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EFFECT OF NEW DELIVERY METHOD ON ANTIBACTERIAL PROPERTIES OF CHLOROHEXIDINE (IN-VITRO STUDY)

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ABSTRACT

Aim: To assess the effect newly introduced delivery method of chlorohexidine versus existing forms on antibacterial properties of the material by applying on two of the most common root canal bacteria; *Enterococcus faecalis and Streptococcus mutans*.

Methodology: A total of ten *Enterococcus fecalis* and ten *Streptococcus mutans* strains were selectively isolated from freshly extracted human teeth on MacConckey's agar and Mitis-Salivarius agar respectively. For each bacterial strain, the antibacterial effect of three chlorohexidine formulations (foam, gel and solution) was tested via in vitro disk diffusion susceptibility assay. Zones of growth inhibition were measured and compared between the three formulations.

Results: When comparing mean ranges of zones of growth inhibition around the three formulations, it was found that the mean range was highest for chlorohexidine foam. The results were significant for both *Enterococcus fecalis* and *Streptococcus mutans* strains as suggested by the P values (0.024 & 0.0003 respectively).

Conclusion: Under the circumstances of this study, it was concluded the chlorohexidine foam better impact on the chlorohexidine antibacterial properties.

KEYWORDS: Chlorohexidine foam, chlorohexidine solution, chlorohexidine gel, dental canal bacteria, *Streptococcus mutans, Enterococcus faecalis*.

INTRODUCTION

A root canal infection, also known as endodontic infection, occurs when bacteria enter and infect the pulp of a tooth. Root canal infections are usually caused by untreated dental decay, deep cavities, cracked or fractured teeth, or dental trauma. Symptoms of a root canal infection may include severe toothache, sensitivity to temperature, swelling and tenderness, and abscess formation. Several types of bacteria can be involved in root canal infections. The most common bacteria implicated include *Enterococcus faecalis, Streptococcus mutans, Streptococcus anginosus*, and various anaerobic

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bacteria such as *Prevotella spp., Porphyromonas spp., and Fusobacterium spp.*[1]

Chlorhexidine is an antibacterial agent that is commonly used in healthcare settings for infection control. It is effective against a wide range of bacteria, including both Gram-positive and Gram-negative bacteria. Chlorhexidine is a cationic compound that works by disrupting the cell membrane of bacteria, which leads to their death. At low concentrations (0.02%-0.06%) chlorohexidine causes displacement of Ca2+and Mg2+ and loss of K+ from the cell wall, resulting in a bacteriostatic effect. At high concentrations (>0.1%) chlorohexidine causes leakage of all the main intracellular components out of the cell, resulting in a bactericidal (cell lysis and death) effect.[2]

It has been shown that chlorhexidine has strong antibacterial properties against many different types of bacteria, including those commonly found in root canals such as *Enterococcus faecalis, Streptococcus mutants, Porphyromonas gingivalis, and Prevotella intermedia*. Studies have shown that chlorhexidine is effective against these bacteria both in vitro and in vivo. It has been used as an irrigant during root canal treatment to help eliminate bacteria and prevent reinfection of the root canal system.[3.4.5]

Chlorhexidine can be delivered in various ways depending on the purpose of use. It can be delivered as a solution, gel, or spray for topical application on the skin or mucous membranes. These formulations differ in terms of their physical properties and potential advantages in certain situations. It's important to note that the choice between various chlorhexidine formulations may depend on the specific application, healthcare setting, and individual preferences. [6]

A new delivery method of chlorhexidine has been introduced in the market by Egyptian company (Shiny Pharma) in foam formulation where chlorhexidine liquid with some additives, Table 1, is delivered in pressed bottle by some type of automizers to incorporate air inside the solution for effervescent foam form. Some potential benefits of chlorhexidine foam over gel include ease of application both periodontally and intra canal through special side perforated needle, reduced dripping and mess, enhanced penetration, long lasting antibacterial effect leading to reduced risk of infection, lack of irritation, reduced staining which provide extra patient comfort and enhance patient compliance.[7]

