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Dr. Neetu Kadu
Reader and HOD, Department of Public Health Dentistry, M. A. Rangoonwala College of Dental Sciences and Research Centre, Pune, Maharashtra, India

Dr. Khatija Memon
Assistant Professor, Department of Conservative Dentistry and Endodontics, M. A. Rangoonwala College of Dental Sciences and Research Centre, Pune, Maharashtra, India

Mustafa Merchant
Intern, M. A. Rangoonwala College of Dental Sciences and Research Centre, Pune, Maharashtra, India

Nooral Huda Khan
Intern, M. A. Rangoonwala College of Dental Sciences and Research Centre, Pune, Maharashtra, India

Corresponding Author:
Mustafa Merchant
Intern, M. A. Rangoonwala College of Dental Sciences and Research Centre, Pune, Maharashtra, India

Antimicrobial efficacy of different irrigants against *Enterococcus faecalis* at two different irrigant temperatures: An *in vitro* study

Neetu Kadu, Khatija Memon, Mustafa Merchant and Nooral Huda Khan

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Abstract
Aim: To evaluate and compare the antimicrobial efficacy of Chitosan, Ethylenediaminetetraacetic acid, Sodium Hypochlorite and Chlorhexidine against *Enterococcus faecalis* at room temperature of the irrigant and after intracanal heating (180º Celsius) of the irrigant.
Materials and Method: A total of 100 extracted single-rooted teeth were infected for 21 days with *E. faecalis* after instrumentation with ProTaper system. Before irrigation procedure, dentinal shavings were collected in 1 ml of sterile broth and incubated. The optical density of each broth was measured using digital colorimeter and initial readings were recorded. Samples were then divided into five groups of 16 teeth in each group– Group A: Sodium hypochlorite irrigation, Group B: EDTA irrigation, Group C: Chitosan irrigation, Group D: Chlorhexidine irrigation, Group E: Saline irrigation. Each group was further divided into two subgroups – (I) Room temperature of irrigant (II) Intracanal heating of irrigant. After irrigation, dentinal shavings were collected and optical density recorded. The values were analysed statistically with Student's t test and analysis of variance followed by Post-Hoc Bonferroni's correction test; *p*-value < 0.05 was considered to be statistically significant.
Results: The post irrigation optical densities in all the groups were significantly lower than pre-irrigation values. Sodium hypochlorite and Chlorhexidine demonstrated better antimicrobial efficacy followed by Chitosan and EDTA, whereas the least efficacy was shown by Saline which was the control group. Differences in optical density using different irrigants were found to be higher by intracanal heating of irrigants followed by room temperature irrigation ($p < 0.0001$).
Conclusion: Chitosan exhibited antimicrobial effect similar to Chlorhexidine and Sodium Hypochlorite. Intracanal heating was most effective for elimination of *E. faecalis* as compared to that of room temperature of the irrigant.

Keywords: *Enterococcus faecalis*, intracanal heating, chitosan, sodium hypochlorite, chlorhexidine

Introduction

Elimination of microorganisms from the root canal system is one of the primary goals of root canal treatment. Infections in endodontics are polymicrobial in nature, but it is dominated by obligate anaerobic bacteria [1]. Even after thorough mechanical and chemical instrumentation there may not be complete elimination of bacteria as the root canal systems are highly complex in nature [2]. *E. faecalis* is one of the most commonly associated organism associated with the etiology of periradicular lesions. It comprises of 6% of total flora in a root canal and is seen in 22-77% root canal failure cases. E. faecalis possesses bound virulence factors as well as lytic enzymes, cytolysin, aggregation substance, pheromones and lipoteichoic acid. It has been shown to adhere to host cells, express proteins that allow it to compete with other bacterial cells and alter host responses. *E. faecalis* is able to suppress the action of lymphocytes, potentially contributing to endodontic failure [1]. Irrigation is one of the ways to impact those areas of the root canal wall that are not touched by mechanical instrumentation. A bigger challenge for irrigation may be the areas untouched by the mechanical instruments, such as fins, isthmuses and large lateral canals. Also, giant areas within the oval and flat canals could stay untouched despite careful instrumentation.

These areas contain pulp remnants and microbial biofilms that only can be removed by chemical means such as irrigation [3]. Sodium Hypochlorite (NaOCl) is considered to be the gold standard irrigant in endodontics.

