Effect of Three Different Pastes on Remineralization of Initial Enamel Lesion: An *in Vitro* Study

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Objective: *Dental caries in enamel is unique as enamel is both acellular and avascular. Fluoride is one of the most effective remineralizing agents in caries prevention. The purpose of the study was to assess whether nano-hydroxyapatite and CPP-ACP can be used as an alternative to fluoride for remineralization of early carious lesions.* ***Study design:*** *Initial artificial carious lesions were prepared in enamel with demineralization solution. The treatment materials used as remineralizing agents were Nano-hydroxyapatite (10%), CPP – ACP (10%), NaF (1000 ppm) and Deionized water (negative control). Surface microhardness (SMH) measurements were performed before/after demineralization and after 3, 6, 9 and 12 days of pH cycling and the percentage surface microhardness recovery (%SMHR) was calculated. The specimens were then examined by scanning electron microscope.* ***Results:*** *Percentage surface microhardness recovery of nano-hydroxyapatite and fluoride was significantly greater than CPP – ACP and negative control. There was no significant difference of %SMHR between n-HA and fluoride at different time periods in the pH cycling. SEM analysis revealed n-HA particles were deposited on the demineralized enamel surface which formed a new surface layer. When observed under SEM CPP – ACP failed to show any significant surface remineralization while surface globular crystal depositions with multiple discrete porosities were evident with fluoride.* ***Conclusion:*** *It was concluded that nano-hydroxyapatite and fluoride had the potential to remineralize initial enamel lesions. CPP – ACP can be used as an effective adjunct to fluoride therapy but cannot be used as an alternative to fluoride.*

Key words: *Casein phosphopeptide-amorphous calcium phosphate, fluoride, nano-hydroxyapatite, pH cycling, surface microhardness.*

INTRODUCTION

Dental caries is an acknowledged global health problem.[1] There exists a delicate balance between the demineralization and remineralization process at the tooth micro-environment. Prolonged multiple exposures to cariogenic food substrates results in an imbalance leading to formation of an early enamel lesion, which may progress into caries or may remain stable and remineralize with time in plaque with a suitable composition.[2] Since enamel lacks cellular repair mechanisms, the events surrounding the development and reversal of caries are dependent upon physiochemical mechanism at the tooth-pellicle/plaque interface.[3]

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The beneficial effects of fluoride in its various vehicles have been regarded for many years, as the mainstay of the non-invasive management of non-cavitated carious lesions. Marinho *et. al.*[4] reviewed various *in vitro* and *in vivo* studies that highlighted the remineralizing effect of fluoride in different concentrations. However, for every two fluoride ions, ten calcium and six phosphate ions are required to form one unit of fluorapatite which are provided by saliva. Availability of these ionic calcium and phosphate from saliva is highly variable and depend on many factors like- basic salivary mineral contents, salivary pH, and buffering capacity of saliva etc.[5] Moreover fluoride, in presence of available salivary ionic calcium and phosphate remineralize enamel surface only, leaving subsurface demineralization unaffected.[6] Also some concerns has been expressed that with the wide array of both prescription and over the counter fluoride products now being marketed in every country, the total fluoride intake has increased to harmful levels.[6] Hence, in-spite of widespread success of fluoride, several new agents have been proposed and recommended for personal and professional applications.[6] Casein Phosphopeptides (CPP), produced from a typtic digest of milk protein "casein", stabilizes amorphous calcium phosphate in solution.[6] They form nanocomplexes at the tooth surface, thus providing a reservoir of non-structurally bound calcium and phosphate ions which favor remineralization during a cariogenic attack.[7] Nano-hydroxyapatite (n-HA) is one of the most biocompatible and bioactive materials and its nano-sized particles

have similarity to the apatite crystal of tooth enamel in morphology, crystal structure and cystallinity.[8] In recent years, an increasing number of reports have shown that nano-hydroxyapatite has the potential to remineralize artificial carious lesions following addition to toothpastes, mouthwashes, etc.[9]

Therefore, in view of wide range of products available for use, clinicians and patients face a dilemma to produce the highest remineralization in enamel. Therefore the aim of this study was to assess whether nano-hydroxyapatite and CPP-ACP can be used as an alternative to fluoride for remineralization of early carious lesion using Vicker's microhardness test and scanning electron microscopy.

MATERIALS AND METHOD

Prior approval for the study was obtained from institutional ethical committee.Twenty six freshly extracted human permanent maxillary incisor teeth were obtained from the Department of Oral and Maxillofacial Surgery, Modern Dental College and Research Centre, Indore after prior permission from the institute. The collected teeth were screened under stereoscope for the presence of cracks, hypoplasia or white spot lesions and were excluded if having any one of these. The selected teeth were cleaned using ultrasonic scaling tips and then stored in 0.1% thymol. The crowns of all the 26 permanent maxillary incisors were separated from the roots at the cemento-enamel junction. Enamel block of 3 x 3 x 2 mm was prepared from flatter labial surface and embedded in polymethyl methacrylate. Superficial surface of enamel was ground flat with water-cooled carborundum discs (1200 grit; Water Proof Silicon Carbide Paper, Struers, Germany) and polished with diamond paste (15 μm Diamond Paste, Struers, Germany), thereby removing approximately 100 μm of the outermost enamel layer and yielding a flat surface as suggested by Huang *et al* [9].

