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Antibacterial Effect of Hyaluronic Acid Compared to Propylene Glycol as Vehicle for Double Antibiotic paste Against Bacterial Strains in Non-Vital Primary Molar (In Vitro Study)

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Aim: This study aimed to evaluate the antibacterial effect of hyaluronic acid versus propylene glycol as a vehicle with double antibiotic (ciprofloxacin and metronidazole) paste against bacterial strains commonly found in non-vital primary molar roots using agar diffusion method.
Materials and Methods: bacterial swap was taken using a sterile paper point size 20 from a non-vital root canal of a lower second primary molar to isolate the most common bacterial strains (staph aureus and Enterococcus faecalis) found in the root canal, The two bacterial strains were cultured in Brain Heart Infusion (BHI) broth to be used in the agar inhibition test. Subsequently, a sterile cork borer was used to aseptically create a hole with a diameter ranging from 6 to 8 mm. Then, a volume of the double antibiotic paste ciprodiazole (consisting of ciprofloxacin 500mg and metronidazole 500mg) combined with various vehicles to get the necessary creamy consistency was inserted into the hole. Inhibition zone formed around the well was measured (diameter) and recorded in millimeters using calipers and mean values were calculated.
Results: For Staphylococcus aureus, values of inhibition zones formed with hyaluronic acid vehicle were significantly higher than those of propylene glycol ($p<0.001$). However, for Enterococcus faecalis, the difference was not statistically significant($p=0.280$).
Conclusion: Using hyaluronic acid as a vehicle with metronidazole and ciprofloxacin could be a good safe substitute for propylene glycol with better antibacterial effect on necrotic root canal bacterial strains.

Keywords: Hyaluronic acid, Nonvital primary molars, Antibacterial effect, Double antibiotic, Propylene glycol.

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Introduction

The most important goal of pulp treatment in primary teeth is to maintain each tooth as a full functional unit in the arch for proper mastication, phonation and natural space maintainer for proper eruption of the permanent successor teeth and prevention of adverse effects due to early tooth loss.[1,2]

Due to the complexity of the root canal system, the difficulty of mechanical debridement, the polymicrobial nature of the infection, the difficulties of complete disinfection of the root canal, and the potential for reinfection, treating infected non-vital primary teeth successfully is difficult. These factors also make the prognosis for teeth with persistent infections less favorable. Therefore, it has been clinically demonstrated that using chemical disinfection methods is crucial for fully sterilizing canals and improving treatment outcomes.[3]

As disinfection together with cumulative effects of mechanical preparation, irrigation, and intracanal medication are the keys for successful endodontic therapy in both primary and permanent teeth especially in resistant cases, there are continuous clinical trials for developing agents that can enhance the activity of the already used intracanal medications or act as their alternatives.[4,5]

The selection of antibacterial medications for intracanal therapy has been made in light of several studies on bacterial isolation from oral sites, such as endodontic lesions of primary teeth. These studies have resulted in the development of the triple antibiotic paste (TAP) which is a formulation consisting of metronidazole, ciprofloxacin, and minocycline. TAP has demonstrated efficacy in fighting bacteria within the root canal, facilitating the growth of secondary dentin, and facilitating the repair of the periodontal ligament.[6]

Minocycline as a tetracycline derivative has a broad-spectrum bacteriostatic effect against wide range of bacteria,[7] but unfortunately it causes chelation reaction with the calcium found in the dentine layers of the root canal causing discoloration and this is the disadvantages of using TAP.[8]

That is why many successful efforts have been made to substitute or even remove minocycline while maintaining the same antibacterial effect of disinfection of the root canals when using intracanal medication what so called double antibiotic paste to be a safer alternative for TAP,[9] Clinical research has demonstrated that using double antibiotic paste, also known as 2-mix paste, has similar antibacterial activity to 3-mix paste, but with a longer residual antibacterial impact and less harmful effects on dental pulp stem cells (DPSCs).[10]

The dental pulp system of primary teeth especially those with periapical pathosis and/or necrosis is rich in both anaerobic micro-organisms as well as facultative anaerobes like Enterococcus faecalis and aerobic bacteria such as Staphylococcus.[11]

