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Dr. Hussain Mookhtiar
Senior Lecturer, Department of Conservative Dentistry and Endodontics, M.A. Rangoonwala Dental College and Research Centre, Pune, Maharashtra, India

Dr. Vivek Hegde
Professor and Head of Department, Department of Conservative Dentistry and Endodontics, M.A. Rangoonwala College of Dental Science and Research Centre, Pune, Maharashtra, India

Dr. Gulnaz Tamboli
Post Graduate Student, Department of Conservative Dentistry and Endodontics, M.A. Rangoonwala Dental College and Research Centre, Pune, Maharashtra, India

Dr. Khatija Memon
Senior Lecturer, Department of Conservative Dentistry and Endodontics, M.A. Rangoonwala Dental College and Research Centre, Pune, Maharashtra, India

Dr. Nishat Nagaonkar
Senior Lecturer, Department of Conservative Dentistry and Endodontics, M.A. Rangoonwala Dental College and Research Centre, Pune, Maharashtra, India

Mubasshira Sheikh
Undergraduate Student, Department of Conservative Dentistry and Endodontics, M.A. Rangoonwala Dental College and Research Centre, Pune, Maharashtra, India

Zahra Shakil Memon
Undergraduate Student, Department of Conservative Dentistry and Endodontics, M.A. Rangoonwala Dental College and Research Centre, Pune, Maharashtra, India

Corresponding Author:
Dr. Hussain Mookhtiar
Senior Lecturer, Department of Conservative Dentistry and Endodontics, M.A. Rangoonwala Dental College and Research Centre, Pune, Maharashtra, India

Comparative evaluation of salivary IgA levels in carious individuals after application of remineralizing toothpastes: An *in vivo* study

Dr. Hussain Mookhtiar, Dr. Vivek Hegde, Dr. Gulnaz Tamboli, Dr. Khatija Memon, Dr. Nishat Nagaonkar, Mubasshira Sheikh and Zahra Shakil Memon

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Abstract
Aim: This study aims to quantify and evaluate Salivary IgA levels in Carious Individuals pre and post-application of Fluoridated, Nano-hydroxyapatite and CPP-ACP toothpaste.
Materials and Methodology: In aggregate, 90 patients were selected randomly from the outpatient department. DMFT and DMFS indices were performed prior to patient selection. After completion of the analysis, 30 patients having a moderate score were selected. A saliva sample was collected before the commencement of the study and was stored. The individuals were then instructed to use different kinds of toothpaste for the next 15 days based on the groups.
They were randomly divided into the following groups based on toothpaste application (n=10):
Group 1: Fluoridated Toothpaste
Group 2: CPP-ACP Toothpaste
Group 3: Nano-Hydroxyapatite Toothpaste
The patients were recalled after 30 days of toothpaste application. The post-application salivary samples were analysed for IgA levels and quantified using ELISA Test.
The Intra-group and Inter-Group mean distribution of salivary IgA levels resulting from the ELISA Test were examined using One-way analysis of variance (ANOVA) in conjunction with Tukey's Post-Hoc test. This analysis was employed for comparing multiple groups, and the SPSS software (Sigma Stats, Version 2.03, SPSS, Chicago) was utilized for this purpose.
Results: It was observed that among the different types of toothpaste, those containing fluoride exhibited the lowest mean salivary IgA levels, while toothpaste containing CPP-ACP demonstrated the highest levels of sIgA.
1) The CCP-ACP group shows the least levels before application whereas the highest IgA level is seen in the same group post-application.
2) Whereas, in the case of fluoridated toothpaste, the IgA levels pre-application were the highest among the three groups but they reduced drastically after the application.
3) Furthermore, the group treated with nano-hydroxyapatite also exhibited a noteworthy rise in IgA levels subsequent to the application.

