

Identification, Quantification and Correlation of Hydrogen Peroxide Present in Saliva to Early Childhood Caries: A Randomized Clinical Trial

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ABSTRACT

Aim: The main objective of the present study was to estimate the production of hydrogen peroxide present in saliva and correlate it to early childhood caries using high-performance liquid chromatography [HPLC] method.

Design: The study was conducted on children aged 3–6 years with a sample size of 20 who had decayed, missing, or filled teeth in accordance to WHO pro forma and were divided into two groups: Study group: Caries active [CA] [n = 10] and Control group caries free [CF] [n = 10]. The whole saliva was collected into the vials with a buffer solution and was stored in cold storage. HPLC was done to estimate, detect, and correlate the amount of production of H₂O₂ in CA and CF groups. To compare age and gender distribution among two groups, Independent student "t" test was used. To compare the mean production of H₂O₂ levels between two study groups with a significance of $p > 0.05$ was done using Mann-Whitney U test. Spearman's correlation was done between caries and H₂O₂.

Results: Comparison of mean H₂O₂ levels [in ppm] between groups was statistically significant at $p = 0.03$, which showed as age increases H₂O₂ production also increases. Age-wise estimation of H₂O₂ obtained a statistically significant result [$p = 0.04$]. However, gender-wise comparison of mean H₂O₂ levels [in ppm] in both the groups showed no difference.

Conclusion: Findings in our study strongly suggested that H₂O₂ levels are more in children without any caries experience. H₂O₂ production is the same among males and females but according to age, H₂O₂ production increases as the child grows with age.

Keywords: ECC, HPLC/UV, Hydrogen peroxide.

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INTRODUCTION

To form dental plaque or biofilm, various bacterial species conquest and exist in the mouth, forming intricate associations of microorganisms. Various commensal organisms are advantageous to the host as they keep out the colonization of pathogenic organisms in oral biofilm.¹ Certain conditional pathogens battle with the commensal flora in a more well organized way on the tooth surface, causing disease.² The most common chronic infectious disease of childhood is dental caries which is caused by the interaction of bacteria, mainly *Streptococcus mutans* [SM], and foods containing sugar on tooth enamel. Transmission of SM from the mother to child is seen during infancy and even predate infants. Microorganisms derive energy from the breakdown of sugars leading to acidic environment in the oral cavity, hence causing dental caries. Early childhood caries [ECC] is a very serious health problem among the public in both developing and developed countries. It begins early in life, which progresses rapidly in a high-risk individuals, and is often untreated. Its consequences can affect the long-term quality of life of the child and family. ECC is defined as "the presence of one or more decayed [noncavitated or cavitated lesions], missing teeth [due to caries], or filled tooth surfaces in any primary tooth in a child 72 of months age or younger."³

Certain streptococci are associated with the initiation and progression of the carious lesion. Though SM has a main role in the caries pathogenesis, the increase in number of SM and the caries presence is controversial.⁴ Furthermore, the production of H₂O₂ is considered as the inhibitory mechanism present in *Streptococcus Sanguinis* [SS] over SM invitro.⁵ Multiple commensal

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oral streptococci cultivate in aerobic conditions utilizing oxygen to generate H₂O₂ and subsequently inhibit SM, conversely, in an anaerobic environment these microorganisms are deprived of oxygen leading to decreased generation of H₂O₂ in turn decreasing the capacity of SS to effectively compete with SM.⁶ The caries risk can be assessed by the ratio between the SM and SS. The lower the ratio and lower is the risk, the high number of SS has been seen associated with caries-free children.⁷ So the quantification of H₂O₂ in saliva can assess the risk of caries in children.

A high-performance liquid chromatography study is an authentic and delicate method for the detection and quantification of individualized species and bacterial inhabitants. Thus the study aims to estimate and correlate the production of H₂O₂ in CA and CF children using high-performance liquid chromatography.

MATERIALS AND METHODS

The present study was carried out in the Department of Pediatric and Preventive Dentistry. The present research protocol was approved by review board of the institution, with the reference number RRDCHET/04PEDO/2020. The period time of the study was from June 2020 to October 2020. Both sample groups will consist of 10 CA and 10 CF children, with a total sample size of 20. Estimation of sample was done using the G Power software v. 3.1.9.2. considering the effect size to be measured (d) at 80% at One-Tailed Hypothesis, power of the study at 80% and the margin of the error at 20%, the total sample size needed is 20.

