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Original Article

Remineralization of white spot lesions by casein phosphopeptide amorphous calcium phosphate (CPP-ACP) and Tri calcium phosphate (TCP) versus fluoride with different application technique by measuring surface micro hardness and surface roughness: An *in vitro* comparative evaluation

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Abstract

Aim: This study aimed to assess the effect of different remineralizing agents with different application technique a on enamel white spot lesion by micro hardness test, surface roughness

Subjects and methods: A total number of 108 specimen obtain from 54 of sound freshly extracted human molar teeth were used in this study. The Specimens were randomly divided into three main groups according to type of varnish used with 36 specimens each, where (A1) demineralized enamel treated with Fluoride remineralizing varnish, (A2) demineralized enamel treated with CPP-ACP remineralizing varnish and (A3) demineralized enamel treated with TCP remineralizing varnish. Each treated group were further divided into four subgroups (B) according to the applied remineralizing paste with 9 specimen each, where (B0) represent no paste application, (B1) represent fluoride paste application, (B2) represent CPP-ACP paste application and (B3) represent TCP paste application

Results: Microhardness recovery, where the highest recovery recorded by TCP Varnish with TCP Paste (95.26%) and the least recorded Fluoride varnish only (15.96%) .Where the highest mean surface roughness recorded by Fluoride Varnish and TCP Paste (0.2535±0.0004) and the lowest mean surface roughness recorded by Fluoride Varnish and Fluoride Paste (0.2513± 0.0011).

Conclusion: 1. TCP varnish is the most effective among other tested varnishes in remineralization of early enamel caries. 2. The Combination of TCP and ACP-CPP paste with different tested varnishes enhance enamel remineralization compared to varnish only. 3. All application techniques of remineralizing agents can improve enamel surface roughness but not all can improve enamel surface micro hardness

Keywords: Remineralization, white spot lesions, CPP-ACP, TCP, fluoride

Introduction

A paradigm shift is emerging in dentistry and dental treatments are now aimed at maximum conservation of tooth structure. Remineralization therapy is preferred in some cases. Many products as caries preventive materials are now available in the market which contains components that have the ability to initiate remineralization. **(Farooq, et al., 2013).**

Fluoride is the most commonly used remineralizing agent through formation fluorhydroxyapatite crystals with more resistant to acid attack and enhancing remineralization. Rapid deposition of fluorapatite forms a firm surface layer, which is more resistant to further demineralization (**Lata, et al., 2010**).

A new remineralization technology based on phosphopeptide from milk protein casein has been developed. The casein phosphopeptides (CPP) contain multiphosphoseryl sequences with the ability to stabilize calcium phosphate in nanocomplexes, where CPP binds to amorphous calcium phosphate ACP in metastable solution preventing the dissolution of calcium and phosphate ions and acts as reservoir for supersaturate solution of bio-available calcium and phosphate thus facilitating remineralization.
(Lata, et al., 2010).

Tri calcium phosphate (TCP) was recently introduced by 3M ESPE. This 1.1% NaF silica-containing paste contains an innovative functionalized tricalcium phosphate (fTCP) ingredient that, when evaluated in development .Formulations, has been shown to boost remineralization performance relative to fluoride-only systems. The fTCP technology solves this problem by protecting its bioavailable calcium with a fluoride-repelling surfactant (sodium lauryl sulfate) an a result, can be readily combined in an aqueous dentifrice formulation with NaF **(Karlinsey, et al., 2010)**

It is expected that combination between remineralizing agents would enhanced remineralization compared to individual application. Therefore this study was conducted to assess the effect of different remineralizing agents with different application technique on enamel white spot lesion by micro hardness test, surface roughness.

Subjects and Methods

This study will be conducted to assess the effect of different remineralizing agents with different application technique a on enamel white spot lesion by micro hardness test, surface roughness.

During treatment of white spot lesion, what are the most effective application techniques of three different remineliazing agents (***TCP, CPP-ACP and fluoride)*** that able to restore the normal hardness of defected enamel? The patients were randomly assigned to either one of two groups by using a special web site concerned with randomization process called research randomizer

1. Materials:

Three types of remineralizing agents available in two form either paste or varnish were used in this study. All Materials' specification, composition, lot number and manufacturers present in.

1.1. Remineralization using *Fluoride:*

a- DuraShield® CV Clear Varnish:

Fluoride Varnish is a topical dental varnish containing 5% sodium fluoride.

b-Colgate™ PreviDent®5000 plus Toothpaste

This toothpaste is a Self-topical neutral fluoride dentifrice that containing 1.1% sodium fluoride and design to be used by both adults and pediatric patients.

1.2. Remineralization using CPP-ACP combined with fluoride:

a- MI Varnish™:

This varnish contains Casein Phosphopeptide-Amorphous Calcium Phosphate (CPP-ACP), and its application leaves a film on tooth surfaces. It also contains 5% sodium fluoride varnish that has a desensitizing action when applied to tooth surfaces.

b- MI Paste Plus™:

It is a water based paste containing CPP-ACP with fluoride; when applied in the oral environment, it bind to biofilms, plaque, bacteria, hydroxyapatite and soft tissue localizing bio-available calcium, phosphate and fluoride **.**

1.3. Remineralization using TCP combined with fluoride:

a- Clinpro™ White Varnish:

It is a solvent based white, viscous varnish that contains 5% sodium fluoride with tri-calcium phosphate ingredient; when it applied to the tooth surface, the solvent system (alcohol and water) evaporates rapidly, leaving behind a hard lacquer-like film. This film adheres well to the surface of the teeth and slowly releases fluoride ion.

b- Clinpro™ 5000 paste:

It is white toothpaste that contains 5000 ppm fluoride and tri-calcium phosphate ingredient. The product is intended to be used once daily in place of conventional toothpaste.

