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Evaluation of Remineralisation Potential of an Indigenously Developed Dentifrice – An In Vitro Study

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Abstract

White spot lesions are a regrettable but frequent side effect of orthodontic treatment that can be avoided by using dentifrices that encourage enamel remineralization. To evaluate the remineralising efficacy of an indigenously developed dentifrice containing green synthesised strontium fluorapatite nanoparticles (SrFAp NPs). SrFAp NPs were green synthesised using plant extracts of Equisetum arvense and Laminariales along with fluorine and hydroxyapatite precursors. Characterisation and cytotoxicity evaluation of the SrFAp NPs was done. Remineralising efficacy was evaluated on 30 extracted non carious teeth separated into 1 control group and 5 test groups of 5 teeth each. All samples underwent demineralisation and remineralisation. The test groups were exposed to the corresponding concentration of SrFAp NPs dentifrice. The pre and post treatment Vickers hardness test and Energy dispersive x-ray analysis were performed. Statistical analysis done using SPSS software. Characterisation and cytotoxicity tests revealed successful formation of SrFAp NPs with good cell viability. Increase in enamel hardness values seen in all test groups post treatment. Highly significant difference in enamel hardness values seen in 0.1%, 0.8% and 1% SrFAp dentifrices. EDAX analysis shows strontium uptake in all the test groups. 0.1 Wt%, 0.2 Wt%, 0.1 Wt%, 0.5 Wt%, 0.2 Wt% of Sr uptake seen in 0.1%, 0.2%, 0.4%, 0.8% and 1% SrFAp dentifrices respectively. SrFAp NPs containing dentifrices have successfully remineralised enamel leading to increased enamel hardness. EDAX shows successful uptake of strontium of highest 0.5 Wt% seen with 0.8% SrFAp NP dentifrice.

Keywords: *Dentifrice, Fluorapatite, Nanoparticle, Strontium, Tooth remineralization*.

Introduction

White spot lesions are a regrettable but typical side effect of orthodontic therapy [1]. As enamel is an acellular and avascular structure, damaged enamel does not remodel. Thus, it is imperative that early carious lesions are arrested and restored by enhancing factors that favour remineralisation of enamel.

Various remineralising strategies have been developed of which fluoride is widely used due to its strong cariostatic property. Fluoride based toothpastes have led to the decline of dental caries world-wide [2]. Several calcium phosphate-based compounds such as tricalcium phosphates (TCP), dicalcium phosphate dihydrate (DCPD), and amorphous calcium phosphates (ACPs) in combination with fluorides have a synergistic action in preventing carious lesions [3]. Other agents such as casein phosphopeptides (CPPs) along with ACPs have shown successful remineralisation of subsurface enamel lesions [4]. Bio active glass (BAG) forms a hydroxyl carbonate apatite (HCA) which attaches to the enamel surface and releases ions for remineralization [5]. However, methods for successfully modifying remineralized crystals to produce nanocrystals that resemble enamel and to attain desired mechanical properties have not yet been realised.

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Nanoparticles (NPs) exhibit superior ion release in comparison to microparticles [6]. They also have accelerated absorption rates, extended half-life, increased concentration, and targeted drug delivery [7-8]. Recently, biomimetic remineralization materials have been used to create NPs that can form apatite crystals inside fully demineralized collagen fibres [3]. Nanohydroxyapatite has bioactive properties as osteoconductivity and cytocompatibility as is the major constituent of mineralised tissue of the human body and can be substituted for repair biomimetically [7]. Since the early 1980's field trials of toothpastes containing nano hydroxyapatite have been carried out in Japan, which led to its approval as an anti-caries agent in 1993 [9,10].

Several other dentifrices containing nanohydroxyapatite have been compared against fluoridated dentifrices and shown to be successful in remineralizing enamel and reducing dentin hypersensitivity [11-16].