A 5.25% sodium hypochlorite is widely recommended as an endodontic irrigant in the treatment of infected root canals, because of its well-known bactericidal action [4]. NaOCl solutions are the most favoured root canal irrigants, because of their tissue dissolving, antibacterial, and lubricating properties. In addition, they are inexpensive and easily available and if stored correctly, they have a good shelf life [5]. But it also possesses certain drawbacks such as its unpleasant taste and periapical tissue irritation potential. This have impelled researchers to find other substitutes. The perpetual rise in antibiotic resistant strains and adverse effects of synthetic irrigants have led to the search for new alternatives [1]. One different approach to enhance the effectiveness of sodium hypochlorite irrigants within the root canal system may be to extend the temperature of low-concentration NaOCl solutions. This appears to improve their immediate tissue-dissolution capacity. At the same time, the systemic toxicity of preheated NaOCl irrigants, once they have reached body temperature, should be lower than the one of more concentrated nonheated counterparts with similar efficacy in the root canal. However, there is only little literature available on features of heated hypochlorite solutions relevant to the endodontics [5].

Preheating and Intracanal heating of NaOCl solution has greater ability to dissolve pulp tissue and cleanse the canal. Woodmansey has shown that sodium hypochlorite at boiling temperature is in a position to disintegrate the pulp tissue at speed 210 times higher compared to same solution at room temperature [6].

Chlorhexidine (CHX) is a broad spectrum antimicrobial agent having substantive antimicrobial activity with relatively low toxic effects. However it does not dissolve organic tissues. *In vitro* studies have shown CHX to exhibit sustained antimicrobial activity in the root canal for some time after being used as an endodontic irrigant. Therefore, CHX has been suggested as a root canal irrigant owing to its unique dentin binding ability, antimicrobial efficacy, and its property of substantivity in the root canal system [7].

Chitin is a natural polysaccharide obtained from crustacean shells, insect cuticles, and from fungal cell walls [8]. Alkaline deacetylation of chitin leads to the formation of chitosan [9]. Chitosan is a natural polysaccharide and is composed of co-polymers of glucosamine and N-acetylglucosamine. It is biocompatible, biodegradable and bioadhesive antimicrobial agent. Its production cost is low which has increased its utility for various applications related to the field of medicine and pharmaceuticals [7].

EDTA is commonly used after NaOCl as a final irrigant and Chelating Agent. EDTA weakens the bacterial cell membrane and exhibits some amount of antimicrobial activity. Some studies have also indicated it to be having antifungal properties [3].

There is limited literature done till date which compares the antimicrobial activity of all these irrigants at three different temperatures. Hence, this study compares irrigants at three different temperatures i.e. room temperature, warm temperature and intracanal heating for their antimicrobial efficacy. This is the first kind of study which evaluates the antimicrobial efficacy of 4 irrigants by intracanal heating of the irrigant.

Materials and Methods

Specimen Preparation

A total of 100 extracted human single rooted mandibular premolar teeth with patent root canals and fully developed root apices, extracted for periodontal or orthodontic reason, were selected for the study. Teeth having cervical caries, cracks in root, immature apex, resorbed roots, and calcified canals were excluded. Each tooth was sectioned below the cementoenamel junction with a diamond disk to obtain a standardized root length of 13 mm.

Specimen Treatment

Canal patency was established using 15 K file and instrumented using Pro-Taper rotary file system (Dentsply Maillefer, Ballaigues, Switzerland) up to an apical size of file F3. A total of 2 mL of 5% NaOCl was used between each instrument during the procedure, followed by irrigation with 17% ethylenediaminetetraacetic acid for 1 minute to remove the smear layer. Apical third of tooth was lined with 2 coats of nail polish. The teeth were steam autoclaved at 121 °C, 15 psi for 15 minutes.