1.4. Demineralizing solution:

The demineralizing solution had the following composition; $CaCl_2$ = 2.2 mM, NaH_2PO = 2.2 mM, Lactic acid = 0.05 M, Fluoride = 0.2 pp, adjusted with 50% NaOH to a pH 4.5**(*Lata, et al., 2010*)**.

1.5. Artificial saliva

The composition of the synthetic saliva is as follows: Na_3PO_4 - 3.90 mM $NaCl_2$ - 4.29 mM KCl - 17.98 mM $CaCl_2$ - 1.10 mM $MgCl_2$ - 0.08 mM H_2SO_4 - 0.50 mM $NaHCO_3$ - 3.27 mM, distilled water, and the pH was set at a level of 7. 2. **(*Comar, et al., 2013*)**

Both artificial saliva and demineralizing solution were fresh prepared in Faculty of Pharmacy, Cairo University.

2. Methods:

2.1. Selection of specimens:

A total number of 108 specimen obtain from 54 of sound freshly extracted human molar teeth were used in this study. The teeth were examined by magnifying loupes[1] to ensure that they were free from caries, cracks, fractures, restorations or any pathological abnormalities. The teeth were then cleaned and washed thoroughly under running water to remove blood followed by gentle scaling[2] to remove any plaque or calculus or any attached periodontal tissue, then stored in a physiological saline solution containing 10%Sodium azide until used.

2.2. Grouping of specimen:

The Specimens were randomly divided into three main groups according to type of varnish used with 36 specimens each, where (A_1) demineralized enamel treated with *Fluoride remineralizing varnish*, (A_2) demineralized enamel treated with *CPP-ACP remineralizing varnish* and (A_3) demineralized enamel treated with *TCP remineralizing varnish*. Each treated group were further divided into four subgroups (B) according to the applied remineralizing paste with 9 specimen each, where (B_0) represent *no paste application*, (B_1) represent *fluoride paste application*, (B_2) represent *CPP-ACP paste application* and (B_3) represent *TCP paste application.*

2.3. Preparation of specimen :

Teeth were sectioned into bucco-lingual direction into two halves (Mesial and distal halves) using gold diamond cutting disc with a low speed handpiece and water coolant. Each half of the sectioned tooth was embedded in acrylic resin[3] block using plastic mold, then initial surface roughness and micro-hardness was measured for all specimens as baseline.

2.4. Preparation of white spot lesion:

The specimen were soaked in the previously

[1] *(3.5X, COXO, China)*
[2] *Jaquette 2/3, Dentsplay, USA))*

[3] *Acrostone, Egypt*

mentioned demineralizing solution in a proportion of 2 ml solution/mm^2 of exposed enamel for 72 hours to induce caries like lesion (***Lata, et al., 2010)***. After that the teeth were removed from the demineralizing solution, washed with water and put on dried absorbent paper, then again both surface roughness and micro-hardness of demineralized specimens were measured.

2.5. Application of remineralizing agent:

All remineralizing agents were applied according to manufacture instructions.

2.5. a. Application of varnish:

All the remineralizing varnish were applied by micro brush then left undisturbed and allowed to set for 4 hours according to manufacturer instruction, which mentioned that patient should be advised not to eat or drink for 4 hours till setting of varnish, after that the toothpaste was applied then subjected to pH cycle protocol.

25. b. Application of remineralizing paste

To standardize the volume of applied material, each paste was inserted into an insulin syringe[4] then 0.2 ml (20 units) of all agents was used to cover the surface of the specimens. Then the paste was rubbed on specimen surface by micro brush and the application time was adjusted to be 5 minutes as recommended by manufacturer. After that the excess paste was removed and washing specimens in distilled water before application of pH cycling protocol.

2.6. PH cycling protocol:

The pH-cycling regimen included: application of remineralizing agent for 3 minutes then placed in remineralizing solution(synthetic saliva) for 30 minutes followed by immersion in demineralizing solution for 3 hours, and finally placed again in remineralizing solution(synthetic saliva) for 8.30 hours. This pH cycle was repeated twice daily for two weeks (***Comar, et al., 2013)***

2.7. Testing procedure:

For each specimen both Microhardness and surface roughness was measured three times; firstly sound enamel as baseline, secondly after demineralization to induce caries like lesion and finally after Ph cycle for two weeks.

2.7.a. Microhardness test:

Surface Micro-hardness of the specimens was determined using Digital Display Vickers Micro-hardness Tester[5] with a Vickers diamond indenter and a 20X objective lens. A load of 200g was applied to the surface of the specimens for 20 seconds; three indentations were made on the specimen surface and placed over a circle and not closer than 0.5 mm to the adjacent indentations. The diagonals lengths of the indentations were measured by built in scaled microscope and Vickers values were converted into micro-hardness values using the following equation:

$HV=1.854\ P/d^2$ where, ***HV*** is Vickers hardness in Kgf/mm^2 , ***P*** is the load in Kgf and ***d*** is the length of the diagonals in mm ***(Fig.11)***.