Demineralizing solution was prepared with 0.05 M lactic acid, 2.2mM of calcium chloride and 2.2mM of sodium dihydrogen orthophosphate. All materials for demineralizing solution were obtained from Metallurgical Lab, Vishvesvaraiya National Institute of Technology, Nagpur, Maharashtra, India. Surface microhardness (SMH) values of the samples were recorded using Vickers microhardness tester under 500 grams of loads for 15 seconds. Early artificial carious lesions were produced in the enamel according to Ten Cate and Duijsters.[10] Distribution of groups and related details are summarized in table-1. All experimental pastes and water were applied on the enamel surface by an applicator brush and left undisturbed for 3 minutes. CPP-ACP and Colgate Total toothpaste were purchased from respective commercial companies, while n-HA 10% in paste form was provided by Group Pharmaceuticles Ltd.

The cycling schedule was designed to approximate the pH dynamics of the oral environment as reported by White[11] which involved two hours of demineralization a day, with the remaining time in artificial saliva acting as remineralizing solution. The demineralizing and remineralizing solutions were freshly made every third day with pH adjusted to 4.5 and 7.2 respectively. Research plan for the methodology is shown in (figure 1).

The percentage surface microhardness recovery (%SMHR)[12] of each treatment material were carried at different time interval (n = 3th ,6th,9th & 12th day). Percentage surface microhardness recovery was calculated [%SMHR= 100 (SMHn – SMH_2)/ (SMH_1- SMH_2); n - mean microhardness value at 3rd , 6th , 9th & 12th day of pH cycling model, SMH_1 - mean baseline microhardness value, SMH_2 - mean microhardness value after demineralization for 72 hours].

SEM analysis was carried out to confirm and compare the surface remineralization after 12 days of application of the treatment materials. One randomly selected sample from group A, group B, group C & group D at 12th day of pH cycling model was selected to check the remineralization under SEM sample analysis.

Statistical analysis was done using SPSS 20 software (IBM, USA). SMH and SMHR were analyzed at 3rd, 6th, 9th & 12th day using KW ANOVA test. Man-Whitney test was done to check the inter-group significance. The significant level (P) was set at 0.05

RESULTS

The mean baseline surface microhardness value before demineralization was statistically non-significant among the groups (table 1). The difference in mean SMH value after demineralization was also statistically non-significant among the groups. SMH value in all the treatment groups had comparatively increased after 12 days of pH cycling when compared to SMH values after demineralization (table-2). Fluoride and n-HA had a significantly greater surface remineralization effect than others at each interval of pH cycling model. Fluoride and n-HA showed no significant difference between them except for the 9th day of pH cycling where n-HA was significantly better than fluoride (table-2). The surface remineralization potential of CPP–ACP was almost similar to negative control group. In the n-HA group, %SMHR was significantly greater than that of the CPP–ACP and control group but was non-significant when compared with fluoride groups at each time point (table-4,3).

SEM observation showed a smooth and intact surface in the normal anatomical enamel surface before demineralization (figure 2). After demineralization, uneven and rough surface with marked increased porosities was evident. Multilayer surface dissolution was noticed with a minor honeycomb pattern of demineralization (figure 3). Distinct surface coatings deposited by different agents were evident by SEM on the treated anatomical enamel surfaces of the specimens (figures 4 to 7). After pH-cycling, in the nano-hydroxyapatite groups, acicular crystals sedimented on the enamel surfaces after demineralization, and the cavities and defects of the enamel surface had decreased (figure 4). Minor honeycomb pattern of demineralization was visible with CPP–ACP group. Partially remineralized interprismatic structure and dissolved prism core were also evident (figure 5). The fluoride group indicated formation of different-sized globular structures with surface irregularities (figure 6). Multiple porosities, irregular surface with slight amorphous surface precipitation were visible with negative control group (figure 7).

