Preparation and pH with Antibacterial Evaluation of Nano Calcium Oxide Based Intracanal Medicament from Natural Products (An In vivo Study)

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Abstract
Background To prepare new calcium based intracanal medicament from egg shell and sea shell powder mixed with pure coconut oil then evaluate the antibacterial activity of the new prepared materials and compare the result with Metapex intracanal medicament. **Materials and Methods** Calcium based intracanal medicament have been prepared from local egg shell and from seashell (Cowrie) in a form of nano calcium oxide powder; each powder was mixed separately with locally made pure coconut oil to form two experimental materials. Antimicrobial activity was evaluated by direct contact inhibition zone test of each experimental material and control with Enterococcus faecalis, after 24 hours of incubation at 37◦C, zone of bacterial growth was observed and measured. **Results** The results showed that both of the prepared materials have antibacterial effect, these results were statistically analyzed using One-way ANOVA & Duncan›s Multiple Range test at p=0.01 levels. **Conclusion** Results have shown that both egg and sea shells contain many types of metal oxides in different concentration that might have antibacterial action in addition to the lauric acid from the coconut oil which clinically proven to have antibacterial activity that aided the experimental materials to have an acceptable antibacterial activity.

Keywords: Antibacterial activity, coconut oil, calcium oxide, Eggshell, intracanal medicament, seashell

Introduction

During endodontic treatment pain, swelling and tenderness might occur which might cause discomfort for the patient and lead to sudden emergent visit to dental office. These complications might happen due to the presence of microbes inside root canal system which cause periapical inflammatory lesion, so the elimination of these microbes might greatly reduce post treatment discomfort and enhance the success rate of endodontic treatment (Siqueira et al, 2002). Many mechanical methods were introduced to eliminate microorganisms from root canal system, including different hand and rotary root canal instrumentation techniques (Siqueira, 2003; Peters et al, 2002). Intracanal medicaments may present such characteristics may act for longer time when remaining in the canal between appointments of endodontic treatment (Himadri

et al, 2019). Calcium hydroxide Ca (OH)2 is commonly used as intracanal medicament because of its antibacterial properties, mainly due to its alkaline pH. However, calcium hydroxide cannot be considered as an ideal canal medicament, as it cannot eliminate all microbes found in root canal (Priyanks et al, 2017). Usually calcium hydroxide is available as powder and it must be mixed with vehicles such as distill water, anesthesia, or glycerin in order to become applicable. Most of these vehicles do not have antimicrobial properties, therefore materials with potent antimicrobial action such as 2% CHX gel, Iodoform, Camphorated Para-monochlorophenol (CPMC) are added as ingredients. Combination of calcium hydroxide with these materials may provoke discoloration of the tooth, irritation of the periapical tissue if extruded and are not easily removed from root canals (Shuping et al, 2007; Kim and Kim, 2014). Concerns are increased about using natural products in dentistry. Coconut oil has been used in dentistry to prevent dental caries due to its ability to destroy most strains of Streptococcus microorganisms, specially Streptococcus mutans, which are responsible for the productions of acids that cause tooth decay (Barnabé et al, 2008). In addition to antimicrobial action, coconut oil has antifungal and antiviral activities and used in many healths care products (Oyi et al, 2010). The main beneficial action of coconut oil is due to the presence of a high natural content of lauric acid (Gopala et al, 2010). Calcium oxide (CaO) has been used as a root canal filling material. Studies showed that CaO is very effective because its biocompatibility (Guigand et al, 1999). It dissolves organic debris which aids in close adaptation of material to the dentin wall (Guigand et al, 1997), induce elevation of alkalinity through dentin thickness (Minana et al, 2001), translocate calcium into dentinal tubules (Guigand et al, 1997) and provide apical sealing that can resist dye penetration (Goldberg et al, 2004). Nano technology is growing very fast in the field of dental biomaterials, it may aid in diagnosis and recovery of oral cancer, stops the accumulation of plaque, prevention of teeth hypersensitivity and applied to enhance esthetic and physical properties of restorative materials to replace missing tooth structure and improve sealing of root canal filling material. The aim of this study is to determine the antimicrobial activity of an experimental root canal medicament prepared from coconut oil and nano CaO powder, all extracted from local natural products.

