EGYPTIAN DENTAL JOURNAL
www.eda-egypt.org
VOL. 68, 3915:3923, OCTOBER, 2022
PRINT ISSN 0070-9484 • ONLINE ISSN 2090-2360
CONSERVATIVE DENTISTRY AND ENDODONTICS
Submit Date : 28-04-2022 • Accept Date : 08-05-2022 • Available online: 01-10-2022 • DOI : 10.21608/edj.2022.136335.2096

ANTIBACTERIAL EFFECT OF CHLORHEXIDINE, NANO-CHITOSAN AGAINST ENTEROCOCCUS FAECALIS WITH AND WITHOUT USING ULTRASONIC ACTIVATION. (AN IN VITRO STUDY)

Hany Samy Sadek* Mohamed Yehia Mohamed Hassan**
and Hisham Mohamed Mohamed Elshishtawy ***

ABSTRACT

Aim: To Compare the antibacterial efficacy of Chlorhexidine, nano-chitosan and their combination against enterococcus faecalis (E. faecalis) with and without ultrasonic activation.

Patients and Methods: 110 extracted teeth were divided into 6 groups according to the antibacterial agent used; 1: Control group, 2: chitosan, 3: chitosan+2% chlorohexidine, 4: 2% chlorohexidine, 5: chitosan extra-strength, 6: chitosan extra-strength+ 2% chlorohexidine. Each group was subdivided into two subgroups, with and without ultrasonic activation (n=10). Microbial samples were collected from all the root specimens and colony forming units were counted and transformed into log CFU. The collected data were statistically analyzed using Kruskal Wallis test and pairwise Mann – Whitney U test with Bonferroni correction.

Results: The control group showed the highest bacterial count while CHX with ultrasonic activation group showed the lowest bacterial count. There was a significant difference in bacterial count between the 6 groups ($p< 0.001$). Pairwise comparison revealed that CHX, chitosan+ CHX and chitosan extra-strength +CHX groups with ultrasonic activation showed significantly lower bacterial count than chitosan with ultrasonic activation group, chitosan extra-strength without ultrasonic activation group and the control group. Chitosan extra-strength with ultrasonic activation group, chitosan extra-strength + CHX, chitosan, chitosan + CHX and CHX groups without ultrasonic activation showed no significant difference in bacterial count from all other groups.

Conclusions: Ultrasonic activation improves bacterial elimination, CHX with ultrasonic activation showed higher antimicrobial effects against E. faecalis among all tested groups.

Key words: Antimicrobial efficacy, Chlorhexidine, E. faecalis, Endodontics, Irrigation, Nano-Chitosan, Ultrasonics.

* Associate Professor of Endodontics , Endodontic Department, Faculty of Dentistry, Cairo University, Cairo, Egypt
** Associate Professor of Endodontics , Endodontic Department, October University for Modern Sciences & Arts (MSA University)
*** Associate Professor of Microbial Genetics Agriculture , Genetic Engineering Research Institute , October University of Modern Sciences & Arts (MSA)

INTRODUCTION

Infected root canals have a diverse microbial flora composed of cocci, rods, spirochetes, filaments, and even fungus [1]. E. faecalis has been identified as a major source of refractory root canal infection, accounting for a significant proportion of clinical treatment failures. It appears to be very resistant to the antibiotics employed during treatment and is one of the few microorganisms that has been demonstrated to resist the antibacterial activity of calcium hydroxide in vitro [2, 3]. As a result, establishing effective and efficient ways for eliminating E. faecalis infection within the root canal or around the apex has long caught the interest of dentistry and material scientists both. [4].

The potential of E. faecalis to colonize dentinal tubules and form a strong bond with collagen, which is prevalent in root dentin and cementum, is the primary cause of endodontic failure. In the nutrient-deficient environment of root-filled teeth, E. faecalis develops biofilms. Antibiotics, phagocytosis, and antibodies do not affect them.