In this study, we aim to assess the effect of different formulations and delivery methods of

TABLE (1) Chlorohexidine Foam Ingredients by Shiny Pharma

Ingredient	**Quantity % (W/V)**	**Function**
Distilled water	100	Solvent
Chlorohexidine Digluconate Solution 20%	0.6	Antimicrobial
Peppermint Oil	0.2	Refreshing agent
Sodium Fluoride (1200ppm)	0.0265	Antiplaque
PEG-40 Castor Oil	1.2	Surfactant - emulsifying agent
Xylitol	5.0	Humectant
Citric Acid	0.05	Buffering agent
Sodium Benzoate	0.25	Preservative
Sodium Lauroyl Sarcosinate	4	Surfactant - cleansing
Glycerin	3	Humectant
Sorbitol 70% Solution	15	Humectant
Zinc Chloride 0.1%	2	Oral care

chlorohexidine on two of the most common root canal bacteria; *Enterococcus faecalis and Streptococcus mutans*, and their impact on antibacterial properties of chlorohexidine.

MATERIALS AND METHODS

Bacterial isolates:

A total of ten *Enterococcus faecalis* and ten *Streptococcus mutans* strains isolated from freshly extracted human teeth were included in this study.

Tested Substances

- Chlorohexidine foam.
- Chlorohexidine solution.
- Chlorohexidine gel.

Culturing media

- **MacConckey's agar and Mitis-Salivarius agar:** MacConckey's agar and Mitis-Salivarius agar are used for selective isolation of *Enterococcus fecalis* and *Streptococcus mutans* respectively. Identification of isolated bacteria was performed by various microbiological techniques as described by ***Patricia, 2021***.[8] Isolation of the desired bacterial strains was performed inside the laboratory of Medical Microbiology and Immunology department at Faculty of Medicine, Ain Shams university. All instruments used during the whole procedure were sterile.
- **0.5 McFarland Standard**: This medium is prepared to adjust the proper bacterial density required for inoculation of Muller-Hinton agar for antibacterial sensitivity testing.
- **Muller Hinton Agar**: This medium is prepared for performance of disk diffusion susceptibility assay to test antibacterial effect of the tested substances on *Enterococcus faecalis*.
- **Mueller Hinton Agar with 5% Sheep Blood**: This medium is prepared for performance of disk diffusion susceptibility assay to test antibacterial effect of the tested substances on *Streptococcus mutans*.

Preparation of McFarland Standard: Following the instructions in CLSI, 2023, a 0.5 McFarland standard was made internally by adding a 0.5-ml aliquot of 0.048 mol/liter BaCl2 to 99.5 ml of 0.18 mol/liter H2SO4 and stirring continuously to maintain a suspended state. A spectrophotometer with a 1-cm light path and matched cuvette was used to measure absorbance in order to confirm that the density of the turbidity standard was accurate. Following that, 4- to 6-ml aliquots of the suspension of barium sulphate were put into screw-cap tubes that were the same dimensions as those used to standardize the bacterial inoculums. Followed by complete sealing of the tubes that were kept at room temperature in the dark.

Preparation of Muller Hinton agar: Muller Hinton agar plates were prepared following the manufacturer's instructions ***(HiMedia, India)***. 38.0 grams of the agar powder were suspended in 1000 ml purified/ distilled water then heated to boiling to dissolve the medium completely. Sterilization of prepared suspension was performed by autoclaving at 121°C for 15 minutes. The suspension was allowed to cool to 45-50°C then well mixed and poured into sterile Petri plates. As for Muller Hinton agar with 5% Sheep Blood, ready-prepared agar plates provided by HiMedia, India were used.