Enterococcus faecalis bacteria has the ability to penetrate the dentinal tubules and the ability to survive long durations without nutrients through utilizing the serum from alveolar bone and periodontal ligament as a source of nutrition, as well as synthesizing stress proteins in adverse environmental conditions that makes it difficult to eliminate such types of bacteria with those surviving abilities as well as morphological and anatomical complexity of the root canal system of primary teeth.[12]

Previous studies have shown that various species of these bacteria remains deep within dentine, cementum, and periapical tissues in infected root canals and periapical tissues especially the furcation area in primary molars.[13,14]

In order to achieve better penetration of dentine layers for better disinfection, proper use of vehicles mixed with these antibiotics should be done, the antibacterial activity of propylene glycol have been clinically investigated showing good antimicrobial and bactericidal activities as well as good handling properties of the resulting paste and diffusion through dentinal tubules.[15,16]

Clinically, the dental use of propylene glycol is considered safe, however many adverse effects have been mentioned in literature in association with prolonged treatment and/or very high doses of propylene glycol such as: nausea, vomiting, hyperosmolality, lactic acidosis; renal dysfunction, and central nervous system depression.[17]

Hyaluronic acid (HA) is an important natural element in the soft periodontal tissues, gingiva, and periodontal ligament, as well as hard tissue, such as alveolar bone and cementum which ensure safety of its use.[18]

Based on the multifunctional roles HA have in all types of wound healing generally, as well as gingival and bone healing which follows similar biological principles, it is possible that HA has comparable roles in the healing of all tissue types of the periodontium as well.[19]

The antibacterial action, viscoelastic nature, biocompatibility and non-immunogenicity of HA were tested clinically against Staphylococcus aureus bacteria and showed good results with no reported cytotoxic as well as effect-controlled release and targeted drug delivery systems.[20]

Accordingly, there are promising results regarding the use of HA as an antimicrobial agent however, a few studies used it as antibiotic vehicle against oral bacteria so, the purpose of our study is to evaluate the antibacterial effect of hyaluronic acid as compared to propylene glycol as a vehicle with double antibiotic (ciprofloxacin and metronidazole) paste against enterococcus faecalis and Staphylococcus aureus found in necrotic primary molar roots.

The null hypothesis (H_0): there is no difference in the antibacterial effect between the Ciprofloxacin, Metronidazole (2-Mix) when mixed with propylene glycol gel and Hyaluronic acid gel as a vehicle against bacterial species present in non-vital primary molars.

Materials and Methods

The study was designed as an in vitro study, antibiotic sensitivity testing was done using agar well diffusion method of the double antibiotic paste with two vehicles Group 1: DAP (Ciprofloxacin, metronidazole, and mixed with propylene glycol gel Group 2: DAP (Ciprofloxacin, metronidazole, and mixed with hyaluronic acid gel. Each antibiotic medicament was tested against E. faecalis and S. aureus. The maximum zone of inhibition was measured by diameter size (mm) for each bacterial strain tested.

Ethics approval

The Faculty of Dentistry's Research Ethics Committee evaluated and approved the study. Ain Shams University with reference number (FDASU-RecID032105).

Sample Size Estimation

A power analysis was designed to have adequate power to apply a statistical test of the null hypothesis that there will be no significant difference between tested groups. By adopting an alpha and beta levels of (0.05) i.e. power=95% and an effect size (d) of (2.10) calculated based on the results of a previous study.[21]

The predicted sample size (n) was found to be (14) samples (i.e. 7 samples per group). Sample size calculation was performed using G*Power version 3.1.9.7.[22]

Pilot Batch

To determine the most appropriate hyaluronic material formulation to be used, a pilot batch was conducted on two forms of hyaluronic acid (gengigel, hyalgan) against three bacterial species (streptococcus mutans, Staphylococcus aureus and Enterococcus faecalis) that were isolated from the swap sample of the necrotic root canal of lower primary second molar tooth. Hyalgan material was found to show more antibacterial effect against the three types of bacteria isolated than does the gengigel form, figure 1 accordingly, it was selected for this study.