Successful root canal treatment needs accurate chemo-mechanical debridement of the pulpal tissue. This procedure includes the removal of dentin debris and infectious microorganisms. Mechanical debridement can be improved by emptying debris, removing tissue, and cleansing the root canal system. Endodontic irrigant is utilized to lubricate, disintegrate pulp remnants, wash away equipment debris, eliminate bacteria (planktonic or biofilm), and clean the smear layer [5].

CHX has a broad spectrum antibacterial action[6]. A study examined the substantivity of a 2% CHX solution inside the root canal system and discovered that the CHX was kept in antimicrobial effective concentrations in the root canal dentine for up to 3 months. Additionally, chlorhexidine's biocompatibility is acceptable [7]. Additionally, the cytotoxic impacts of CHX, sodium hypochlorite and hydrogen peroxide, were investigated; CHX was found to be the least hazardous antiseptic agent [8].

Chitosan is a biopolymer composed of natural polysaccharides formed when chitin, a key ingredient of crustacean outer skeletons, is alkaline deacetylated. The versatile hydrophilic polymer generated from chitin, has a wide antibacterial range to which gram -ve, gram +ve bacteria and fungi are extremely sensitive [9].

Ultrasonic energy has a long history in endodontics for cleaning and disinfecting root canals [10]. When used in conjunction with an irrigant, ultrasonic irrigation leads to a more thorough cleansing of the root canal system than irrigation by syringe. Ultrasonic irrigation has demonstrated a high level of root canal system cleansing effectiveness [11].

Rather than utilizing either alone, mixing chlorhexidine gluconate and chitosan may enhance the antibacterial action of chlorhexidine against E. faecalis in vivo [12, 13]. Therefore, this study examined the antibacterial activity of chlorhexidine, nano-chitosan, and their mixture against E. faecalis with and without ultrasonic activation.

MATERIALS AND METHODS

1. Materials

a. ***Nano-chitosan:***

10% Chitosan solution (Nanotech, Giza, Egypt)

b. ***Chitosan extra strength:***

20% Chitosan solution **(Nanotech, Giza, Egypt)**

c. ***Chlorhexidine and Nano-chitosan combination***

The ratio of concentration between chitosan and chlorhexidine is 1:1.

2. Methods

Experimental designing and sample grouping

One hundred and ten extracted teeth were

classified into 6 groups based on the antimicrobial agent utilized; 1: Control group (saline solution), 2: chitosan, 3: chitosan+2% chlorhexidine, 4: 2% chlorhexidine, 5: chitosan extra-strength, 6: chitosan extra-strength+ 2% chlorhexidine. Then each group was split into two subgroups, with and without ultrasonic activation (n=10).

Teeth preparation

Single-rooted teeth with single canals were employed. Using a diamond stone, teeth were decoronated to a standard 16 mm from the root tip. To prevent bacterial penetration and material diffusion through the dentin, tray adhesive was applied on the exterior surface of the roots. After that, the roots apex was sealed with adhesive to prevent bacterial and irrigation leakage. Teeth were sterilized at 121ºC for 20 minutes

Preparation of E. faecalis

E. faecalis (ATCC 29212) was acquired and cultured in brain-heart infusion (BHI) broth. The inoculum density was regulated to 0.5 McFarland (1.5 108 bacteria/ml) turbidity.

Teeth contamination with Enterococcus faecalis

By plating on blood agar medium, the bacterial strain E. faecalis from the stock was revived. Colonies isolated from sterile brain heart infusion broth were transferred and cultured for a further 12-14 hours. 5 µL of E. faecalis microbial suspension calibrated to McFarland standard no. 1 was injected into the previously autoclaved teeth using a syringe. This treatment was done daily for five days throughout the connective tissue phase. Throughout this time period, the teeth were maintained in a 37°C oven.

After five days, each tooth was irrigated with 100 µL of sterile saline and a size-20 sterile absorbent paper tip was placed into the root canal and left for five min. The paper points were then transmitted to a test tube filled with 1 mL saline solution, from which four serial dilutions (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} CFU) were made. Aliquots of 25 µL of each dilution were plated onto Mueller–Hinton agar plates. Colony forming units (CFU-1) were recorded after 1 day incubation.