Conclusion: Both CPP-ACP and nano-hydroxyapatite-infused toothpaste demonstrated a substantial decrease in salivary IgA levels when compared to toothpaste containing fluoride. When compared to Nano-Hydroxyapatite based toothpaste, CPP-ACP toothpaste showed a better result.

Keywords: Remineralization, CPP-ACP, Nano-hydroxyapatite, fluorides, caries, toothpastes

Introduction
Dental caries is fundamentally driven by bacterial mechanisms. The progression of this ailment stems from the interaction of bacterial byproducts with dietary fermentable carbohydrates, culminating in the infiltration of tooth structure and subsequent mineral dissolution, referred to as demineralization. Pathogenic factors, including acidogenic bacteria like mutans streptococci and lactobacilli, as well as issues with salivary function and dietary carbohydrate intake, contribute to the advancement of caries.

On the other hand, protective elements such as salivary proteins, calcium, and phosphate, along with optimal salivary flow and the presence of fluoride within saliva, collectively work to establish equilibrium, prevent, or even reverse the occurrence of dental caries [1].

As indicated by the WHO report, dental caries persists as a significant public health issue in numerous developed nations, impacting 60–90% of school-age children and the vast proportion of adults. This rise can be attributed primarily to increased sugar consumption and insufficient exposure to fluoride [2].

Fluorides are recognized as effective agents in managing dental caries, offering benefits for both prevention and treatment. The primary mechanism through which fluoride aids in caries prevention is by mitigating demineralization while encouraging enamel remineralization. Additionally, fluoride exerts an influence on microorganisms. In regular conditions, the susceptibility of enamel to acid-buffered dissolution changes in relation to fluoride concentration. Once fluoride concentration reaches 0.05 mg/l, the solubility of enamel diminishes. Fluorine can bind with free hydroxyapatite (HA) to generate fluorohydroxyapatite (FHA) within a saturated hydroxyapatite solution, which can then be re-incorporated into enamel. This phenomenon is termed remineralization. Common methods of topical fluoride application include fluoride toothpaste, fluoride mouth rinse, fluoride varnish, fluoride gel, and fluoride foam [4].

As knowledge, technology, and research have advanced over time, numerous novel products have emerged as effective agents in preventing dental caries. Examples of such products encompass casein phosphopeptide amorphous calcium phosphate (CPP-ACP) and nano-hydroxyapatite, among other options.

Employing milk and its derivatives as a preventive measure against dental caries has introduced an innovative approach to the concept of remineralization. The safeguarding impact of milk against caries is attributed to the existence of casein, calcium, and phosphate, elements that bolster resistance against acid-induced dissolution. A prominently investigated and acknowledged remineralizing substance in this context is casein phosphopeptide amorphous calcium phosphate (CPP-ACP) [5].

Nanotechnology has seen notable progress, resulting in particle sizes typically ranging from 0.1 to 100 nm. Coupled with alterations in particle morphology, this advancement has led to the creation of profoundly bioactive calcium and phosphate compounds. These compounds exhibit enhanced potential for infiltrating the pores within demineralized regions, thus fostering the likelihood of remineralization. The introduction of nanohydroxyapatite crystals into enamel pores serves as a framework during the precipitation process, actively promoting the growth and structural solidity of crystals [6].

Saliva plays a pivotal role in maintaining oral well-being, acting as a key regulator. It serves as a reflection of the body's physiological condition, encapsulating emotional, endocrine, nutritional, and metabolic changes, often referred to as "The Body's Mirror." Critical salivary constituents such as immunoglobulins, salivary proteins, salivary calcium, inorganic phosphorus, and alkaline phosphatase levels, in conjunction with factors like flow rate, viscosity, buffering capacity, and pH, collectively contribute significantly to the onset and advancement of dental caries [7].