Subject Selection

The present study was conducted on 20 children with age-group 3–6 years visiting the Department of Pediatric and Preventive Dentistry. Children were selected using random sampling technique who fulfilled the criteria. Twenty children with CA and CF were selected by briefly explaining the clinical procedures, involved risks, and clarifying all the doubts raised by the patients. Informed consent has been obtained from parents or guardians of the children participating in this study. The study samples were based on the inclusion and exclusion criteria.

Inclusion Criteria

Children with four or more than four cavitated restorable lesions and children with no caries.

Exclusion Criteria

Children who took antibiotic medications, past 3 months, who received fluoride topical application during last 48 hours, who require special health care needs, with existing restorations on any surface of a tooth, pulpal involved teeth, and children undergoing any kind of interceptive orthodontic treatment.

Clinical Examination

One calibrated examiner carried out a comprehensive dental examination of each participant with optimal light using a mouth mirror and explorer. The presence of carious teeth, missing teeth, and restored teeth were diagnosed and scored based on the modified WHO criteria. Children were allocated randomly to one of the groups: CA group [$n= 10$]- children with four or more than four caries, CF group [$n= 10$]- children with no caries.

Saliva Sample

Saliva samples were collected from each participant. The unstimulated whole saliva was collected into the vials with a buffer solution and was stored in cold storage. Saliva sampling was performed early in the morning from 9 to 11 am and the children were refrained from eating or drinking for 1 hour prior to sampling. Children were also asked not to brush their teeth in the morning before saliva sampling. Estimation of production of H_2O_2 in each sample of unstimulated saliva in CA and CF children was determined using an HPLC/UV method.

METHOD

Standard Solution Preparation

Stock standard solution of 1000 ppm was made ready by dissolving a standard of 10 mg in 10 mL HPLC grading acetonitrile. The stock

was serially diluted in the mobile phase to obtain different levels of calibrations [25, 50, 100, 200] ppm.

Sample Preparation

Hydrogen peroxide in sample/standard was used to oxidize Triphenylphosphine into Triphenylphosphine oxide. Sample/standard [100 μ l] was mixed with 0.5 mL acetonitrile, 0.3 mL distilled water, and 0.1 mL Triphenylphosphine [0.01M solution prepared in acetonitrile]. The reaction mixture was stirred in a vortexer for 30 seconds and allowed to stand for 2 hours at room temperature before analyzing by high performance liquid chromatography [HPLC].

High-performance Liquid Chromatography

Analysis for HPLC was performed on a prominence 20AT system [Shimadzu LC. JAPAN] set-up with a diode array detector. A reverse-phase Nucleosil® C18 Chronachemei [INDIA] 250 mm x 4.6 mm, 5 microns, 100A column was used. A 10 μ L injection volume was present and was detected at 225 nm. The mobile phase contained a gradient elution of water [50%] and acetonitrile [50%]. The column temperature was kept as 25 0 C and the mobile phase flow rate was kept as 1.0 mL min⁻¹. H_2O_2 was quantified using the peak area of samples.

The calibration plot of H_2O_2 is obtained by plotting a graph of peak areas of that standard against its injected concentration. Linearity with calibrants with correlation co-efficient of ≥ 0.99 will be established and used for quantification.

H_2O_2 content in the sample will be calculated using the calibration curve generated using the formula $Y = mX + c$, Where, $Y =$ Peak area (or height), $X =$ Concentration of drug, $m =$ Slope of the calibration curve, $c =$ Intercept, Concentration of drug is determined by $X = Y - c/m$.

Statistical Analysis

Statistical Package for Social Sciences for Windows Version 22.0 Released 2013. Armonk, NY: IBM Corp., is used to perform statistical analysis.

Descriptive Statistics

The frequency distribution is expressed as number and percentage, and continuous data, is expressed in terms of frequency, mean, and standard deviation [SD]. Inferential Statistics: Mann Comparison of mean levels between the two groups was done using Whitney U test, with the level of significance $p > 0.05$.

RESULTS

The study group consisted of five females and five males [CA], four females and six males were included in the control group [CF]. The 20 participants included in the study fulfilled the inclusion criteria.