Materials and Methods:

Sample preparation

The powder of the material consists of calcium oxide, prepared either from egg or sea shell.

Preparation of the egg shell powder

Egg shell powder was prepared according to (Alkhalidi et al, 2014). Egg shells were collected from local chickens which were fed the same provender. Clean shells were collected, washed in distilled water then were boiled for 15 minutes to eliminate microbes and left in water for 12 hours to facilitate the removal of the inner protein layer by the use of a fine tweezers. The inner protein layer was rechecked with the aid of magnifying loops (Denshine, China). Following, the shells were immersed in water so that all the fine remnants float at top surface of water and shells were left to dry for 24 hours. The shells were heated in a furnace (Manfredi, Italy) to 900º C for one hour (Figure 1), after letting the shells to cool for three hours they looked chalky white in color and became very brittle and porous, this is because that CaCO3 decomposed

and gave CaO and CO2 according to the following reaction (Engin et al, 2006).

$CaCO_3 = CaO + CO_2$

Each 110g of egg shell gave 84g of CaO, the heated shells were grounded into powder using coffee grinder (Braun, Mexico) and nanoparticles were gained according to (Amer et al, 2015). The particles sized were standardized using 25 Mm sieve. Nano particles were evaluated using Transmission Electron Microscope (Philips CM10, Holland). All powder was stored in a tightly sealed container.

Figure 1: Egg shells in furnace.

Preparation of the sea shell powder

Preparation of sea shell powder was done according to (Neam et al, 2015). Sea shells (Cowrie) were bought from local market and kept moist under water for 24 hours then cleaned using ultrasonic cleaning machine (Link Instrument, Chine) and brushed with dental brush to remove any remnant debris. The seashells were heated in a furnace (Manfredi, Italy) to 1000º C for two hours (Figure 2), and left to cool for four hours they looked chalky white in color and became very brittle. Each 67g of seashells gave 41g of CaO. The production procedure and the storage of the powder was the same as for the egg shells powder.

Figure 2: Sea shell in furnace.

The liquid

The liquid consisted of virgin coconuts oil (VCO) without any preservatives or an additive which was prepared from mature coconut according to (Divina and Keith, 2006). After removing the outer shell of coconut fruit, the coconut meat was washed thoroughly with water then sliced into small pieces. Warm water is added to the coconut pieces (about 1 cup of warm water is added to 1 cup of coconut pieces) and mixed in blender (Modex, UK) until a homogenous, smooth creamy mixture was gained. The collected creamy mixture was sieved by pouring a small amount of the coconut mixture onto a muslin cloth then squeezed into a bowl to collect pure coconut milk. The bowl was covered and left for 24 hours. This will make the coconut milk to separate from water and float on the surface. The thickened milk is collected from the surface and put in a clean dry pot and heated very slowly for 60 minute in 60ºC temperature with stirring to coagulate the cream and release the oil then left to cool. The mixture was sieved again using muslin cloth to separate the oil. The oil should be dried to ensure prolong shelf life of the virgin coconut oil. This is done by heating the extracted oil until the color of oil change from turbid to water-clear color. Temperature should not exceed 65ºC. This oil should be filtered through muslin cloth to ensure removal of fine particles and getting pure oil. The collected oil stored into tightly sealed glass bottles.

Preparation of the experimental intracanal medicament

Different ratios of liquid and powder from egg and sea shells were mixed separately in a powder/liquid ratio of (1:1,1:2,1:3,1:4,1:5,1:6,2:1, 3:1,4:1,5:1 and 6:1). Mixing of the ingredients was performed using glass slab and spatula for 40 seconds at 23±1ºC and 41±5% relative humidity. Small increments of powder were added to liquid gradually to ensure complete wetting of powder until powder and liquid are totally consumed. The criteria for selecting this ratio was based according to the consistency of the tested material, the selected ratio gave paste like material and according to antibacterial property which gave us the best antibacterial property. Metapex paste intracanal medicament was used as control in this study. A 2% titanium oxide was added to each powder to give radio-opacity. In whole study the egg shell powder mixed with coconut oil was given the name (experimental material 1) while powder of sea shell mixed with coconut oil was given the name (experimental material 2).