The communicable character of dental caries implies that the activity of caries is influenced by host immunity. The particular immune defense against mutans streptococci is facilitated by the common mucosal immune system (CMIS). Immunoglobulin A (IgA) is predominantly discharged by the common mucosal immune system into various bodily secretions, including saliva [8, 9] Saliva inherently contains IgA antibodies targeting various streptococcal antigens, which play a significant role in guarding against dental caries [10].

Hence, the objective of the investigation was to measure and assess salivary IgA levels in individuals with dental caries before and after the application of fluoridated, nano-hydroxyapatite, and CPP-ACP toothpaste.

Materials and Method

The study was designed and carried out in the Department of Conservative Dentistry & Endodontics, M.A. Rangoonwala College of Dental Sciences and Research Centre, Pune, in collaboration with the Department of Microbiology. After obtaining institute ethical clearance, a total of 90 patients were randomly selected from the outpatient department. DMFT and DMFS indices were performed prior to patient selection. After completion of the analysis, 30 patients having a moderate score were selected and informed about the study.

Saliva Collection

Prior to gathering saliva samples, participants were directed to chew paraffin wax for a period of 5 minutes. Subsequently, around 3 ml of stimulated saliva was collected. The collected samples were placed into sterile containers, appropriately labelled, and then stored in preparation for testing.

The individuals were then instructed to use different toothpaste for the next 15 days based on the groups.

They were randomly divided into the following groups based on toothpaste application (n=10):

Group 1: Fluoridated Toothpaste

Group 2: CPP-ACP Toothpaste

Group 3: Nano-Hydroxyapatite Toothpaste

The patients were recalled after 30 days of toothpaste application. Stimulated, whole saliva samples were obtained in sterile containers. The salivary samples were analysed for IgA levels and quantified using ELISA Test.

Pre-processing of samples

Saliva samples collected pre-operatively were processed. They were placed on a shaker so that the debris present in the saliva comes up and can be filtered out easily, as shown in Fig. 1. Subsequently, the samples were preserved in a freezer at a temperature of -68 °C. (Fig. 2). The samples were then taken in a test tube and centrifuged (Eppendorf Centrifuge 5702 A) at 3000 rpm for ten minutes to separate the supernatant (Fig. 3), which was then collected and transferred in a microfuge tube for ELISA test (Enzyme Linked Immunosorbent Assay)

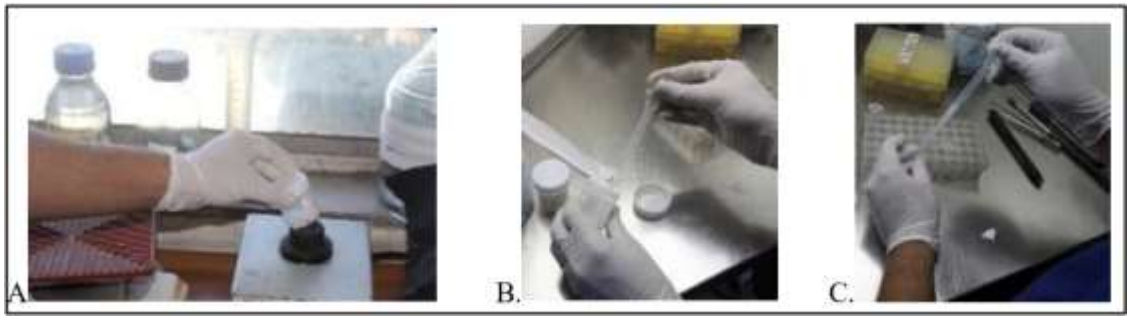

Fig 1: Pre-processing of saliva samples–saliva samples are placed on a shaker to separate the debris