Recent trends towards strontium substituting calcium in hydroxyapatite have resulted in increased crystallinity and bioactivity [17]. Strontium exhibits osteoinduction and high solubility. Strontium also acts synergistically with fluoride leading to its increased ion release and enhanced enamel remineralisation [18]. Thus, the rationale behind this study was to evaluate the remineralisation efficacy of strontium and fluorine doped hydroxyapatite NPs in a indigenously developed dentifrice.

The downside of synthetically developed NPs includes environmental pollution, high energy consumption, toxicity and other adverse health effects. In attempts to reduce such drawbacks, green synthesis method of producing NPs has led the frontier of research. Environmentally friendly materials, such as bacteria, fungi, algae, and plants, are used in green synthesis as reducing agents to convert metal ions into metallic nanoparticles (NPs) [19]. Strontium fluorapatite nanoparticles (SrFAp NPs) were green synthesised using plant extracts of *Equisetum arvense* and *Laminariales* along with fluorine and hydroxyapatite precursors based on a previous study by the same author [20]. This study evaluated the remineralising efficacy of an indigenously developed dentifrice containing green synthesised SrFAp NPs.

Materials and Methods

Study Setting

The research was a single-centre in vitro study carried out in March and April of 2023 at the Saveetha Dental College and Hospital research facility in Chennai, Tamil Nadu. Ethical clearance was granted by the Saveetha Institute of Medical and Technical Sciences (SIMATS) ethics committee.

Synthesis and Characterisation of Strontium Fluorapatite Nanoparticles (SrFAp NPs)

SrFAp NPs were developed based on a previous study by the same author using green synthesis method from plant extracts of Equisetum arvense (Horsetail) and Laminariales (Kelp) along with fluoride and hydroxyapatite precursors [20]. The developed NPs were characterised by visual observation of colour change, Ultraviolet–visible spectroscopy (Perkin Elmer Lamba 35), Fournier transform infrared spectroscopy (Alpha Bruker's ll FT-IR) and scanning electron microscopy (SEM) with energy dispersive X-ray analysis (EDAX) (JSM–IT800 Nano SEM).

Cytotoxicity Testing of Strontium Fluorapatite Nanoparticles (SrFAp NPs)

SrFAp NPs were tested for cell viability using MTT assay against human gingival fibroblast (HGF) cell lines in a previous study by the same author [21]. The HGF cell lines were cultured and treated with varying concentrations of 10, 20, 30, 40, and 80 μg/mL of the SrFAp NPs. The MTT reagent was added to the media and the reaction product

transferred to a 96-well ELISA plate was measured with UV-Spectrometer ELX800.

Enamel Remineralisation Analysis

Sample Collection

A total sample size of 30 non carious, permanent premolar teeth with intact crowns, freshly extracted for orthodontic purposes were used in the study. All the teeth were cleaned in saline and blotted dry. All samples were separated into 6 groups of five teeth each: 1 control group and 5 test groups with different concentrations of SrFAp NPs. A window of size 5 mm x 5mm was created on the enamel surface by using adhesive tape. The rest of the enamel surface was coated with nail varnish. The adhesive tape was removed after drying the varnish and the square area for testing of microhardness and EDAX was identified.

Formulation of Dentifrice

All the constituents were weighed and amalgamated using a vortex mixer for homogeneity as follows:

Calcium carbonate: 35 - 50 %w/v
Sodium lauryl sulphate: 0.5 - 3 %w/v
Glycerine: 30 %w/v
Carboxymethyl cellulose: 0.1 - 5 %w/v
Carrageenan: 0.01 - 1 %w/v
Methyl paraben: 0.1 %w/v
Titanium dioxide: 0.5 %w/v
Saccharine: 0.3 %w/v
Menthol: 1.5 %w/v
Strontium Fluorapatite: 0.1 – 2 %w/v
Purified water: q.s

SrFAp dentrifices were synthesised with different concentrations of the NPs and separated into 5 test groups. Group 1 dentifrice contained 0.1% SrFAp NPs, while groups 2, 3, 4 and 5 dentifrices contained 0.2%, 0.4%, 0.8% and 1% SrFAp NPs respectively. (Figure 1).