Bacterial inoculum preparation: For each bacterial isolate to be tested, fresh broth culture was made. Bacterial inocula were prepared through dilution of the broth culture to match a 0.5 McFarland turbidity standard ***(CLSI, 2023)[9].***

Disk Diffusion Susceptibility Assay: This assay was conducted following guidelines of ***CLSI, 2023***:

- **Implantation of bacterial isolates on Muller Hinton agar plates:** A sterile swab was inserted into the inoculum tube for each created bacterial

inoculum. The excess fluid was then removed by rotating the swab firmly against the tube's wall (above the fluid level). The Muller Hinton agar plate's dried surface was inoculated by streaking the swab across it three times, rotating the plate each time by around 60 degrees to achieve a uniform dispersion of the inoculum. The swab was then used to clean the plate's rim of any extra liquid. The surface of the agar plate was ultimately allowed to dry for at least 3 to 5 minutes, but no longer than 15 minutes, before moving on to the following stage.

- **Mixing and application of tested substances:** Using a metal punch, a total of three holes of 4 mm in diameter were punched on the agar surface, leaving around 10-15 mm from the petri dish's edge. To prevent zones of inhibition from overlapping, these holes were spaced apart by a distance of at least 20 mm. Each hole was labelled as follows: hole I for chlorohexidine foam 0.6%, hole II for chlorohexidine solution 0.2%, and hole number III for normal saline 9%. Each hole contained one of the tested substances. Prior to presenting the findings, all of the agar plates were left to incubate for up to 24 hours in aerobic conditions at 37°C.

Method of evaluation: after observation period of 24 hours, a poly gauge millimeter ruler was used to quantify the zone of microbial growth inhibition (lack of bacterial colonization) around the holes at its biggest diameter.

Statistical analysis:

SPSS software (version 16.0, SPSS, Chicago, IL, USA) was used to analyse the data. The ANOVA and Kruskal-Wallis tests were used to compare the data in each group. The significance level was set at 0.05.

RESULTS

Determination of the inhibition zones for the three tested materials against *Enterococcus faecalis* and *Streptococcus mutans*: The antibacterial activity of tested formulations (chlorohexidine foam, solution, and gel) was examined against all bacterial isolates after 24-hour incubation and the mean values of inhibition zones were recorded.

The results are illustrated in Table 2. This table illustrates the range and mean range of zones of bacterial growth inhibition in mm around the three chlorhexidine formulations. According to the values presented in the provided table, chlorohexidine foam had higher mean range of zone of growth inhibition (21.3) than other chlorohexidine formulations (18.6 and 19.7 for chlorohexidine solution and gel respectively), denoting the superior antibacterial effect of chlorohexidine foam. The results were significant for both Enterococcus faecalis and Streptococcus mutans strains as indicated by the P value.

TABLE (2) Range and Mean of Bacterial Growth Inhibition Zones in mm around the Three Chlorhexidine Formulations

	***Enterococcus faecalis* Zone of Growth Inhibition**			***Streptococcus mutans* Zone of Growth Inhibition**		
	Range in ***mm***	***Mean (±SD)***	***P Value***	***Range***	***Mean (±SD)***	***P Value***
Chlorohexidine foam	19-24	21.3 (±1.76)		16-21	18.2 (±1.75)	
Chlorohexidine solution	16-21	18.6 (±2.11)	0.024	12-16	14.1 (±1.37)	0.0003
Chlorohexidine gel	18-22	19.7 (±1.41)		14-19	15.9 (±1.66)	

DISCUSSION

Irreversible pulpitis and periapical periodontitis are caused by bacteria entering the root canal system, including Gram-positive *Enterococcus faecalis* and *Streptococcus mutans*, which is arguably the most resistant bacteria to disinfection and unresolved periapical infections. The use of chlorohexidine in dentistry and oral healthcare is widespread and therefore it is of utmost importance that dentists understand, based on its various mechanisms of action on different microbes, that appropriate clinical and dental use of chlorohexidine should be oral disease specific.[10]