Irrigation procedure

The working length (WL) was adjusted to 15 mm, and filing was carried out with the Pro-Taper rotating NiTi system up to size F3. Between the files of each subgroup, roots were irrigated with 2mL of the irrigant. For subgroups of ultrasonic activation, stimulation was delivered for 5 seconds. In all subgroups, irrigation was done using a 30-gauge needle with an end-closed and double side vent linked to a 3 mL plastic syringe. The needle was shorter than the working length by roughly 1 mm.

Final sampling procedure

Microbial samples were obtained by inserting paper points (ISO 20) for 30 sec. into root canals before to and immediately following the rinse processes. Serial dilutions of each specimen were performed, and aliquots were deposited on agar plates. Colony-forming units (CFUs) were measured and documented after two days of incubation.

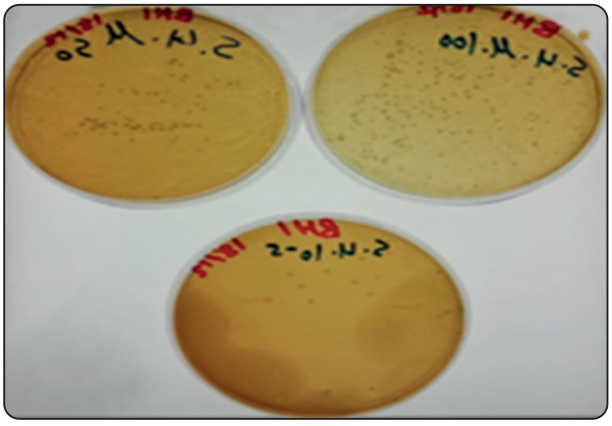

Fig. (1): Microbial samples placed in agar plates

Statistical Analysis

Based on a previous study [20], a Cohen's d effect size that indicates the difference between two groups

2% chlorhexidine and 0.2% chitosan regarding E. faecalis count in CFUs was found to be 2.16. Using the mentioned effect size, a type I error of 0.05 and a power of 0.8, a sample of 5 samples per group (55 samples total) is required to detect a significant difference between the two groups regarding E. faecalis count in CFUs. The sample was doubled to 10 samples per group (110 total samples) to further detect the small differences between the different groups. The sample size was calculated using the P.S. software version 3.1.6.

Descriptive statistics of bacterial counts were presented as mean, standard deviation (SD), median, minimum and maximum values. Between-groups comparison was performed using Kruskal Wallis test with 0.05 significance level followed by Mann – Whitney U test with Bonferroni correction for pairwise comparison.

RESULTS

Bacterial Count:

The control group showed the highest bacterial count while CHX group with US activation showed the lowest bacterial count. There was a significant difference in bacterial count between the 11 groups ($p< 0.001$). CHX, chitosan+ CHX and chitosan extra-strength +CHX groups with US activation showed significantly lower bacterial count than chitosan with US activation group, chitosan extra-strength without US activation group and the control group. Chitosan extra-strength with US activation group, chitosan extra-strength + CHX, chitosan, chitosan + CHX and CHX groups without US activation showed no significant difference in bacterial count from all other groups.

Pairwise comparisons:

Chitosan with US activation group showed significantly higher bacterial count than CHX, chitosan+ CHX and chitosan extra-strength +CHX groups with US activation. There was no significant difference between this group and the remaining groups.

Chitosan+ CHX with US activation group showed significantly lower bacterial count than Chitosan with US activation, Chitosan extra-strength without US activation and the control groups. There was no statistically significant difference between this group and the remaining groups.

CHX with US activation group showed significantly lower bacterial count than Chitosan with US activation, Chitosan extra-strength without US activation and the control groups. There was no statistically significant difference between this group and the others.

Chitosan extra-strength with US activation group showed no significantly different bacterial count than all other groups.

Chitosan extra-strength+ CHX with US activation group showed significantly lower bacterial count than Chitosan with US activation, Chitosan extra-strength without US activation and the control groups. There was no significant difference between this group and the remaining groups.