Figure 1: Schematic Representation of Methodology

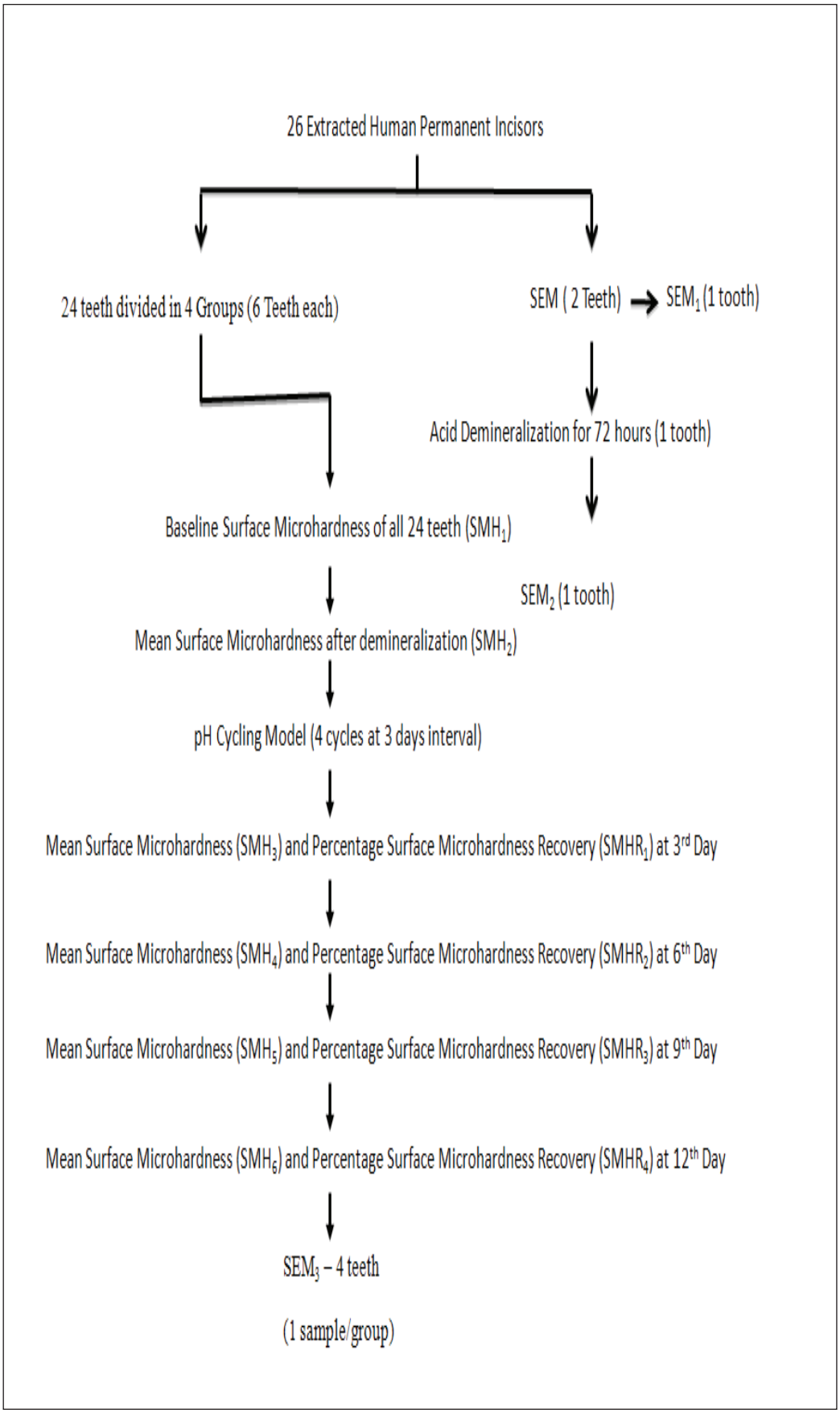


Figure 2: SEM Pictures of enamel before demineralization A – 200x Magnification B – 3500x Magnification

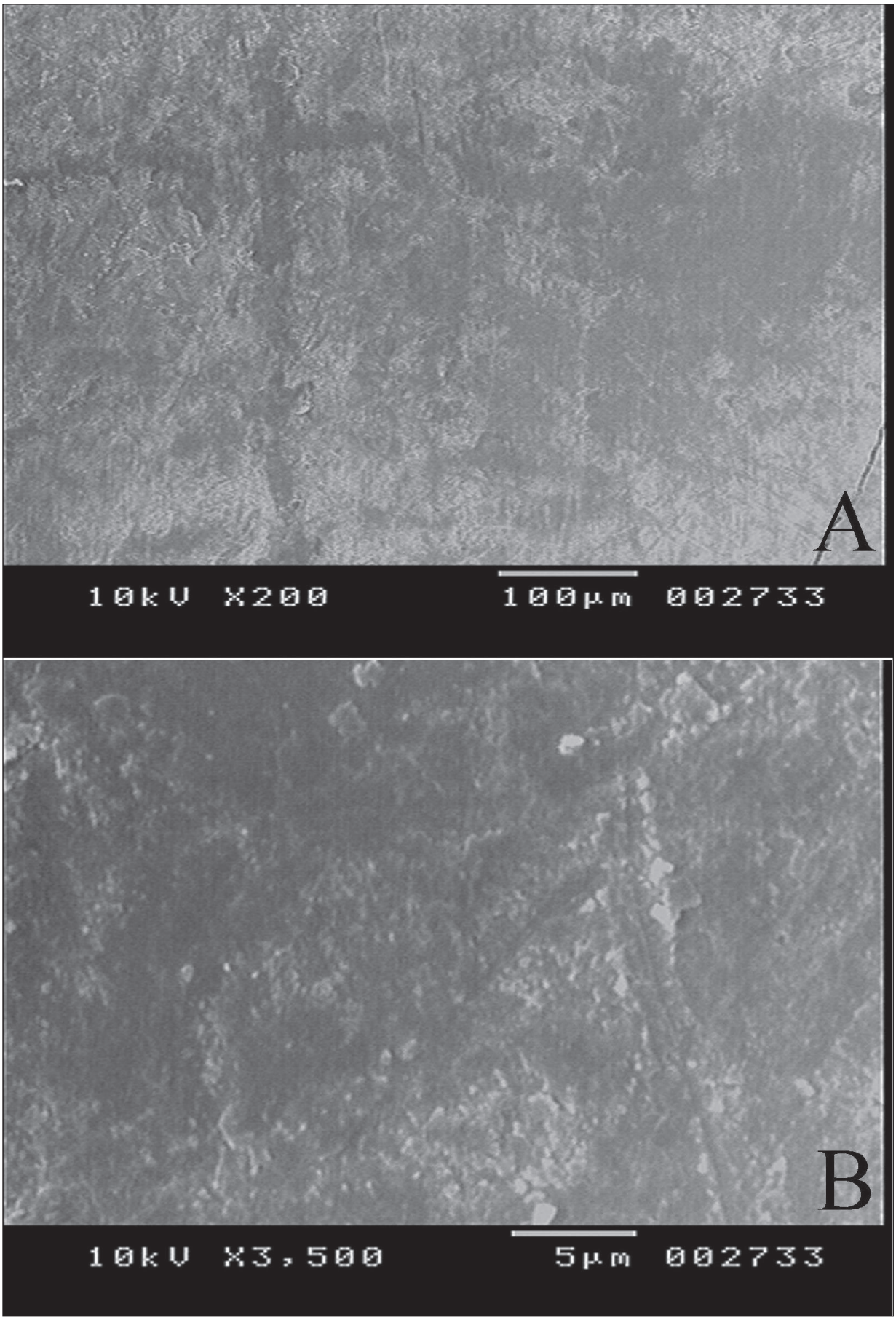


Figure 3: SEM Pictures of Enamel after Demineralization A – 200x Magnification B – 3500x Magnification

