Original Research Article

IN VITRO ANTIMICROBIAL ACTIVITY OF SODIUM HYPOCHLORITE, NANO SILVER AND CHLORHEXIDINE AGAINST SELECTED SINGLE-SPECIES BIOFILMS

ABSTRACT

Background: Amongst the actions involved in the control of endodontic infection, instrumentation and irrigation are important factors in eliminating microorganisms from the root canal system. However, mechanical debridement alone does not result in total or permanent reduction of bacteria. It is recommended to use antimicrobial agents as an adjunct to mechanical devices to reduce the number of microorganisms. The most common irrigation solution is sodium hypochlorite (NaOCl). It is an effective antimicrobial agent and an excellent organic solvent for vital, necrotic and fixed tissues.

Aim: To investigate the antimicrobial activity of 2.5% and 5.25% sodium hypochlorite, 2.0% chlorhexidine liquid and 60mg/L Nano-silver liquid as endodontic-irrigating substances against selected single-species biofilms.

Methods: Single-species biofilms of *Enterococcus faecalis*, *Staphylococcus aureus*, *pseudomonas aeruginosa* and *Candida albicans* oral sources were produced on a cellulose nitrate membrane placed on agar medium. The biofilms were then immersed in endodontic irrigators for 30 s and also for 5, 10, 15, 30 and 60 min. Sterile saline was used as a control. After each time period, membrane filters were transferred to tubes containing 2 mL of fresh broth medium plus neutralizers (in order to prevent residual effect of the tested materials). Microorganisms were suspended using a vortex, and the inoculum was serially diluted 10-fold. Aliquots of the dilutions were plated on 5% blood agar medium and incubated under appropriate gaseous conditions. Colony forming units were calculated.

Results: The antimicrobial agents in liquid presentation, especially 5.25% NaOCl, 2% chlorhexidine, and 2% nano-silver liquid killed the tested microorganisms more rapidly with mean rank equal to 1.4, 1.9, and 1.6, respectively. Saline did not inhibit the growth of any of the tested microorganisms, as it was statistically ($p < 0.05$) different for NaOCl, Nano-silver and chlorhexidine. *Enterococcus faecalis, Pseudomonas aeruginosa and Staphylococcus aureus* were eradicated within 30 seconds by all antimicrobial agents, unlike *Candida albicans* were eliminated in 30 seconds with 5.25% NaOCl and 60 mg/L silver nano-fluid but with 300 seconds with 2.5% NaOCl, and 2% chlorhexidine.

Conclusions: Preferring agents in liquid supply to get rid of biofilm microorganisms, especially 5.25% NaOCl and 60mg/L nanosilver liquid.

Keywords: *Candida albicans*, chlorhexidine, endodontic-irrigating, *Enterococcus faecalis*, nanosilver, *Pseudomonas aeruginosa*, sodium hypochlorite, *Staphylococcus aureus*

INTRODUCTION

Root canal treatment (endodontics) is a sequence of treatment of an infected tooth's pulp that aims to eliminate infection and protect an antiseptic tooth from future microbial invasion.[1] Root canals, and the associated pulp chamber, are the somatic cavities within a tooth that are normally inhabited by nerve tissue, blood vessels, and other cellular entities. Together, these elements make up the dental pulp [2]. Endodontic treatment includes removal of these structures, disinfection, consequent shaping, cleaning, decontamination of cavities using small files and irrigation solutions, and blockage (filling) of the antiseptic canals. Clean, disinfected ducts are filled with an inert filler such as gutta-percha and usually eugenol zinc oxide-based cement. Epoxy resin is used to bind gutta-percha in some root canal procedures. Another option is to use an antiseptic filler that contains paraformaldehyde such as N2.[3] Endodontic treatment includes both primary and secondary endodontic treatments as well as periradicular surgery that is generally used for teeth that still have salvage potential[4].

