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Rana Amr Mahmoud Elkhashab
ranakasho@gmail.com

Medhat Abdelrahman Kataiab
medhat.abdelrahman@fue.edu.eg

Wael Hussien Kamel
waelhussien@yahoo.com

Marwa Shaaban
marshabban@yahoo.com

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Comparison of antibacterial effect and smear layer removal of herbal versus traditional irrigants – An in vitro study

Rana Amr Mahmoud Elkhashab[a,*], Medhat Abdelrahman Kataia[b], Wael Hussien Kamel[c], Marwa Shaaban[d]

[a] Endodontic Department, Faculty of Oral and Dental Medicine, Future University in Egypt, Cairo, Egypt
[b] Head of Endodontic Department, Faculty of Oral and Dental Medicine, Future University in Egypt, Cairo, Egypt
[c] Head of Endodontic Department, Faculty of Dentistry, Al-Azhar University, Cairo, Egypt
[d] Department of Medical Microbiology and Immunology, Faculty of Medicine, Ain Shams University, Egypt

1. Introduction

Endodontic infections are polymicrobial in nature dominated by obligate anaerobic bacteria. The microbial invasion prompts the host to respond with a combination of nonspecific inflammatory processes and specific immunologic response [1].

Eradication of root canal infection using chemo-mechanical preparation is of prime importance to remove vital or necrotic tissues from root canal system. Among the phases of endodontic treatment, the choice of instrumentation and irrigating solution that permit bacterial neutralization and toxin inactivation without negative interference with the healing process is fundamental to the success of treatment [2]. Numerous solutions have been used in endodontics to achieve the desired chemical effect since sodium hypochlorite was introduced by Walker in 1936 [3].

An amorphous, irregular layer known as smear layer has been shown to form on root canal walls following mechanical preparation. Smear layer is shown to consist of dentin and necrotic tissue, including remnants of odontoblastic processes, pulp tissue microorganisms and their by-products [4].

Sodium hypochlorite has remained a popular root canal irrigant because of its antimicrobial potential and its ability to dissolve organic material. However, sodium hypochlorite is not only irritant to the periapical tissues, but also inherently possesses certain disadvantages like staining of instruments, burning of surrounding tissues etc. [5].

Chitosan is a natural, cationic amino-polysaccharide copolymer of glucosamine and *N*-acetyl-glucosamine obtained by the alkaline, partial de-acetylation of chitin which is obtained from shells of crustaceans and shrimps. This polysaccharide has properties of biocompatibility, biodegradability, bio-adhesion and antimicrobial activity [6].

Miswak is a chewing stick commonly used as a brush to clean the teeth. It is made from the aromatic root of a small bush known as the Arrak (Salvadora Persica). Many studies have demonstrated that extracts of Salvadora Persica possess various antiplaque, antiperiopathic, anticaries, anti-inflammatory and antimycotic effects. Research studies show that miswak contains certain natural chemical compounds which have high efficacy against oral pathogens [7].

Therefore, it's of prime importance to shed a light on the efficacy of natural irrigants versus sodium hypochlorite.

2. Materials and methods

2.1. Selection and preparation of the samples

Seventy-five freshly extracted human single-rooted teeth (upper anteriors and lower premolars) with fully formed root apices were selected to be used in this study. The selected teeth were decapitated at CEJ using Ear low-speed precision diamond saw1 under copious irrigation to standardize their length at 17 mm. Patency of each canal was verified by passing sterile a #15 K file into the root canal by watch winding motion until the tip was visible at the apical foramen. The working length was determined visually by subtracting 1 mm from this measurement. The Apical portion of roots was enlarged with K-files (up to size 25) While preparing the teeth, their root canals were rinsed with normal saline.

2.2. Sterilization of samples

All samples were inserted into sterilization packs and sterilized by autoclave for 20 min at 121˚c.

2.3. Bacterial infusion

The sterilization bags containing the samples were opened and the

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* Corresponding author.
E-mail addresses: ranakasho@gmail.com (R.A.M. Elkhashab), medhat.abdelrahman@fue.edu.eg (M.A. Kataia), waelhussien@yahoo.com (W.H. Kamel), marshabban@yahoo.com (M. Shaaban).

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samples were handled with sterile gloves. Each sample was infused with 50 µL of E. faecalis suspension using a sterile pipette. Infused samples were placed inside Eppendorf, closed then inserted inside a rack and placed in the incubator at 37°c to prevent accumulation of bacterial by-products; fresh bacterial suspension was prepared and replaced every 72 h. This procedure was done for a period of 3 weeks [8].