PH Measurement

Each tested material was mixed and subjected to PH measurement in addition to the control. Each of the experimental materials were mixed in clean glass slab and 0.1ml ml of each three tested materials were placed in 10ml of deionized water in screw cap closed vial and measurements were done by immersing the probe of the PH meter inside the tested material and wait until reading become stable (Figure 3). Each reading was repeated for three times and the mean of measurements was recorded. Measurements were done immediately, half an hour, 1 hour, 12 hours, 24 hours, 3 days, 7 days and 2 weeks.

Figure 3: *Enterococcus Faecalis* appeared as reddish-pink colonies on the surface of selective media.

Microbiological study

The microbiological study was conducted under the supervision of specialist in Laboratory of Microbiology, Department of Dental Basic Science, College of Dentistry, University of Mosul. Three materials were evaluated:

1st group: Experimental material I. (Nano CaO powder prepared from egg shell with coconut oil).

2nd group: Experimental material II. (Nano CaO powder prepared from sea shell with coconut oil).

3rd group: Metapex (META BIOMED, Korea) as control.

Microbial isolation

Enterococcus Faecalis was selected for the microbiological study. The oral Enterococcus Faecalis were isolated from tooth The oral Enterococcus Faecalis were isolated from premolars during primary root canal treatment with periapical lesion by inserting a sterilized stainless steel reamer (Dentsply Maillefer, Ballaigues, Switzerland) into the canal with slight instrumentation of canal and inserting the reamer into sterilized screw capped vial containing 5 ml brain heart infusion broth (BHI, Lab M,UK) and vortexed for 20 second to disperse the adhering bacteria and incubated for 15 minutes at 37ºC. Using sterilized cotton swab a loop full of this suspension was streaked into the surface of Enterococcus Faecalis selective media (Himedia, India); media was incubated at 37ºC for 24 hours. Enterococcus Faecalis is the only microorganism that grows in the Enterococcus Faecalis selective media which appeared as reddish-pink colonies on the surface of media (Figure 3). The culture was examined microscopically (Olympus, Japan) by selecting random colonies and making smear slide using Gram stain technique which appeared as reddish pink chained cocci. A single colony was transferred into brain heart infusion broth BHI broth (BHI, Lab M, UK) and incubated (Electro. Mag, Turkey) at 37°C for 24 hours. and further identifications were done by Gram›s stain and colony morphology. McFarland 2 turbidity tubes were used to prepare suspensions of the strains at approximately 6×108 organisms/ml.

Measurement of direct contact inhibition zone

Preparation of discs

Fifty discs of 6mm in diameter were prepared from filtering paper using paper plunger, discs were sterilized using autoclave (Hirayama MFG, CORP, Japan). Two materials were mixed under aseptic condition; nano powder from sea shell with coconut oil and nano powder from egg shell with coconut oil. Using disposable insulin syringe, 10µl of each material and control (Metapex) were placed on a sterile glass slab under aseptic condition and in each material a disc was immersed for 10 minutes, during that time, ten plates of Enterococcus agar media were utilized, on each plate 100µl of inoculated broth was spread using sterilized swab. Two discs of each tested material and one from control were placed on the surface of the media with gentle adaptation using sterilized tweezers (Derfla, Germany) (Figure 4A) and incubated for 24 hours at 37ºC. After the end of incubation period, inhibition zones surrounding tested materials and control were measured using a digital Caliper Accuracy 0.01mm (GeprufteSicherheit, Germany) as shown in (Figure 4B). Three measurements of the diameter of inhibition zone at different points was evaluated and the mean of these three measurements was recorded. The data were subjected to One Way ANOVA Test for evaluation of inhibition zone for each type of material, Duncan›s Multiple Range Test used to compare the results.

