mechanical rinsing, buffering action, and calcium phosphate binding proteins as well as antimicrobial activities including microorganism aggregation and clearance from the oral cavity, immune surveillance, and the secretion of antimicrobial peptides.[11]Antimicrobial peptides are natural antibiotics that provide a first line of defence against a wide spectrum of pathogens.[12]Saliva contains 3 α defensins (HNP 1–3) of almost identical antimicrobial activity, 4 β defensins (hBD 1–4), and the only biologically active human cathelicidin, LL-37 peptide.[10]
Human neutrophil peptides 1–3 (HNP1-3) are small cationic antimicrobial peptides that provide the first line of host defence against a broad spectrum of microorganisms. HNP1-3 are expressed in ductal epithelial cells of submandibular salivary glands and secreted into saliva.[13]The preventive role of HNP1-3 against dental caries has been suggested by the significantly higher salivary HNP1-3 levels in children who are caries free than those experiencing caries.[12]

Liquid chromatography–mass spectrometry (LC–MS) techniques is one of the recent advances and have been widely useful in peptide analysis. However,the application of LC–MS for quantitative analysis of peptides in complex biological samples are few.[14] This is due in part to the amphipathic nature of peptides, which makes routine extraction and LC–MS analysis challenging.[15] Hence the aim of this pilot study is to evaluate the salivary HNP1-3 profile in children with and without ECC using LC-MS.

2. Materials and Methods:

This pilot study was conducted among 10 children of age three to six years who reported to the Pediatric dental OPD of a privateteaching institution in Chennai, Tamilnadu, India.Informed consent was received by the parents of the participating children.

Oral Examination:
Children from both genders of age 3 to 6 yearswere screened by a single qualified dentist for presence or absence of Early Childhood caries and were divided into two groups. Group 1 (n=5) children with Early Childhood caries (dmft> 1), and Group 2 (n=5) children without Early Childhood caries (dmft< 1). Children who did not show enough cooperation for dental examination, saliva sample collection or those who were using any medications during saliva collection period and all children with interfering systemic or congenital diseases were excluded from the study. Dental examination was performed using visual and tactilemethods with dental explorer and mouth mirror on a dental chair with dental light and the mean dmft score was recorded. [16] The teeth were identified as being decayed when at least one surface presented with clinical signs of cavitated lesion.

Saliva Collection:
2 ml of unstimulated saliva was collected from both the groups through simple spitting into a wide mouth sterile plastic container. All samples were collected between 9 am and 1 pm. Children were asked not to eat, drink, or brush their teeth for at least 1 hour before the sample collection, however, they were advised to rinse the mouth with water just before sample collection. Sample collection was performed while the child was sitting in an upright and relaxed position under natural light.

Sample Preparation:

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Salivary peptide analysis was performed by liquid chromatography–mass spectrometry (LC-MS), a method of choice for the quantitative analysis of antimicrobial peptides. The collected samples were centrifuged at300 rpm for 10 minutes to remove debris and were re-suspended in 300 lL of double-distilled water by centrifuging for 2 min. The samples were then transferred to microcentrifuge tubes and deep freezed at -80° C for future peptide analysis.

Peptide Analysis:

The frozen saliva samples were defrosted before analyses, where a repeat centrifugation was performed on each sample (10 min, 2000g) to obtain a clear fluid. The individual samples were then re-suspended in volumes of 50 and 100 lL, respectively, by adding double-distilled water containing 0.1% trifluoroacetic acid (TFA) and were centrifuged for 2 min, placed into the LC-MS-certified vials containing 100-lL inserts, and 10 lL of each sample wasinjected into the LC-MS system from Analytical Technologies Limited (Gujarat, India). The samples were first loaded onto LC Packings PepMap C18 precolumn (300 mm 6 1 mm; particle size 5 mm) and washed for 5 min with the loading solvent, 3% Acetonitrile /97% double distilled water/0.1% formic acid (FA)/0.01% TFA (Trifluoroacetic acid). The samples were then injected onto a LC Packings C18 column (75 mm 6 150 mm;particle size 5 mm) for nano-LC separation at a flow rate of 180 nL/min. The eluents used for the LC were (A) 5% Acetonitrile/ 95% H2O/0.1% FA/0.01% TFA and (B) 95% Acetonitrile /5% Double distilled water/ 0.1% FA/0.01% TFA. Chromatograms of the UV spectra were recorded at 230 nm using a diode array detector. The total ion chromatograms consisting of the mass spectra from all points were obtained by continuously scanning the masses of 100–3000 m/z. Peptide identification was accomplished utilizingthe data from a previous study[17]Verification of these peptides consisted on assessment NCBI (http://www.ncbi. nlm. nih.gov/entrez/query.fcgi) databases for identification of the indexed peptides that expressed the same molecular masses as the ones extracted in this study by the LC-MS system. Pool chromatogramsof the peptide profile between children with and without Early childhood caries were used for comparison. Differences between the observed peaks of the pool chromatograms and the individual chromatograms were also analysed. Depending on the time that these masses were presented in the pools, the correspondent peaks were identified and matched in the chromatogram of each individual sample.