Bacteria play an essential aetiological role in the development of necrotic pulp, periapical pathology and post-treatment diseases after root canal therapy [5]. A critical factor for the success of treatment is the elimination of microorganisms and their byproducts from the root canal system [6]. Among the actions involved in the control of endodontic infection, instrumentation and irrigation are important factors in eliminating microorganisms from the root canal system. [7]. Though, mechanical debridement lonely does not result in total or permanent diminution of bacteria. It has been recommended that antimicrobial agents be used as an adjunct to mechanical devices to reduce the number of microorganisms[8]. The most common irrigation solution is sodium hypochlorite (NaOCl). It is an effective antimicrobial agent [9, 10] and an excellent organic solvent for

vital, necrotic and fixed tissues. Alternatively, it is very irritating to periapical tissues, especially at high concentrations. Chlorhexidine gluconate is recommended as root canal irrigation therapy [8]. It is a strong antimicrobial agent [10], bears intrinsic[11], and a low degree of toxicity. However, chlorhexidine is not able to dissolve pulp tissue and debris may remain on the canal walls, obstructing the dentinal tubules. Even after careful mechanical actions associated with antimicrobial agents, bacteria can still be recovered from the ducts.

The most persistent genera include *Enterococcus* [12], *Staphylococcus*[13], Gram-negative enteric bacilli, and *Pseudomonas* [13]. Much research has been done to test the efficacy of antimicrobial irrigation agents in vitro, using various methodologies, such as: (1) direct contact method: microorganisms and materials tested in close contact [10]; (2) Agar diffusion method: the tested material diffuses through the medium, resulting in areas of microbial growth inhibition around the tested material[14] and (3) prosthetic infection of the extracted teeth with selected bacteria and on-site irrigation with antimicrobial agents[11]. In spite of this, diverse results have been reported even when using the similar microorganisms and the similar antimicrobial agents. These differences are attributed to the difference in contact between microorganisms and irrigation[15]. The circumstances used in laboratory tests do not reflect circumstances in vivo, where bacteria grow on the surface of the tooth producing a biofilm. Biofilms can be defined as communities of microorganisms bound to a surface, embedded in an extra cellular matrix of polysaccharides. Within these microcolonies, bacteria evolved into organized communities with functional heterogeneity[16]. It forms a protected pattern of growth that allows survival in a hostile environment. Bacteria in such an environment differ greatly in phenotype when compared to their planktonic counterparts, and are less likely to be killed by antimicrobials [17]. Nevertheless, the clinical significance of bacterial biofilm formation in root canal therapy has not been extensively evaluated.

Although there are various studies on oral and dental problems in Yemen[18-29]. However, there is not even a single study to test the efficacy of commonly used endodontic-irrigating substances in vitro including the new nano-oral mouthwash liquid in Yemen or the Arab region. Therefore, the purpose of this study was to use a simple one-type biofilm model for 4 bacterial species to evaluate the efficacy of commonly used endodontic-irrigating substances against selected single-species.

MATERIALS AND METHODS

The tested materials were NaOCl (2.5% and 5.25%), chlorhexidine gluconate liquid at 2% concentration and 60 mg/L colloidal silver nanoparticles liquid.

Sodium hypochlorite: a chemical compound with the formula NaOCl or NaClO, comprising a sodium cation (Na +) and a hypochlorite anion (OCl or ClO)[30]. Anhydrous sodium hypochlorite can be prepared, but, like many hypochlorites, it is very unstable and decomposes explosively upon heating or friction. Decomposition is accelerated by carbon dioxide at atmospheric levels. It is a white solid with a rhombic crystal structure [30].

Chlorhexidine (CHX): Commonly known by the salt forms chlorhexidine gluconate and chlorhexidine digluconate (CHG) or chlorhexidine acetate) (Shopee, Malaysia).

Silver Nano liquid: Colloidal silver nano-liquid was prepared by India MART, (Nanosil Silver Nano liquid, Rs 490/L Sanosil Biotech private Limited, ID; 2239159331).

It has diluted NaOCl, silver nanoparticles, and chlorhexidine liquid in sterile water without preservatives. The solutions were prepared 24 hours before the start of the experiment, always in small portions. Sterile saline (0.89%) was used as a control. The types of microorganisms used in this experiment were: (1) *Candida albicans*. (2) *Staphylococcus aureus*. (3) *Enterococcus faecalis*; (4) *Pseudomonas aeruginosa*. Tubes containing 5 ml of sterile BHI suspension were individually inoculated with aerobic strains (*C. albicans*, *pseudomonas aeruginosa*, and *S. aureus*) and a facultative strain (*E. faecalis*). The suspension was then modified by a spectrophotometric method according to Koo *et al.* [31] who used an optical density at 800 nm (OD800) to match the turbidity of $1.5 \bullet 10^8$ CFU mL) (colony forming unit, CFU), which is equivalent to the standard 0.5 McFarland.