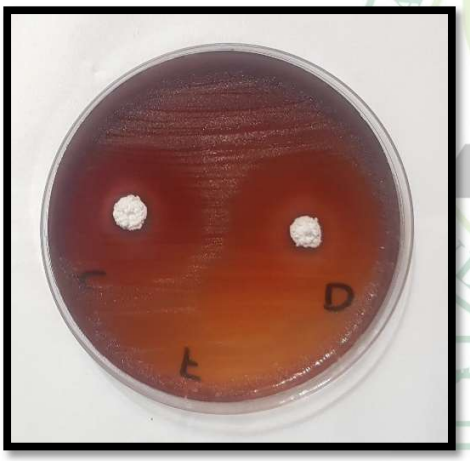

Figure1 : Showing the bilot patch of the Staph aureus bacterial inhibition zones of Gengigel (C) and Hyalgan (D) materials

Sample collection

A necrotic second primary molar tooth with intact roots was chosen for the sample, After disinfection of the oral cavity with 10 mL of 0.12% chlorhexidine digluconate for 1 min.[23]Coronal access was performed with high speed spherical diamond burs cooled with air and water and pulp chamber irrigation with sterile physiological saline had been performed.[24] Bacterial samples were obtained immediately after crown access by inserting three consecutive sterile absorbent paper points of appropriate size into the root canal up to the working length. After a duration of 30 seconds, the paper points were transferred into a test tube containing 2 mL of Reduced Transport Fluid (RTF) that was prepared following the method described by Syed and Loesche.[25]

Isolation & identification

Isolation of bacteria from the paper points were done on a specific media and identification of the bacteria were made according to microbiological standard, two strains of bacteria were identified (staph aureus and E.faecalis) staph aureus were identified by gram stain, biochemical reaction and coagulase test, E. Faecalis bacteria were identified by gram stain, Bile Esculin media. The two bacterial strains were standardized to optical density of 0.5 McFarland standard and were cultured in Brain Heart Infusion (BHI) broth to be used in the agar inhibition test.

Preparation of double antibiotic pastes

Commercially available pharmacological agent ciprodiazole (ciprofloxacin 500mg and metronidazole 500mg) was used in this study. After removal of outer coating, the drugs were crushed using sterile mortal and pestle. Two scopes of the powdered drug were mixed with one drop of propylene glycol and similarly with hyaluronic acid to obtain thick, creamy consistency of both pastes.[26]

Agar well diffusion method

The microbial inoculum was dispersed evenly over the whole surface of the blood agar plate. Then an aseptic hole with a diameter of 6 to 8 mm was created using a sterile cork borer. Then, a volume of the double antibiotic paste, ranging from 20 to 100 UL, was introduced into the well at the desired consistency. Subsequently, the agar plates were placed in an incubator set at a temperature of 37°C for a duration of 24

hours. The antimicrobial agent disperses throughout the agar medium and hinders the growth of the tested microbiological strain.[27]

The diameter of the inhibitory zone surrounding the well was measured using calipers and recorded in millimeters. Subsequently, the mean values were determined.[28] Figure 2,3

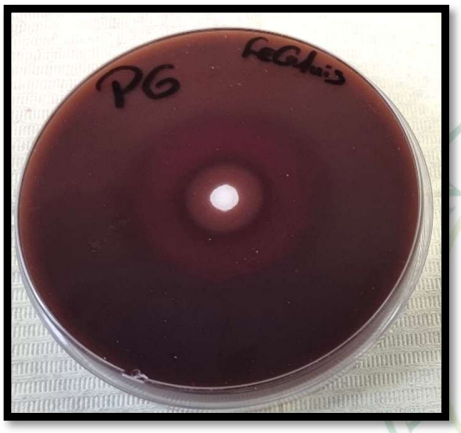

Figure 2: Double antibiotic mixed with PG (after incubation)

Figure 3: Double antibiotic mixed with HA (after incubation)

