ORIGINAL RESEARCH

Role of Salivary Protease Enzymatic Activity in Saliva of Children with and without Early Childhood Caries: A Randomized Clinical Trial

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Abstract

Background/introduction: Early childhood caries (ECC) is one of the most prevalent diseases in children worldwide. Early childhood caries is driven by a dysbiotic state of oral microorganisms, mainly caused by a sugar-rich diet. Additionally, poor oral hygiene or insufficient dental plaque removal leads to the rapid progression of ECC. Early childhood caries leads not only to dental destruction and pain in children but also affects the quality of life of the caregivers.

Additionally, upon neutrophil activation at inflammatory locations, these proteases are externalized in an active state, aiding in the control of inflammatory and immunological responses. Any enzyme that catalyzes proteolysis reactions is known as a protease. Proteases are produced by human glands or derived from microbes in the oral cavity. Additionally, the oropharyngeal mucosae and crevicular fluids are sources of protease.

Aim: This study is aimed at the estimation and correlation of salivary protease enzymatic activity in the saliva of children with and without ECC.

Materials and methods: A total of 50 children were included in the study, which was divided into two groups: group I (caries-active) and group II (caries-free)—each consisting of 25 children. Unstimulated saliva samples were collected and subjected to a spectrophotometer for analysis. Salivary protease levels were estimated and correlated between caries-active and caries-free children.

Results: The correlation between caries score and salivary protease activity was statistically significant with a moderate correlation. The comparison of mean salivary protease activity between caries-active and caries-free groups was statistically significant. However, the comparison of salivary protease activity based on different age-groups was not statistically significant, whereas gender and caries scores in group A were statistically significant.

Conclusion: In conclusion, there is a substantial correlation between salivary protease enzyme levels and the severity of dental caries, and an increase in salivary protease enzyme levels is linked to a considerable rise in caries severity. As a result, prevention may be possible with early detection.

Keywords: Early childhood caries, Protease enzyme, Saliva.

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Introduction

Early childhood caries (ECC) is one of the most common diseases in children worldwide. Early childhood caries is caused by abnormal oral microbiomes, mainly due to a high-sugar diet. Furthermore, poor oral hygiene or inadequate plaque removal can rapidly progress to ECC. Early childhood caries not only causes tooth decay and pain in children but also impacts the quality of life of caregivers.[1]

The first cells drawn to inflammatory regions are polymorphonuclear neutrophils, which also serve as the initial line of defense against invasive microbes. They aid in the degradation of ingested bacteria inside phagolysosomes by working in concert with reactive oxygen species. Additionally, upon neutrophil activation at inflammatory locations, these proteases externalize in an active form, aiding in the control of immunological and inflammatory responses.[2]

Any enzyme that catalyzes proteolysis reactions is called a protease. Proteases are either released by human glands or obtained from microbes in the oral cavity. Additionally, the oropharyngeal mucosae and crevicular fluids can be sources of proteases. Research has indicated that lower levels are linked to more severe dental caries.[3]

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To facilitate the dentin matrix's early breakdown in tandem with its acid denaturation, the enamel and dentin become demineralized, exposing the dentin matrix and triggering host-derived proteases.[4]

A spectrophotometer is based on the amount of light and its wavelength absorbed by the sample, and components in the sample solution will be identified very specifically. Additionally, it

will support the customer workflow with a fast, easy-to-use, and trustworthy analytical instrument.[5]

Since there is a deficiency in the literature regarding salivary protease enzyme and its correlation to ECC among the population of India, the study's objective is to estimate and correlate salivary protease enzyme activity in ECC using the spectrophotometer method. Hence, the study aims to estimate and correlate salivary protease enzymatic activity in the saliva of children with and without ECC using a spectrophotometer.

Materials and Methods

A total of 50 children between the ages of three and six participated in the current study while attending the Department of Pediatric and Preventive Dentistry at RajaRajeswari Dental College and Hospital, Bengaluru, Karnataka, India. To lower the possibility of bias, patients were chosen using a nonrandom sampling method and distributed using a computer-generated random allotment sequence. A proforma for the study was created that included information about the major complaint, demographics, and a list of all subjective and objective symptoms, as well as the clinical and radiological results.

Experimental Group (Caries-active)

Around 25 patients with severe ECC participated in the study. After receiving informed consent from the parents or guardians of the children taking part in the trial, 25 patients without caries between the ages of three and six were considered for the control group (caries-free group).

Inclusion Criteria

Children aged 3–6 years, children with four or more active carious lesions, children with no caries, and parents giving consent for the study.

