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The Effect of Amorphous Calcium Phosphate Nanoparticles Loaded in Chlorhexidine as an Intra-canal Medicament on Radicular Dentin Microhardness (An In-Vitro Study)

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Abstract

Objectives: This study evaluated the effect of 20% amorphous calcium phosphate nanoparticles loaded in chlorhexidine as an intra-canal medicament, on root canal dentin microhardness.

Methods: A total of 30 human permanent single rooted teeth were used in this study. Teeth were decoronated and roots were split longitudinally into two equal halves, the better one half was kept and the other was discarded (30 specimens). The specimens were randomly divided into 3 groups according to the medicament used: 20% amorphous calcium phosphate nanoparticles in chlorhexidine (NACP+CHX), 2% chlorhexidine (CHX) and calcium hydroxide (Ca(OH)₂). Each medicament was applied for two weeks immediately after initial baseline microhardness determination. The initial baseline microhardness determination utilized Vickers indenter under a 200-g load and a 15-second dwell time. Post treatment microhardness values were obtained utilizing the same settings. The change in radicular microhardness was calculated as a percentage. Data were statistically analyzed utilizing 1-way analysis of variance (P=.05) and post hoc Tukey test for the multiple comparisons at the same level of significance. The difference between the pre-treatment and the post-treatment microhardness values were statistically analyzed utilizing t-test with a P < .05.

Results: 20% amorphous calcium phosphate nanoparticles in chlorhexidine was the only medicament to result in a statistically significant increase in radicular dentin microhardness, while both 2% chlorhexidine and calcium hydroxide resulted in a statistically significant decrease, with calcium hydroxide having the most detrimental effect.

Conclusion: On the contrary of 2% chlorhexidine and calcium hydroxide, 20% amorphous calcium phosphate nanoparticles in chlorhexidine demonstrated a reinforcing effect on radicular dentin microhardness.

Key Words: Amorphous calcium phosphate, Intra canal medication, Microhardness, Nanoparticles.

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Introduction

The main objectives of root canal treatment are to eliminate bacteria and disrupt bacterial ecology, create a clean root canal space, seal the root canal system preventing leakage of microorganisms and ingress of periapical fluids into the root canal, provide a bacterial tight coronal seal, prevent and intercept pulpal and periradicular pathosis and preserve the natural dentition. (1). The chemo-mechanical preparation of root canal removes necrotic pulp tissues and creates a space for delivery of disinfectants, eliminating microbes not reached by shaping procedures.

Bacteria are the predominant microorganisms in root canals. They can penetrate into dentinal tubules, lateral and accessory canals. They can be found between the root canal filling and the root canal walls, and they can also form extra-radicular biofilms (2). Some bacterial strains such *E. faecalis* show resistance to the traditional antimicrobial interventions due to their ability to form a biofilm where bacteria can exchange their genetic material, communicate, have metabolic and growth diversity and protect themselves against nutritional depletion and antimicrobial agents (3).

Intra-canal medications are a key solution to the situations where resistant microorganisms are irresponsive to conventional antimicrobial interventions. They are introduced into the hectic environment of the root canal space, remaining in direct contact with pathogenic microorganisms for prolonged periods of time, enhancing their antibacterial effect (4).

The preservation of the natural tooth structure is of outmost importance for a long term success. Unfortunately prolonged periods of contact needed for the available antibacterial irrigants and medicaments to exert their antibacterial action have a

detrimental effect on radicular dentin mineral content and microhardness (5), such depleting effect increases as the agent's concentration and duration of contact increases.

Calcium phosphates are considered the most important constituents of biological hard tissues including bone and teeth. Previous research stressed on the remineralization potential of calcium phosphates and their ability to initiate remineralization of demineralized hard tissues, owing to the increased calcium release, especially from the more soluble amorphous state and their ability to increase the pH of acidic media. (6,7)

The introduction of materials in the nanoscale allowed the development of novel properties from their bulk counterparts. Such novel leap in medicine, allowed the development medicaments with enhanced and prolonged release of its major constituents, providing an enhanced overall effect, whether in terms of antimicrobial efficacy or promoting remineralization in affected hard tissues. (8)

The use of nano-particles of calcium phosphate in a vehicle of chlorhexidine, was intended to solve the dilemma of the negative influence of antimicrobial agents on radicular dentin microhardness.