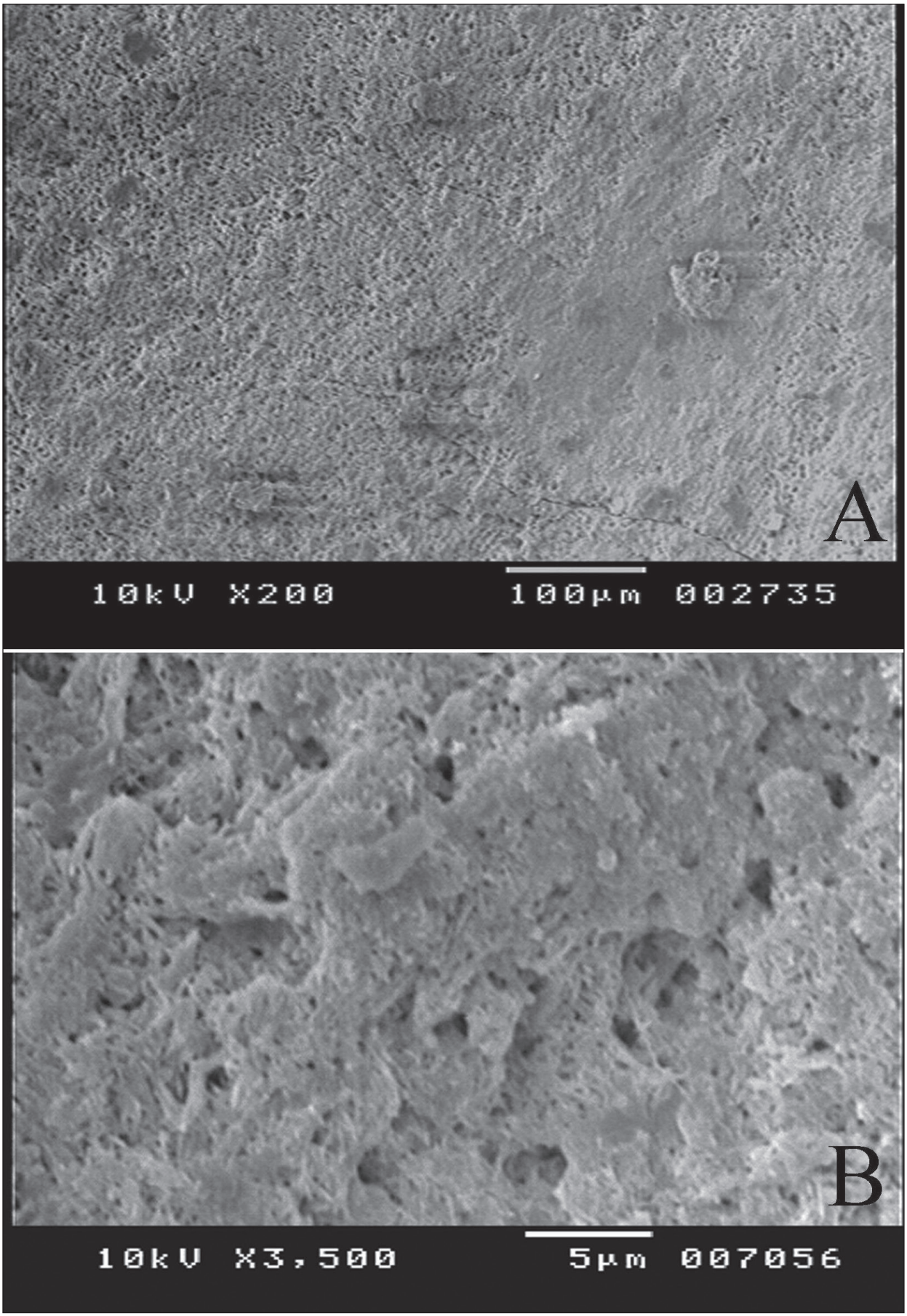


Table-1: Distribution of groups with respective active ingredients

Groups	Reminerelizing agents	Active ingredients	Company
A	Nano hydroxyapatite	10% nano hydroxyapatite Crystal size: 20-40 nm	Group Pharmaceuticals Ltd. (Chennai, India)
B	CPP-ACP	-Casein phosphopeptide -Amorphous calcium phosphate	GC Tooth Mousse (Recaldent GC Corporation, Tokyo, Japan)
C	Fluoride	-Sodium fluoride: 0.24% (0.15w/fluoride) Available fluoride 1000ppm -Triclosan: 0.30%	Colgate Total toothpaste (Colgate –Palmolive Ltd., Solan, India)
D	Control group	Deionized water	-

Table 2: **Microhardness of different groups after pH cycling**

Microhardness Groups	Baseline	After Deminerlization (72 Hrs)	After pH Cycling			
			3 days	6days	9days	12 days
Group A - Nano hydroxyapatite 10%	336.50 ±19.89	61.17 ±11.44	99.92 ±14.12	163.50 ±15.44	203.03 ±14.73	235.33 ±13.02
Group B - CPP-ACP	351.40 ±19.49	62.17 ±13.64	86.33 ±10.37	115.00 ±25.23	149.77 ±8.58	180.98 ±7.57
Group C - Fluoride 1000 ppm	359.33 ±12.80	63.17 ±18.69	115.33 ±15.95	176.17 ±15.42	232.13 ±16.04	243.82 ±9.57
Group D - Deionized Water	329.78 ±38.43	56.33 ±8.26	71.83 ±16.34	99.43 ±13.34	131.95 ±14.21	163.45 ±7.80