Contamination of Specimen

Enterococcus faecalis American Type Culture Collection 29212 (National Chemical Laboratory, Pune, India) was streaked out on Blood agar, incubated for 48 hours at 37 °C. Histological slides were prepared with Gram's stains to confirm the presence of bacteria. A suspension was prepared by inoculating *E. faecalis* from pure culture into Tryptic soy broth, incubated at 37 °C for 24 hours, and adjusted to an optical density of 1nm with sterile Tryptic soy broth. Each root canal was completely filled with the infected broth by using sterile syringes. Samples were divided into five groups of 24 teeth each, and incubated at 37 °C for 21 days. Fresh broth was added to the canal every 48 hours. After 21 days, saline irrigation was done to eliminate the broth from the canals. Dentin was collected with H file and Gates Glidden drill No. 2 and dentinal shavings transferred into 1 mL of broth for each specimen and incubated for 24 hours at 37°C. The optical density of the broth was measured using digital colorimeter and initial readings were recorded. All the procedures were carried out in laminar air flow chamber.

Irrigation of the Specimens:

All the teeth were then subjected to irrigation under following groups:

- Group A: Irrigation with Sodium hypochlorite (n = 20)
- Subgroup I: Room temperature of the irrigant. (n = 10)
- Subgroup II: Intracanal heating of the irrigant. (n = 10)

- Group B: Irrigation with EDTA (n = 20)
  – Subgroup I: Room temperature of the irrigant. (n = 10)
  – Subgroup II: Intracanal heating of the irrigant. (n = 10)

- Group C: Irrigation with Chitosan (n = 20)
  – Subgroup I: Room temperature of the irrigant. (n = 10)
  – Subgroup II: Intracanal heating of the irrigant. (n = 10)

- Group D: Irrigation with Chlorhexidine (n = 20)
  – Subgroup I: Room temperature of the irrigant. (n = 10)
  – Subgroup II: Intracanal heating of the irrigant. (n = 10)

- Group E: Irrigation with Saline (n = 20)
  – Subgroup I: Room temperature of the irrigant. (n = 10)
  – Subgroup II: Intracanal heating of the irrigant. (n = 10)

Intracanal Heating of Irrigant

The samples in subgroup II were filled with the respective irrigants and an Obturating Heating Device (Elements, Sybron Endo) was used for intracanal heating of the irrigant. (Fig. 1)

Fig 1: Obturating Heating Device (Elements, Sybron Endo)

Irrigation in Subgroup I was performed by constant back and forth motion of needle from 2 to 4 mm from the working length for 30 seconds. Irrigant was left in the canal for 60 seconds. Irrigation in Subgroup II was done by attaching a tip of ISO 15/04 to the Obturating device (Elements, Sybron Endo) which was kept 3 mm short of the working length and heated at 180 degree Celsius for 3 seconds. 10 such cycles were repeated with replenishing the irrigant before each cycle. The excess irrigant was removed from the canal of all the samples by using absorbent paper points. In all the groups, 2 mL of irrigating solution was used for each sample. After irrigation procedure, dentinal shavings were collected from root canal of each tooth as previously mentioned and incubated for 24 hours at 37 °C. The optical density of the broth was measured using digital colorimeter and post irrigation readings were recorded.

Statistical analysis

The data on continuous variables was presented as Mean and Standard deviation (SD) across five study groups. The inter-group statistical comparison of continuous variables was done using analysis of variance with Bonferroni's Post-Hoc correction for multiple group comparisons. The intra-group statistical comparisons were done using paired t test in each study group. The underlying normality assumption was tested before subjecting each variable to t test and ANOVA

In the entire study, the p-values less than 0.05 were considered to be statistically significant. The entire data was statistically analyzed using Statistical Package for Social Sciences (SPSS ver 22.0, IBM Corporation, USA) for MS Windows.

Results

The post irrigation optical densities in all the groups were significantly lower in comparison with the preirrigation values when compared with ANOVA for independent samples ($p< 0.0001$) (Table 1).

Sodium hypochlorite and Chlorhexidine demonstrated better antimicrobial efficacy followed by Chitosan and EDTA, whereas the least efficacy was shown by Saline which was the control group.

Differences in optical density using different irrigants were found to be higher by intracanal heating of irrigants followed by room temperature irrigation ($p< 0.0001$) by Student's t-test, but the difference failed to reach statistical significance for all the groups (Table 2) (Fig. 2,3).