Agar diffusion method is an in vitro antimicrobial susceptibility test that is easy to perform and cost effective in comparison to dilution methods. Using this method, various antimicrobials and natural extracts can be screened for antimicrobial activity against pathogenic microbial species. Results are easy to interpret; the larger the zone of organism growth inhibition, the more susceptible the organism is to the tested antimicrobials. Though agar diffusion method provides qualitative results, it allows the simultaneous testing of various antimicrobials which makes it easier to compare the antimicrobial effect of different materials against a pathogenic microbial species at the same time. [11]

Cochrane advised that chlorohexidine, compared to other antiseptics, is the superior irrigant of choice for root canal infection therapy.[12,13] Another study concluded that 2% chlorohexidine had superior bactericidal properties to 2.5% sodium hypochlorite on *Enterococcus faecalis* based on an in-vitro culture study.[14]

Another in vitro study showed that gutta-percha points containing chlorhexidine showed larger growth inhibition zones with various microorganisms implicated in root canal infection when compared to calcium hydroxide.[15]

Our study revealed that chlorohexidine foam had a more potent in-vitro antibacterial effect based on range of zones of growth inhibition of both *Enterococcus faecalis and Streptococcus mutans* around the three tested formulations. The mean range around chlorohexidine foam was 21.3 and 18.2 for *Enterococcus faecalis and Streptococcus mutans* respectively.

The use of chlorohexidine foam was also proved superior over other chlorohexidine formulations by various studies. According to a study by *Jones et al.,* the use of chlorohexidine foam was found to reduce biofilm formation by oral microbiota. Moreover, *Zachary et al* mentioned in his study that chlorohexidine foam is superior to other formulations and recommended its general use to prevent and control infections in various medical conditions.[16,17] This difference in the magnitude of the antimicrobial efficacy of the foam can be attributed to microbial characteristics of both bacteria.[18,19]

According to Haraji et al., chlorohexidine gel was reported to be more effective than chlorohexidine solution.[20] Another study by Wang et al. suggested that a 2% chlorohexidine gel is an effective root canal disinfectant based on in-vitro and in-vivo study results.[21] This study confirmed the superior in-vitro effect of chlorohexidine gel over other chlorohexidine formulations, which is further supported by various in-vivo studies. Lee et al mentioned in his in-vivo study that chlorohexidine gel had several advantages over chlorohexidine solutions such as relieving gingivitis, inhibition of dental plaque formation, and reduction of bacteria that cause periodontal disease. In addition, there were no subjects who complained of side effects of the chlorohexidine gel during the study period. Accordingly, the use of the chlorohexidine gel in dental clinics could be further expanded.[22]

It was also revealed in our study that chlorohexidine foam had a more potent antibacterial

effect on both bacteria than other tested formulations (chlorohexidine solution and gel). The mean ranges of zone of growth inhibition around chlorohexidine solution were 18.6 and 14.1 for *Enterococcus faecalis* and *Streptococcus mutans* respectively, while mean ranges around chlorohexidine gel were 19.7 for *Enterococcus faecalis* and 15.9 for *Streptococcus mutans*. The more potent effect of chlorohexidine foam can be attributed to several factors as suggested by several studies. The foam formulation allows for increased contact time and distribution of the antibacterial agent resulting in better bioavailability. It also has better and deeper penetrative ability providing better coverage and efficacy against bacterial infections.[23,24.] Moreover, it can be attributed to the effervescent effect and the release of energy of rupturing bubbles of the foam and the incorporation of air inside the liquid under pressure. Additionally, the foam form of chlorhexidine has a higher concentration of the active ingredient than gel and solution form, which further increases its antimicrobial efficacy. These findings also coincided with an article that concluded the foam to be superior to the gel form in terms of antimicrobial efficacy due to the same factors described previously. [25]

CONCLUSION

Within the limitations of the present study, it was concluded that chlorohexidine foam exhibited a stronger antimicrobial activity against bacteria that are commonly implicated in root canal infections. Overall, foam formulations can provide an effective and convenient method for delivering oral antibacterial therapy.

RECOMMENDATIONS

More in-vivo studies on the delivery methods to confirm these revolutionary results and get better understanding about the foam.

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