Chitosan extra-strength without US activation group showed significantly higher bacterial count than Chitosan+ CHX, CHX and Chitosan extra-strength+ CHX all with US activation. There was no statistically significant difference between this group and the remaining groups.

Chitosan extra-strength + CHX, Chitosan, Chitosan + CHX and CHX groups without US activation showed no statistically significant difference bacterial count than all other groups.

The control group showed significantly higher bacterial count than Chitosan+ CHX, CHXand Chitosan extra-strength + CHXwith US activation groups. There was no statistically significant difference between the control group and the remaining groups.

TABLE (1): Descriptive statistics and the results of Kruskal Wallis test and pairwise Mann – Whitney U test with Bonferroni correction:

	Control	Chitosan		Chitosan + 2%CHX		2% CHX		Chitosan extra strength		Chitosan extra strength + 2% CHX	
Ultrasonic	X	√	X	√	X	√	x	√	X	√	x
Mean	341.7[a]	99.0[a]	68.0[ab]	10.8[b]	57.4[ab]	0.0[b]	67.8[ab]	73.0[ab]	90.0[a]	10.6[b]	16.0[ab]
SD	70.0	23.3	6.7	4.4	7.2	0.0	5.1	10.4	7.3	1.9	3.2
Median	361.0	93.0	65.0	11.0	55.0	0.0	68.0	76.0	90.0	10.0	17.0
Minimum	264.0	71.0	60.0	5.0	50.0	0.0	62.0	60.0	80.0	8.0	11.0
Maximum	400.0	129.0	76.0	15.0	66.0	0.0	75.0	86.0	98.0	13.0	19.0

P-Value <0.001**Significant at p < 0.05. Values in Colony forming units (CFU)

Different small letters indicates statistical significance by Mann-Whitney U test with Bonferroni correction.

DISCUSSION

The present study evaluated and compared the antibacterial efficiency of chlorhexidine, nano-chitosan, and chitosan extra strength irrigating solutions against enterococcus faecalis.

Chlorhexidine is a cationic compound that is used in medicine. It is one of the most effective irrigants against E. faecalis. CHX interacts with phospholipids and lipopolysaccharides on bacteria's surface before entering the cell via active or passive transport. The molecule's positive charge interacts with the negative charged phosphate groups on microbial cell walls, affecting the osmotic balance of the cells. It enhances the cell wall's porosity, enabling CHX to enter the bacteria.[14].

The concentration of chlorhexidine selected was 2% since it is the most often used concentration in endodontic practice [15]. This is because a 2 percent chlorhexidine concentration shown superior anti-bacterial action against E.faecalis for 72 hours [16] and all concentrations of NaOCl [17]. Additionally, chlorhexidine at a concentration of 2% is bactericidal, since it causes cytoplasmic precipitation, which results in cell death [18].

Chitin is a key component of the outer skeletons of crustaceans. It is a natural polysaccharide comprised of copolymers of glucosamine and N-acetylglucosamine. Chitosan is produced when chitin is partially deacetylated. Chitosan possesses the qualities necessary for successful usage as an irrigant; it is biocompatible due to its efficacy as a chelating agent with minimal change to radicular dentine [19], biodegradable, bioadhesive, and potent against E.faecalis and C.albicans, with no known toxicity [20, 21]. Additionally, its cheap manufacturing costs have expanded its applicability for a variety of medical and pharmaceutical applications [20].

Chitosan's antibacterial action has been attributed mostly to its polycationic nature, which is transmitted electrostatically via positively charged amino acids that interact with the anionic parts of the bacterial cell surface. This leads to the collapse of cell membrane, intracellular leakage, metabolic imbalance, disrupted ionic homeostasis, impairment of essential bacterial functions, and ultimately cell death. Gram +ve bacteria are more sensitive than gram -ve bacteria due to their unique cell membrane designs. Additionally, chitosan suppresses DNA transcription, RNA synthesis, and protein synthesis [22, 23].