Variables such as age and gender distribution between two groups were done with CA group consisting of age ranging from 4 to 6 years, mean and SD 5.14 and 0.86, respectively. CF group consisting of age ranging from 3.5 to 6 years, mean and SD of 4.85 and 0.91. For gender, the CA group with five males and five females and the CF group with six males and four females were allocated [Table 1].

The assessment of the production of H_2O_2 was derived from the calibration plot of H_2O_2 obtained by plotting a graph of peak areas of the injected concentration. It is obtained for different levels of calibrations ranging from 25 to 200 ppm [Fig. 1].

Different levels of calibration were obtained from 25 to 200 ppm. High-performance liquid chromatography for H₂O₂ standard at 25 ppm is shown in [Fig. 2].

The H₂O₂ estimation was done for all samples. Significant higher levels of H₂O₂ were estimated from the saliva of CF subjects when compared to that of CA participants. In the CA group, the values of production of H₂O₂ ranged from a minimum of 24.78 to 267.88 ppm suggesting that as the number of caries increases the production of H₂O₂ decreases. Conversely, in the CF group as the number of caries decreases the production of H₂O₂ increases [Table 2].

Spearman's correlation between H₂O₂ levels and caries scores showed a strong negative correlation [rho- 0.83] between caries and H₂O₂ production that is, the number of caries increases, levels of H₂O₂ decreases, and which is statistically significant [$p = 0.003^*$] [Table 3].

Scatterplot depicting the relationship between H₂O₂ levels and caries scores. Scores in the CA group suggested reducing levels of H₂O₂ production observed as caries increases, inversely as caries incidence reduced H₂O₂ production increased [Fig. 3].

When the intergroup comparison between CA and CF group for H₂O₂ production was done, a statistically significant result was seen $p = 0.03^*$ [Fig. 4].

The results were further evaluated based on age. For age-wise comparison children were divided into CA [< 6 years and ≥ 6 years], CF [< 6 years and ≥ 6 years]. Age-wise estimation of H₂O₂ among the groups were significant statistically, $p = 0.04$ [Table 4].

Gender-wise comparison of mean H₂O₂ levels [in ppm] in both the groups between males and females, the results were not statistically significant [Table 5].

DISCUSSION

Antioxidant Capacity [AC] is indicated as one of the important responsible causative factors in many of the oral inflammatory diseases and probably dental caries is not an exception.⁷⁻⁹ Antagonistic effect of SS against SM is of specific interest in caries research, and it has been considerably investigated. Studies confirm that the production of H₂O₂ in the CF group prevails over that of the CA group.¹⁻⁴ A study¹ strongly suggested that SM may be prevailing with respect to SS in the saliva of adults with a high caries index. However, SS appears to be more in number than SM in the saliva of adults who are caries free. SS from caries free subjects produced more H₂O₂ than the same species from high caries individuals. But evidence of this opposed effect in children is not sufficient. Hence,