Discussion

This study evaluated the antimicrobial efficacy of five different irrigants on *Enterococcus faecalis* and the effect of different temperatures for reduction in the bacterial count of *Enterococcus faecalis*. Microorganisms and their products are considered to be the primary etiological agents in endodontic diseases. Success of endodontic treatment relies on complete elimination of bacteria and their toxic byproducts from the root canal system. Hence it is necessary to completely eliminate bacteria from the root canal system and prevention of recolonization or propagation of residual micro-organisms. This is done effectively by means of chemo-mechanical preparation and irrigation. Amongst these bacteria E- faecalis is the most dominant bacteria since it is the most commonly detected species in root filled teeth with persistent periapical lesions [10].

Chemical reaction rates accelerate with increases in temperature, pressure, and concentration. Since intracanal pressure cannot be increased, only the concentration or temperature of the irrigant can be increased to accelerate the chemical debridement. Increased irrigation temperatures can be achieved by preheating solutions before irrigation or by positioning heated instruments into the canal. Preheated solutions have limited usefulness due to their rapid equilibration to a temperature between body temperature and room temperature [11]. On the other hand, with similar short-term effectiveness in the immediate environment, i.e. the root canal system, the systemic toxicity of pre-heated NaOCl irrigants is considered to be lower than one of the more concentrated non-heated counterparts as temperature equilibrium is reached relatively quickly [12, 13]. Caution must be exercised to prevent overheating of the tooth's periodontal ligament (PDL). Exterior root surface temperatures above 47 ° C for more than 1 minute are considered to endanger the health of the PDL [14]. According to Eriksson and Albrektsson, the threshold temperature for bone survival is 47 °C for 1 min [15]. In a study conducted by Alfredo Iandolo, during intracanal heating of the irrigant, with an infrared thermometer (resolution: 0.1degree) the temperatures on the outer surface of the root were measured and were not found to be higher than 42.5 degree [14]. For the Continuous Wave of Condensation technique with the System B Heat Source, Buchanan recommends heating the plugger for less than 4 seconds for safety. When Hosoya, et al used intracanal heated Buchanan Pluggers to dry canals, 2 applications of 200 °C for 5 seconds were separated by a 5-second cooling interval. In general, to minimize the risk of PDL overheating with this irrigation technique, the Buchanan System B Plugger must remain passive and not be wedged against the canal walls. The plugger should only be heated in 3- to 5-second bursts and not continuously activated [11].

Sodium hypochlorite is a considered to be quite effective irrigant for all presentations of *E. faecalis* including its biofilm form. This antimicrobial action is because of the high pH (>11) and presence of OCl– ions (equivalent to hypochlorous acid), which facilitates its penetration into bacterial cell wall, chemical combination with protoplasm, and disruption of metabolic activities and deoxyribonucleic acid synthesis [16]. The findings of a study by Gambarini et al showed that heating NaOC1 has no adverse effect on the chemical stability of the solutions [17].

Chlorhexidine digluconate (CHX) has been suggested as a root canal irrigant owing to its unique ability to bind to dentin, its effectiveness as an antibacterial agent against *E. faecalis* and its substantivity in the root canal system [7]. CHX acts by absorbing into the cell wall of the microorganism and causing leakage of cytoplasmic substances [18].

In a study conducted by Jaiswal N, Chitosan was used for the first time as root canal irrigating solution and it exhibited good antimicrobial effect against E. faecalis. The possible reason for the antimicrobial action of chitosan might be due to the mechanism of action of chitosan that possesses the positively charged NH3 + groups of glucosamine that interacts with negatively charged surface components of bacteria, resulting in extensive cell surface attraction, leakage of intracellular substances, and causing damage to vital bacterial activities [7].

The present study demonstrates the antibacterial efficacy of chitosan almost equivalent to 5% NaOCl, This possible animal extract can well be substituted as an endodontic irrigant to mitigate the adverse effects of traditional irrigants (NaOCl and chlorhexidine) on dentin. NaOCl is considered to be cytotoxic to tissues and a need for replacement with a more biocompatible irrigant is necessitated [19]. Saline was taken as negative control which as expected has least antimicrobial activity.

In this study, all the groups showed reduction in the bacterial count of *Enterococcus faecalis*, however complete eradication of this refractory organism was not achieved. However, even though complete eradication was not achieved, intacanal heating still proved to be much better in reducing the bacterial count of *Enterococcus faecalis*. The results suggest that difference in optical density for all irrigants was higher in intracanal heating (180 ^{0}C) group followed by warm irrigation group (60 ^{0}C), but EDTA group showed better efficacy in warm irrigation group. Hence, future research is required to explore this behaviour of EDTA. The intracanal heating of different irrigants can be considered as a research project in near future.