Table 3: KW ANOVA Test for Percentage Microhardness Recovery of Each Treatment Materials at Different Time Interval ($p \leq 0.05$)

		N	Mean	Std. Deviation
Percentage Surface Microhardness Recovery at 3 days (p =0.017*)	Group A - Nano hydroxyapatite 10%	6	14.07	5.74
	Group B - CPP-ACP	6	8.31	3.37
	Group C - Fluoride 1000 ppm	6	17.60	4.90
	Group D - Deionized Water	6	6.28	8.97
Percentage Surface Microhardness Recovery at 6 days (p =0.003*)	Group A - Nano hydroxyapatite 10%	6	37.06	7.32
	Group B - CPP-ACP	6	18.17	7.70
	Group C - Fluoride 1000 ppm	6	37.68	7.06
	Group D - Deionized Water	6	16.52	8.77
Percentage Surface Microhardness Recovery at 9 days (p =0.001*)	Group A - Nano hydroxyapatite 10%	6	51.49	6.72
	Group B - CPP-ACP	6	30.37	6.69
	Group C - Fluoride 1000 ppm	6	56.72	7.19
	Group D - Deionized Water	6	28.44	8.71
Percentage Surface Microhardness Recovery at 12 days (p =0.002*)	Group A - Nano hydroxyapatite 10%	6	63.62	9.14
	Group B - CPP-ACP	6	41.17	6.13
	Group C - Fluoride 1000 ppm	6	60.89	4.50
	Group D - Deionized Water	6	40.17	10.67

Table 4: Intergroup Comparison of Percentage Surface Microhardness Recovery at Different Intervals of pH Cycling

Intergroup / Days	3 days	6 days	9 days	12 days
A vs B	0.025	0.010	0.004	0.004
A vs D	0.019	0.010	0.006	0.016
C vs A	0.337 NS*	0.631 NS*	0.109 NS*	1.000 NS*
C vs B	0.010	0.010	0.004	0.004
C vs D	0.016	0.010	0.006	0.010
B vs D	0.423 NS*	0.631 NS*	0.423 NS*	0.423 NS*

NS* - Non-Significant

DISCUSSION

Artificial carious lesions are considered to be more reproducible than natural carious lesions and thus make the experimental model more reliable.[13] They facilitate the testing of multiple areas in any lesion at different time intervals, in order to assess the remineralizing phenomena.[13] We had removed the surface aprismatic layer, also known as critical layer, with fine polishing agent of 100 grit in order to create a more uniform surface to compare demineralization and demineralization effect on enamel, although removal of this layer is not advocated clinically. This method was based on the method employed in several published manuscript [7,9] and experience given by Pashley and Tay[14] and Lai SCN et al [15] Again, in the interest of standardization; all of the specimens in the present study were subjected to 12 days of pH cycling.

The results of the present study confirmed that 10% n-HA and 1000 ppm fluoride has the ability to remineralize initial enamel lesions at each point time in the pH cycling model. In a previous study on the remineralization effect of n-HA toothpaste on artificial caries, the solubility properties of n-HA were found to play a significant role in remineralization when the demineralized specimens were subjected to the treatment solutions continuously for several days.[9] However, in the present study, n-HA was applied for only a short period during pH-cycling, and due to the low solubility of pure hydroxyapatite, not enough Ca^{2+} and PO_4^{3-} were available to increase the stability of hydroxyapatite in the enamel and to prevent dissolution of the dental enamel.

In this study, CPP–ACP did not show significant surface remineralization. This is probably due to the mechanism of action of CPP–ACP which causes subsurface remineralization. CPP binds to the apatite crystal faces in the surface of the lesion keeping the diffusion pathways open to allow ions to penetrate more deeply, which results in remineralization throughout the body of the lesion rather than just in the surface layer.[7]

Surface enamel remineralization was also observed in control after 12 days of pH cycling. Artificial saliva has the potential to remineralize initial enamel lesions which goes in accordance with the study done by Huang et al.[9]

In the current study, the enamel surfaces in different treatments were examined by a scanning electron microscope. The different enamel surface morphologies after the corresponding treatments may be due to different mechanisms for promoting remineralization. As shown in the figure-3, initial enamel lesion has been formed on the surface and shows significantly more porosity than the sound enamel. This allows a greater penetration of solution ion constituents and a larger surface area for a subsequent reaction of enamel mineral. n-HA is said to penetrate the enamel pores acting as a template in the precipitation process and attracting a large amount of Ca^{2+} and PO_4^{3-} from the remineralization solution to the enamel surface to fill the vacant positions of the enamel calcium crystals.[8] Huang et al[9] compared remineralization potential of 1%, 5%, 10% and 15% n-HA under scanning electron microscope and found similar results with 10% and 15% concentration of n-HA.

SEM picture with CPP–ACP showed minor honeycomb pattern of demineralization indicating that very little or no remineralization occurred on the surface (figure 5). In a study done by Vashisht *et al* [16] CPP–ACP showed significant remineralization as compared to the control group (deionized water). This difference was probably due to their study was conducted in an *in vivo* environment and saliva, perhaps, promoted surface remineralization along with subsurface remineralization done by CPP–ACP.