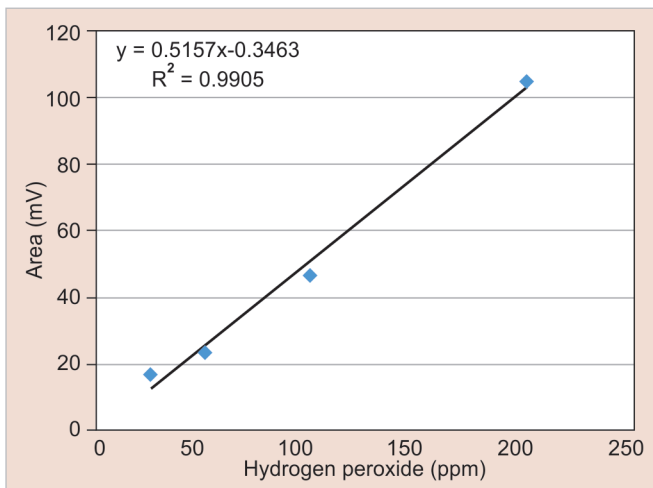


Fig. 1: Calibration graph of H₂O₂ obtained for linear standard

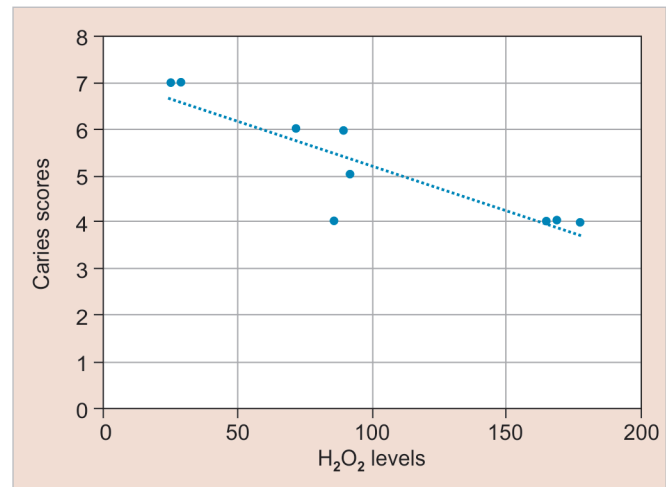


Fig. 3: Scatterplot depicting the relationship between H₂O₂ levels and caries scores

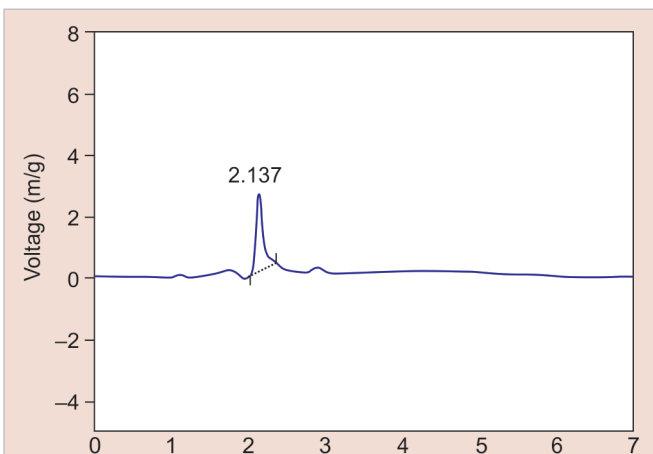


Fig. 2: HPLC chromatogram for H₂O₂ standard (25 ppm)

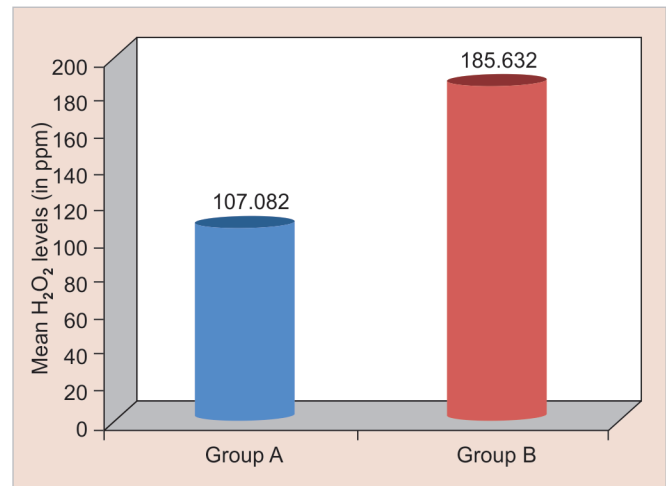


Fig. 4: Graph representing mean hydrogen peroxide levels (in ppm) between 2 groups.

Table 1: Showing age and gender-wise distribution among study subjects

Variable	Category	CA		CF		p-value
		Mean	SD	Mean	SD	
Age	Mean & SD	5.14	0.86	4.85	0.91	0.39 ^a
	Range	4-6		3.5-6		
Sex		N	%	N	%	0.65 ^b
	Males	5	50%	6	60%	
	Females	5	50%	4	40%	

Note: a. Independent Student t Test; b. Chi-square Test

Table 2: Hydrogen peroxide estimation of all the subjects

Group	Sample	Retention Time	Area	Hydrogen peroxide (ppm)
(CA)—A	S1	2.177	91.232	177.58
	S2	2.207	47.045	91.90
	S3	2.16	45.577	89.05
	S4	2.177	36.58	71.60
	S5	2.113	136.11	264.60
	S6	2.16	12.431	24.78
	S7	2.177	86.725	168.84
	S8	2.177	14.493	28.78
	S9	2.143	95.478	185.81
	S10	2.09	137.8	267.88
	S11	2.107	101.977	198.42
	S12	2.157	76.733	149.47
	S13	2.123	149.067	289.73
	S14	2.19	75.449	146.98
(CF)—B	S15	2.093	121.859	236.97
	S16	2.097	172.268	334.72
	S17	2.153	46.15	90.16
	S18	2.197	49.807	97.25
	S19	2.147	6.65	13.57
	S20	2.187	93.538	182.05