Since there is scarce information about the effect of such combination on radicular dentin, the aim of the present study is to evaluate comparatively the action of 20% NACP+CHX, 2%CHX and Ca(OH)₂ on radicular dentin microhardness.

Materials and Methods:

• Samples Selection and Preparation

Thirty extracted mature permanent single rooted teeth were collected from the oral and maxillofacial surgery department, Faculty of Dentistry, Ain Shams University. These teeth were extracted for periodontal or non-restorability reasons. Bone, calculus and periodontal tissues were removed

mechanically using an ultrasonic scaler, teeth were then inspected to exclude fractures, cracks or craze lines. Extracted teeth were placed in 5.25% NaOCl for 15 minutes for disinfection purposes and to remove soft tissue remnants. Crowns were removed below the cemento-enamel junction and root lengths were standardized to 16mm using a water cooled high speed wheel stone. Roots were then stored in distilled water until use. Root sections were cleaned and shaped using rotary files up to F4 (ProTaper; Dentsply Maillefer) while maintaining apical patency and using sodium hypochlorite between each consecutive file.

- **Specimens Preparation**

Thirty roots were sectioned vertically into two equal halves buccal and lingual, using a 0.3-mm IsoMet saw under constant cooling with sterile distilled water. This resulted into 60 halves; the better one half of each root was selected and embedded in autopolymerizing acrylic resin. The exposed root dentin surface was ground flat and smooth with a sequence of fine polishing papers (400, 600, 800 & 1200 grit polishing papers) under a flow of sterile water to remove any surface scratches.

- **Specimens Classification**

30 specimens were equally divided and classified according to the type of medicament applied into three groups (N=10).

- Group (A): 20% Amorphous calcium phosphate nanoparticles in chlorhexidine.
- Group (B): 2% Chlorhexidine gel.
- Group (C): Calcium hydroxide paste.

- **Baseline Microhardness**

- **Determination**

The baseline microhardness measurement of all specimens was performed initially before medicaments application utilizing Vickers Diamond Micro-hardness tester (Wilson Buehler., USA). Three measurements were

made at the coronal, middle and apical thirds, at a depth of 1000 μ m from the lumen, using a 200g load and a dwell time of 15s. The average of the values was obtained to produce one hardness value at each of the three levels (coronal, middle, apical) for each sample. Measurements obtained were considered as pre-treatment baseline values.

- **Medicaments preparation**

The 20% amorphous calcium phosphate nanoparticles in chlorhexidine (Nanotech, Giza, Egypt) were manufactured by discontinuous batch precipitation technique, where continuous mixing of two aqueous solutions (2L each) with a ratio 1:1 was performed at room temperature. The first solution was calcium chloride + sodium citrate, while the second was disodium hydrogen phosphate. Alkalinity was maintained by the addition of 320 mL of sodium hydroxide 0.1M. After maturation of the suspension at room temperature, centrifugation at 4500 RPM for 10 min was performed and resultant calcium phosphate nanoparticles were pelleted, washed with ultrapure water and dried in a ventilated oven at 70°C for 72h. The obtained particles were then embedded in an injectable gel of 2% chlorhexidine and methylcellulose, maintaining a ratio of 20% for ACP NPs. (9) 2% Chlorhexidine gel was prepared by mixing chlorhexidine (JK Dental, Egypt.) with methylcellulose as a thickening agent, where 4mg of polymer were added for every 1 ml of chlorhexidine to obtain an injectable gel form.

Calcium hydroxide was prepared by mixing powder (JK Dental, Egypt.) with sterile saline in a ratio of 1.5:1 (wt/vol) to obtain a paste consistency.

- **Medicaments application**

Specimens from each group were arranged on separate sterile petri dishes, where 0.3 ml of each corresponding medicament was applied from sterile syringes onto each

specimen. A sterile cement spatula was used to ensure even distribution over the entire surface. A storage period of 2 weeks was done at 37°C and 100% humidity.

- **Post Medication Microhardness Determination**

After two weeks of application, the samples were washed with distilled water and blotted dry. The change in microhardness was measured by Vickers Diamond Micro-hardness tester in Vickers Hardness Units (VHN).

Three measurements were taken at each of the coronal, middle and apical thirds, at a depth of 1000 µm from the lumen.

Measurements were done using a 200 g load for 15 s dwell time.