2.7. b. Surface roughness test:

Specimens were photographed using USB Digital microscope with a built-in camera6 connected with an IBM compatible personal computer using a fixed magnification of 120X.The images were recorded with a resolution of 1280 × 1024 pixels per image. Digital microscope images were cropped to 350 x 400 pixels using Microsoft office picture manager to specify/standardize area of roughness measurement. The cropped images were analyzed using WSxM software7. Within the WSxM software, all limits, sizes, frames and measured parameters are expressed in pixels. Therefore, system calibration was done to convert the pixels into absolute real world units. Calibration was made by comparing an object of known size (a ruler in this study) with a scale

[4] BD micro-fine plus, Franklin Lakes, USA
[5] Model HVS-50, Laizhou Huayin Testing Instrument Co., Ltd. China

[6] Scope Capture Digital Microscope, Guangdong, China
[7] Version 5 develop 4.1, Nanotec, Electronica, SL

generated by the software.Subsequently, a 3D image of the surface profile of the specimens was created. Five 3D images were collected for each specimen, in the central area and in the sides at area of 10 µm × 10 µm, then WSxM software was used to calculate average of heights (Ra) expressed in µm

2.8. <u>Statistical analysis</u>

Statistical analysis was performed with IBM®SPSS[8], the significance level was set at P ≤ 0.05, and data presented as mean and standard deviation (SD) values. Data explored for normality using Kolmogorov-Smirnov and Shapiro-Wilk tests. Roughness and Microhardness showed parametric distribution, so One Way-ANOVA test used to compare between Different Varnish within each different Paste and vice versa on mean Microhardness and followed by Duncan's post hoc test. Percent % Microhardness recovery showed nonparametric distribution, so Kruskal Wallis test used to compare between Different Varnish within each different Past and vice versa on mean % of Microhardness recovery followed by Mann Whitney U-test test for pair-wise comparison.

Results

The result of current study show that the highest microhardness recovery record by TCPgroups either varnish or paste followed by CPP-ACP and finally the least recovery recorded by fluoride either paste or varnish. This result was in agreement with (Karlinsey, et al., and 2010a) who clarified that remineralization benefits of TCP is achieved by its ability to penetrate deep into the subsurface lesion. (Elkassas and Arafa, 2014) also reported that fTCP is produced by functionalizing β-TCP with silica, which provides linking opportunities with hard tissue defects under acidic conditions. It can permeate throughout enamel without attacking the inter-prismatic organic material, which may encourage greater calcium, phosphate, and fluoride uptake in demineralized lesions

1. Microhardness

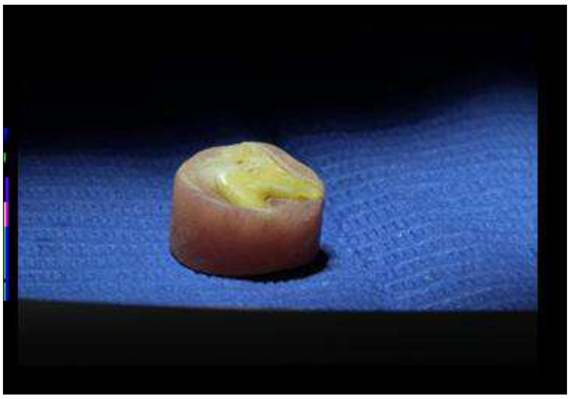
show ranking of percent % Microhardness recovery, where the highest recovery recorded by TCP Varnish with TCP Paste (95.26%) than TCP varnish with CPP-ACPpaste (80.65%) than TCP varnish only (70.80%) than Fluoride Varnish with TCP Paste (70.76%) than Fluoride Varnish with CPP-ACPPaste (70.70%) than CPP-ACPvarnish with CPP-ACPpaste (60.16%) than CPP-ACPVarnish with TCP Paste (59.52%) than Fluoride Varnish with Fluoride Paste (50.52%) than CPP-ACPvarnish only (50.37%) than CPP-ACPvarnish with Fluoride paste (39.55%) than TCP varnish with Fluoride paste (20.19%) and the least recorded Fluoride varnish only (15.96%) with significant difference between them. **(Table 1)**

2. Surface Roughness

show ranking between variables, where the highest mean surface roughness recorded by Fluoride Varnish and TCP Paste (0.2535±0.0004) than CPP-ACP Varnish and Fluoride Paste (0.2532±0.0004) than Fluoride Varnish and CPP-ACP Paste (0.2531±0.0012) than TCP Varnish and Fluoride Paste (0.2530±0.0004) than TCP Varnish Only (0.2530±0.0004) than CPP-ACP Varnish Only (0.2529±0.0010) than CPP-ACP Varnish and CPP-ACP Paste (0.2529± 0.0005) than CPP-ACP Varnish and TCP Paste (0.2526 ± 0.0004) than TCP Varnish and CPP-ACP Paste (0.2525± 0.0004) than TCP Varnish and TCP Paste (0.2521± 0.0008) than Fluoride varnish only (0.2519± 0.0009) and the lowest mean surface roughness recorded by Fluoride Varnish and Fluoride Paste (0.2513± 0.0011) with significant difference between them. **(Table 2)**

[8] *Statistics Version 23 for Windows, SPSS Inc., IBM Corporation, NY, USA.*

(Figure of specimen preparation)



1- low speed contra gold diamond disc **2-specimen impeded in acrylic resin**

(Figure of material application)