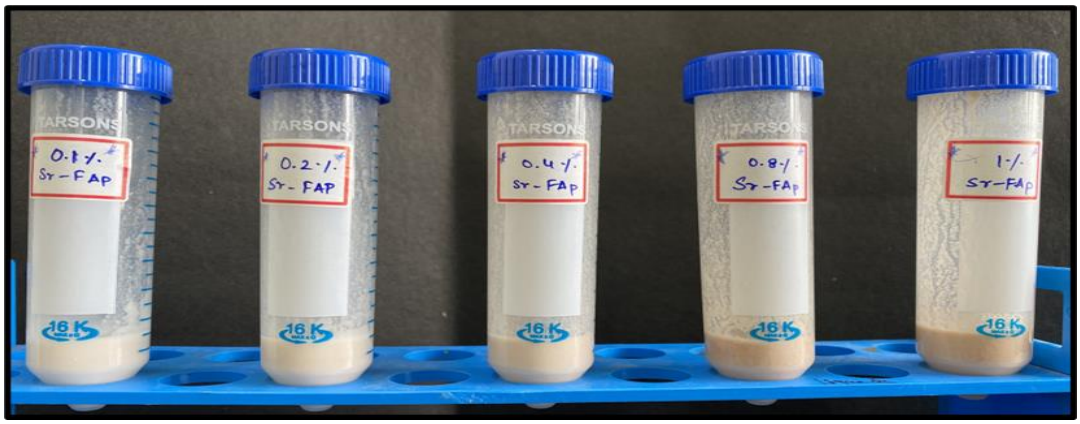

Figure 1. Varying Concentrations of Srfap Dentifrices

Demineralising Solution

Demineralising solution was prepared for 1000ml using 323.4mg of calcium chloride, 450µl of acetic acid and 300mg of potassium dihydrogen phosphate. pH was adjusted to the range of 4.4-4.7 using sodium hydroxide or hydrochloric acid.

Remineralising Solution

Remineralising solution was prepared for 1000ml using 136mg of potassium dihydrogen phosphate, 74.56mg of potassium chloride and 147.02mg of calcium chloride. pH was adjusted above 7 using sodium hydroxide or hydrochloric acid.

pH Cycling

All the test groups and the control group were immersed in 15 ml of demineralising

solution each for 72 hours. (Figure 2) The control group was exposed to demineralising solution and remineralising solution but not the SrFAp dentifrice. The pretreatment Vicker's hardness test was done for all the samples. Three indentations were made in each tooth and the average microhardness was calculated. All the samples of the test groups and the negative control group were then maintained in 15 ml of remineralising solution each for a period of 10 days. Each sample of the test groups was then treated with the corresponding concentration of SrFAp dentifrice for 3 minutes every day and rinsed. (Figure 3) It was then placed back in freshly replaced remineralising solution. Post treatment Vicker's hardness test and Energy dispersive x-ray analysis was repeated for all the samples. Three indentations were again made as close to the initial indentations as possible to precisely calculate the difference in remineralisation as the enamel varies in hardness at different regions of the tooth.