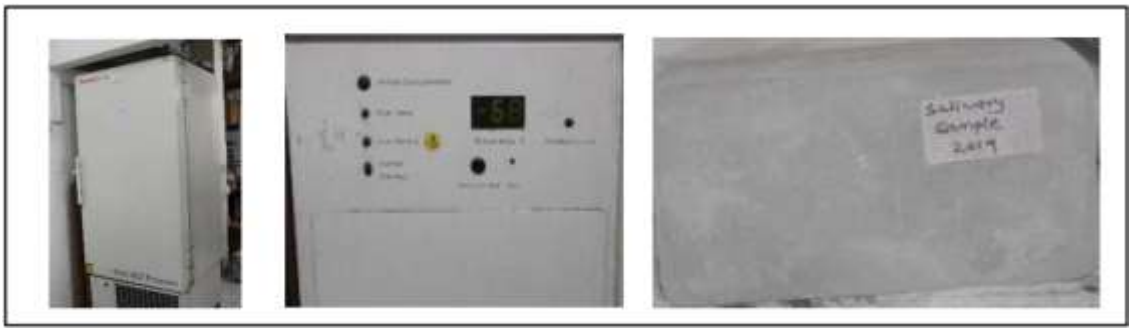

Fig 2: Storage of samples in a freezer at–680 C until they are analysed.

Fig 3: (A)-Samples placed in centrifuge tubes, (B)-The samples were subjected to centrifugation at 3000 rpm for a duration of 10 minutes, (C) Saliva after centrifugation

ELISA processing

The supernatant was examined by enzyme-linked immunosorbent assay (ELISA) using Human IgA ELISA Quantitation kit (Catalog No: E80-102; Bethyl Laboratories, Montgomery, Texas, USA-Fig. 4(A))

All steps were performed at room temperature. The buffer preparations were done (according to the manual provided by the kit) before the ELISA procedure. The standards, blanks, study and control samples were analyzed in duplicate. Ninety-six microliters of capture antibody were diluted to 9600 µl coating buffer and coated on each well. The coated plate was incubated for 60 min (Fig. 4 (B)). After incubation, the capture antibody was aspirated from the solution from each well with the Skan washer instrument. Each well was filled with wash solution and was removed by aspiration. This was repeated for three washes. Next 200 µl of blocking (post coat) solution was added to each well and incubated for 30 min at room temperature. After incubation, the blocking solution was removed from each well and washed three times. Since our samples were expected to have high salivary IgA levels than the standard range given by the company, the samples were diluted in sample diluents. The standards which were supplied along the kit were also diluted in sample diluents. One microliter of saliva sample was diluted to 100 µl in sample diluents. One hundred microliters of the samples were transferred to assigned wells and incubated for 60 min at room temperature. After incubation, samples and standards were removed and each well was washed 5 times. Then, 100 µl of horseradish peroxidase (HRP) conjugate was transferred to each well and incubated for 60 min and each well was washed five times. Next, 100 µl of tetramethyl benzidine (TMB) peroxidase substrate solution was transferred to each well and incubated for 15 min at room temperature. Enzyme substrate reaction resulted in blue colour formation. To stop the TMB reaction, 100 µl of 2 M sulfuric acid was added to each well and the blue colour changed to yellow. Using a microtiter plate reader, Versamax molecular device (USA), the plate was read at a wavelength of 450 nm.

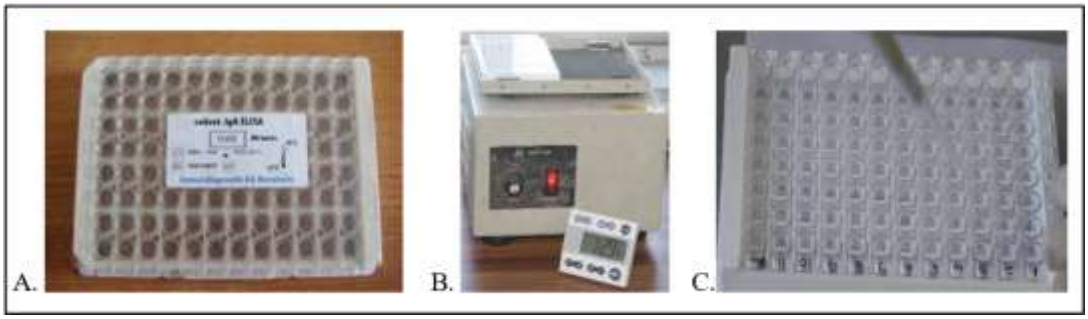

Fig 4: (A) ELISA plate before placement of saliva samples and processing (B) Incubation at Room Temp. for 60 mins (C) Salivary sample with Reagent placed in ELISA wells.