After the application of 1000 ppm fluoride, globular structures sedimented on the demineralized surface consisting mainly of calcium fluoride causing crystal growth (figure 6) which support the studies done by Hoffman et al,[17] Karlinsey *et al* [18] and Featherstone[19]. Deionized water failed to show any significant surface remineralization when observed under SEM (figure 7).

CONCLUSION

This study encompassed various aspects of surface remineralization on early artificial carious lesion brought about by three different treatment materials on enamel samples with the help of Vicker's microhardness test and scanning electron microscopy. As 10% n-HA was found having similar remineralization effectiveness as that of 1000 ppm fluoride, it can be considered as an effective alternative to 1000 ppm fluoride for promoting remineralization on a daily basis. As CPP-ACP was not found remineralizing enamel surface, it cannot be recommended to be used as an alternative to fluoride.

Figure 4: SEM Pictures of Enamel treated with 10% Nano-Hydroxyapatite A – 200x Magnification B – 3500x Magnification

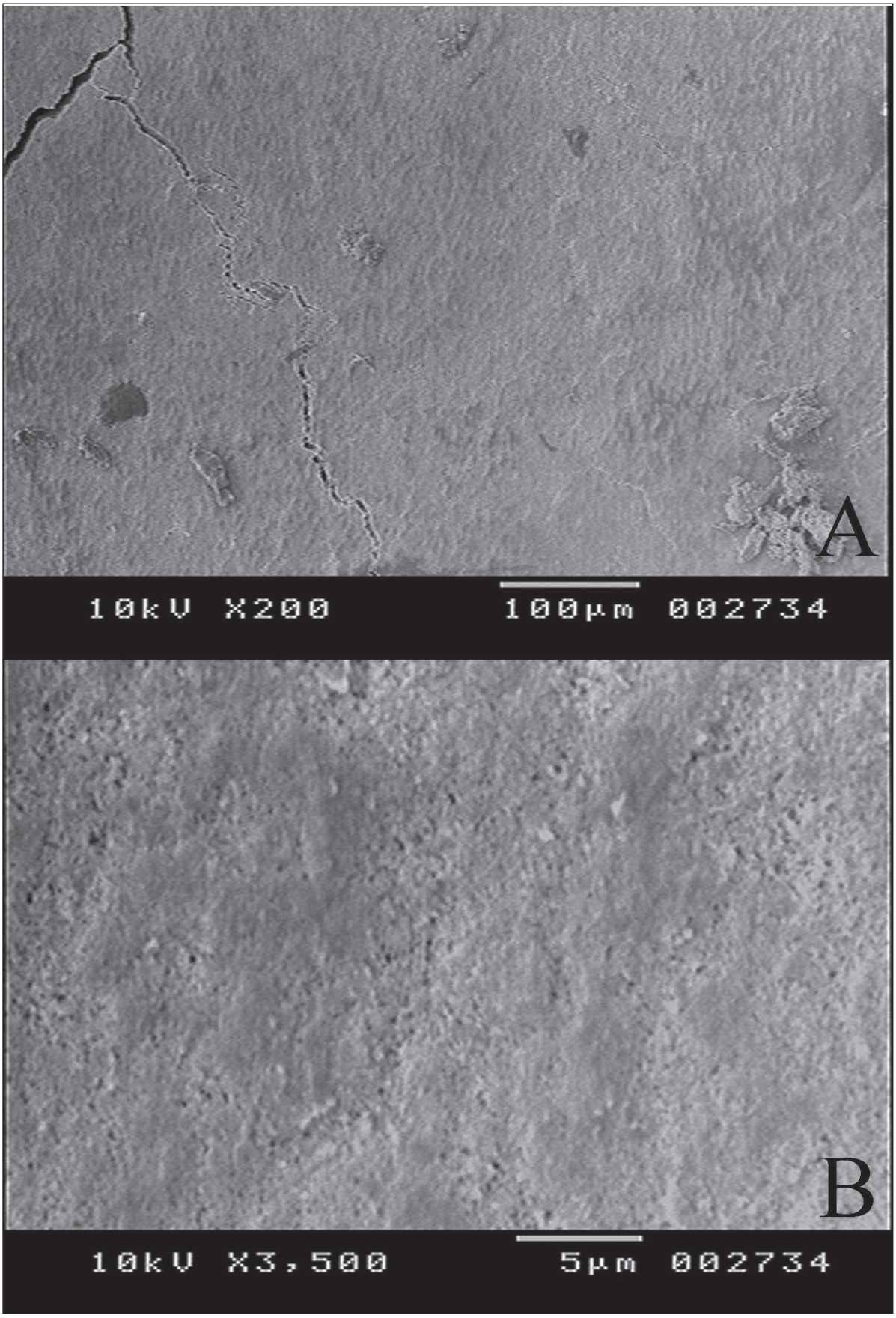


Figure 5: SEM Pictures of Enamel treated with CPP-ACP A – 200x Magnification B – 3500x Magnification