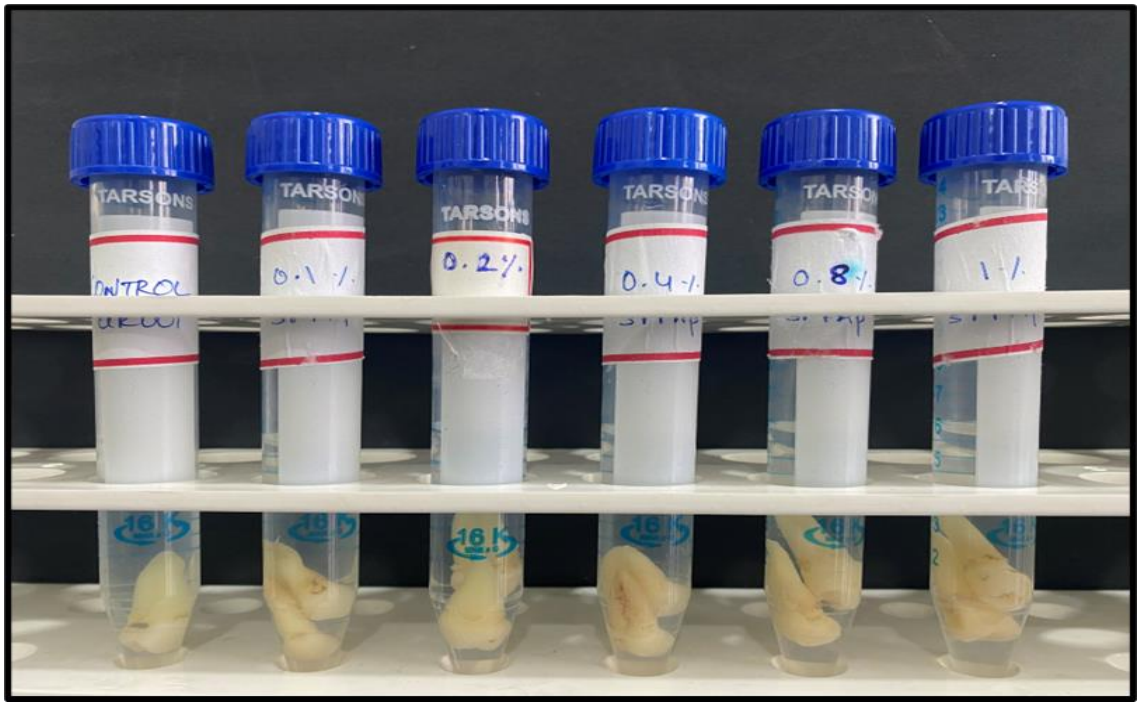

Figure 2. Samples immersed in demineralising and remineralising solutions

Figure 3. Samples treated with the corresponding dentrifice

Microhardness Test

Pre and post treatment enamel microhardness were determined using Shimadzu HMV-G31D microhardness tester. Vickers elongated diamond pyramidal indentations were made for 10 seconds using 500g weight. Three indentations were placed, and the average vickers hardness value was calculated. (Figure 4).

Figure 4. Vicker's Hardness Test

Statistical Analysis

Data was collected and statistically analysed using SPSS software (version 23, IBM Statistical Package for the Social Sciences). Kolmogorov-Smirnov test was used to evaluate the normality of data. The mean and standard deviation of the Vicker's hardness number and the EDAX analysis was calculated using descriptive statistics. Paired sample t test was conducted to assess the significance of the difference between the pre and post treatment enamel hardness values.

Results

Characterisation of the SrFAp NPs

The formation of the NPs is indicated visually by a change in color from pale brown to dark brown. UV Vis Spectroscopic analysis observed the formation of NPs at wavelengths of 420 and 410 nm correlating to the surface plasmon resonance (SPR) band of the strontium NPs and hydroxyapatite NPs, respectively. The creation of the SrFAp NPs was validated by a second measurement taken at 375 nm. It was possible to identify peaks in the FT-IR mid-infrared spectrum that corresponded to calcium salts, carboxylic acid salts, amino and imino compounds, fluoro compounds, phosphates, and strontium. SEM analysis identified 20–50 nm-sized sharp, rod-like particles that represented metallic elements like strontium. There were observed spherical agglomerates with sizes between 30 and 80 nm, which correspond to hydroxyapatite. Using elemental analysis (EDAX), the composition was found to be 7.0 wt% strontium, 5.4 wt% fluorine, 9.4 wt% calcium, 4.2% phosphorus.