2.4. Sample classification

All teeth were classified into five groups; four experimental groups and one Control group according to the type of irrigant solution.

Group I: Root canals were irrigated using 12.5% alcoholic extract of miswak. (n = 20).
Group II: Root canals were irrigated using 0.2% chitosan. (n = 20).
Group III: Root canals were irrigated using 5.25% Sodium hypochlorite (n = 20).
Group IV: Root canals were irrigated using saline. Positive control (n = 15).

2.5. Experimental procedures

2.5.1. Preparation of salvadora persica

Alcoholic extract of miswak was prepared by taking 800 g of Salvadora persica chewing sticks and cutting them with a sharp knife then ground into powder with a blender then 120 ml of 60% ethanol was added to 40 g of powder in a sterile well capped flask, left for 5 days at room temperature and then filtered using filtration system.

2.5.2. Preparation of chitosan

0.2%chitosan solution was prepared using 0.2 g of chitosan powder (90% degree of de-acetylation), It diluted in 100 ml of 1% acetic acid, and the mixture was stirred for 2 h using a magnetic stirrer [9]. The solution was used within one week after preparation and saved in the refrigerator.

2.5.3. Root canal preparation

Root canal treatment was performed using rotary files (Protaper Universal rotary system) in crown-down manner: the speed of the electric motor was adjusted to be 250–300 rpm and the torque of each file was adjusted according to the manufacturer instructions.

The canals were thoroughly irrigated using 3 ml of irrigating solutions according to its group between every two successive instruments using plastic disposable syringe with the aid of a 25-gauge side perforated needle.

2.6. Methods of evaluation

2.6.1. Counting bacteria

Root canals were sampled using 2 sterile paper points size 30. Those paper points were transferred to a tube containing 1 ml of distilled water. The tube was vortexed for 30 s serial 10-fold dilutions (1:10, 1:100 and 1:1000) were made in distilled water.

0.1 ml of each dilution was smeared onto the (Brain heart infusion agar) the media plates with cultivated bacteria were incubated aerobically at 37°c and 100% humidity for 48 h. At the end of the incubation period, the number of colonies was counted, all of the plates containing less than 300 colonies were selected, because the greater than 300 colonies on plate leads to a high degree of error. Then the number of bacterial colonies forming units (CFUs) per milliliter were calculated by multiplying the number of colonies by the dilution factor multiplied by the amount of specimen added to bile esculin plate. **Number of colonies (CFUs) = bacteria X ml dilution X amount plated.**

2.6.2. SEM evaluation

Two opposed longitudinal grooves were made in the teeth using metallic disk at low speed under cooling without penetrating the canal, the roots then split in two halves with a chisel. For each root, the half containing the most visible part of the apex and best represented the total canal length were selected. The specimens were secured on metal stubs and examined under SEM at X3000 magnification. The Smear layer was assessed at the coronal, middle, and apical regions of each root half of each specimen. Smear layer was evaluated using a three-point scoring system reported by Torabinejad et al., in 2003 [10].

3. Results

3.1. Antibacterial results

A statistically significant difference was found between Miswak, Chitosan, Sodium hypochlorite and Positive control where ($p < 0.001$). A statistically significant difference was found between Positive control and each of Miswak, Chitosan and Sodium hypochlorite where ($p < 0.001$). No statistically significant difference was found between Miswak and Chitosan where ($p = 0.845$), while a statistically significant difference was found between Miswak and Sodium hypochlorite where ($p = 0.019$). A statistically significant difference was found between Chitosan and Sodium hypochlorite where ($p = 0.019$). The highest mean value was found in Positive control followed by Chitosan and Miswak, the lowest mean value was found in Sodium hypochlorite (Fig. 1).

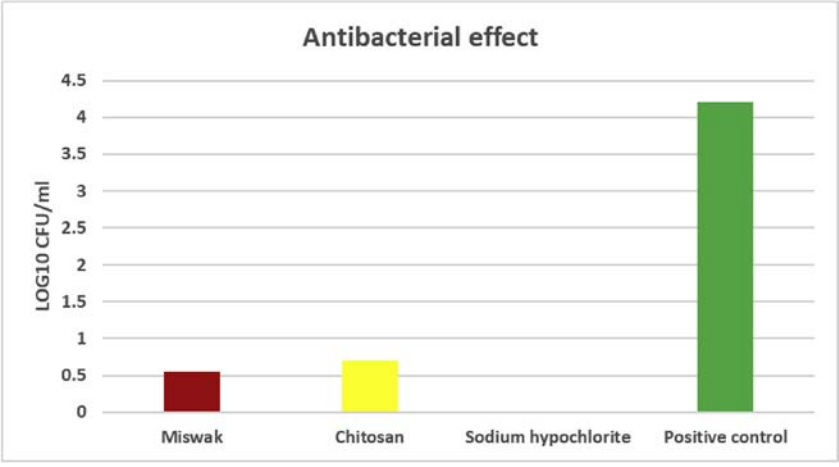

Fig. 1. Bar charts representing antibacterial effect.