Fig 5: (A) & (B) Plate Washing (C) Substrate Placement

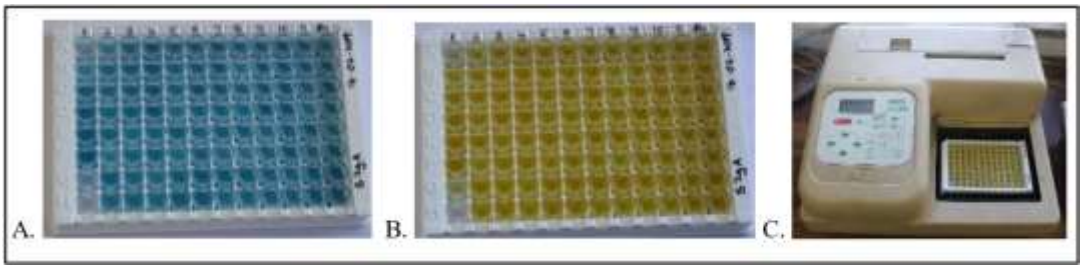

Fig 6: (A) & (B) After incubation for 90 mins at room temp. (C) Plate placed in ELISA reader

Statistical Analysis

One-way analysis of variance (ANOVA), coupled with Tukey's Post-Hoc test, was employed to assess the distribution of mean salivary IgA levels within both Intra-group and Inter-Group contexts. This comprehensive analysis facilitated comparisons across multiple groups, and to facilitate this, the statistical tool SPSS software (Sigma Stats, Version 2.03, SPSS, Chicago) was utilized.

Results

Table 1: Intergroup Mean Salivary IgA levels

Toothpaste agent used	Mean salivary IgA levels (µg/ml)
Fluoridated	175.37
CPP- ACP	242
Nano hydroxyapatite	212

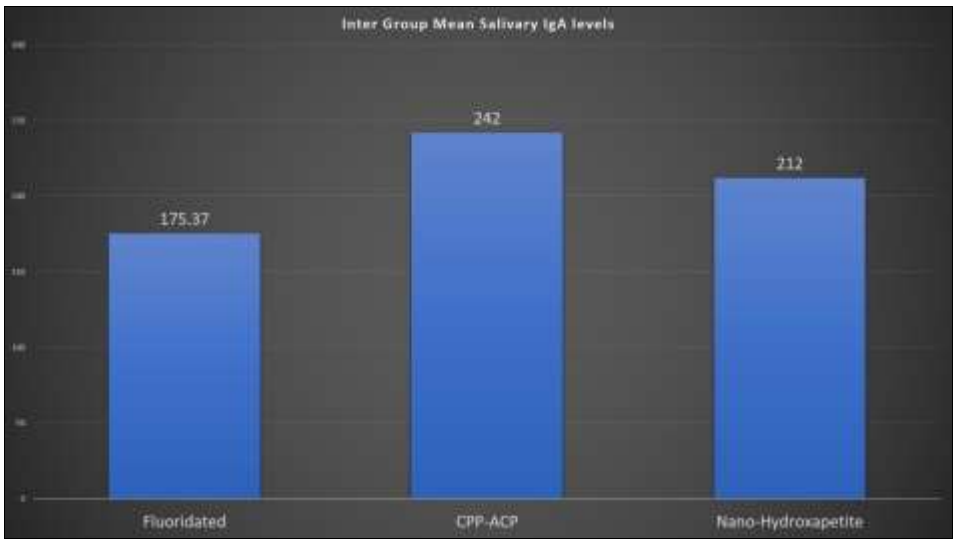

Fig 7: Graphical representation of Inter Group Mean Salivary IgA levels

As indicated in Table 1, the fluoridated toothpaste group recorded the lowest mean salivary IgA levels at 175.37 µg/ml, while the CPP-ACP toothpaste group demonstrated the highest sIgA levels at 242 µg/ml.

