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Evaluation of Antimicrobial Activity of Newly Prepared Calcium Oxide Based Nanosealer Using Agar Diffusion Test

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Abstract

Aim of the study: The antimicrobial activity of a root canal sealer can increase the success rate of endodontic treatments. The key of antimicrobial properties of calcium based sealers laid in their composition and alkalinity. The invention and applications of newer materials are quintessential in every field especially in endodontics. This reality has led to growing interest in the use of biomaterials products that comprised of both mineral and organic components as those in the teeth. This study was performed to assess the antibacterial activity of newly prepared calcium oxide based nanosealer and compare the result with BioRoot sealer

Materials and methods: The CaO nanopowder was firstly prepared from calcinated cowrie seashell using ball milling and ultrasonic irradiation methods, and then the CaO nanopowder was used as the main component in the preparation of an experimental nanosealer powder part. After several pilot studies, the final formula for the prepared sealer that gave the best clinical sealer consistency and properties within the limits of ANSI/ADA Specification No.57/2012 was as the following. The powder part consists mainly of nano CaO (44%) in addition to glutamic amino acid, zirconium oxide (20nm), and silica oxide (15-20) nm, while the liquid part consists mainly from distilled water in addition to propylene glycol. The Antimicrobial activity of the prepared sealer was evaluated by agar diffusion test against *E.faecalis* and *E.faecium*, after 1, 2, 3, and 7 days of incubation at 37˚C. Zone of bacterial inhibition was measured and analyzed statistically using Independent sample t – test at (p=0.05) levels.

Results: The experimental sealer had significantly higher antibacterial effect against both bacteria spp. than BioRoot sealer ($p \leq 0.05$) at all observation times, and the antibacterial effect was higher against *E.faecium* than *E.faecalis* for both sealers ($p > 0.05$). The values of inhibitions zones decreased over time for both sealers.

Conclusion: within the limitations of the present study, the outcomes showed that the newly prepared nanosealer represented higher antibacterial activity than BioRoot sealer against most highly resistance bacteria that lead to endodontic treatment failure.

Keywords: antibacterial activity, nanoparticles, calcium - based sealer.

Introduction

Bacteria and their byproducts play an essential role in the endodontic treatment failure. Bacteria may remain even after complete chemo-mechanical preparation of the root canal in the ramifications, apical deltas and dentinal tubules. The antimicrobial activity of a root canal sealer can increase the success rate of endodontic treatments by inhibiting bacterial growth or eliminating residual intraradicular infections (1,2). Many researchers reported that the production of sealers with nanosize level can improve their physicochemical characteristics, increasing the biocompatibility and biomineralization abilities, enhancing their antibacterial property, and provide good sealing ability (3.4).

One of the major applications of nanotechnology is the synthesis of metal and non-metal oxide (ceramic) nanoparticles that can be used in different medical and dental fields like zinc oxide (ZnO), zirconium oxide (ZrO), silica oxide (SiO), and calcium oxide (CaO). Calcium is the most important natural constituent of bone and teeth; one of the calcium derivatives is the CaO, which is considered as an imperative inorganic compound. It has excellent antimicrobial potential and adeptness to highly resistance microbial endotoxin and it is considered as a safe material to human beings and animals (5,6). Calcium oxide was introduced as an endodontic filling material by Bernard in 1952. *In vitro* studies have established that CaO based root fillings are biocompatible and due to their high alkalinity produce high antimicrobial activity, dissolve organic predentin and translocation of calcium into dentinal tubules resulting in an intimate bonding to the root dentin wall that lead to provide tightly apical seal (7,8).

According to the community of materials technologists, the greatest present and future need is for the recycling of cheap, abundant, and biologically safe natural waste materials. Seashells are considered as biomass waste; they composed mostly of calcium carbonate (95%), protein and polysaccharides (2%), and variable amounts of silicon, sodium, aluminum, and magnesium which are reported to be as a raw materials for producing natural bioceramics (9,10).

Many efforts have been made to use seashell for many purposes, one of which is to consider them as natural sources for CaO production rather than limestone which are usually associated with greater environmental pollution and lower purity CaO production (11,12). Various methods can by employed for the synthesis of nanoparticles, however these methods are broadly divided into two main classes the top-down approaches like mechanical milling, sputtering, nano-lithography, laser ablation, and thermal decomposition, and bottom-up approaches like chemical vapor deposition, sol-gel (sonication),

spinning, pyrolysis, and biosynthesis (13,14).