A binary logistic regression using individual peptide data was used to evaluate the simultaneous contribution of the dependent and independent variables which include presence and absence of Early childhood caries and Peptides.

3. Results

A total of 10 samples were collected for the study with equal distribution of 5 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). (Table 1).

Pool chromatograms showed qualitative differences in HNP1-3 (1744 m/z), noted in peaks 14 and 21, were present in the caries free pool, but absent in the ECC pool (Figure 1).There were few peaks which differed in height, but were not qualitatively identified because they did not express previously identified masses. These results suggest the existence of HNP1-3 peptide pattern among children who had and those who had not experienced dental caries. Based on the comparison of the theoretical mass values available in the data banks, with experimental monoisotopic mass(1744 m/z), HNP1-3 peptide was suggested as being present in either the ECC or Non-ECC pool.The association between the presence/ absence of early childhood caries and presence/absence of the peptides HNP1-3 was among5 children with absence of these peptides, 4 had experienced early childhood caries and among 5 children who presented with these peptides, none had experienced ECC.

4. Discussion:

Salivary peptides are chemical barriers to protect oral environment in innate immune responses.[18]Among the various salivary peptides HNP 1–3, the α -defensins are expressed in neutrophils and duct cells of the submandibular gland and participate in non-oxidative microbial death. [18,19]HNP 1-3 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 by binding to the bacterial outer membranes.[20]As salivary compounds can undergo changes in the presence of oral diseases, such as dental caries, this study aimed to find a possible association between salivary peptides HNP1-3 and the presence/ absence of early childhood caries using Liquid Chromatography Mass Spectrometry.

In literature there are many studies that have identified peptides in saliva,[12,13,20-27]out of which associations have been found between salivary

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peptidesand dental caries[12,13,20,22,24-27]and a few only with early childhood caries.[20,24,25,27] In the present study unstimulated saliva was collected for the peptide analysis as it was preferred over stimulated saliva considering the age of the children. [20]

In the present study, the salivary peptide HNP1-3 was assessed using LCMS and had found its presence in children without early childhood caries. This is in accordance with the studies done by Tao et al [12], Ribeiro et al [20], and Jayakaran TG et al, [24]where the levels of HNP1-3 were significantly high in caries free individuals when compared to children with ECC.Almoudi MM et al suggested that low salivary peptide LL37 level and higher S. mutans and S. sobrinus count in ECC supported the protective role of salivary peptide LL37 against dental caries.[25]

Wattanarat Oet al, studied the severe-ECC status in children where, daily or triweekly consumption of L. paracasei SD1 significantly increased salivary HNP1-3 levels, but reduced S. mutans levels, possibly resulting in reduction of caries progression.[13]HNP1-3 in saliva may contribute to resistance of dental caries by direct antimicrobial properties, either alone or in combination with other salivary components or by preventing biofilm formation on the tooth surface through its ability to bind to bacterial outer membranes.[20]

The identification of caries risk has been of high interest and is very essential for the development of new protocol for caries prevention. Preschool children and children with special health care needs can be a key target for this. [27] Saliva being an easily available fluid, can be collected non-invasively and be used to measure and monitor the risk for caries. Based on the present study, the inverse correlation of HNP1-3 with early childhood caries suggests its possible protective effect. Conversely, low levels of HNP1-3 may result in increased susceptibility to early childhood caries.

This pilot study opens gateways to add peptides into dental materials, topical fluoride and xylitol products, tooth paste and mouth rinses or may be used independently providing beneficial anti-cariogenic effects.

5. Conclusion:

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

- The salivary peptide HNP1-3 level was significantly lower in children with ECC when compared to children without ECC,thus salivary peptides may allow children at high risk of developing caries to be identified and establish specific preventive and treatment protocols.

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Table 1. Distribution of children and dmft Score

Group	Mean Age	Mean dmft
Group 1 (With ECC) (n=5)	5.12 ± 0.851	7.41 ± 12.16
Group 2 (Without ECC) (n=5)	4.88 ± 0.879	0

Figure 1. Pool chromatograms of Children in Group 1 and Group 2

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