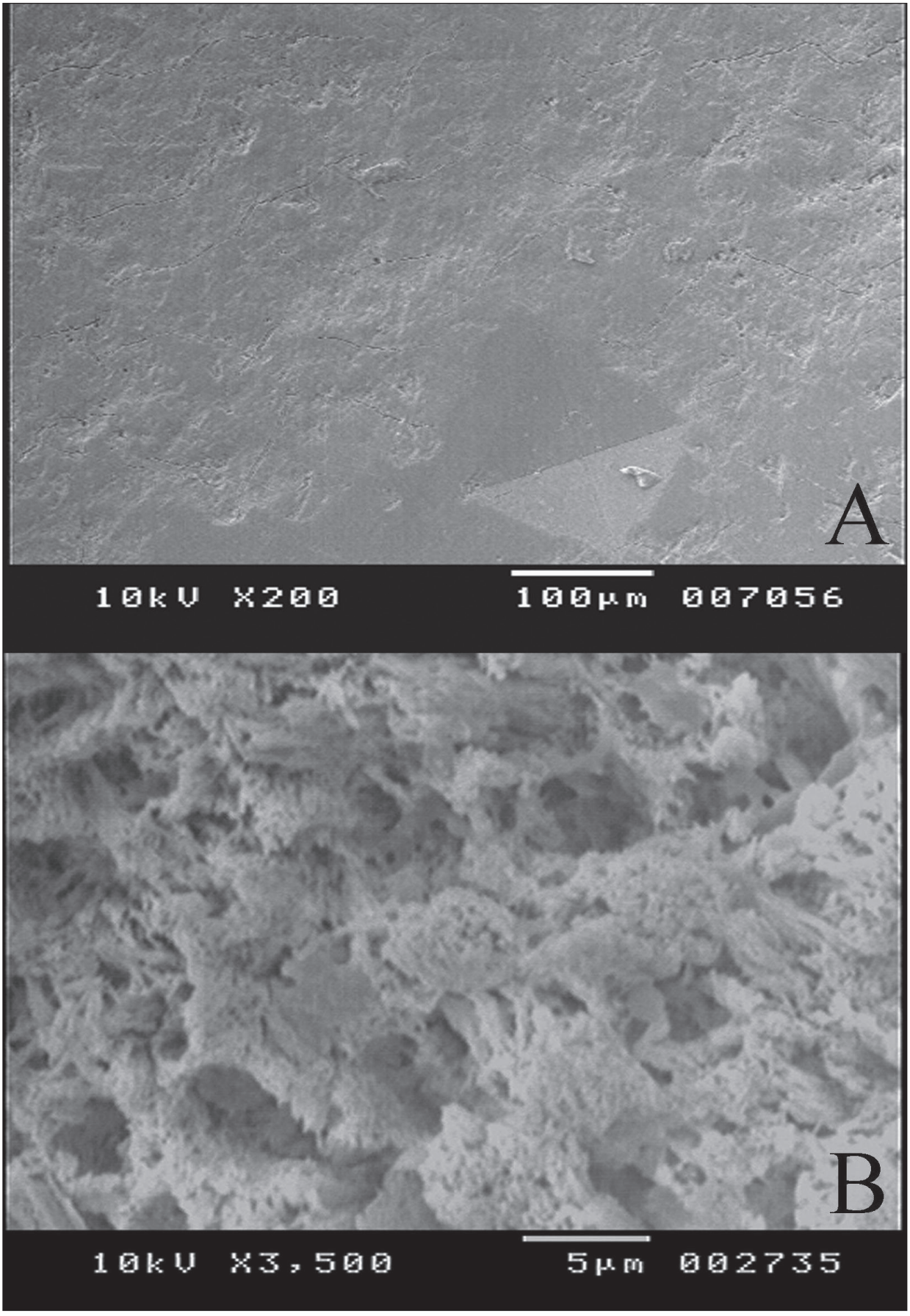


Figure 6: SEM Pictures of Enamel treated with 1000 ppm Fluoride A – 200x Magnification B – 3500x Magnification