Statistical analysis

Numerical data was represented as mean, standard deviation (SD), median, and interquartile range (IQR) values. They were analyzed for normality by checking data distribution and using Shapiro-Wilk's test. Enterococcus faecalis data were normally distributed and were analyzed using independent t-test. However, Staphylococcus aureus data were non-parametric data and were analyzed using the Mann-Whitney U test. The significance level was set at $p<0.05$ within all tests. Statistical analysis was performed with R statistical analysis software version 4.3.3 for Windows.[29]

Results

Table 1, figure 4,5. For Staphylococcus aureus, values of inhibition zones formed with HA were significantly higher than those of PG ($p<0.001$). However, for Enterococcus faecalis, the difference was not statistically significant ($p=0.280$).

Table 1: Summary statistics, inter and intragroup comparisons of bacterial inhibition zones. SD Standard deviation, IQR Interquartile range.

Bacteria	*Measurement*	*PG*	*HA*	*Test statistic*	*p-value*
Staphylococcus aureus	*Mean±SD*	3.79±0.10	3.96±0.05	92.00	<0.001*
	Median (IQR)	3.75 (0.20)	4.00 (0.10)		
Enterococcus faecalis	*Mean±SD*	3.62±0.42	3.83±0.42	1.11	0.280
	Median (IQR)	3.75 (0.70)	3.75 (0.50)		

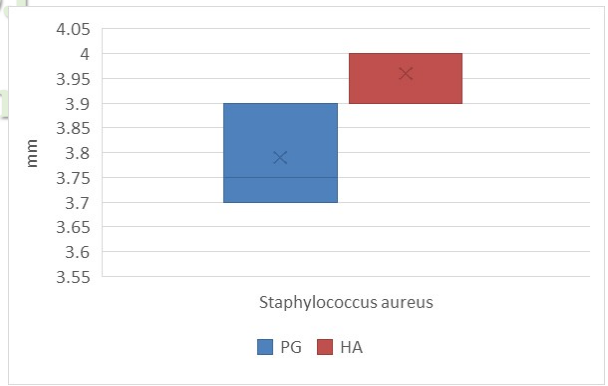

Figure 4: Box plot for Staphylococcus aureus inhibition zone values

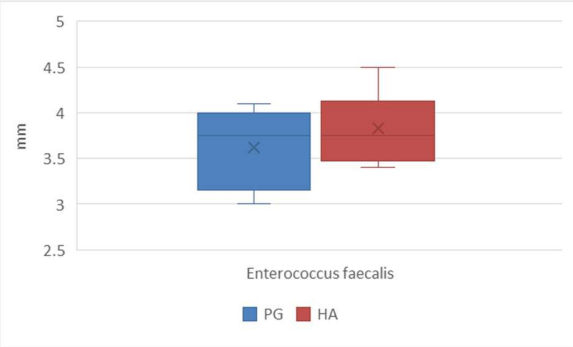

Figure 5: Box plot for Enterococcus faecalis inhibition zone values.

Discussion

It was validated that mechanical preparation of the root canals, together with appropriate antibacterial irrigation, can effectively eliminate 50% to 70% of infectious bacteria from the roots. However, certain vital bacteria may remain in the deep layers of the root canals.[30]

This carries special importance in primary teeth where the anatomy of the dental pulp is highly complicated with several branching and ramifications that makes complete bacterial eradication rather more difficult than normal, as proved by many studies root canal system of primary teeth is poly microbial in nature exhibiting large number of bacterial strains especially anerobic bacteria (Enterococcus faecalis) specifically those teeth with necrotic pulp and /or periapical pathosis.[31]

In accordance to Mohammadi et al[7] the selection of metronidazole and ciprofloxacin drugs (DAP) to be used as intracanal medications were based on their antibacterial actions, ex: metronidazole drug was based on its antibacterial power of disrupting bacterial DNA, that action proposed against most bacteria found in the root canal which is obligate anaerobes found in the deeper dentin layers of infected root canals.