1-Application of paste **2-Application of varnish**

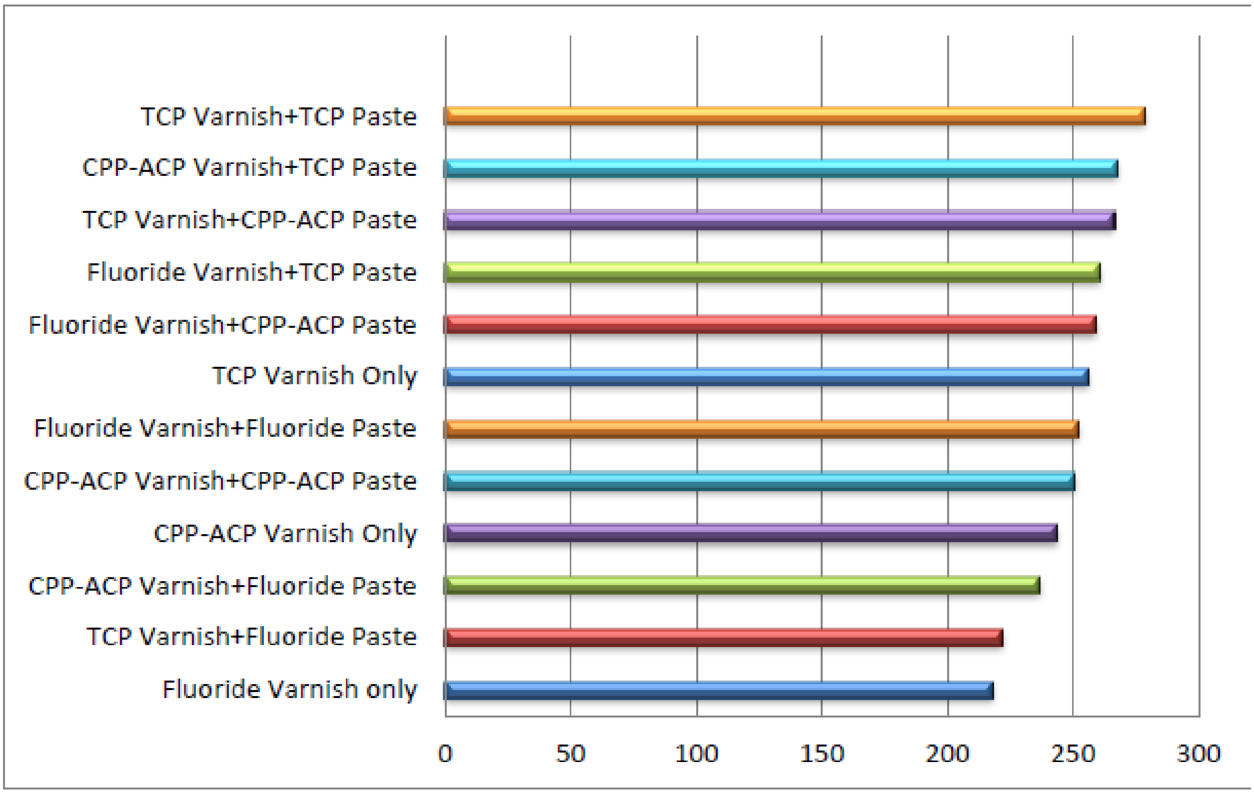
(Figure of tester devices)

1- Vickers Micro-hardness Tester **2-USB Digital microscope connected to computer**

	Microhardness		Rank	p-value
	Mean	SD		
Fluoride Varnish only	218.03	12.30	fg	≤0.001*
TCP Varnish+Fluoride Paste	222.03	.31	ef	
CPP-ACPVarnish+Fluoride Paste	236.58	6.42	de	
CPP-ACPVarnish Only	243.76	10.43	cd	
CPP-ACPVarnish+CPP-ACPPaste	250.36	13.27	bcd	
Fluoride Varnish+Fluoride Paste	251.84	27.62	bcd	
TCP Varnish Only	255.77	19.49	bc	
Fluoride Varnish+CPP-ACPPaste	259.08	4.97	bc	
Fluoride Varnish+TCP Paste	260.35	11.73	bc	
TCP Varnish+CPP-ACPPaste	266.29	2.27	ab	
CPP-ACPVarnish+TCP Paste	267.36	51.22	ab	
TCP Varnish+TCP Paste	278.41	2.51	a	

Means with the same letter within each column are not significantly different at p=0.05.
**= Significant, NS=Non-significant*