Table 2: Intragroup Mean Salivary IgA levels

Toothpaste agent used	Mean salivary IgA levels (µg/ml)	
	Pre-application	Post-application
Fluoridated	181.75	175.37
CPP-ACP	165.75	242
Nano hydroxyapatite	169.75	212

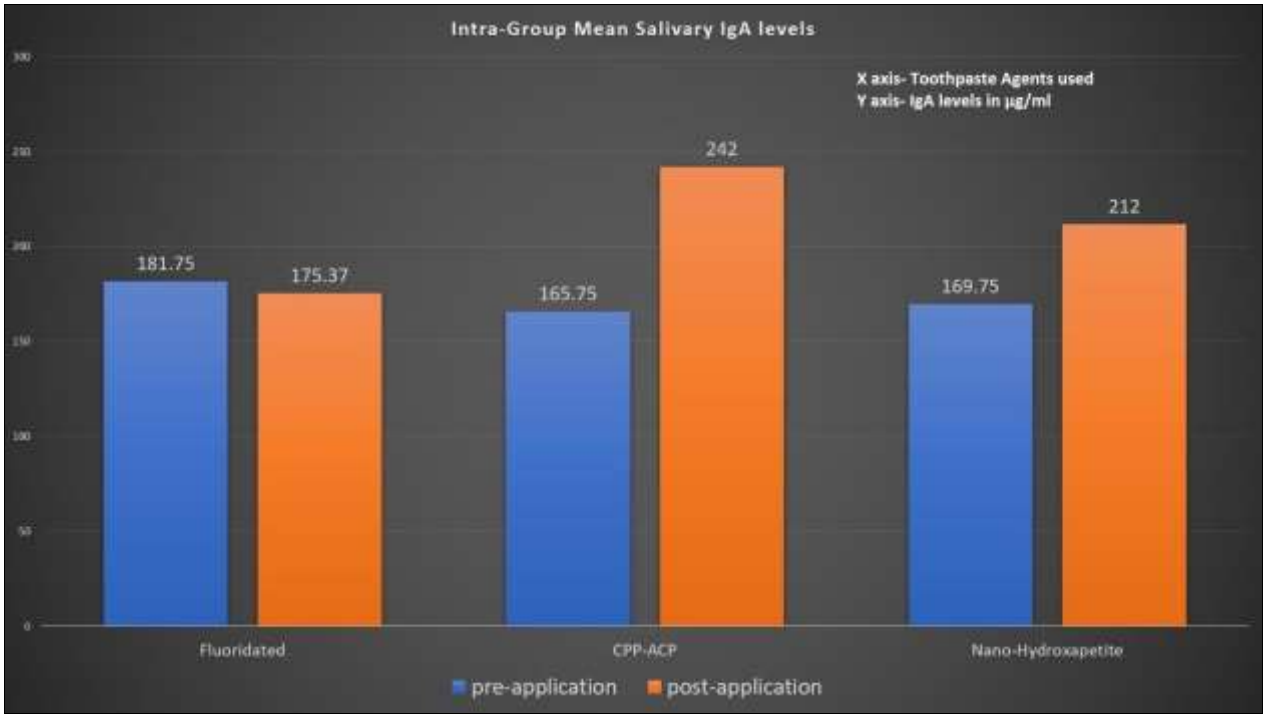

Fig 8: Graphical representation of Intragroup Mean Salivary IgA levels

As demonstrated in Table 2, the Intragroup Mean salivary IgA levels are depicted both before and after the application of the three distinct kinds of toothpaste. The CCP-ACP group shows the least levels before application whereas the highest IgA level is seen in the same group post-application. Whereas, in the case of fluoridated toothpaste, the IgA levels pre-application were the highest among the three groups but they reduced drastically after the application. The nano-hydroxyapatite group also showed a significant increase in the IgA levels post-application