Exclusion Criteria

Children with pulpally impacted teeth, children who received topical fluoride application within the last month, children in need of special medical attention, children who have been on antibiotic therapy within the last 3 months, children with genetic disorders or syndromes, and children taking any kind of medicine. Ethical clearance number: RRDCH/IEC/23/20.

The decayed, missing, and filled teeth (dmft) index was used to record caries. The children were categorized based on whether the disease was present (dmft ≥ 4) or not (dmft = 0). Unstimulated saliva was collected using the passive drool method (Fig. 1). The samples were kept in an ice-filled, hermetically sealed case. They were brought to the lab and stored at −80°C for an hour after collection. Each sample was assigned a number, and this process continued until the laboratory examination was completed. To prevent bias, the sample investigator was blinded. A spectrophotometer was used to measure salivary protease enzyme activity in all 50 samples (Fig. 2).

Statistical Analysis

Statistical Package for the Social Sciences (SPSS) for Windows, Version 22.0 (released 2013; Armonk, New York: IBM Corp.) was used to perform statistical analyses. Descriptive analysis of all explanatory and outcome parameters was done using frequency and proportions for categorical variables, and mean and standard deviation (SD) for continuous variables using the Mann–Whitney *U* test. Spearman's correlation test was used to assess the relationship between caries scores and salivary protease activity levels. The Mann–Whitney *U* test was used to compare gender and age distribution between the two groups. The level of significance was set at $p < 0.05$.

Results

The results show that the comparison between both caries-active and caries-free was statistically significant with a mean dmft of 2.87 ± 1.07; salivary protease levels exhibit significantly higher activity in the caries-active group than in the caries-free group (Table 1). The correlation between caries score and salivary protease

Table 1: Showing comparison of salivary protease activity between caries-active and caries-free subjects

Comparison of mean salivary protease activity b/w caries-active and caries-free using the Mann–Whitney U test

Parameter	*Groups*	*N*	*Mean*	*SD*	*Mean difference*	*p-value*
Protease activity	Caries-active	25	2.87	1.07	0.82	<0.001*
	Caries-free	25	2.05	0.84		

*Statistically significant

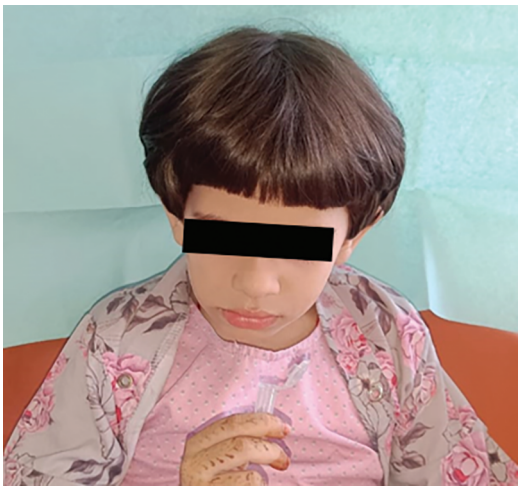

Fig. 1: Collecting saliva into Eppendorf tubes by spit method

Fig. 2: Spectrophotometer for estimation of protease activity in saliva

level activity was statistically significant with a moderate correlation of ρ = 0.53 at $p < 0.007$ (Table 2). In the present study, we compared salivary protease levels activity based on gender in the caries-free group showed significant results at $p < 0.01$ (Table 3), and between different age-groups, it was not statistically significant (Table 4). A comparison of mean salivary protease activity levels based on caries scores in the caries-active group was done and found that for ≥6, the mean was 3.46 ± 1.27 and ≤6, the mean was 2.40 ± 0.56 and was statistically significant at $p < 0.01$ (Table 5).

Discussion

The salivary glands and other sources, including the oropharynx, stomach reflux, gingival crevicular fluid, and blood, combine to form the complex mixture known as saliva. Analysis of salivary component composition has long been recognized as a highly useful method for tracking health status. Salivary compound variation has emerged as a "window" reflecting the physiological and pathological conditions of the body. Therefore, tracking the onset, progression, recurrence, and therapy of systemic and oral diseases may be aided by the examination of chemicals found in saliva.[6]

Table 2: Showing Spearman's correlation between caries score and protease activity between caries-active subjects

Spearman's correlation test to assess the relationship b/w caries scores protease activity levels in caries-active group			
Groups	*Variable*	*Values*	*Salivary protease levels*
Caries-active	Caries scores	ρ	0.53
		p-value	0.007*

*Statistically significant

Table 3: Showing protease activity levels based on different ages in both caries-active and caries-free subjects

Comparison of mean salivary protease activity levels based on different age-groups, each group using the Mann–Whitney U test						
Groups	*Age*	*N*	*Mean*	*SD*	*Mean difference*	*p-value*
Caries-active	4–5 years	9	2.98	1.02	0.17	0.46
	6 years	16	2.81	1.12		
Caries-free	4–5 years	11	2.18	0.87	0.24	51
	6 years	14	1.94	0.83		