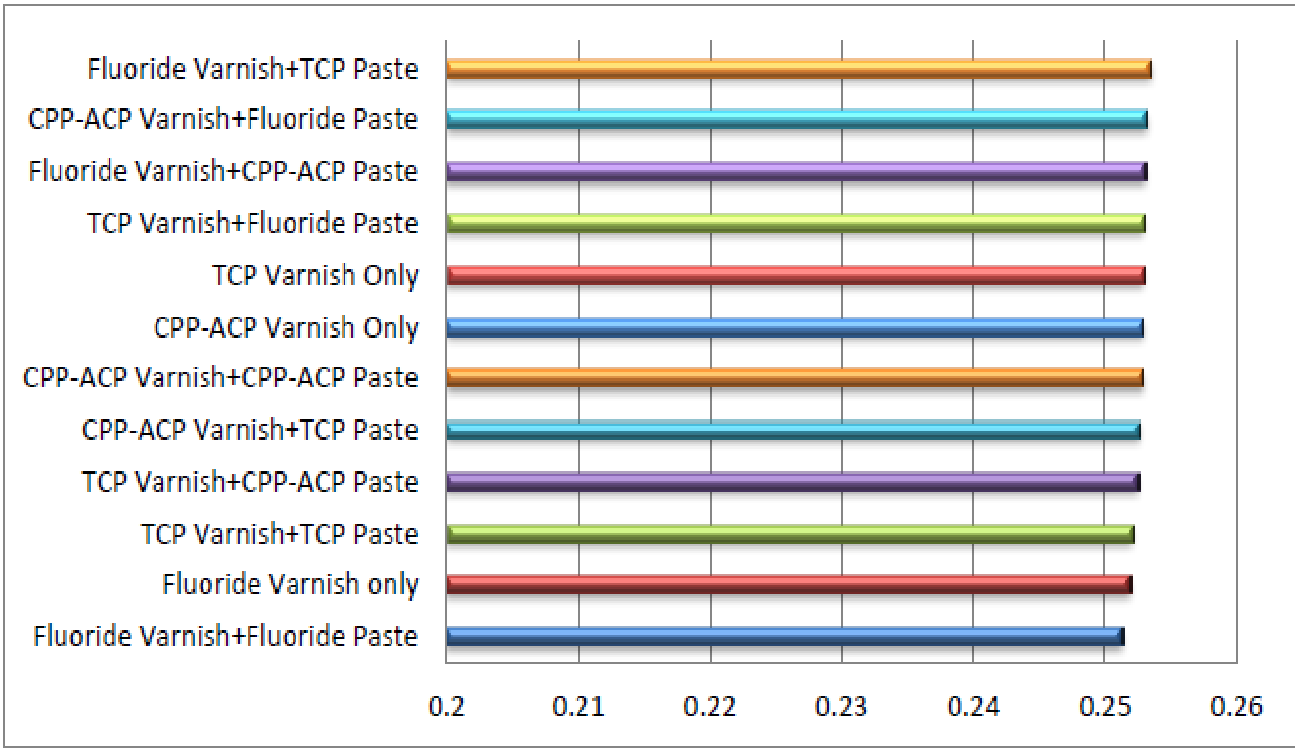
1. **Table (1):**show ranking of percent % Microhardness recovery


TCP Varnish+TCP Paste
CPP-ACP Varnish+TCP Paste
TCP Varnish+CPP-ACP Paste
Fluoride Varnish+TCP Paste
Fluoride Varnish+CPP-ACP Paste
TCP Varnish Only
Fluoride Varnish+Fluoride Paste
CPP-ACP Varnish+CPP-ACP Paste
CPP-ACP Varnish Only
CPP-ACP Varnish+Fluoride Paste
TCP Varnish+Fluoride Paste
Fluoride Varnish only
0
50
100
150
200
250
300

	Surface Roughness		Rank	p-value
	Mean	SD		
Fluoride Varnish+Fluoride Paste	0.2513	0.0011	d	≤0.001*
Fluoride Varnish only	0.2519	0.0009	c	
TCP Varnish+TCP Paste	0.2521	0.0008	c	
TCP Varnish+CPP-ACPPaste	0.2525	0.0004	bc	
CPP-ACPVarnish+TCP Paste	0.2526	0.0004	bc	
CPP-ACPVarnish+CPP-ACPPaste	0.2529	0.0005	ab	
CPP-ACPVarnish Only	0.2529	0.0010	ab	
TCP Varnish Only	0.2530	0.0004	ab	
TCP Varnish+Fluoride Paste	0.2530	0.0004	ab	
Fluoride Varnish+CPP-ACPPaste	0.2531	0.0012	ab	
CPP-ACPVarnish+Fluoride Paste	0.2532	0.0004	ab	
Fluoride Varnish+TCP Paste	0.2535	0.0004	a	

Means with the same letter within each column are not significantly different at p=0.05.
**= Significant, NS=Non-significant*

2. Table (2): show ranking of percent % surface roughness recovery



Discussion

The goal of modern dentistry is to manage non-cavitated carious lesions non-invasively in an attempt to prevent further disease progression and preserve integrity of healthy tooth substrate**(Goswami, et al., 2012)**. Non-invasive management of white spot carious lesions takes several strategies; among these is healing the demineralized lesions by using remineralizing agents like pastes and resinous varnishes **(Elkassas and Arafa, 2014)**.

Nevertheless, the substance may also exert undesirable effects such as fluorosis and toxicity in high doses. Therefore, efforts to find effective anti-caries compounds with minimal side effects have been intensified (**Tavassoli-Hojjati, et al., 2012**). In consequence this study was conducted to assess the effect of different remineralizing agents with different application technique on enamel white spot lesion by micro-hardness and surface roughness test.

Three different remineralizing agents with different form either paste or vanish were used in this study; the first one is the fluoride remineralizing paste and varnish. Fluoride is believed to prevent dental caries through several mechanisms inducing reduction of acid production by microorganisms, inhibition of intracellular and extracellular enzymes, and replacement of hydroxide ions in hydroxyapatite with fluoride ions (resulting in acid-resistant fluorapatite crystals).