Chitosan's antibacterial effect is also attributed to its chelating activity, since it preferentially binds important trace metals, limits nutrition availability,

and inhibits the development of bacterial enzymes and toxins. Additionally, increased chitosan absorption in bacterial biofilms results in enhanced contact not only with individual bacteria, but also with the negatively charged polymeric matrix of the biofilm structure [24].

The antibacterial impact of several root canal irrigating materials was evaluated in the current investigation using E. faecalis. This is because it is widely believed to be the main reason of root canal therapy failure. According to earlier investigations, it is frequently discovered in asymptomatic and chronic endodontic infections [25].

E. faecalis's resistance can be linked to a variety of survival and pathogenicity mechanisms, including its capacity to compete with other microbes, its ability to infiltrate dentinal tubules, and its resilience to nutrient deficiency [26]. Additionally, these species may be pathogenic due to the existence of secreted factors such as toxic cytolysin, gelatinase, adhesins (e.g. aggregation substance, enterococcal surface protein, and collagen adhesin), capsular polysaccharide, extracellular superoxide production, and the presence of potential adaptive mechanisms [27]. Additionally, culture and modification are extremely simple at the experimental level [28, 29].

According to several prior in vitro researches investigating the antibacterial activity of E. faecalis [30-32], the CFU counts of bacteria utilized in this study are regarded the gold standard approach for measuring disinfection efficacy. The CFU technique has two notable advantages: It can count any amount of germs using dilutions or concentrations if they are too numerous or excessively few. Second, this method counts only living bacteria, whereas the CFU method counts both living and dead bacteria and debris [33]. The agar diffusion technique was omitted from this investigation since it is deemed unreliable in irrigation comparison studies due to the unknown chemical interaction between the medium and the irrigation material [34].

Extracted teeth were employed to imitate oral cavity conditions in the current investigation. The present study examined single-rooted teeth with single canals because they have less anatomical complexity and variability [35, 36] and because they limit the microbiological analysis to a single ecological setting. The teeth were decoronated with a diamond stone, leaving a specified 16 mm from the root tip [37]. The root's outer surface was then sealed with tray adhesive to prevent bacterial penetration and substance diffusion through the dentin, and the root's apex was then sealed with glue to prohibit bacterial and irrigation leakage, simulating the root being sealed by the periodontal ligament and alveolar bone socket, resulting in the canal acting as a closed-end channel [38].

The ultrasonic activation method was employed in this study; ultrasonic energy and an irrigating fluid interacting is referred to as a "synergistic system." Ultrasonication imparts biological-chemical properties to the irrigating fluid. The primary ultrasonic effects are cavitation and acoustic streaming. Transient cavitation is defined as an ultrasonic bubble that grows to a point before collapsing. This collapse creates a suction that cleans and disinfects canals. Resonant or stable cavitation is the oscillation motion of the ultrasonic instrument that actively moves the irrigating fluid. Cavitation phenomena cause physical acoustic (sound wave) streaming which is supposed to aid in the cleaning and disinfection processes [39].

In terms of antimicrobial activity, the current investigation discovered that CHX, Chitosan+CHX and Chitosan extra-strength + CHX with ultrasonic activation groups significantly increased antimicrobial action against E. faecalis compared to the other groups investigated. This is consistent with the outcomes of a comprehensive research, which proved that ultrasonic irrigation speeds up the operation and increases the removal of germs and the smear layer throughout the canal system, leading

to greater endodontic treatment rates of success [10]. Previous laboratory research comparing various irrigation techniques demonstrated that ultrasonic activation is more successful than irrigation by needle in clinical settings for eliminating germs from root canals [39-41]. Two recent investigations compared the bacterial reduction efficiency of ultrasonic activation approaches to that of traditional needle irrigation. In both instances, ultrasonic irrigation resulted in a statistically significant reduction in bacterial load [42, 43]. Numerous investigations have established that ultrasonic activation methods are crucial for irrigant effectiveness [44]. On the other hand, two investigations comparing ultrasonic vs syringe irrigation found no statistically significant change in bacteria counts between the two groups [45, 46].