Table 4: Showing protease activity levels based on gender in both caries-active and caries-free subjects

Comparison of mean salivary protease activity levels based on gender using the Mann–Whitney U test						
Groups	*Gender*	*N*	*Mean*	*SD*	*Mean difference*	*p-value*
Caries-active	Males	14	2.79	1.18	−0.19	0.43
	Females	11	2.97	0.95		
Caries-free	Males	9	1.48	0.67	−0.88	0.01*
	Females	16	2.36	0.77		

*Statistically significant

Table 5: Showing protease activity levels based on caries scores in caries-active subjects

Comparison of mean salivary protease activity levels based on caries scores in caries-active group using Mann–Whitney U test						
Groups	*Caries*	*N*	*Mean*	*SD*	*Mean difference*	*p-value*
Caries-active	≤6 numbers	14	2.40	0.58	−1.06	0.01*
	>6 numbers	11	3.46	1.27		

*Statistically significant

The requirement for early detection instruments to be accessible and noninvasive makes salivary diagnostics a viable substitute for blood testing. When saliva is utilized as a diagnostic tool instead of serum or tissues, there are several benefits. Salivary diagnostics offers advantages such as a noninvasive collection method, a smaller sample volume, strong patient compliance, cost-effectiveness, ease of storage and transportation, increased sensitivity, and correlation with blood levels.[7]

Proteases are significant molecules that can break proteins at either the N- or C-terminal end, resulting in smaller peptides. Normally, they serve a purpose inside the cell or contribute significantly to the extracellular environment through secretion. Based on their structure and proteolytic process, human proteases are divided into five groups—metalloproteinases, aspartate proteases, cysteine proteases, serine proteases, and threonine proteases.[8] Pathogenic processes in cells, tissues, and organs may result from abnormal protease activation, secretion, or release because protease signaling pathways are strictly regulated. Since the extracellular matrix surrounding tumors can be broken down by a variety of proteases, they are crucial to the invasion and spread of cancer. Proteases found in biological fluids or within cells could be helpful indicators for early detection, screening, and monitoring.

Our results suggested that the level of protease activity within saliva samples from subjects with caries was higher than that in subjects who were caries-free, indicating a high level of caries severity.

In our study, the correlation between caries score and salivary protease activity was assessed and found to be statistically significant, with a moderate correlation (ρ = 0.53). This indicates that as caries severity increases, protease enzyme activity also increases. In comparison, a study concluded that low levels of protease inhibitors were detected by LC-MS in saliva from healthy individuals without any systemic disease. Hyposalivation leads to an increased risk of dental caries or periodontitis, oral mucosa or tongue pain, and bacterial and fungal infections.[9] A different study that examined salivary protease as a biomarker for oral cancer concluded that salivary protease profiles varied significantly based on oral health. Individuals with oral squamous cell carcinoma (OSCC) had a considerably greater protease profile and levels in their saliva than those without cancer.[8] In contrast to our research, another study found that the submandibular saliva of caries-resistant subjects had 3.8 times higher levels of protease activity than the saliva of caries-susceptible individuals. This finding raises the possibility that the expression of the enzyme may be associated with caries resistance.

Contrary to our study, another study concluded that protease PR3 is associated with the severity of dental caries, with low levels being linked to greater caries severity.[2] The initial breakdown of the dentin matrix, coincident with its acid denaturation, may be facilitated by bacterial acids that demineralize enamel and dentin, reveal the dentin matrix, and activate host-derived proteases. According to *in vitro* research, endogenous proteases present in the matrix can self-degrade the demineralized dentin matrix without the need for bacteria.[4]

In both enamel and dentin caries, bacterial acid production initiates the processes that lead to caries. Additionally, in dentin caries, host-derived proteases play a significant role in these

processes. In our study, a comparison of salivary protease enzyme activity across different age-groups was not statistically significant. However, in a gender-wise comparison, protease enzyme activity was statistically significant in the caries-free group.

The comparison of caries scores in the caries-active group in our study was statistically significant. Conversely, a higher number of caries was associated with a higher concentration of protease enzyme activity.

Since salivary protease levels rise in caries-active patients, measuring salivary protease levels could be a useful method for identifying individuals at high-risk for dental caries. Saliva can also be considered an important diagnostic fluid, providing a noninvasive method for evaluating various biomarkers and opening up new avenues for preventive dentistry.

Conclusion

This study concluded that the high level of salivary protease enzyme has an important role in developing ECC when compared with caries-free children. As a result, prevention may be possible with early detection.

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