Fluoride Varnish *(DuraShield CV)* with 5% sodium fluoride in an alcohol solution of synthetic resin was used. Each unit-dose package contains an average of 20 mg of sodium fluoride (NaF), equivalent to 9 mg fluoride ion. According to the manufacturer, the non-drip formula stays on the brush, making it easy to apply. It's virtually invisible on the patients' teeth and does not discolor. It has a neutral pH and easy application compared to fluoride gel. Varnish fluoride hardens after exposure to saliva; consequently, sticking to the teeth result in increase contact time of fluoride with the teeth. Also the high fluoride concentration help in prevention of tooth decay **(Tavassoli-Hojjati, et al., 2012)**

The Fluoride toothpaste *(Colgate PreviDent®)* was also used in this study with 1.1% (5000 ppm) sodium fluoride. It is well established that 1.1% sodium fluoride is safe and effective in caries preventive when frequently applied. It has been documented in many studies that toothpaste containing 5000 ppm fluoride can further reduce demineralization and enhance remineralization specially for high risk caries patients. Recently, it was suggested that patients undergoing orthodontic treatment should brush twice a day with 5000-ppm fluoride toothpaste to provide greater prevention than the daily use of low sodium fluoride paste **(Guzmán-Armstrong, et al., 2010)**

The second remineralizing agent in this study was the Casein phosphopeptide amorphous calcium phosphate (CPP-ACP) either paste or varnish. Casein phosphopeptides (CPP) naturally occur in milk. In the oral cavity, CPP binds to surfaces such as teeth, dentin, oral mucosa and biofilm while Calcium and phosphate ions are the building blocks for healthy teeth. **(Esfahani, et al., 2015)**

CPP-ACP varnish *(MI Varnish)* is a 5% sodium fluoride varnish with CPP-ACP. MI Varnish is unique formula compared to all the other fluoride varnishes; CPP-ACP Varnish brings bio-available calcium and phosphate to the tooth surface together with releasing high levels of fluoride from the sodium fluoride in order to remineralized and prevent of initial caries lesion. The Amorphous Calcium Phosphate (ACP) is the source of calcium and phosphate, which strengthens enamel. **(Bayrak, et al., 2016)**

CPP-ACP toothpaste (*MI Paste Plus*) was also used in this study. It is a water based tooth crème containing CPP-ACP with incorporated 0.2% (900ppm) fluoride (CPP-ACPF: Casein Phosphopeptide-Amorphous Calcium Phosphate Fluoride). Upon CPP-ACPF application, it stabilize and localize calcium, fluoride, and phosphate at the tooth surface in a slow release amorphous form, thus enhancing deeper remineralization of initial carious white spot lesion **(Huang, et al., 2013).**

The Tri-Calcium Phosphate (TCP) was the third remineralizing agent tested in this study, applied either in paste or varnish form. Tri-Calcium Phosphate varnish (*Clinpro White Varnish*) is a proprietary formula that contains fluoride, calcium and phosphate components naturally found in saliva. Clinpro white varnish is a white, viscous fluid that contains 5% sodium fluoride (NaF). When applied to the tooth surface in a thin layer, the solvent system (alcohol and water) evaporates rapidly, leaving behind a hard lacquer-like film. This film adheres well to the surface of the teeth and slowly releases fluoride ion **(Karlinsey, et al., 2010)**. The resin used in most cavity varnishes is typically yellow in color. Although the yellow color does not have a negative effect on the performance of a cavity varnish, many patients prefer a white or invisible film, rather than yellow on their teeth, especially as they leave the dental office and return to their daily activities. Clinpro white varnish contains specially designed resin that is white or tooth colored. After Clinpro white varnish is applied to the teeth, the product is virtually invisible. Evidence showing that TCP improves the fluoride uptake of permanent enamel treated with fluoridated dentifrices **(AlAmoudi, et al., 2013)** .

Furthermore TCP toothpaste (*Clinpro™ 5000)* contains 1.1% (5000 ppm) sodium fluoride was also used in this study. This 1.1% sodium fluoride (NaF) silica-containing paste contains an innovative functionalized tricalcium phosphate (fTCP) ingredient that, able to boost remineralization performance relative to fluoride-only systems **(Karlinsey et al., 2009)**. The fTCP technology protect bioavailable calcium with a fluoride-repelling surfactant (sodium lauryl sulfate) and as a result, can be readily combined in an aqueous dentifrice formulation with NaF **(Karlinsey, et al., 2010)**. This Anti-Cavity Toothpaste was developed specifically for patients who need the benefits of higher concentration fluoride tooth paste **(Karlinsey et al., 2013)**.

In this study, every effort was exerted to standardize the methodology and simulate the clinical conditions. Intact human molars were selected in this study to standardize anatomic variations and to overcome heterogeneous nature of tooth structure.

Teeth were sectioned into bucco-lingual direction into two halves (Mesial and distal halves) for testing whole surface of enamel due to proximal surface in premolars and molars morphology near flat than buccal and lingual surface so its suitable for microhardness test and without flattening due to our main concept in this study not to harm normal structure of enamel for simulation of real life situation as possible **(Heymann, et al., 2014)**.

Artificial carious lesions were obtained by immersion of the specimens in demineralizing solution for 72 hours to create a subsurface demineralization area of approximately 150 microns width with an intact surface simulating an early enamel lesion**(Ekambaram, et al., 2011)**. The 50% concentration of both calcium and phosphates, together with fluoride in order to prevented surface demineralization by forming fluorapatite at the surface, which simulated the naturally occurring early enamel lesions that characterized by subsurface demineralization with intact surface layer **(Lata, et al., 2010).**

The in-vitro pH cycling models are still broadly used because they mimic the dynamics of mineral loss and gain involved in caries process **(Buzalaf, et al., 2010)**. In the current study, the pH-cycling regimen was applied that consist of application of remineralizing paste for 3 minutes then placed in remineralizing solution (synthetic saliva) for 30 minutes as the manufacture recommended that patient should avoid eating or drinking for 30 minute after application to avoid rapid drop in pH. After that the specimens were immersed in demineralizing solution for 3 hours which simulate the duration of demineralization that occurs in the oral cavity and finally placed again in remineralizing solution(synthetic saliva) for 8.30 hours to mimic the normal day of patient dietary habits. This pH cycle was repeated twice daily for two weeks **(Lata, et al., 2010 &Comar, et al., 2013)**.