Single-species biofilms of *E. faecalis*, *S. aureus*, *C. albicans* and *Pseudomonas aeruginosa* were produced on a cellulose nitrate membrane (pore size 0.2 lm, diameter 13 mm - Whatman International Ltd, Maidstone, UK). Membranes were placed on the surface of 5% defibrillated BHI blood agar plates (for aerobic and optional anaerobic microorganisms) and further inoculated with 20 µL of each microorganism test suspension. The plates, each containing four membrane filters, were incubated at 37 °C again under the appropriate gaseous conditions: aerobic and facultative anaerobic in a CO_2 incubator. Membrane filters were aseptically removed from the agar plate and carefully transferred to tubes containing 5 mL of antimicrobial test agent and saline for the control group, which were incubated for 30 s, as well as for 5, 10, 15, 30 and 60 min. After each time period, membrane filters were transferred to tubes containing 2 ml of fresh broth medium plus neutralizers (Tween 80 plus 0.07% lecithin was used for chlorhexidine and nanosilver and 0.06% sodium thiosulfate for NaOCl) in order to prevent residual work of the materials [32]. Then it was rotated for 30 sec to resuspend the

microorganisms. Serial tenfold dilutions of the bacterial suspension were made and plated on blood agar plates . The plates were then incubated at 37 °C under appropriate gaseous conditions for 24 h (aerobes), and 48 h (facultative anaerobic). The number of CFU per membrane was calculated. The tests were performed in triplicate for each antimicrobial agent and microorganism, and the survival curve was calculated. Samples were analyzed statistically, indicating that the data were non-parametric. Because of the high SD of the CFU, a rank shift was indicated. This is a statistical tool that produces a table containing the ordinal arrangement of each value in a data set, in other words, transforms the classification of the dependent variable. In the present analysis, high rank averages indicate significant CFU means. Then, samples were compared using the Friedman and Tukey test, when necessary, at a significance level of $P < 0.05$. All data were converted to seconds for comparisons.

RESULTS

Table 1 shows the contact time in seconds and mean order required for NaOCl, chlorhexidine, and colloidal silver nanoliquid to produce negative cultures against all tested microorganisms. *C. albicans* and *S. aureus* took 300 seconds (5 minutes) to be killed by 2% CLX, while *S.aureus*, *Pseudomonas aeruginosa* and *E. faecalis* were eradicated in 30 seconds, and the average rank of this factor was 1.9. *E. faecalis* took over 3600 seconds (over 60 minutes) to be killed by 2.5% NaOcl and *Pseudomonas aeruginosa* took 900 seconds (15 minutes) to be killed by 2.5% NaOCl, and *C. albicans t*ook 300 seconds (5 minutes) to be killed by 2.5% NaCl while *Staphylococcus aureus* was eradicated in 30 seconds. Mean rank of 2.5% NaOCl was 4. *S.aureus*, *Pseudomonas aeruginosa*, *E. faecalis* and *C. albicans* took 30 s to be killed by 60 mg/L **:** Nano colloidal Silver liquid, with the mean rank for this agent equal to 1.6. *S.aureus*, *Pseudomonas aeruginosa*, *E. faecalis* and *C. albicans* took 30 s to be killed by 5.25% NaOCl liquid, with the mean rank for this agent equal to 1.4. There was no statistically significant difference between 5.25% NaOCl, 2.0% liquid chlorhexidine and 60mg/L Nano-siliver liquid needed more time to eliminate all bacteria and yeast (mean rank 1.63). However, it was statistically different ($P < 0.05$) from the 2.5% NaOCl and the control group (saline).