In this study CaO nanopowder was synthetized from seashell, then incorporated it as the main component to prepare root canal nanosealer, and then evaluated the antibacterial activity of the prepared nanosealer using agar diffusion test.

Materials and method

The cowrie shells were bought from local market, cleaned with sodium hypochlorite followed by distilled water ultrasonic baths (5min each), then dried for three days at room temperature. Then they were calcinated to 1000 ºC for 2h using digital muffle furnace at heating rate of 10 ºC/min and left to cool down for two hours. Then ground into fine powder and sieved using 25µm pore size sieve (15). The calcination and sieving were done in the College of Basic Education/ Science Department/ Mosul University.

The resultant micronized CaO was first milled with high energy ball milling machine in the Ministry of Science and Technology/ Department of Nano/ Baghdad for 24 h with 2:1 balls to powder mass ratio (13). Then the obtained milled powder was underwent further size reduction using high intensity ultrasonic machine in the College of Science/ Department of Biology/ Mosul University.

Approximately 10 g of milled CaO powder was added to 200 ml deionized water inside cylindrical glass bottle under constant magnetic stirring at room temperature to prepare the $Ca(OH)_2$ suspension. The ultrasonic machine was preset at 20 kHz and 500 watt. Then the suspension was ultrasonically irradiated at room temperature with titanium oscillator at 1/20,000s speed (16,17).

The resulted suspension was further diluted with deionized water and filtered by a cellulose nitrate ester millipore membrane with 0.20 µm (CHMLAB Group, Spain) and then with 0.10 µm (Sartorius/Germany) pore size using vacuum filtration system (18).

The filtered $Ca(OH)_2$ suspension was traveled to 13 ml sterile falcon tubes and centrifuge at 4000 rpm for one hour. After that the supernatant aqueous medium was discarded and left behind 2 ml of filtered $Ca(OH)_2$ suspension in each tube. The tubes with the remaining suspension were dried using freeze drying by first freezing the samples using deep freeze refrigerator at (-80)˚C, and then drying at low pressure using Lyophiliyzer machine (15,19). The filtration and Lyophiliyzation was being done in the College of Basic Education/ Science Department/ Mosul University.

The freeze dried powder was then calcinated in a muffle furnace at 1000 ºC for 2h. Finally, the synthesized CaO nanopowder was stored in tightly seal Eppendorf tubes and kept inside a desiccator until further use.

The obtained CaO nanopowder was characterized using Field Emission Scanning Electron Microscopy (FE-SEM) to evaluate the surface morphology, shape and size of the nanoparticles coupled with elemental analysis using Energy-Dispersive X-ray spectroscopy (EDX).

The structural phases and crystallite size were identified by X-Ray diffraction system (XRD) at $2\theta = (10^{\circ} - 80^{\circ})$ and the phases were identified using the International Centre for Diffraction Data (ICDD).

Then after; we prepared the nanosealer that consists of powder part and liquid part; after several pilot

studies, the final formula for the prepared sealer that gave the best clinical sealer consistency and properties within the limits of American National Standards Institute/American Dental Association (ANSI/ADA) Specification No.57/2012 and International Organization for Standardization (ISO) 6876/2012 for dental root canal sealing materials was as the following; the powder part consists mainly of prepared nano CaO (44%) in addition to glutamic amino acid, ZrO (20nm), and SiO (15-20 nm); while the liquid part consists mainly from distilled water in addition to propylene glycol. The powder/liquid ratio was 1g powder part to 0.3ml liquid part and the mixing time was 38 second.

The antimicrobial assay was carried out in the Laboratory of Microbiology/ Department of Biology/ College of Science/ Mosul University under aseptic conditions in a laminar air flow cabinet.

BioRoot sealer (Septodont, France) was used as a control in this study and *Enterococcus faecalis* (*E.faecalis*) and *Enterococcus faecium* (*E.faecium*) were used for evaluation the antibacterial activity of sealers.

The bacteria were isolated from the patients with failed endodontic treatment and persist periapical lesion with no post placement using paper points under aseptic condition that immediately transferred into tube containing sterile brain heart infusion (BHI) broth. The tubes with their contents were incubated at 37°C for 24h (1,2).