Microhardness was obtained using the following equation:

$$HV=1.854 P/d^2$$

HV = Vickers hardness (Kgf/mm²).

P = Load (kgf).

D = Average length of diagonals (mm).

Mean percentage of change in microhardness, was calculated and statistically analyzed using the following equation (10):

$$V1-V2/V1 \times 100$$

Where V1 = Preoperative VHN and V2 = Postoperative VHN

- **Statistical Analysis**

The differences between pre-treatment and post-treatment microhardness values were analyzed statistically using t-test with a P-value of .05. The comparisons between the three experimental groups were performed utilizing one-way analysis of variance, proceeded by Tukey honestly significant difference test at p= 0.05.

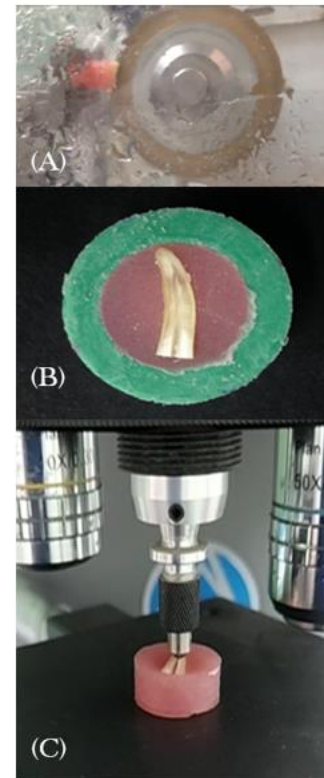


Figure 1. (A) Photograph showing root sample sectioning utilizing Isomet saw, (B) Photograph showing root specimen embedded in autopolymerizing acrylic resin for test procedures, (C) Root specimen undergoing Vickers microhardness test.

Results:

Vickers microhardness values (mean ± standard deviation) for all groups at base line showed no statistically significant difference between them (Table 1). After two weeks of medicaments application, all groups showed a significant decrease in radicular microhardness except NACP+CHX group, which showed a significant increase (P < .05) (Table 1).

Table 1: Mean (Kgf/mm²) and standard deviation (SD) values of microhardness before and after 2 weeks of medicaments application

	NACP+CHX	CHX	CaOH	P-Value
Coronal				
Pre	53.17 ±6.16	53.20 ±4.28	53.49 ±7.13	0.05 10 ¹⁴
Post	58.63 ±1.884 ^a	47.73 ±1.297 ^b	43.79 ±2.342 ^c	
Difference	5.46	-5.47	-9.7	
Middle				
Pre	52.78 ±6.24	51.37 ±3.45	49.49 ±6.98	0.03 10 ¹²
Post	57.97 ±2.105 ^a	46.35 ±1.859 ^b	40.17 ±2.025 ^c	
Difference	5.19	-5.02	-9.32	
Apical				
Pre	45.90 ±5.50	43.91 ±4.73	42.61 ±6.28	0.05 10 ¹²
Post	50.2 ±1.305 ^a	39.55 ±1.502 ^b	34.20 ±2.610 ^c	
Difference	4.3	-4.36	-8.41	

Means that do not share same letter in the same row are significantly different.

Mean percentage of change after two weeks of medicaments application in Vickers microhardness values (mean ± standard deviation) is summarized in Table 2.

Table 2: Mean (Kgf/mm²) and standard deviation (SD)

	NACP+CHX	CHX	CaOH	P-Value
Coronal	-10.268% ^c	10.282% ^b	18.134% ^a	0.01x 10 ⁻¹⁴
Middle	-9.833% ^c	9.772% ^b	18.832% ^{ca}	0.05x 10 ⁻¹⁵
Apical	-9.368% ^c	9.929% ^b	18.85% ^a	0.01x 10 ⁻¹⁶

Means that do not share same letter in the same row are significantly different.

values of percentage of change in microhardness after 2 weeks of medicaments application.

Discussion:

Ideally, in vivo evaluation should be performed, as it's the most accurate representation of the actual clinical situation. However in vivo studies present difficulties in standardization, ethical considerations as well as huge time consumption, for these reasons this study was carried out In vitro (11).