DISCUSSION

The primary objective of chemical irrigation is to kill microbes and dissolve pulp tissue. Some irrigants, such as sodium hypochlorite and chlorhexidine, have proven antimicrobial efficacy in vitro and are widely used during root canal therapy universal. In harmony with a systematic review, good evidence to support the use of one irrigator over another in terms of short- and long-term treatment prognosis is lacking [1-4]. Microorganisms and their products are the main cause of endodontic and periapical diseases, and can adhere to root canal walls forming biofilms. Since it is difficult to ensure adequate removal of the biofilm on the dentin wall using only mechanical procedures, it is necessary to use materials with antimicrobial properties [34]. In the laboratory, planktonic microorganisms are generally cultured, but the utopian microcosms created in culture vessels are designed to maximize microbial growth rates and not to replicate the natural growth conditions of microorganisms, which typically use biofilm placement for growth in an outside laboratory [17]. Most in vitro tests use plankton cultures to test the antimicrobial efficacy of endodontic irrigation materials.

Depending on the concentration of the tested substance and the sensitivity of the microorganism, the latter can be eliminated in seconds using planktonic cells and the direct contact method. Such a fatal effect may not occur clinically [33]. The organization of bacteria within biofilms confers a set of unclear phenotypic properties in and among other planktonic analogues, and also confers lower susceptibility to antimicrobial agents [35]. Therefore, the use of the biofilm model can more accurately reproduce the conditions in vivo. The biofilm model was used in this study to evaluate the antimicrobial efficacy of materials used during chemical-mechanical preparation, against selected microorganisms commonly found in root canals. The methodology performed was based on the method used by Sena *et al.*[33] and Spratt *et al.*[15], which allows biofilms to grow on cellulose nitrate membranes. The antimicrobial agent was tested in direct contact with a single biofilm. However, differently from Spratt *et al.* [15], In this study, the time taken to produce a consistent biofilm was longer, which may explain the increased resistance of biofilms, possibly due to the regulation of biofilms. Also in this study Nano-siliver was tested in addition to other endodontic irrigation materials.

In this investigation, 5.25% NaOCl and 60 mg/L liquid nanosilver killed microbial cells more rapidly than 2.5% NaOCl and 2% liquid chlorhexidine, which is different from Sena *et al.* [33], Where the effect of 2.5% NaOCl was similar in that with 5.25% NaOCl and 2% CLX.

This study showed that different microorganisms are susceptible to different degrees of the antimicrobial agents tested, and the exposure time can be critical to the efficacy of the substance. All microorganisms were grown in contact with saline solution (positive control). 5.25% NaOCl, eliminates all microorganisms in 30 seconds. Similar results were found by Sena *et al.*[33] and Spratt *et al.* [15]. Viana *et al.* [10], using 5.25% NaOCl, *P. intermedia, P. gingivalis, P. endodontalis, C. albicans, S. aureus* and *E. faecalis* were eradicated within 15 seconds using the direct contact method. Sina *et al.* [33], Gomes *et al.* [32] and Senia *et al.* [36], they were also

found that 5.25% NaOCl killed *E. faecalis* cells in 15 seconds using the direct contact method. Radcliffe *et al.* [37] Vianna *et al.* [10], they were found that 2.5% NaOCl inhibited the growth of all tested microorganisms at 5 and 10 minutes, respectively. In this study, 2.5% NaOCl was effective against *S.aureus* biofilm at 30 sec but against *E. faecalis* biofilm was >3600 sec, *Pseudomonas aeruginosa* biofilm was 900 sec and *C. albicans* biofilm was 300 sec. Vianna *et al.* [10] and Gomes *et al.* [32], which used planktonic cells, showed that the time required for a 2% chlorhexidine gluconate liquid to produce negative cultures was 1 minute for aerobic and facultative microorganisms. *Candida albicans* is the most common fungal species isolated from infected root canals [33, 38], and has been associated with post-treatment disease cases [39-42]. In this study, C. albicans, under biofilm formation, required 30 to 300 sec to be eliminated by all tested antimicrobial agents. This time was lower than that reported by Sena *et al.* [33], where *C. albicans*, under mechanical stimulation, required eradication time by all tested antimicrobial agents from 30 to 900 seconds. Radcliffe *et al.* [37], using the direct contact method they were found that after 10 seconds of contact between *C. albicans* and 0.5% NaOCl, no cells could be detected. Vianna *et al* . [10] reported a maximum growth inhibition time of 30 min for NaOCl and 10 min for all chlorhexidines tested (gel and liquid presentation*). Enterococcus* and *pseudomonas aeruginosa* are most resistant to low concentration of NaOCl (2.5%) in the current study, and are also usually the most resistant bacterial species to antiseptic agents, and thus can be expected to persist more frequently in the root canal after inadequate root canal preparation and obscuration [33]. Persistent microorganisms or their byproducts can maintain an infectious process and cause treatment failure. According to Molander *et al.* [43], *E. faecalis* can live in a quiescent phase with low metabolic activity for a period of time, and factors such as coronal leakage can alter nutritional conditions and contribute to bacterial growth. A possible factor for virulence is their ability to survive within polymorphous leukocytes (PMN) and macrophages. Aggregate substance (AS) has been indicated as being responsible for the internalization of *E. faecalis* bacteria within the PMN, making it resistant to killing [44, 45]. The results of this study also found that *P. aeruginosa* and *E. faecalis* were the most resistant microorganisms tested.