The Enterococcus spp. were identified by selective agar base, biochemical analysis (Vitek2 System), and polymerase chain reaction (PCR) analysis (20).

The antimicrobial effects were assessed with agar diffusion tests. The broth culture of the bacterial strain compared to McFarlan's standard 0.5 was prepared. About 80 selective agar plates (40 plates for each bacteria type) were prepared and divided randomly into four test groups (n=10) according to time intervals of incubation (1, 2, 3, and 7) days. Then 0.1 ml of each bacterial suspension (1.5×10^8 CFU/ml) using micropipette was added to the surface of the corresponding plates and spread homogeneously on the selective agar base (HiCrome™, India) using a sterile cotton swabs. The plates were allowed to dry for 3-5 minutes, then two wells measuring (6 mm in diameter) each one corresponding to a single tested sealer, were made and remove the agar at equidistant points and to full agar depth and then they filled immediately with freshly mixed BioRoot and experimental sealers using disposable syringe (1,2 and 21).

All plates were maintained at room temperature for 2h to allow diffusion of the sealer into the agar and then incubated at 37ºC for (1, 2, 3 and 7) days.

The diameters of inhibition zones of bacterial growth around each well of both sealers for *E. faecalis* and *E. faecium* were then measured by digital vernia at the vertical and horizontal diameters and the mean value of these measurements was calculated and recorded. Independent sample t - test was used at $p \leq 0.05$ for evaluating the results of inhibition zone for each sealer and bacterial types at different time interval.

Results

The FE-SEM images (figure 1a) revealed that the prepared CaO powder has nanoparticles with nearly spherical in shape with some agglomeration areas. The EDX analysis (figure 1b) showed that the purity of prepared CaO

was (95.6%). Carbone element usually results from carbonization of CaO surface during sample preparation for FE-SEM analysis also Sodium can be detected in small quantity.

The XRD analysis (figure 2) represented that all the peaks were confirmed that the prepared powder was CaO. The XRD profile observed peaks that are in good agreement with the ICDD standard data for CaO (ICDD Cards No. 00-004-0777).

The average crystallite size of the prepared CaO nanopowder was calculated from the XRD data using the Debye-Scherrer's formula and it was found to be (41.25) nm which is highly matches the average particle size that calculated from the FE-SEM image (42.95) nm.

The inhibition zones diameters in (mm) for the experimental and BioRoot sealers against *E.faecalis* and *E.faecium* were represented in figures 3 and 4 respectively, and the mean and standard deviation values were represented in (table 1) at different observation periods.

Independent sample t - test represented that the experimental sealer has statistically significantly larger inhibitions zones against *E.faecalis* and *E.faecium* than for BioRoot sealer ($p \leq 0.05$) at all observation times.

Independent sample t - test represented that the BioRoot and experimental sealer has larger inhibitions zones against *E.faecium* than against *E.faecalis* at all observation times ($p > 0.05$). The values of inhibitions zones decreased over time for the experimental and BioRoot sealers against *E.faecalis* and *E.faecium* however still found at day 7 as shown in (figure 5).

Discussion

Seashells have been used as a raw material for various dental and medical applications, and compounds from seashells have been used as a natural chemical product applied in biomedicine and biodentistry fields (10-13). Utilization of nanoparticles in the production of endodontic sealers has become favorable for many researchers (3,4).

In this study, the mechanical attrition depending on the shear plastic deformation principles and the high intensity ultrasonic irradiation depending on cavitation principles were used to prepare nanometer CaO powder. Both methods were considered simple and efficient procedure for the production of nanoparticles at ambient temperature (13,14).

One of the commonly used methods to separate the nanosized from the microsized particles in suspension, is the use of water resistance membranes with selective nano-pores sized that only permit the passage of particles that are equal or less than the membrane pore size (18).

Lyophilization is a low temperature dehydration process mostly used for drying of various pharmaceutical and biological products. In contrast to the heat dehydration; the low temperature dehydration make the quality of the material's rehydration is excellent and maintain the primary physical and chemical characteristics of the materials (15,19).

Although the role of *E. faecalis* in endodontic infections had been extensively studied, little is known about the presence of *E. faecium* and its role in endodontic infections. Some studies verified a high prevalence of *E. faecium* in mixed infections with *E. faecalis* in persistent endodontic infections through their identification using molecular methods (2,20, and 22).