Cytotoxicity Testing

Cell viability in the control group was 100%. The viability of cells declined as the NPs' concentration increased. Cell viability at the lowest concentration of 10 μg/mL was 83%; at 20, 30, 40, and 80 μg/mL, it was 81, 79, 74, and 70%, respectively. 70% of the cells remained viable even at the maximum concentration of 80 μg/mL of NPs.

Enamel Remineralisation Analysis

Kolmogorov-Smirnov test revealed normal distribution of data. Mean and standard deviation of all groups are tabulated in Table 1. There is an increase seen in the mean enamel hardness value from baseline levels in all the test groups. This indicates successful

remineralisation of the enamel. There is a decrease in the enamel hardness value in the control group. This indicates the efficiency of the demineralising solution and the absence of remineralising effect of the remineralising solution used. Therefore, the remineralisation occuring in the test groups is due to the efficiency of the SrFAp dentifrice alone, the remineralising solution plays no part in increasing the enamel hardness value. The mean hardness value gradually increases with increase in the concentration of the SrFAp NP dentifrice. There is a slight decrease in hardness in Group 2 and Group 3 corresponding to 0.2% SrFAp dentifrice and 0.4% SrFAp dentifrice respectively. A sharp increase in hardness values is seen with Group 4 and Group 5 corresponding to 0.8% and 1% SrFAp dentifrices respectively.

Table 1. Descriptive Statistics of All the Samples

Group	**Pre treatment (Mean ± SD) VHN**	**Post treatment (Mean ± SD) VHN**
Control	280 ± 19.38	194.8 ± 34.62
Group 1	294.2 ± 36.89	358 ± 30.52
Group 2	306.2 ± 23.12	318.8 ± 32.37
Group 3	277.6 ± 35.68	299.8 ± 41.14
Group 4	289.4 ± 27.55	373.2 ± 17.08
Group 5	287.8 ± 35	380.4 ± 13.64

Intragroup comparison using paired samples to test is tabulated in Table 2. A highly significant difference is seen between the pre and post treatment values in the control group and groups 1, 4 and 5. A significant difference is seen in the control group indicating the strength of the demineralising solution in reducing the enamel hardness and absence of a remineralising effect of the remineralising solution. A highly significant difference seen in 0.1%, 0.8% and 1% dentifrice indicate the true remineralising potential of the dentifrice.

Table 2. Paired t test for Intragroup Comparison

Group	**T**	**Significance (2 – tailed)**
Control	9.091	.001
Group 1	-6.705	.003
Group 2	-.517	.632
Group 3	-1.074	.343
Group 4	-8.235	.001
Group 5	-5.444	.006

Energy dispersive x-ray analysis was conducted to evaluate the elemental composition of the enamel surface. EDAX testing was performed for pre and post for the control group and post treatment for all the test groups. There is an absence of strontium uptake in the control group pre and post treatment. There is a reduction in calcium wt% seen from pre-treatment to post treatment of the control group. EDAX analysis shows strontium uptake in all the test groups.

0.1 Wt%, 0.2 Wt%, 0.1 Wt%, 0.5 Wt%, 0.2 Wt% of Sr uptake seen in 0.1%, 0.2%, 0.4%, 0.8% and 1% SrFAp dentifrices respectively. 0.8% dentifrice shows the highest Sr uptake. (Figures 5-11).