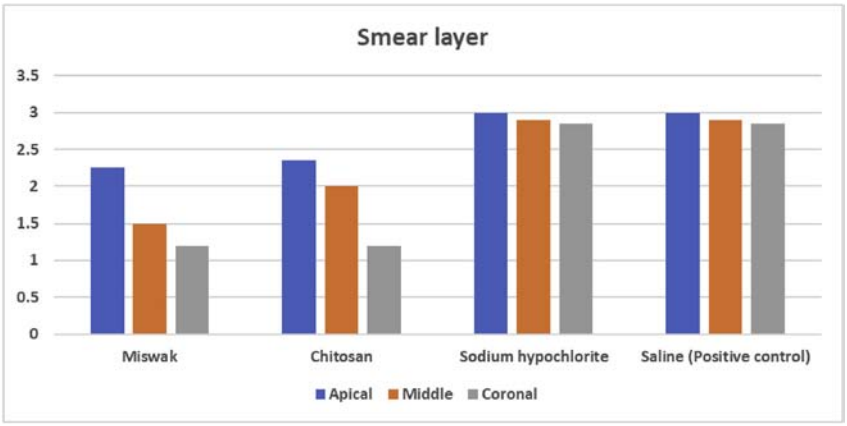

Fig. 2. Bar Chart representing smear layer in different groups.

3.2. Smear layer results

A statistically significant difference was found between (Miswak), (Chitosan) and (Sodium hypochlorite & saline) where ($p < 0.001$). A statistically significant difference was found between (Sodium hypochlorite & saline) and each of (Miswak) and (Chitosan) where ($p < 0.001$). No statistically significant difference was found between (Miswak) and (Chitosan) where ($p = 0.093$). The highest mean value was found in (Sodium hypochlorite & saline) followed by (Chitosan), while the lowest mean value was found in (Miswak) (Figs. 2–6).

4. Discussion

Micro-organisms in the root canals are the prime causative factors in the development of pulp and periapical lesions [11]. A major objective in root canal treatment is to disinfect the entire root canal system, which requires that all contents of the root canal system be eliminated as possible sources of infection [12]. Various materials have been used during and immediately after mechanical instrumentation to help eliminate microorganisms that cannot be eliminated by mechanical instrumentation alone due to the complex anatomy of the root canal system [13].

Root canal instrumentation procedures produce a layer of organic and inorganic material called the smear layer. This layer is composed of two zones: the first zone is 1–2 μm thick and consists of organic matter and dentin particles, and the second zone extends into dentinal tubules to a depth of 40 μ (smear plugs) and is formed largely of dentine chips [14].

Removal of smear layer is essential to improve the fluid-tight seal of the root canal system whereas other factors such as the obturation technique or the sealer, did not produce significant effects [15].

Sodium hypochlorite one of the most successful and widely used endodontic irrigant were chosen to assess the antibacterial agent [16].

NaOCl solution is, to date, the most commonly employed root canal irrigant, as it has strong tissue solvent action especially in high concentrations [17] has a broad spectrum of antimicrobial activity and provides good lubrication for instruments. Despite of all these advantages, NaOCl still has several drawbacks as its cytotoxic effect if injected into the periapical tissues, it's bad smell and taste, its toxic effect to stem cells, and its corrosive activity as well as its potential for producing allergic reactions.

Salvadora Persica (Miswak-Siwak), Its chewing sticks contain trimethyl amine, salvadorime chloride and fluoride in large amounts, showed significant antimicrobial effect against aerobic and anaerobic bacteria which make it possible to be used as irrigant solution in endodontic treatment against the endodontic pathogens, it can be used as a substitute for sodium hypochlorite and chlorhexidine as root canal irrigant [18] due to its biocompatibility with soft tissues [19]. Moreover, alcoholic S. persica solution can help in smear layer removal.

Chitosan is a natural polysaccharide which is characterized by biocompatibility, biodegradability, bio-adhesion and a-toxicity to human cells [20] and it has high chelating capacity for different metallic ions and its low cost, made it preferred as an alternative to Sodium hypochlorite [21].