In the present study, both sealers exhibited larger inhibition zone against *E.faecium* than against *E.faecalis*. In addition both of sealers exerted strong antimicrobial effect against *E.faecalis* and *E.faecium* at day 1 which was diminished on day 7, however they still exhibited inhibition zones against *E.faecalis* and *E.faecium*.

Silica and zirconia nanoparticles reported to have greater antibacterial activity in many biological applications; this may be due to their large surface area that proposed to produce a large amount of reactive oxygen which in turn leads to damage of cell membrane and disturbance of the cellular components activities (5,6).

The experimental sealer has significantly larger inhibitions zones against *E. faecalis* and *E. faecium* than for BioRoot sealer at all observation times.

The key of antimicrobial properties of calcium based sealers lie in their alkalinity which in turn depends on the amount of $Ca(OH)_2$ byproducts. Since the experimental sealer consists of nano CaO, therefore large surface area has been available to react with distilled water to form the $Ca(OH)_2$ byproduct which in turn provides more ion exchange and higher amount of OH release (7,8).

From other point, studies found that propylene glycol exhibited strong antibacterial action against common microorganisms found in the infected root canals like *E. faecalis* through its ability to cause plasmolysis and cell wall collapse. In addition, the propylene glycol reported to extend the release of Ca^{++} and OH ions periods (23,24). This may explain the higher and the extending antibacterial activity of the experimental sealer.

Conclusion

The outcome of the present study showed that the newly prepared nanosealer represented higher antibacterial activity than BioRoot sealer against most highly resistance bacteria that lead to endodontic treatment failure.

Conflicts of Interest

The authors reported that there is no conflict of interest.

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Table (1): Mean and standard deviation of the inhibition zones diameters of experimental (Exp) and BioRoot sealers against *E.faecalis* and *E. faecium* at different observation times

Observation Periods	Mean ± Sd. (*E.faecalis*)		Mean ± Sd. (*E.faecium*)	
	Exp. (10)	BioRoot (10)	Exp. (10)	BioRoot (10)
1 days	18.42 ± 0.03	17.41 ± 0.17	20.84 ± 0.02	17.84 ± 0.06
2 days	17.34 ± 0.08	15.11 ± 0.08	17.36 ± 0.05	16.22 ± 0.49
3 days	14.28 ± 0.01	9.64 ± 0.40	15.51 ± 0.03	14.76 ± 0.10
7 days	11.82 ± 0.04	5.24 ± 0.39	12.41 ± 0.05	10.11 ± 0.10

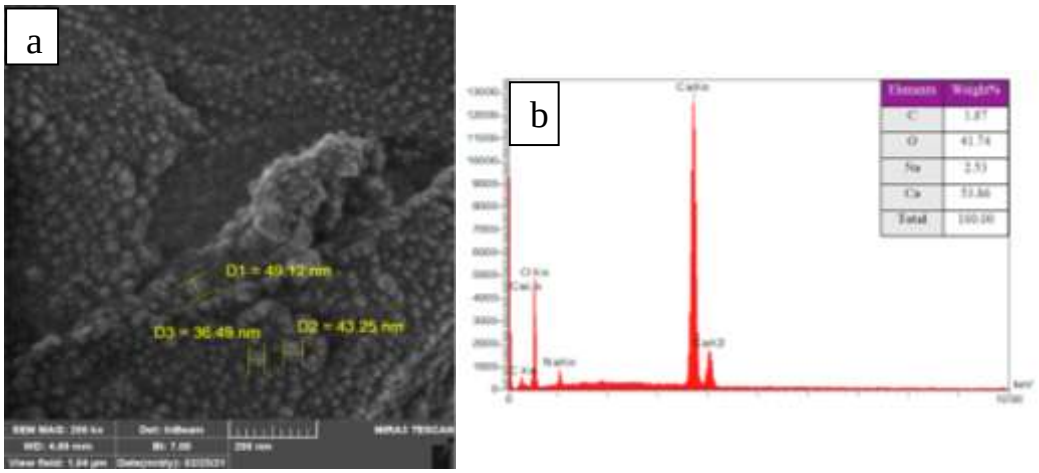

Figure (1): Representative FE-SEM image of prepared CaO nanopowder (a), the EDX spectrum (b).