Ciprofloxacin is a very effective broad-spectrum bactericidal antibiotic specially against gram negative organisms mainly by inactivating the bacterial enzymes,[32] when compared to other drugs ciprofloxacin was found to have the highest antibacterial action against E. faecalis, Palasuk et al[33] has proven that the use of ciprofloxacin together with metronidazole have great efficacy against E. faecalis, P. gingivalis and other bacterial species without adversely affect cell viability.

Regarding the type of bacteria (E.faecalis and S. aureus) that specifically isolated to be used for our study it is because E. Faecalis bacteria was clinically proven to survive under very difficult environmental conditions as well as resisting a lot of now used intracanal medications as well as its ability to occupy the dentinal tubules, as for S.aureus bacteria has the ability to cause primary as well as persistent endodontic infection and the ability of the DAP to eliminate such resistant type of bacteria would mean elimination of other less resistant types of bacteria present in the root canal system. [34-36]

To optimize the antibacterial action of the DAP an appropriate vehicle must be mixed with the drugs as it helps the medicine to penetrate through the infected dentin and eliminate the bacteria from the deeper layers of the radicular portion of the root.[37]

In the present study we intended to evaluate a new vehicle (hyaluronic acid HA) versus propylene glycol when mixed with double antibiotic against E.faecalis and S.aureous bacteria to determine which vehicle gives the better antibacterial action as well as to overcome disadvantages of PG as it has been linked in the literature to a number of negative consequences, including lactic acidosis, nausea, vomiting, hyperosmolality, renal failure, and depression of the central nervous system.[17]

The findings of this study found that hyaluronic acid showed superior antibacterial effect as a vehicle when compared to propylene glycol which align with the research conducted by Nalawade et al[38] who investigated the use of PG as a carrier for

endodontic medication and found that it exhibited strong antibacterial activity against various types of bacteria, including S. aureus, E. coli, and E. faecalis, as it enhance the drug diffusion and prolong the duration at which medications release gradually.

Concerning HA results in our current study, it was found to have good antibacterial effect against both S aureus and E.faecalis bacteria when used as a vehicle for double antibiotic paste, Mandras et al[39] conducted a study on ciprofloxacin, metronidazole, and minocycline (3-MIX); ciprofloxacin, metronidazole, and clarithromycin (3-MIXC); and ciprofloxacin and metronidazole (2-MIX), which matches the findings of our study. Each antibiotic formulation was combined with either macrogol (MG) or hyaluronic acid (HA) vehicles. The findings revealed that when coupled with HA, each antibiotic mixture exhibited a greater ability to kill bacteria compared to when combined with MG.

Also, in accordance with our findings regarding the antibacterial impact of hyaluronic acid on S. aureus, Zamboni et al[40] conducted a study to assess the antimicrobial activity of HA derivatives on Staphylococcus aureus. The outcomes demonstrated that films made from HA exhibit antibacterial properties against S. aureus. This material has the potential to be used on surfaces in clinical settings where there is currently a problem with staphylococcal contamination. It also promotes the usage of these films for creating wound healing dressings.

Study strengths and limitations

The strength points in our study are the novelty and scarcity of similar studies as well as, the strict adherence to standardization measures in bacterial isolation, identification and agar diffusion method in detecting the antibacterial zone of inhibition. Moreover, all procedures were carried out with the same operator in the same manner.

Concerning the limitations of our current study is that the results of the double antibiotic pastes obtained under strict laboratory conditions might be affected when used in biological environmental condition of the human body subjected to a wide variability of human immune response as well as the wide variety of bacterial strains present intraorally.

Conclusion

Using hyaluronic acid as a vehicle with metronidazole and ciprofloxacin could be a good safe substitute for propylene glycol with better antibacterial effect on non vital root canal bacterial strains

Funding

This research received no external funding.

Data Availability Statement

Complete data is available upon request.

Conflicts of Interest

The authors declare no conflict of interest.

Declarations

All procedures performed in the study were in accordance with CRIS guidelines and regulations.

Ethics approval

The Faculty of Dentistry's Research Ethics Committee evaluated and approved the study. Ain Shams University with reference number (FDASU-RecID032105).

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