Correlating with our results, a study by **Arathi et al.** [47] showed that CHX solution with ultrasonic agitation resulted in the smallest bacterial colonies with the greatest penetration into dentinal tubules when compared to CHX solution without ultrasonic activation and Chitosan solution with and without ultrasonic activation; however, this study did not combine the two solutions.

In summary, ultrasonic active irrigation is clinically superior in that it delivers the irrigant to the whole working length of the canal, guaranteeing that the canals are cleaned during endodontic treatment. It is unknown, however, if ultrasonic irrigation techniques effectively eradicate germs from root canals. Additional clinical investigations are required to reach clinically significant conclusions.

CONCLUSION

Ultrasonic activation improves bacterial elimination. Combining CHX with ultrasonic activation has superior antimicrobial effects against E. faecalis among all tested groups.

REFERENCES

1. Sakko M, Tjäderhane L, Rautemaa-Richardson R. Microbiology of Root Canal Infections. Prim Dent J 2016; 5:84-9.
2. Sundqvist G, Figdor D, Persson S, Sjogren U. Microbiologic analysis of teeth with failed endodontic treatment and the outcome of conservative retreatment. Oral Surg Oral Med Oral Path Oral RadiolEndod 1998; 85:86-93.
3. Vivacqua-Gomes N, Gurgel-Filho ED, Gomes BP, Ferraz CC, Zaia AA, Souza- Filho FJ. Recovery of Enterococcus faecalis after single- or multiple-visit root canal treatments carried out in infected teeth ex vivo. Int Endod J 2005;38: 697– 704.
4. Fan, W. et al. (2016) 'Calcium-silicate mesoporous nanoparticles loaded with chlorhexidine for both anti-Enterococcus faecalis and mineralization properties', Journal of Nanobiotechnology, 14(1), pp. 1–12.
5. Campos-Ibarra P, de la Fuente-Hernández J, Tenorio-Rocha F, Acosta-Torres L. Biocompatible Antimicrobial Irrigants and Nanoparticles-Sealers for Endodontics. Entreciencias 2013; 1:9-28.
6. Delany GM, Patterson SS, Miller CH, Newton CW. The effect of chlorhexidine gluconate irrigation on the root canal flora of freshly extracted necrotic teeth. Oral Surg Oral Med Oral Pathol 1982; 53:518-23.
7. Rosenthal S, Spangberg L, Safavi KE. Chlorhexidine substantivity in root canal dentine. Oral Surg Oral Med Oral Pathol Oral RadiolEndod 2004; 98:488– 92.
8. Tatnall, FM, Leigh, IM, Gibson, JR. Comparative study of antiseptic toxicity on basal keratinocytes, transformed human keratinocytes and fibroblasts. Skin Pharmacol Phys 1990; 3:157– 63.
9. Tanikonda R, Ravi RK, Kantheti S, Divella S. Chitosan: Applications in Dentistry. Trends Biomater. Artif. Organs 2014: 28;74-8.
10. Mozo S, Llena C, Forner L. Review of ultrasonic irrigation in endodontics: Increasing action of irrigating solutions. Med Oral Patol Oral Cir Bucal 2012;17:e512-6.
11. Jiang LM, Lak B, Eijsvogels LM, Wesselink P, van der Sluis LW. Comparison of the cleaning efficacy of different final irrigation techniques. J Endod2012;38:838-41.
12. Ballal V, Kundabala M, Bhat KS, Acharya S, Ballal M, Kumar R, Prakash PY. Susceptibility of Candida albicans and Enterococcus faecalis to Chitosan, Chlorhexidine

gluconate and their combination in vitro. Aust Endod J 2009; 35:29–33.

13. Barreras US, Méndez FT, Martínez RE, Valencia CS, Rodríguez PR, Rodríguez JP. Chitosan nanoparticles enhance the antibacterial activity of chlorhexidine in collagen membranes used for periapical guided tissue regeneration. Mater SciEng C Mater Biol Appl 2016; 58:1182-7.