The chelating behavior of chitosan demonstrated in the present study indicates that this solution acted on the inorganic portion of the smear layer, favoring its removal.

The biological marker used in this study was Enterococcus faecalis, which is a facultative anaerobic gram-positive coccus, has been implicated in the persistent root canal infections [22]. As the pathogenicity of E. faecalis in endodontic infections is well documented [23]. E. faecalis has demonstrated the capacity to survive in an environment in which there are scant available nutrients and in which commensality with other bacteria is minimal. E. faecalis has been found suitable for experimental penetration into dentinal tubules as it has been reported that bacteria can enter the dentinal tubules as deep as 500–1000 μm. This is considered to be one of the major causes for failure in endodontic treatments [24].

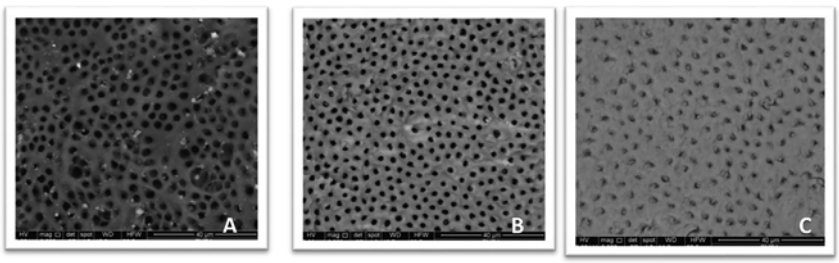

Fig. 3. The effect of irrigation using 12.5% Salvadora persica solution. The smear layer is removed and the tubule openings are visible on the coronal and middle thirds (A&B) (3000x). the smear layer is seen to occlude the openings of the dentinal tubules in the apical third (C) (3000x).

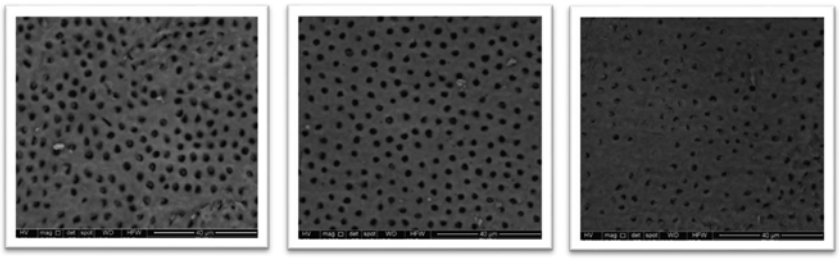

Fig. 4. The effect of irrigation using 0.2% chitosan solution. The smear layer is removed and the tubule openings are visible on the coronal and middle thirds (A&B) (3000x). A moderate amount of smear layer is observed in the apical third (C) (3000x).

E. faecalis is known to survive periods of starvation in water for over 4 months and in nutrient-limited media for extended periods [25]. A capacity to endure starvation is a distinctive characteristic that might allow E. faecalis to survive until an opportunity for acquiring suitable nutrition becomes available.

The biofilm model was chosen, because of the well documented resistance of biofilms to disinfection as opposed to planktonic bacteria [23].

Furthermore, the Bile-esculin is a selective medium for the detection of enterococci, it hydrolyzes esculin to 6, 7-dihydroxycoumarin, which reacts with iron to produce a characteristic blackening of the medium, thus E. Faecalis was grown up in a Bile-esculin plate [26], and all procedures were done in a laminar air flow cabinet as it is a carefully enclosed bench designed to prevent contamination of biological samples [27].

All root canals were mechanically prepared in a crown-down approach using the rotary ProTaper Universal instruments. The crown-down technique is highly advantageous, it reduces stresses during instrumentation and improves root canal debridement since coronal enlargement provides a reservoir for the irrigating solution allowing for better irrigant penetration in the apical third [28]. Dalton et al. Reported a significant bacterial reduction with Ni-Ti instrumentation and substantial decrease in bacterial count with progressively larger instrument sizes [29].

The in vitro model used in this study aimed to simulate the in vivo conditions as close as possible, that's why bacteria were grown inside root canals and convention cleaning and shaping was performed.

Bacteriologic sampling is accomplished in this study by inserting two sterile paper points size #30, both passively by imbibition and actively by circumferential filing to prevent false culture calculation through sampling planktonic bacteria in contact with the paper points, leaving the sheltered biofilm micro-biota untouched [30].

The use of paper points has the advantage that it is simple method and can be performed in vitro as well as in vivo but has a limitation in which only microorganism that are in root canal can be sampled, while that located inside dentinal tubules cannot loaded [31].