Nano-particles are gaining an increased interest in the literature owing to their unique anti-bacterial mechanisms where they can interact with the peptidoglycan of the cell wall and cell membrane causing cell lysis, interact and disrupt the synthesis of bacterial proteins, interact with and disrupt bacterial DNA (12) and induce oxidative

stresses through free radical formation. These mechanisms allow nano-particles to inhibit the bacterial ability to form biofilms and drug resistance. (13)

Nano-particles of amorphous calcium phosphate also have the ability to induce remineralization and increase the hardness of demineralized dentin to a level comparable to that of normal dentin through calcium and phosphate ions release & raising the pH. (14)

Chlorhexidine demonstrates very effective broad-spectrum antibacterial properties, due to its ability to adsorb onto the cell wall of microorganisms, resulting in the leakage of intracellular components, precipitation and coagulation of cytoplasm and hence cell death. The most important property of chlorhexidine is its substantivity owing to its ability to bind to the hydroxyapatite of tooth enamel as well as the pellicle. (15)

Calcium hydroxide is one of the most commonly used and researched medicaments in endodontics. It exerts its anti-bacterial effect through its ability to dissociate into calcium and hydroxyl ions, which results in an increased pH and inhibition of enzymes essential for microbial metabolism. (16)

Vickers diamond microhardness tester was used to measure radicular microhardness. Microhardness is the resistance to local deformation and it's based on inducing permanent surface deformation that remains after load removal. (17) Vicker's test was used because previous studies showed the accuracy and practicality of the Vickers indenter for evaluation of dentin microhardness of radicular dentin.

Vickers hardness number calculations are based on the mean of two diagonals compared to only one in the knoop test, thus giving more accurate readings. (18) Also the Vickers hardness

tester has a smaller indenter tip suitable for small specimens. 200g loads and 10 seconds were used for measurements according to Mathew et al (19).

The changes in the mean of microhardness were recorded utilizing Vickers microhardness test. The two weeks of application of calcium phosphate nanoparticles in chlorhexidine resulted in a significant increase, while chlorhexidine and calcium hydroxide resulted in a significant decrease, with calcium hydroxide recording the least values

The increase in microhardness following the the two weeks of application of amorphous calcium phosphate nanoparticles in chlorhexidine can be attributed to that the amorphous nano-sized particles of calcium phosphate, release significantly higher amounts of calcium and phosphate ions, when compared to conventional hydroxy-apatite crystalline phases of calcium phosphate (6, 7), in addition to its ability to neutralize the low pH acidic environment. This inhibits demineralization and initiates re-mineralization. The results of our study are in agreement with several studies (20-26).

The application of 2% CHX for two weeks resulted in a moderate reduction in the mean of microhardness of radicular dentin as CHX tends to cause alterations in radicular dentin, by its ability to disrupt the links between collagen fibers and hydroxyapatite crystals, resulting in a decrease in dentin microhardness. The results of our study is in agreement with Oliveria et al. (27) who found that the application of chlorhexidine for 15 minutes significantly reduced the microhardness of radicular dentin, as well as Prabhakar et al. (28) who demonstrated the adverse effects of 2% CHX on radicular microhardness at 1, 3 and 7 days of application.

The application of calcium hydroxide for two weeks resulted in the

highest reduction in the mean of microhardness of radicular dentin. This could be explained by the proteolytic action of calcium hydroxide, in addition to the pH increase that results in reduced organic support of the dentin matrix, resulting in breakdown of the protein structure and the disruption of the links between collagen fibers and hydroxyapatite crystals that has a negative influence on the mechanical properties of dentin (29).

Another reason can be the penetration into the intrafibrillar structure of collagen fibrils due to their very minute molecular size, which changes the three-dimensional confirmation of tropocollagen, resulting in a diminished microhardness of the radicular dentin (30). The results of our study are matching with those of Parashar et al.(31), Naseri et al.(32), Pacios et al. (33) and Yassen et al. (34) who have demonstrated the deleterious effects of calcium hydroxide on radicular dentin microhardness.

Finally, combining the properties of amorphous calcium phosphate nanoparticles and chlorhexidine can provide a novel and a promising solution to the damage that radicular dentin has to withstand for the conventional intra canal medications to exert their antibacterial actions.

In conclusion, our present study provides an insight into the effect on radicular microhardness of various intracanal medicaments. Calcium hydroxide had the most detrimental effect, followed by chlorhexidine, yet amorphous calcium phosphate nanoparticles in chlorhexidine had a significant enhancing effect. Further studies should be carried out to verify the benefits and risks to humans.

Acknowledgments

The authors deny any conflicts of interest related to this study.

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