This biofilm model appears to be more realistic than the direct contact method for testing antimicrobial agents, as it allows microorganisms to grow as biofilms on the cellulose nitrate membrane, which are more resistant to treatment. Further studies should test the sensitivity of antimicrobials against mixed biofilms. Within the limits of this in vitro study, the biofilm model gave a simple means to determine the antimicrobial efficacy of irrigation used in root canal treatment. This methodology may be more representative clinically than methods that do not take into account the microorganisms in biofilms. However, it still does not produce what clinically occurs in the root canal. In such an environment, several mechanisms allow the growth and selection of many microorganisms, even after treatment.

The best result of the current study was recorded with a high concentration of NaOCl (5.25%), as it is known that diluted NaOCl has been used for decades to treat moderate to severe eczema in humans, but it is not clear why it is effective. According to work published by researchers at Stanford University School of Medicine in November 2013, a very dilute solution of sodium hypochlorite in water was successful in treating skin damage with an inflammatory component caused by radiation therapy, excessive sun exposure, or aging. in laboratory mice. Mice with radiation dermatitis were subjected to bathing for 30 minutes daily in bleach solution with less severe skin damage and better healing and hair re-growth compared to animals dipped in water [46,47]. Therefore, this substance is promising in medicine, the former President of the United States of America said in one of his speech, “People who are afraid of Covid 19 should drink bleaching solution,” despite his sarcastic and irresponsible words, but it should prompt us to conduct more research for this compound and it needs further studies, in terms of its effect not only as an endodontic irrigation material, but also the possibility of using it systemically to treat infections as Covid 19.

Conclusion

The favoring the agents in liquid presentation to eliminate micro-organisms biofilms , especially 5.25% NaOCl and 2% nanosilver liquid.

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CONFLICT OF INTEREST

No conflict of interest associated with this work.

AUTHOR’S CONTRIBUTIONS All authors co-wrote the article and reviewed the results. Laboratoty parts and data analysis were performed by Omar Ahmed Ismael Al-dossary and Hassan Abdelwahab Al Shamahy.

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Results

Table 1: Contact time in seconds and mean rank required for chlorhexidine liquid, sodium hypochlorite liquid and Nanosilver liquid to produce negative cultures against all tested micro-organisms

Micro-organisms	Antimicrobial agents Time of exposure/log of count CF/ml				
	2% CLX liquid	2.5% NaOCl liquid	60mg/L Nanosilver liquid (600 PPM)	5.25% NaOcl liquid	Normal saline
E. faecalis	30 (1.5)	>3600 (4.0)	30 (1.5)	30 (1.0)	>3600 (5.0)
S. aureus	30 (2.0)	30 (2.0)	30 (2.0)	30 (2.0)	>3600 (5.0)
Pseudomonas aeruginosa	30 (1.5)	900 (3.0)	30 (1.5)	30 (1.5)	>3600 (5.0)
Candida albicans	300 (2.5)	300 (2.5)	30 (1.5)	30 (1.0)	>3600 (4.5)
Mean Rank	1.9	4	1.6	1.4	4.9
Rank	A	B	A	A	B