Table 3: Spearman's correlation between H₂O₂ levels and caries scores

Group	Variable	Values	Caries
CA		rho	-0.83
	H ₂ O ₂	p-value	0.003*
		N	10

*Significant

the hypothesis was tested in children with opposite caries histories, where H₂O₂ level shows an inverse relationship. In our study, we found the inverse correlation between H₂O₂ production and caries that is higher levels of H₂O₂ was seen in children without incidence of caries when compared to that of caried individuals.

Samples like the whole saliva, plaque are the source for analysis and quantification. Saliva represents the universal colonization of the mouth and gives a general conclusion about the colonization by the organisms.¹ So in our study we used unstimulated samples of whole saliva to estimate the relative production of H₂O₂ in the groups. Various studies¹⁰⁻¹⁸ studied Total antioxidant capacity [TAC] in unstimulated and stimulated saliva samples and concluded that TAC was higher in unstimulated saliva. Hence unstimulated saliva is more accurate to evaluate the anti-oxidant properties.

The unstimulated whole saliva was collected into the vials with a buffer solution and was stored in cold storage. Saliva sampling was performed early in the morning from 9 to 11 am and the children were asked to refrain from eating or drinking anything for 1 hour before sampling. Children were also asked not to brush their teeth in the morning prior to saliva sampling.

In a 1994 workshop, the Centres for Disease Control and Prevention, coined the term "early childhood caries" with an attempt to concentrate on the various factors [i.e., socioeconomic, behavioral, and psychosocial] that leads to caries at such early ages. ECC is defined as "the presence of one or more decayed [noncavitated or cavitated lesions], missing teeth [due to caries], or filled tooth surfaces in any primary tooth in a child 72 of months age or younger. In children younger than 3 years of age, any sign of smooth-surface caries is indicative of severe ECC [S-ECC]."³

In our study, we have selected children with ages ranging from 3 to 6 years as ECC most commonly affects the children of 6 years or younger. They were subjected into two groups CA [n = 10] and CF [n = 10]. The distribution of gender and age between groups were done consisting of age ranging from 4 to 6 years and 3.5 to 6 years, respectively. For gender, in the CA group with five males and five females and CF group with six males and four females were

Table 4: Age-wise comparison of mean H₂O₂ levels (in ppm) in CA and CF

Group	Age	N	Mean	SD	Mean diff	p-value
CA	< 6 years	6	78.452	51.205	-71.576	0.04*
	≥6 years	4	150.028	43.034		
CF	< 6 years	8	153.984	50.084	-158.241	0.04*
	≥6 years	2	312.225	31.813		

*Statistically Significant

Table 5: Gender-wise comparison of mean H₂O₂ levels (in ppm) in CA and CF

Group	Gender	N	Mean	SD	Mean diff	p-value
CA	Male	5	87.824	51.605	-38.156	0.18
	Female	5	126.340	64.420		
CF	Male	6	173.980	74.304	-29.130	0.52
	Female	4	203.110	98.307		

allocated. The whole saliva was collected among all the samples and estimation of H₂O₂ was done under HPLC/UV method.

HPLC is a technique in analytical chemistry used to isolate, recognize, and measure each component in a mixture.¹⁹ It's based on pumps to permit a pressed liquid solvent consisting of the sample mixture through a column occupied with a solid adsorptive material. Every part of the sample interacts slightly in a different way with the adsorptive material, leading to different flow rates for the various components and thus leading to the parting of the components as they flow out. Every part of the sample interacts slightly in a different way with the adsorptive material, leading to different flow rates for the various components and thus leading to the parting of the components as they flow out.²⁰

It has the capacity to isolate and recognize compounds that are existent in any sample that can be melted in a liquid in small concentrations as low as parts per trillion. Due to its flexibility, HPLC has many scientific applications. HPLC method has advantages such as speed, efficiency, accuracy, versatility, and extremely precise when it comes to identifying and quantifying chemical components.