Discussion

Saliva has become a non-invasive systematic sampling measure for medical diagnosis and research. Its status in the oral cavity is considered at par with that of blood. Salivary IgA inhibits adherence by preventing the colonisation of microorganisms on surfaces of teeth [11].

This research aimed to evaluate salivary IgA levels in individuals with dental caries and those without. As noted by Senpuku *et al*., their findings indicate elevated salivary IgA levels in the caries-free group in contrast to the group with caries, reinforcing the recognition of salivary IgA in medical literature [12].

Limited research has delved into investigating the impact of salivary IgA levels on dental caries within the Indian population in this specific context. Notably, salivary IgA functions as a potent agglutinin due to the possession of four antigen binding sites by each molecule [13].

Fluorides have been traditionally used as a remineralisation agent and various evolutions of fluorides have been produced for the same purpose. According to Guzel *et al*., elevated levels of sIgA and sIgG have the potential to impede the advancement of caries in individuals. However, in this study, there was a fall in sIgA levels, thus questioning the role of fluorides in the decrease in caries [14].

However, in recent years, the evolution of remineralising agents has focused on more calcium and phosphate- based products which result in the replacement of the lost tooth structures when compared to fluorides which result in the formation of fluorapatite crystals.

Within this study, both CPP-ACP and Nano-hydroxyapatite toothpaste demonstrated a noteworthy rise in salivary IgA levels when contrasted with the fluoridated toothpaste. However, when compared with Nano-hydroxyapatite toothpaste CPP-ACP showed better results.

Casein, a protein derived from milk, is recognized for its affinity towards calcium and phosphate, constituting a natural component of food. It exhibits a facile binding capability to various surfaces including the saliva pellicle, dental plaque, soft tissues, and even the hydroxyapatite component of enamel. According to Shadha *et al*., CPP-ACP has purported efficacy in caries due to its suggested antimicrobial action. Microbial analysis revealed that the CPP-ACP molecule had a direct effect on micro-organisms preventing their adherence to the tooth surface [15].

Furthermore, an analysis undertaken by Tao *et al*. revealed that CPP-ACP showcases notable effectiveness in addressing early caries lesions located on occlusal surfaces of teeth [16].

Nano-hydroxyapatite comprises calcium and phosphate ions that closely resemble those present in enamel and dentin. Nano-hydroxyapatite adheres to the pores created by demineralisation thus resulting in the replacement of loss structure rather than remineralisation [17, 18, 19].

As per Daas and Issa Nooreddin, the utilization of nano-hydroxyapatite paste as a potential alternative remineralizing agent holds promise due to its comparatively lower fluoride concentration in comparison to fluoride varnish. This could prove advantageous for specific groups such as children, pregnant individuals, and those at an elevated risk of dental fluorosis [20]. In line with Anil Aiswarya *et al*., toothpaste formulations containing nano-hydroxyapatite (nHAp) exhibit a range of therapeutic and preventive benefits. However, comprehensive and extended studies adhering to standardized protocols are essential to arrive at conclusive determinations regarding the impact of nHAp dentifrices on both primary and permanent dentitions [21].

Conclusion

Given the constraints of this study, it was observed that both CPP-ACP and nano-hydroxyapatite-infused toothpaste variants displayed a notable decrease in salivary IgA levels when compared to toothpaste containing fluoride.

However, when compared to Nano-Hydroxyapatite-based toothpaste, CPP-ACP toothpaste showed a better result.

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