14. Athanassiadis B, Abbott PV, Walsh LJ. The use of calcium hydroxide, antibiotics and biocides as antimicrobial medicaments in endodontics. Aust Dent J 2007; 52:64–82.

15. Mohammadi Z, Abbott PV. The properties and applications of chlorhexidine in endodontics. Int Endod J 2009:42; 288-302.

16. Mahendra A, Koul M, Upadhyay V, Dwivedi R. Comparative evaluation of antimicrobial substantivity of different concentrations of chlorhexidine as a root canal irrigant: An in vitro study. journal of oral biology and craniofacial research. 2014 Sep 1;4(3):181-5

17. Gomes BP, Ferraz CC, Vianna ME, Berber VB, Teixeira FB, Souza-Filho FJ.In vitro antimicrobial activity of several concentrations of sodium hypochlorite and chlorhexidine gluconate in the elimination of Enterococcus faecalis. Int Endod J 2001; 34:424-8.

18. Gomes BPFA, Souza SFC, Ferraz CCR. Effectiveness of 2% chlorhexidine gel and calcium hydroxide against Enterococcus faecalis in bovine root dentine in vitro. Int Endod J 2003; 36:267–75.

19. Mathew SP, Pai VS, Usha G, Nadig RR. Comparative evaluation of smear layer removal by chitosan and ethylenediaminetetraacetic acid when used as irrigant and its effect on root dentine: An in vitro atomic force microscopic and energy-dispersive X-ray analysis. Journal of Conservative Dentistry: JCD. 2017 Jul;20(4):245.

20. Jaiswal N, Sinha DJ, Singh UP, Singh K, Jandial UA, Goel S. Evaluation of antibacterial efficacy of Chitosan, Chlorhexidine, Propolis and Sodium hypochlorite on Enterococcus faecalis biofilm : An in vitro study. J Clin Exp Dent. 2017; 9:1066–74.

21. Shaik J, Garlapati R, Nagesh B, Sujana V, Jayaprakash T, Naidu S. Comparative evaluation of antimicrobial efficacy of triple antibiotic paste and calcium hydroxide using chitosan as carrier against Candida albicans and Enterococcus faecalis: An in vitro study. J Conserv Dent 2014; 17:335-9.

22. El-Sharif AA, Hussain MH. Chitosan-EDTA new combination is a promising candidate for treatment of bacterial and fungal infections. CurrMicrobiol 2011;62:739-45.

23. Younes I, Sellimi S, Rinaudo M, Jellouli K, Nasri M. Influence of acetylation degree and molecular weight of homogeneous chitosans on antibacterial and antifungal activities. Int J Food 2014; 185:57-63.

24. Raafat D, Sahl HG. Chitosan and its antimicrobial potential – a critical literature survey. MicrobBiotechnol 2009;2: 186-201

25. Prada I, Micó-Muñoz P, Giner-Lluesma T, Micó-Martínez P, Collado-Castellano N, Manzano-Saiz A. Influence of microbiology on endodontic failure. Literature review. Med Oral Patol Oral Cir Bucal. 2019; 24:364:72

26. Stuart CH, Schwartz SA, Beeson TJ, Owatz CB. Enterococcus faecalis: its role in root canal treatment failure and current concepts in retreatment. J Endod. 2006;32:93-8.

27. Marina George, Romana Ivančaková. Root Canal Microflora. Acta Medica 2007; 50:7-15.

28. Shabahang S, Torabinejad M. Effect of MTAD on Enterococcus faecalis– Contaminated Root Canals of Extracted Human Teeth. J Endod 2003 29;576-9.

29. Basmaci F, Oztan MD, Kiyan M. ex vivo evaluation of various instrumentation techniques and irrigants in reducing e. faecalis within the root canals. Int Endod J 2013; 46:823-30.

30. Shen Y, Gao Y, Lin J, Ma J, Wang Z, Haapasalo M. Methods and models to study irrigation. Endod Topics 2012; 27:3-34.

31. Williamson AE, Cardon JW, Drake DR. Antimicrobial susceptibility of monoculture biofilms of a clinical isolate of Enterococcus faecalis. J Endod 2009: 35: 95-7.