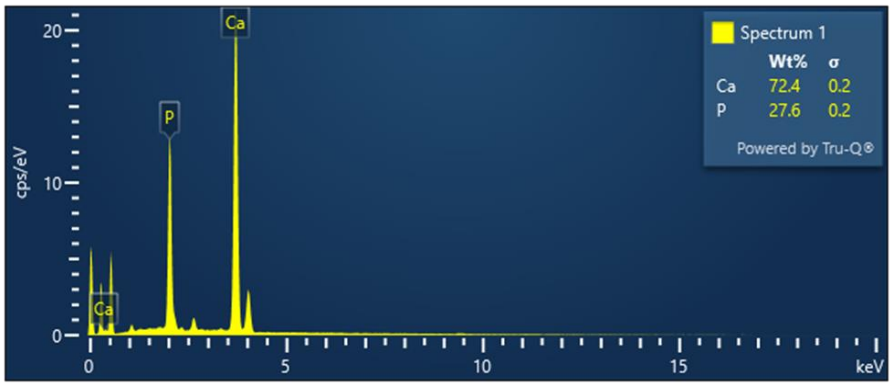

Figure 5. Pre-treatment Control Group EDAX

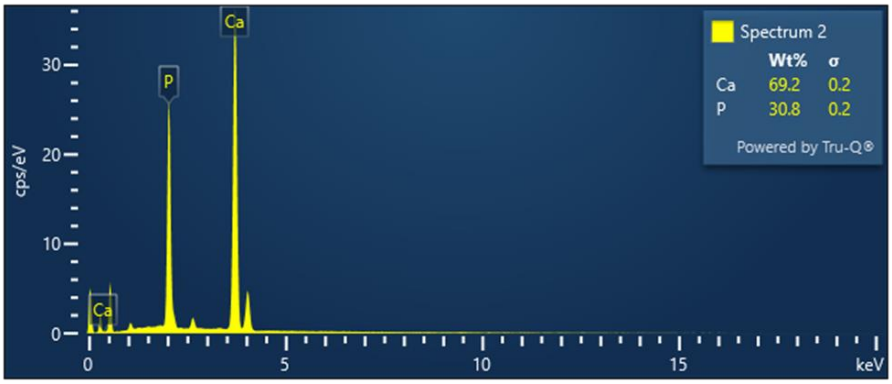

Figure 6. Post treatment Control Group EDAX

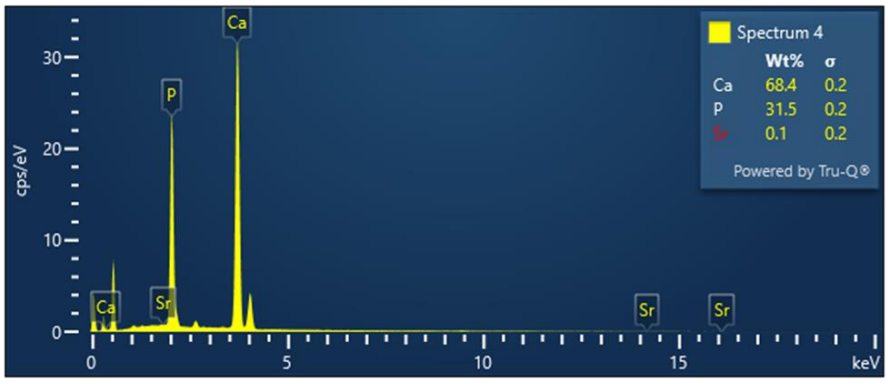

Figure 7. Group 1- 0.1% SrFAp dentifrice EDAX

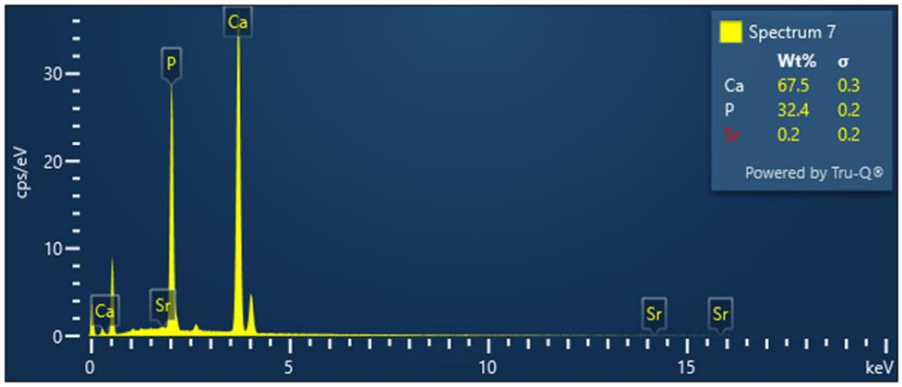

Figure 8. Group 2- 0.2% SrFAp Dentifrice EDAX

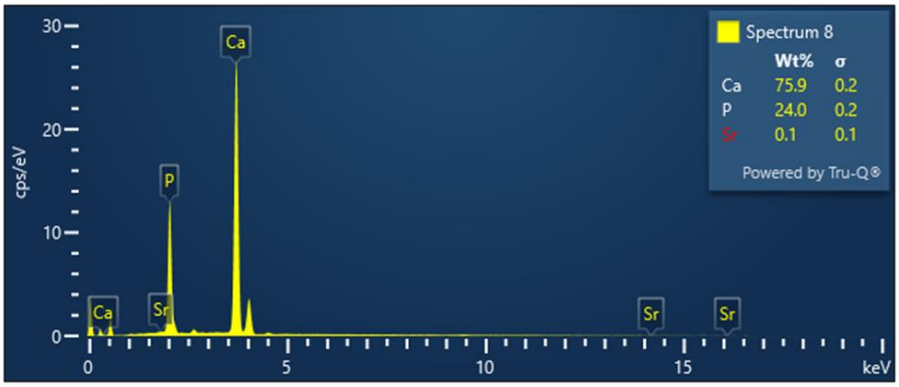

Figure 9. Group 3- 0.4% SrFAp Dentifrice EDAX

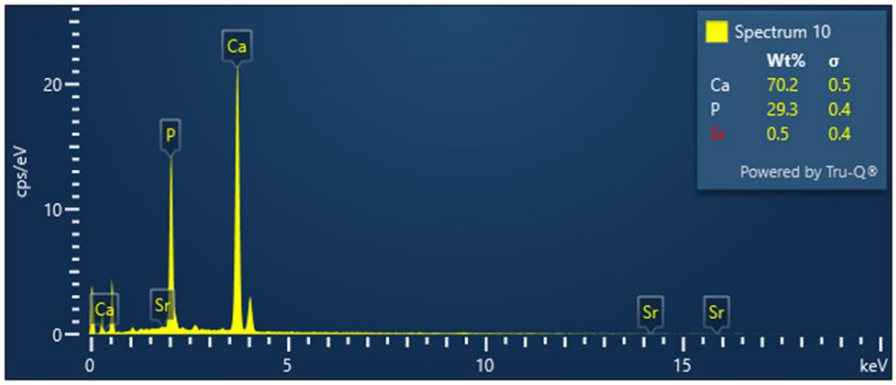