As there are limited studies in the literature pertaining to the use of this method in dentistry hence we used this method of HPLC/UV for estimating H₂O₂ in saliva. The assessment of the production of H₂O₂ was derived from the calibration plot of H₂O₂ was obtained by plotting a graph of peak areas of the injected concentration.

Saliva consists of various biochemical systems that are involved in repair of soft tissue, and many antibacterial enzymes such as lysozyme, lactoferrin, and salivary peroxidase. Saliva has a compound peroxidase system, the components of which include various forms of lactoperoxidase produced by the salivary glands and myeloperoxidases from polymorphonuclear leukocytes. The most important function of salivary peroxidase is to regulate oral bacteria forming dental plaque which leads to dental caries. Salivary peroxidase catalyzes the peroxidation to form oxidation products which restrict the growth and metabolism of microorganisms.¹⁷

As the levels of H₂O₂ increase, the incidence of caries decreases conversely as the H₂O₂ levels decrease the incidence of caries increases. Hence, this hypothesis was tested in children with opposite caries histories. Studies^{20,21} stated that increased caries severity, increases the salivary antioxidant activity causing a reduction in the salivary oxidative damage, in accordance with that in our study we found where H₂O₂ levels show an inverse relationship. Significant higher levels of H₂O₂ were estimated from the saliva obtained from CF subjects in comparison to that of CA subjects.

Commonly seen organisms in caries individuals are SM and in caries-free individuals are SS. Although, molecular mechanisms of the opposition between SS and SM have been reported and accomplished in vitro. H₂O₂ production is the key mechanism of SS to prevent colonization by SM on tooth surface. Alternatively, SM can outperform the commensals by production of bacteriocins or mutacins. SM produces mutacins I and IV under some environmental conditions, thus inhibiting SS. These competitive mechanisms have a high influence on the composition of the oral biofilm and explains the prevalence of cariogenic species proved in clinical studies.¹

Clinical samples were used in the current study to assess the production of H₂O₂ levels. Although H₂O₂ production rather appears among streptococci, SS is resistant to its H₂O₂, while shows an repressive effect on cariogenic SM. It is difficult to entitle that H₂O₂ production is the only factor, but numerous variables affecting the changing aspects of oral biofilm. Interestingly, the repressive effect of SS over SM is associated with the inhibition of numerous SM genes related to its virulence. Therefore, H₂O₂ is not only involved in the killing but also in regulating the expression of virulence genes in SM.¹

Likewise, H₂O₂ production was more ample in CF than in CA children. CF children evidently show higher quantity of H₂O₂ in their saliva, but study subjects with caries show the reverse trend. Author concluded that salivary lactoperoxidase plays an important role in caries prevention at high concentrations. On confirmation of this association, we found increased levels of H₂O₂ in CF children.²²

Munther et al. concluded that the growth-inhibiting nature of H₂O₂ might help in reducing caries lesions.²³ In accordance with previous studies conducted in children, we found increased levels of H₂O₂ in children with no history of caries in comparison with subjects with caries.

In our study when a correlation between H₂O₂ levels and caries scores was done by Spearman's method a negatively strong correlation [rho- 0.83] was found between caries and H₂O₂ production, that is, as a number of caries increases levels of H₂O₂ decreases, however as the number of caries increase levels of H₂O₂ decreases which is statistically significant [*p* = 0.003*].

Zhu et al. reported that the enzymatic activity of the lactoperoxidase-thiocyanate system inhibits bacterial growth.²⁴ Supporting this in our study we obtained a scatterplot depicting the relationship between H₂O₂ levels and caries scores which intern decreases caries, which is in accordance with our study.



Comparison of mean H₂O₂ levels [in ppm] between two groups was done using Mann Whitney U test. In our study, intragroup comparison indicates that children with a history of caries have in general less production of H₂O₂ in their saliva than children without caries experience and is statistically significant.

The results in our study were further evaluated based on age-wise evaluation for production of H₂O₂ between CA and CF. Age more than or equal to 6 and less than 6 were compared between two groups and were statistically significant [$p = 0.04$] which showed that as age increases H₂O₂ also increased.