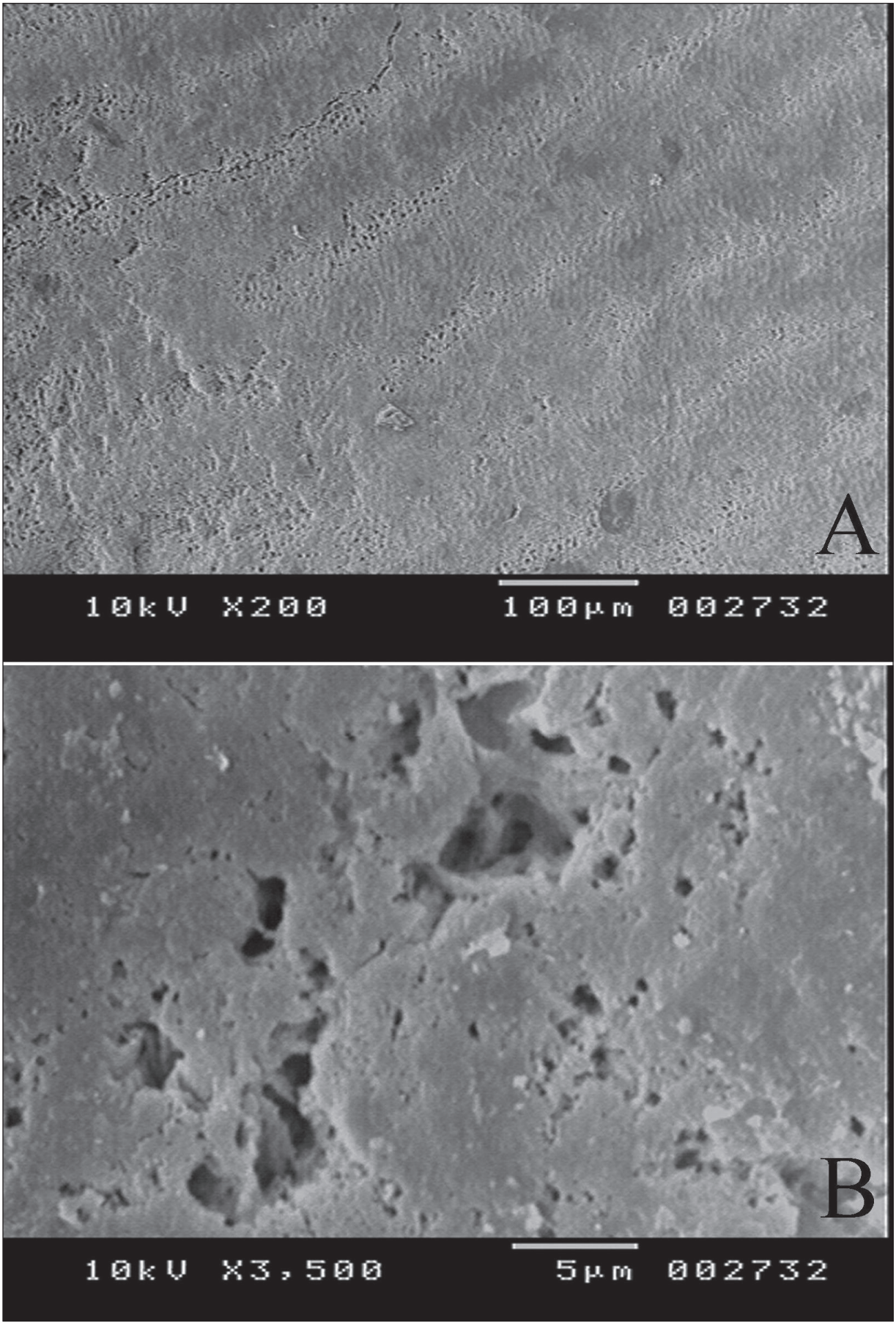
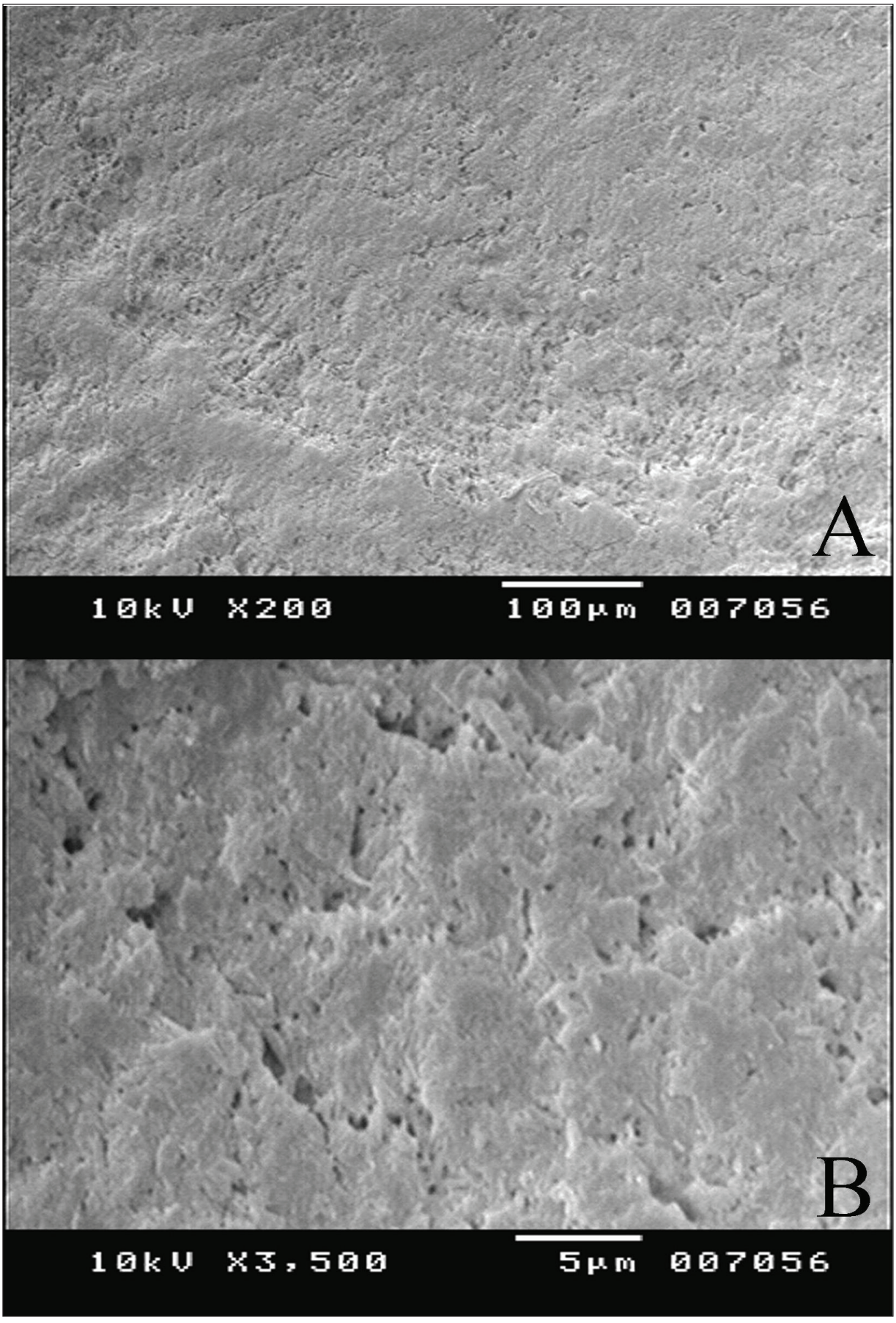


Figure 7: SEM Pictures of Enamel treated with Deionized Water (Negative Control) A – 200x Magnification B – 3500x Magnification



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