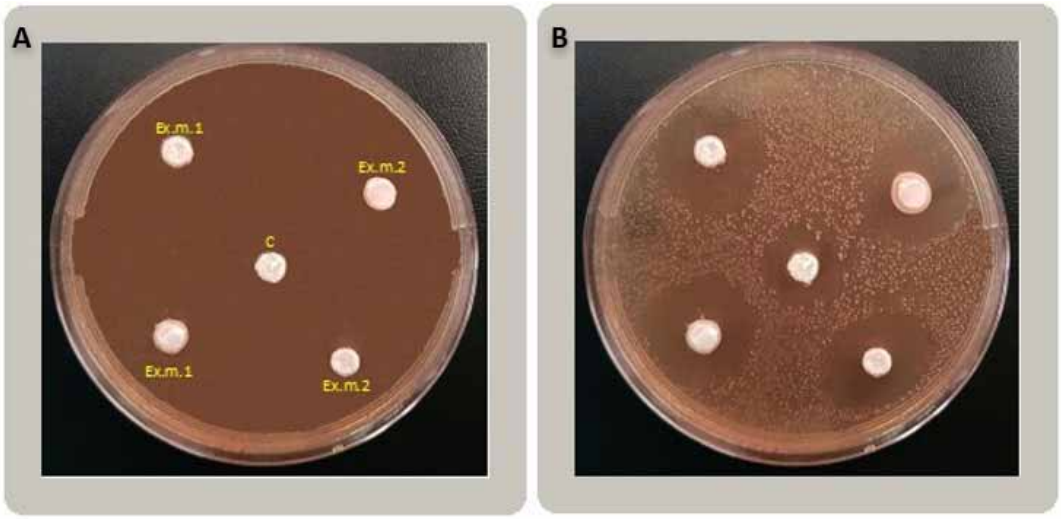

Figure 4: A. Placement of discs before incubation. Left: Two discs experimental materials I (Ex. M.1). Right: Two discs experimental material II (Ex. M.2). Middle: One disc control (C). B. Discs of the tested materials surrounded by the inhibition zone.

Results

PH Measurements

The mean of pH measurements of the two experimental materials and control (Metapex) are listed in table (1).

Table (1): The pH of different materials at different periods.

Time	Exp.Mat.1	Exp.Mat.1	Metapex
0 hour	9.71	10.88	9.05
½ hour	10. 70	11.53	10.33
1 hour	11.28	12.06	10.82
12 hours	11.30	12.09	11.03
24 hours	11.43	12.10	11.23
3 days	11.67	12.07	11.70
1 week	11.97	12.06	12.13
2 weeks	11.91	12.04	12.18

All the tested materials showed gradual increase in pH with time until reached highest pH at period of 1 week. The experimental material 1 showed immediately highest pH value at zero hour periods. Experimental material 2 showed lowest pH values at period of 2 week.

Antibacterial

The mean and standard deviations of the diameter of the inhibition zone of the bacterial growth of the experimental material I and II and control are revealed in (Table 2). Results showed that the experimental material I and II have higher mean values compared to the control. The diameter of the inhibition zone was measured in mm which is represented in Figure 4. The control in the middle showed the least size of inhibition zone against faecalis strain compared to experimental materials. The statistical analysis showed that there was statistically no significant difference between the diameter of inhibition zones induced by the two experimental materials but both provided significantly higher zone compared to control at $P < 0.001$ (Table 3 and Table 4).

Table 2: Shows the diameter of inhibition zone of tested materials on bacterial growth.

Material	Mean	SD
Experimental Mat.1	21.47	0.75
Experimental Mat.2	21.68	0.74
Control	16.11	0.48

Table 3: One Way ANOVA test of the inhibition zones produced by tested materials on bacterial growth.

	Sum of Squares	Df	Mean Square	F	*P*-value
Between Groups	239.37	2	119.68	241.73	.00
Within Groups	23.27	47	0.49		
Total	262.64	49			

Table 4: Duncan,s Multiple Range tests show the effect of tested material against the bacterial.

Group	No.	Duncan's Grouping	
		A*	B*
Experimental material I	20	21.47	
Experimental material II	20	21.68	
Control	10		16.11

*Different letters mean significant difference.