32. Liu H, Wei X, Ling J, Wang W, Huang X. Biofilm formation capability of Enterococcus faecalis cells in starvation phase and its susceptibility to sodium hypochlorite. J Endod 2010; 36: 630–5.

33. Hazan R, Que YA, Maura D, Rahme LG. A method for high throughput determination of viable bacteria cell counts in 96-well plates. BMC microbiology. 2012 Dec;12(1):1-7.

34. Editorial Board of the Journal of Endodontics. Wanted: a base of evidence. J Endod 2007; 33:1401:2.

35. Nakamura VC, Candeiro GTM, Cai S, Gavini G. Ex vivo evaluation of three instrumentation techniques on E. faecalis biofilm within oval shaped root canals. Braz Oral Res 2015; 29:1-7.

36. Athanassiadis B, Abbott PV, Walsh LJ. The use of calcium hydroxide, antibiotics and biocides as antimicrobial medicaments in endodontics. Aust Dent J 2007; 52:64–82.

37. Saber Sel-D, Hashem AA.. Efficacy of Different Final Irrigation Activation Techniques on Smear Layer Removal. J Endod 2011; 37:1272-5.

38. Parente JM, Loushine RJ, Susin L, Gu L, Looney SW, Weller RN, Pashley DH, Tay FR. Root canal debridement using manual dynamic agitation or the EndoVac for final irrigation in a closed system and an open system. Int Endod J 2010; 43:1001–12.

39. Townsend C, Maki J. An in vitro comparison of new irrigation and agitation techniques to ultrasonic agitation in removing bacteria from a simulated root canal. Journal of endodontics. 2009 Jul 1;35(7):1040-3.

40. Nakamura VC, Pinheiro ET, Prado LC, Silveira AC, Carvalho AP, Mayer MP, Gavini G. Effect of ultrasonic activation on the reduction of bacteria and endotoxins in root canals: a randomized clinical trial. International Endodontic Journal. 2018 Jan;51:e12-22.

41. Cachovan G, Schiffner U, Altenhof S, Guentsch A, Pfister W, Eick S. Comparative Antibacterial Efficacies of Hydrodynamic and Ultrasonic Irrigation Systems In Vitro. J Endod. 2013; 39:1171-5.

42. Ballal NV, Gandhi P, Shenoy PA, Shenoy Belle V, Bhat V, Rechenberg DK, et al. . Safety assessment of an etidronate in a sodium hypochlorite solution: randomized double-blind trial. Int Endod J. (2019) 52:1274–82. 10.1111/iej.13129

43. Orozco EIF, Toia CC, Cavalli D, Khoury RD, Cardoso F, Bresciani E, et al. . Effect of passive ultrasonic activation on microorganisms in primary root canal infection: a randomized clinical trial. J Appl Oral Sci. (2020) 28:e20190100. 10.1590/1678-7757-2019-0100

44. Ballal NV, Gandhi P, Shenoy PA, Dummer PMH. Evaluation of various irrigation activation systems to eliminate bacteria from the root canal system: a randomized controlled single blinded trial. J Dent. (2020) 99:103412. 10.1016/j.jdent.2020.103412

45. Beus C, Safavi K, Stratton J, Kaufman B. Comparison of the effect of two endodontic irrigation protocols on the elimination of bacteria from root canal system: a prospective, randomized clinical trial. J Endod. 2012;38:1479–1483.

46. Cohenca N, Silva LA, Silva RA, Nelson-Filho P, Heilborn C, Watanabe E, Saraiva MC. Microbiological evaluation of different irrigation protocols on root canal disinfection in teeth with apical periodontitis: an in vivo study. Braz Dent J. 2013;24:467–473.

47. Arathi G, Rajakumaran A, Divya S, Malathi N, Saranya V, Kandaswamy D. Comparison of penetrating depth of chlorhexidine and chitosan into dentinal tubules with and without the effect of ultrasonic irrigation. Journal of Oral and Maxillofacial Pathology: JOMFP. 2019 Sep;23(3):389.