In the present study counting bacteria was performed by calculating the number of bacterial colonies forming units (CFU) as it is simple and easy method [32].

The results of our study showed that Sodium hypochlorite group had significantly higher antibacterial action than Miswak and Chitosan groups and this could be due to high concentration of un-disocciated hypochlorous acid (HCIO) in sodium hypochlorite solution that exerts its antimicrobial effect by an oxidative action on sulfhydryl groups of bacterial enzymes and this come in agreement with several studies [33].

Furthermore, the results clearly demonstrated that the antibacterial effect of Miswak and Chitosan groups was significantly higher than the Saline group (positive control) and these results are in accordance with previous study which found that saline is completely ineffective as an antimicrobial agent [34]. Regarding the inhibition of Enterococcus faecalis it was found that 12.5% S. persica is effective but less than the inhibition effect of 5.25% NaOCl. Antimicrobial effect of alcoholic extract of Salvadora Persica is believed to be due to its high chemical contents of chlorides, tannins, trimethylamine, salvadorine, nitrate, thiocynate and sulpher [35]. Chitosan's antibacterial nature is due to the interaction between positively charged chitosan and a negatively charged bacterial cell which transmutes the bacterial cell permeability, leading to the leakage of intercellular components and cell death [36].

Regarding results of smear layer removal, it was found that Chitosan and Miswak groups significantly higher than Sodium hypochlorite and saline groups. These results are in accordance with the work of Kamble AB et al. [37]. Under the Scanning electron microscopic view, 0.2% chitosan was found to be effective in removing the smear layer in all the three regions (coronal-middle-apical) of the root surface which was agreed with the work of Darrag AM [9] and this may be attributed to adsorption, ionic exchange and chelation are probably the mechanisms responsible for the formation of complexes between chitosan and metal ions. The type of interaction that occurs depends on the ions involved, the chemical structure of chitosan and the pH of the solution [38].

The ability of S. persica to remove the smear layer may be attributed to its acids content, which may react with calcium in the dentin and act as chelating agent. The scanning electron microscopic pictures of NaOCl in the present study showed the absence of superficial debris with the presence of smear layer at all root thirds, signifying the inability of 5.25% NaOCl in complete removal the smear layer. These results were similar with the work of Baumgartner JC et al. [39].

Throughout the study, all the groups showed less or no removal of smear layer at the apical third of the root canal. This is because, the flow ability and backflow of the fluid are poor at the apical third due to

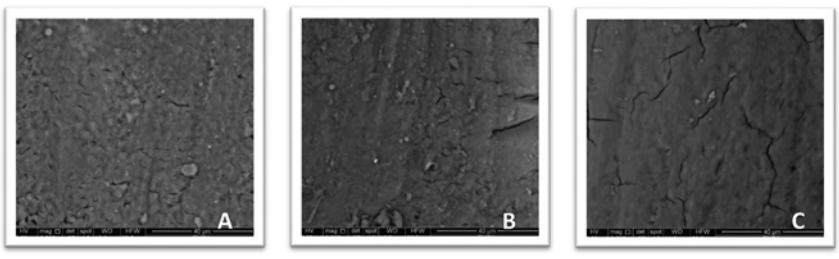

Fig. 5. The effect of irrigation using 5.25% NaOCl showing completely covered surface with thick smear layer without any open dentinal tubules on coronal, middle and apical thirds. (A, B&C).

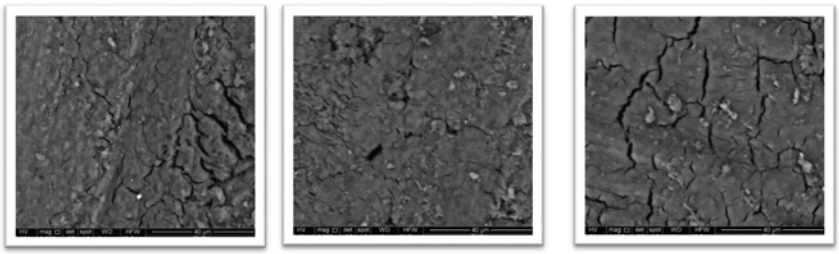

Fig. 6. The effect of irrigation using Saline showing completely covered surface with thick smear layer without any open dentinal tubules on coronal, middle and apical.

the reduced diameter and the increase in depth of the root canal and this come in accordance with Wu L et al. [40].

5. Conclusion

From the results of the current study, it could be concluded that: The use of herbal alternatives as root canal irrigating solutions might prove to be advantageous considering several unfavorable properties of NaOCl. It seems that chitosan and miswak are promising materials to remove smear layer from root canal.

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