In this study, both microhardness and surface roughness of each specimen were measured three times; firstly sound enamel as the baseline, and after induction of white spot lesion (demineralization) and finally after application of tested materials under pH cycling regime; this method allows repeated measurements of the same specimen over a given period of time thus reducing the experimental variation **(Mohd Said, et al., 2016).**

Moreover the Vickers microhardness was used in this study, as it is considered one of the most important tests that measure surface layer in caries progression. This Microhardness measurement is appropriate for a material having fine microstructure, non-homogenous or prone to cracking like enamel. Surface microhardness indentation provides a relatively simple, non-destructive and rapid method in demineralization and remineralization studies **(Arends, et al., 1980; Lata, et al., 2010).**

Furthermore the Surface roughness test was also measured in the present study, as it is an important clinical aspect not only affect aesthetic properties but also reflects the bacterial adhesion and plaque formation potentials in the oral environment. The 3D Non-Contact Optical Profile techniques tend to fulfill the need for quantitative characterization of surface topography without contact**(Elkassas and Arafa, 2014)**. Also this methods fulfill the need for quantitative characterization of surface topography without contact and capable of measuring an area of the surface rather than a single line; additionally since the surface roughness was measured three times on each specimen so it was preferable to use the non-contact method to avoid the removal of any applied materials by the measuring stylus used in contact method in order to obtain accurate testing results **(Bui and Vorburger, 2007)** , where as the highest mean surface roughness indicated a rough surface while the lowest mean indicated more surface smooth **(Cavalli, et al., 2004)**.

Moreover with the purpose of not to harm enamel surface aiming to get accurate readings of microhardness and surface roughness test, Varnish wasn't removed by blade but we let it to be removed automatically under brushing teeth with each paste to mimic intraoral conditions **(Souza, et al., 2010)**. As in the oral cavity, the varnish is removed by the action of the cheeks and tongue, salivary flow, mastication and oral hygiene procedures **(Cochrane, et al., 2014)**.

The result of current study show that the highest microhardness recovery record by TCP groups either varnish or paste followed by CPP-ACP and finally the least recovery recorded by fluoride either paste or varnish. This result was in agreement with **(Karlinsey, et al., 2010a)** who clarified that remineralization benefits of TCP is achieved by its ability to penetrate deep into the subsurface lesion. **(Elkassas and Arafa, 2014)** also reported that fTCP is produced by functionalizing β-TCP with silica, which provides linking opportunities with hard tissue defects under acidic conditions. It can permeate throughout enamel without attacking the inter-prismatic organic material, which may encourage greater calcium, phosphate, and fluoride uptake in demineralized lesions. In other word **(Ulkur, et al., 2014)**explained that TCP is insoluble crystalline form of calcium phosphate and is similar to apatite calcium phosphate in tooth enamel. When TCP comes into contact with the tooth surface and combines with saliva, the protective barrier breakdown making calcium and phosphate available.

Furthermore **(Mohd Said, et al., 2016)** mentioned that CPP-ACP exhibited the greatest release of inorganic phosphate and this may be unfavorable for the formation of loosely bound fluoride reservoirs as they can reduce the formation of calcium fluoride ion and favour the formation of ectopic fluorapatite. The formation of fluorapatite ectopically will dramatically decrease the fluoride ion activity and may promote calculus formation. Therefore, the high inorganic phosphate release may be disadvantageous in terms of localizing bioavailable fluoride ions in the oral environment.

However this result was in contradicted by **(Schemehorn et al., 2011 and Cochrane et al. 2014)** who concluded that CPP increased enamel microhardness significantly more than fTCP, as CPP released more calcium and fluoride than fTCP. Additionally **(Memarpour, et al., 2015)** clarified that once CPP stabilizes ACP on the enamel, it allows free calcium and phosphate to diffuse to the subsurface lesion and form new crystals. Moreover **(Cochrane, et al., 2014)**explained that the low release of calcium and inorganic phosphate ions from Clinpro White may be due to the low amount of fTCP being added to the varnish or due to the low solubility of tricalcium phosphate.

Additionally **(Kumar, et al., 2008)**stated that CPP-ACP varnish are more effective in reducing lesion depth. This finding can be attributed to very fine particles of amorphous calcium phosphate (nanocomplexes) present in CPP-ACP, i.e. calcium and phosphate ions can readily diffuse into the porous lesion and penetrate deep into the demineralized lesion. As a result of long-term treatment and adequate exposure time, apatites crystals will be formed again even in deeper parts.

In this study, the fluoride only recorded the least percentage of recovery. This result was agreement with **(Lata, et al., 2010)**who explained that remineralization by fluoride is a self-limiting surface phenomenon that prevents penetration of ions into the depth of the lesion as the newly deposit fluorapatite crystal are larger in size and forms a firm surface layer, which is more resistant to further demineralization and the same time, it limits the penetration of calcium and phosphate ions required to rebuild the lesion in depth. However this was contradicted by **(Elkassas and Arafa, 2014)** who reported that remineralization and enamel microhardness increase as fluoride concentration increase. The High Fluoride gradient (22,600ppm) in fluoride varnish promotes driving fluoride deeper into the lesion.