Figure 10. Group 4- 0.8% SrFAp Dentifrice EDAX

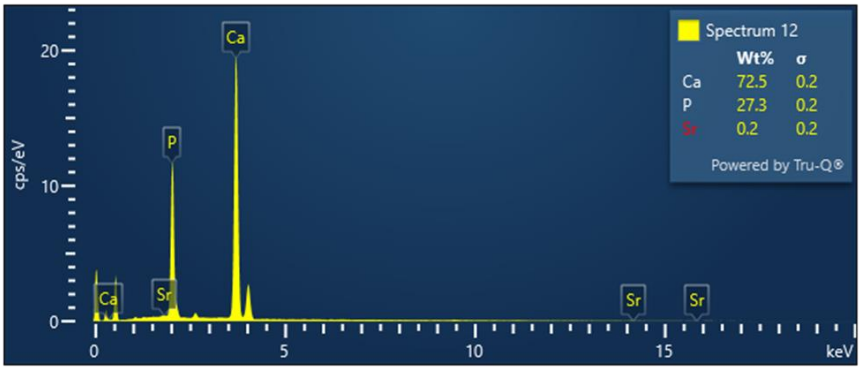

Figure 11. Group 5- 1% SrFAp Dentifrice EDAX

Discussion

The present study aimed at developing an effective remineralising dentifrice composed of SrFAp NPs produced using green synthesis method. SrFAp NPs were successfully synthesised and characterised. Even at the highest concentration of 80 μg/mL NPs, the SrFAp NPs demonstrated good cell viability and were not cytotoxic. The cell viability was 70%.