Author reported that, total proteins and total antioxidants were increased with activity of caries and concluded that as age increases caries activity decreases.¹³ The credible reasons suggestive of increase in AC in children with age is recommended to be nutrition. Children belonging to the younger age, consume less volume diet with nutritious benefits, hence lower volumes of micronutrients with higher level of antioxidants when compared to the older age-group. As age increases, the antioxidant levels also increase, which is a normal finding. Also, the immune status of children improves with age and could therefore account for the increase in AC of saliva in children belonging to the older age-group. As caries activity increases, AC of saliva also increases. Another probable factor for the changes in the levels of TAC in relation to age and gender could be, a substantial change in the hormones, as the child goes through pubertal growth spurts.¹³⁻¹⁴

The change of salivary TAC takes place with age can be due to enhanced activity of neutrophils and monocytes in the oral cavity which generates reactive oxygen species [ROS] in the presence of bacteria. That is, as the production of ROS increases, there is decreased TAC production in saliva.^{9,18}

Gender wise comparison of mean H₂O₂ levels [in ppm] in both the groups was estimated and found no much difference between gender in our study. On the contrary, few studies^{25,26} showed that the prevalence of SM was more in females [62.9%] than in males [37.1%] further concluded that SM occurred more within the age-group of 11 to 25 years.

Further studies should explore the expression of genes and its virulence in actual oral biofilm. A new approach by taking saliva as a biomarker for CF children instead of the SM sums as a caries risk assessment factor. In some individuals the properties of both species SM, SS in assuring risk factor. The type of possible approach should be explored in future studies. In the current study clinical samples were used to assess the production of H₂O₂ levels in saliva and intended to discover the correlation between caries and H₂O₂. Our study indicates higher H₂O₂ in CF subjects compared to CA subjects, supportive of H₂O₂ in maintaining a oral biofilm in health.

Furthermore, the results propose a correlation between the capability to produce H₂O₂ in CF status. Future studies on SS characteristics may explain some innovative anticaries strategies.

CONCLUSION

Our results strongly propose that significantly higher levels of H₂O₂ were estimated from the CF children when compared to that of CA children. A correlation between H₂O₂ levels and caries scores showed a strong negative correlation between caries and H₂O₂ production that is, as the number of caries increases, levels of H₂O₂ decreases which is statistically significant. A statistically significant result was seen when an intergroup comparison between CA and CF group for H₂O₂ production was done. As age increases significantly level also increased with age and H₂O₂ was statistically significant, whereas gender-wise comparison

of mean H₂O₂ levels [in ppm] in both the groups between males and females showed no much difference.