Discussion

In this study the antibacterial activity of the experimental intracanal medicaments was evaluated because the anti-bacterial action is a principle requirement for efficient intracanal medicaments.

Direct contact inhibition zone is usually used to evaluate the antimicrobial action of dental materials. This method allows evaluation of the antimicrobial properties of different substances (cements, intracanal medications, and irrigating solutions) against a large number of microbial strains, at various concentrations (Hasan et al, 2009). Enterococcus faecalis was selected as the microorganism in this study because many previous studies showed that it is the main pathogen that are responsible for post endodontic treatment failure due its presence in endodontically treated teeth with apical periodontitis (Portenier et al, 2003). Studies also showed that *E .Faecalis* can persist in root canal even in very difficult environment due to its ability to resist several antibiotics, irrigations, intracanal medicaments and elevated alkaline atmosphere. In addition, it can penetrate deeply into dentinal tubules (Molander and Dahlén, 2003). Although the first introduction of CaO as a root canal filling material was in 1952 as a root canal filling material, but inadequate investigations has been performed regarding its properties. In this study we prepared two recent CaO based intra canal medicaments by utilizing nano powder of CaO with coconuts oil all extracted from natural materials. The prepared experimental materials showed a larger inhibition zone of

bacterial growth compared to control (Metapex) which might be attributed to a number of factors. The common method for the production of nanoparticles is frequently expensive and usually utilizes chemicals that are potentially harmful to the human body and environment. In this study the nanoparticles (NPs) powder was produced from low cost natural products in an easy, fast and without any harmful effects. The prepared powder particles size ranged between 1-100nm when examined under SEM which is considered nanomaterial according to Kishen, (2015). NPs nanoparticles with active molecules and nano-sized particles are capable to eliminate resistant bacteria, since they have the benefit of a huge surface area to mass ratio due to too small sizes and also very good reactivity (Lins et al, 2013). The bacterial cell wall has a negative charge while the nanoparticles of the prepared CaO powder have positive charge which will lead to electrostatic interaction between the different charges and lead to accumulation of the NPs in the bacterial cell membrane and disruption of permeability of this membrane and further bacterial elimination (Salata, 2004). These nanoparticles can have penetrated deep into the dentinal tubules to eliminate bacterial strains that persist inside dentinal tubules and cannot be reached by normally sized particles intracanal medicaments. Furthermore, the powder of the prepared experimental materials has many mineral oxides which have antimicrobial activity against different types of bacteria. Previous study on the antibacterial activities of metallic oxide powders revealed that the antibacterial activity of CaO was higher than MgO and ZnO (Sawai, 2003). Both egg shells and seashells contain large amounts of CaO that are capable of eliminating microbes inside root canals with aids of other mineral oxides present. The coconut oil that is mixed with prepared experimental powders contain C12 (lauric acid) in high concentration, which is considered the most bacterial inhibitory saturated fatty that is present, it may enhance the effect of the NPs and the mineral oxides of the powder to eliminate microorganisms. Therefore, in the present study the bacterial inhibition zones of the experimental material were significantly greater than that of the control (Metapex) due to the synergistic antibacterial effect from CaO and coconut oil. These differences in the results could be attributed to the size of the molecules of the prepared powder (Nano particles), the presence of high concentration of calcium and other minerals, the ability of the material to diffuse the aqueous agar medium and the presence of coconut oil which is a natural antibacterial agent. All these combined bacterial inhibitory factors make the experimental materials a good substitute for an intracanal medicaments.

Conclusion

CaO powder prepared from locally available, low cost materials (egg shell and seashell) are very effective antibacterial agent specially when mixed with pure coconut oil. The powder of the experimental intracanal medicament can be prepared in a nano particles size in a fast, easy and low-cost procedure. Although the two experimental materials were prepared from two raw materials with difference in minerals, they displayed similar bacterial inhibition activity. Further studies are required such as (biocompatibility, remnants amount after application and effect on smear layer and apical seal) to evaluate the experimental materials before its being used as an intracanal medicaments.

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