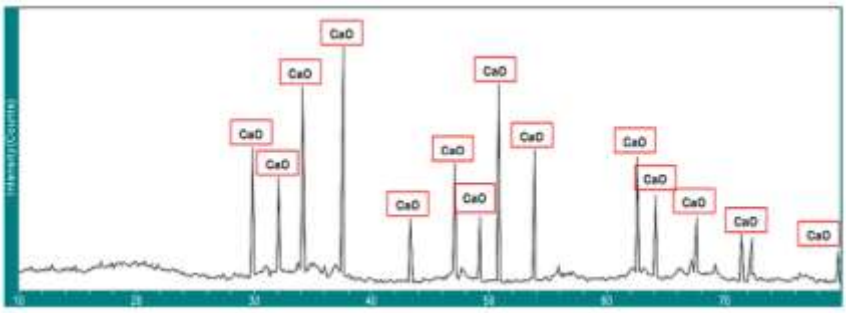

Figure (2): The XRD spectrum of the prepared CaO nanopowder.

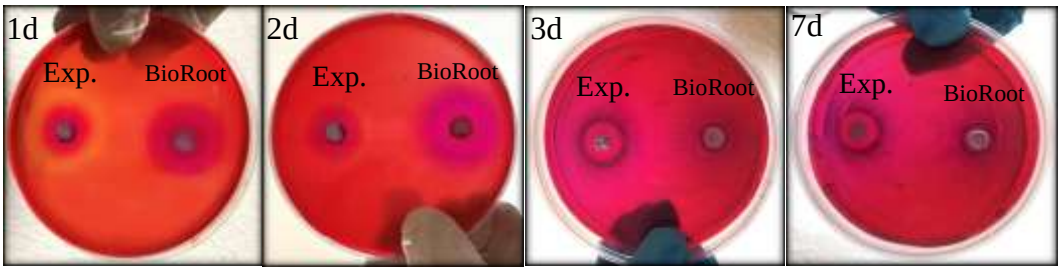

Figure (3): Inhibition zones of experimental (Exp.) and BioRoot sealer against *E.faecalis* at different observation times.

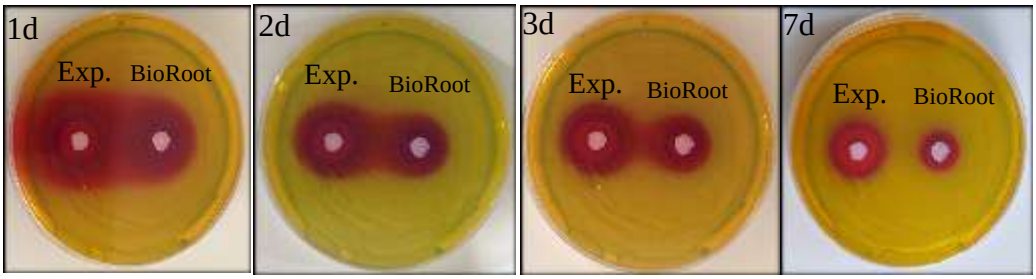

Figure (4): Inhibition zones of experimental (Exp.) and BioRoot sealers against *E.faecium* at different observation times.

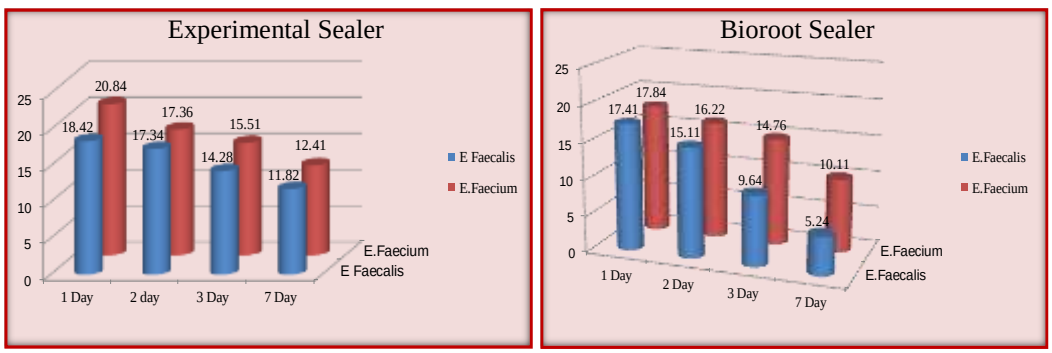

Figure (5): Histograms representing the reduction of inhibitions zones over time for experimental and BioRoot sealer against *E.faecalis* and *E.faecium*.