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REFERENCES

- Giacaman RA, Torres S, Gómez Y, et al. Correlation of streptococcus mutans and Streptococcus sanguinis colonization and ex vivo hydrogen peroxide production in carious lesion-free and high caries adults. Arch Oral Biol 2015;60(1):154-159. DOI: 10.1016/j.archoralbio.2014.09.007
- Marsh PD. Microbiology of dental plaque biofilm and their role in oral health and caries. Dent Clin N Am 2010;54(3):441-454. DOI: 10.1016/j.cden.2010.03.002
- Colak H, Dülgergil CT, Dalli M, et al. Early childhood caries update: A review of causes, diagnoses, and treatments. J Nat Sci Biol Med 2013;4(1):29-38. DOI: 10.4103/0976-9668.107257
- Loesche WJ. Role of streptococcus mutans in human dental decay. Microbial Rev 1986;50(4):353-380. DOI: 10.1128/mr.50.4.353-380.1986
- Kreth J, Merritt J, Schi W, Qi F. Competition and coexistence between streptococcus mutans and streptococcus sanguinis in dental biofilm. J bacteriology 2005;187(21):7193-7203. DOI: 10.1128/JB.187.21.7193-7203.2005
- Kreth J, Zhang Y, Herzberg MC. Streptococcal antagonism in oral biofilms: streptococcus sanguinis and Streptococcus gordonii interference with Streptococcus mutans. J Bacteriol 2008;190(13):4632-4640. DOI: 10.1128/JB.00276-08
- DeStoppelaar JD, VanHonte J, BackerDirks O. The relationship between extracellular polysaccharide producing streptococci and smooth surface caries in 13 year old children. Caries Res 1969;3(2):190-199. DOI: 10.1159/000259582
- Battino M, Bullon S, Wilson M, et al. Oxidative injury and inflammatory periodontal diseases: The challenge of antioxidants to free radicals. Crit Rev Oral Biol Med 1999;10:458-476. DOI: 10.1177/10454411990100040301
- Battino M, Ferreira MS, Gallardo I, et al. The antioxidant capacity of saliva. J Clin Periodonol 2002;29:189-194. DOI: 10.1034/j.1600-051x.2002.290301x.x
- Tenovuo J, Lehtonen OP, Aaltonen AS, et al. Antimicrobial factors in whole saliva of human infants. Infect Immun 1986;51(1):49-53. DOI: 10.1128/iai.51.1.49-53.1986
- Moore S, Calder KA, Miller NJ, et al. Antioxidant activity of saliva and periodontal disease. Free Radic Res 1997;26:209-234. DOI: 10.3109/10715769409056594
- Pereslegina IA. The activity of antioxidant enzymes in the saliva of normal children. Lab Delo 1989;11:20-23.
- El-Sherbiny, Antimicrobial susceptibility of bacteria detected from the root canal infection (Before and after) root-filled teeth : an in vitro study GM. Int J Dent Sci Res 2015;3(1):4-9. DOI: 10.12691/ijdsr-3-1-2
- Tulunoglu O, Demirtas S, Tulunoglu I. Total antioxidant levels of saliva in children related to caries, age, and gender. Int J Paediatr Dent 2006;16(3):186-191. DOI: 10.1111/j.1365-263X.2006.00733.x
- Dodwad R, Anupama V.B, B. P. Preeti. Estimation of total antioxidant capacity levels in saliva of caries-free and caries-active children. Contemp Clin Dent 2011;2(1): 17-20. DOI: 10.4103/0976-237X.79296
- Ge Y, Caufield PW, Fisch GS, et al. Streptococcus mutans and Streptococcus sanguinis colonization correlated with caries experience in children. Caries Res 2008;42(6):444-448. DOI: 10.1159/000159608
- Caufield PW, Dasanayake AP, Li Y, et al. Natural history of Streptococcus sanguinis in the oral cavity of infants: evidence for a discrete window of infectivity. Infect Immun 2000;68(7):4018-4023. DOI: 10.1128/IAI.68.7.4018-4023.2000

18. Rahmani M, Ghoorchi V, Rezaei F, et al. Evaluation of total antioxidant capacity of saliva in high school students. *Glob J Health Sci* 2015; 8(4):89–94. DOI: <https://dx.doi.org/10.5539/gjhs.v8n4p89>
19. Krawczyk D, Błaszczak J, Borowicz J, et al. Life style and risk of development of dental caries in a population of adolescents. *Ann Agric Environ Med* 2014;21(3):576–580. DOI: 10.5604/12321966.1120605
20. Thammana M. A review on high performance liquid chromatography (HPLC). *Res Rev J Pharm Anal* 2016;5(2):22–28.
21. Gornowicz A, Tokajuk G, Bielawska A, et al. The assessment of sIgA, histatin-5, and lactoperoxidase levels in saliva of adolescents with dental caries. *Med Sci Monit* 2014; 20: 1095–1100. DOI: 10.12659/MSM.890468
22. Araujo HC, Nakamune AC, Garcia WG, et al. Carious Lesion Severity Induces Higher Antioxidant System Activity and Consequently Reduces Oxidative Damage in Children's Saliva. *Oxidative Medicine and Cellular Longevity* 2020.
23. Munther. The impact of salivary lactoperoxidase and histatin-5 on early childhood caries severity in relation to nutritional status. *Saudi Dent J* 2020;32(8):410–416. DOI: 10.1016/j.sdentj.2020.01.010
24. Zhu L, Kreth J. The role of hydrogen peroxide in environmental adaptation of oral microbial communities. *Oxid Med Cell Longev* 2012;2012:1–10. DOI: 10.1155/2012/717843
25. R. Schmid, A. R. Firestone, H. R. Muhlemann. Effects of topical hydrogen peroxide on caries incidence and bacterial agglomerate formation in rats. *J Dent Res* 1980;59(7):1173. DOI: 10.1177/00220345800590072001
26. I.B. Enweani, A.R. Akpe, N.J. Inyang J.O. Ihenyen. Effect of fluoride, hydrogen peroxide and chlorhexidine on staphylococcus mutans. *J. Sci. Engr. Tech* 2006;13(1):6597–6602.