All five test groups of dentifrices having increasing concentrations of the SrFAp dentifrices exhibited remineralisation efficacy with improved hardness. There was a dose-dependent increase in hardness with increasing concentrations of the SrFAp NPs. An increase in the mean vicker's hardness value was seen between pre and post dentifrice application in all the test groups. There was a decrease in the hardness value in the control group after exposure to the pH cycling alone. This signifies that the remineralising solution has no effect on the enamel hardness. Increase in enamel hardness in the test groups is solely due to the SrFAp dentifrice. Significant difference in the hardness value was seen in the Group 1, Group 4 and Group 5 corresponding to 0.1%, 0.8% and 1% SrFAp dentifrices. Group 5 corresponding to 1% SrFAp dentifrice showed the highest vicker's hardness value. Strontium uptake on the enamel surface was demonstrated by EDAX analysis. 0.8% SrFAp dentifrice shows the highest strontium uptake of 0.5 Wt%.

Incipient caries creates cavities that are less than 50 μm deep. These cavities cannot be filled with conventional restorative techniques that use an invasive technique. Additionally, due to differences in their chemical constitution and crystal structure, the restorative materials available today do not conform to these early cavitations [22]. Therefore, the search for a material which can initiate the reparative process lead to successful supplements of fluoride, calcium as well as hydroxyapatite.

According to reports, pure but non-stoichiometric hydroxyapatite can be formed when strontium is added to calcium in hydroxyapatite [23]. It is this partial substitution of strontium ions for calcium ions in the hydroxyapatite matrix that causes an increase in hydroxyapatite's insolubility [24]. Hydroxyapatite with increased lattice dimensions is formed due to the higher atomic radius of strontium compared to calcium [7]. Strontium was doped in different concentrations to hydroxyapatite for this purpose.

Thus, biomimetic remineralisation of the enamel was achieved through SrFAp NPs dentrifices. Hydroxyapatite toothpastes prove to be more advantageous than fluoride toothpastes as there is lesser probability of toxicity and other adverse effects [25,26,27]. This developed dentifrice combines the advantages of both strontium apatite as well as fluorine. The advantages of the developed dentifrice are many including aiding to combat white spot lesions. It could prevent caries development in patients undergoing fixed orthodontic treatment who are unable to maintain proper oral hygiene [28]. Since the NPs are developed using green synthesis method, the dentifrice is cost effective and environmentally friendly.

Limitations include the in vitro nature of the study where there is a lack of saliva, plaque and salivary pellicle. The samples were subjected to demineralisation and remineralisation as part of the pH cycling which was more aggressive than that occurs in the oral cavity. Hence, further studies involving in vivo testing are required, whereas in vitro studies can be used to test newly developed products. Dentifrices may serve as a limitation as it is dependent on user dexterity and the reach in spread may be less extensive when compared to a mouthwash. Future studies can involve emerging techniques to understand remineralisation better with advanced biomimetic techniques.

Conclusion

SrFAp NPs containing dentifrices have successfully remineralised enamel leading to increased enamel hardness. A dose dependent relationship exists between the concentration of SrFAp NPs and enamel hardness. EDAX shows successful uptake of strontium of highest 0.5 Wt% seen with 0.8% SrFAp NP dentifrice. Future in vivo studies to understand the biomimetic remineralisation technique further are required.

Source of Support

Self-funded Study

Conflict of Interest

Nil

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