Regarding The surface roughness result of current study, the best surface roughness recorded by Fluoride Varnish followed by Fluoride Paste that means remineralizing agents contain Fluoride able to restore and surpass the normal enamel surface roughness making more smooth surface, that gradual decreased when fluoride combined with another remineralizing agents like CCP-ACP and TCP groups was near to normal surface roughness this result was in agreement with **(Lee, et al., 2010)** who mentioned that fluoride incorporates into enamel crystals, thereby forming a fluoro-apatite like mineral that improves the ability of the enamel to remineralize, also **(Esfahani, et al., 2015)** found that the effects of fluoride (either varnish or pastes) on remineralization to be similar to or greater than those of CPP-ACP, and they explained that the presence of fluoride ions in the oral cavity causes the precipitation of fluoro-apatite from existing calcium and phosphate ions in the saliva, and with the increased in pH will then lead to the formation of larger acid resistant crystals containing fluoride (fluoro-hydroxyapatite). Consequently a strong remineralized surface layer will be developed, and finally the resistance of the dental structure to demineralization will be enhanced.

Also **(Pinto, et al., 2004 & Elkassas and Arafa, 2014)** there is a relation between micro hardness and surface roughness and micro porosities the loss of mineral content and organic matrix decreased enamel microhardness and increased surface roughness and increased micro porosities; in our study fluoride contained remineralizing agent have ability to improve surface roughness in short time more than other remineralizing agents but more time is needed to give the opportunity for the fluoride ion to penetrate deeply into demineralized surface thus increase microhardness.

Conclusion:

This study was conducted to assess the effect of different remineralizing agents with different application technique on enamel white spot lesion by micro hardness test, surface roughness.

56 extracted molars were collected, divided into three main groups according to type of varnish used with 36 specimens and each treated group were further divided into four subgroups according to the applied remineralizing paste with 9 specimen one group covered with varnish only and other three groups covered with varnish then each different paste will be applied on , and Teeth were sectioned into bucco-lingual direction into two halves (Mesial and distal halves) then initial surface roughness and micro-hardness was measured for all specimens as baseline.

The specimens were soaked in demineralizing solution to induce caries like lesion then again both surface roughness and micro-hardness of demineralized specimens were measured.

All remineralizing agents' varnishes and pastes were applied according to manufacture instructions with standardization of amount of them when applied then all specimen entered ph cycling process and pH cycle was repeated twice daily for two weeks .

For each specimen both Microhardness and surface roughness was measured three times; firstly sound enamel as baseline, secondly after demineralization to induce caries like lesion and finally after Ph cycle for two weeks.

Surface Micro-hardness of the specimens was determined using Digital Display Vickers Micro-hardness Tester with a Vickers diamond indenter and a 20X objective lens. A load of 200g was applied to the surface of the specimens for 20 seconds. For Surface roughness of the specimens was determined by using photographed non-contact method by using USB Digital microscope with a built-in camera connected with an IBM compatible personal computer using a fixed magnification of 120X , were analysed using WSxM software.

Microhardness results showed that for in fluoride varnish group the highest recovery recorded with TCP paste (70.76%) followed by CPP-ACP paste (70.70%) then fluoride paste (50.52%) and least the varnish only (15.96%) with significant difference between them. Also in TCP varnish group the highest mean microhardness recorded with TCP paste (95.26%) followed by CPP-ACP paste (80.65%) then varnish only (70.80%) and least the fluoride paste (20.19%) with significant difference between them. However in CPP-ACP varnish group the highest mean microhardness recorded with CPP-ACP paste (60.16%) followed by TCP paste (59.52%) then varnish only (50.37%) and least the fluoride paste (39.55%) with no significant difference between them.

The Surface Roughness results showed that for different paste within each varnish, where in fluoride varnish group the highest mean Surface Roughness recorded with TCP paste followed by CPP-ACP paste then varnish only and least the fluoride paste with significant difference between them. Furthermore in TCP varnish group the highest mean Surface Roughness recorded with varnish only followed by fluoride paste then CPP-ACP paste and least the TCP paste with significant difference between them. However in CPP-ACP varnish group the highest mean Surface Roughness recorded with fluoride paste followed by varnish only then CPP-ACP paste and least the TCP paste with no significant difference between them.

Under limitation of current study the following conclusion could be:

1. TCP varnish is the most effective among other tested varnishes in remineralization of early enamel caries.
2. The Combination of TCP and ACP-CPP paste with different tested varnishes enhance enamel remineralization compared to varnish only.
3. The use of fluoride paste with different tested varnish does not provide any additive remineralization potential for these varnishes.
4. Fluoride varnish only or when combined with paste will improve surface roughness faster than other remineralizig agents.
5. All application techniques of remineralizing agents can improve enamel surface roughness but not all can improve enamel surface micro hardness

Recommendation:

Regardless the type of active ingredient in Varnish applied to enamel, it is advisable to daily use TCP paste in order to prevent and treat the initial carious white spot lesion in enamel.

Further investigation:

1. Given that all remineralizing agents have the ability to improve both surface roughness and microhardness; so further investigation are considered necessary in order to detect the correlation between surface roughness and microhardness.
2. At invitro studies, the remineralization are different from that usually occurs in oral cavity due to dynamic complex nature of oral environment. Thus more in vivo studies are also required to assess the clinical performance of different remineralizing agent for a long period of time.

Conflict of Interest:

The authors declare no conflict of interest.

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Ethics:

This study protocol was approved by the ethical committee of the faculty of dentistry- Cairo university

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