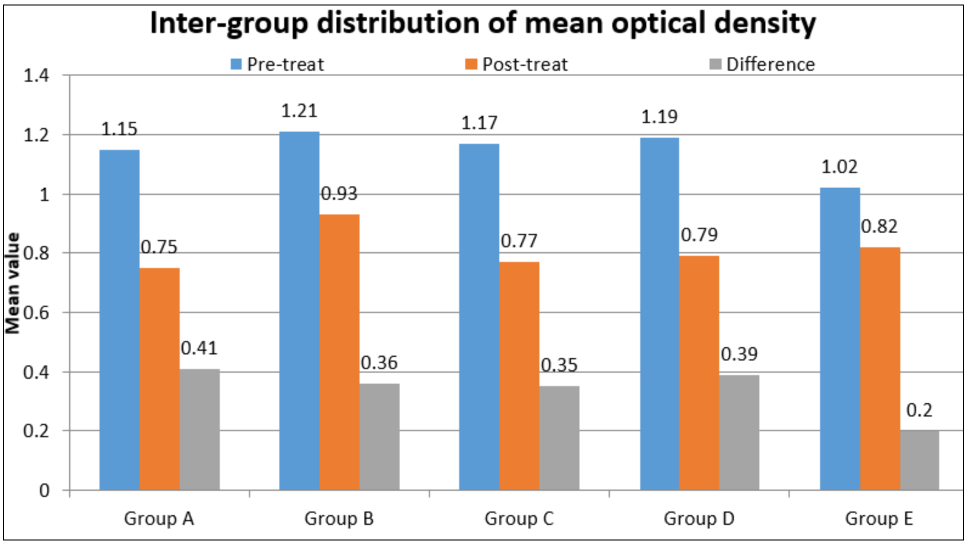

Fig 2: Intergroup comparison of mean optical density

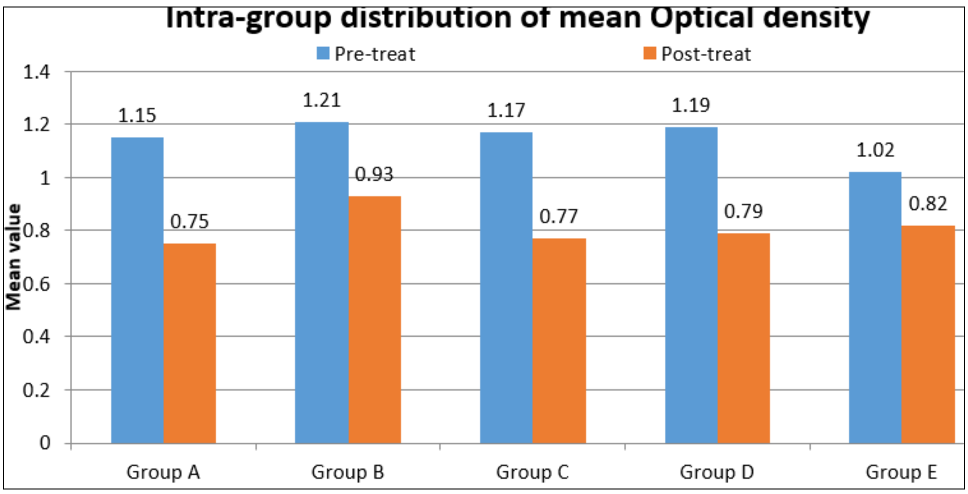

Fig 3: Intra group comparison of mean optical density

Table 1: Intergroup distribution of mean optical density

	Group A (n=20) [NAOCL]		Group B (n=20) [EDTA]		Group C (n=20) [CHITOSAN]		Group D (n=20) [CHLORHEXIDINE]		Group E (n=20) [SALINE]	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Pre-treat	1.15	0.11	1.21	0.18	1.17	0.16	1.19	0.16	1.02	0.09
Post-treat	0.75	0.12	0.93	0.19	0.77	0.21	0.79	0.14	0.82	0.09
Difference	0.41	0.09	0.36	0.26	0.35	0.07	0.39	0.07	0.20	0.05
P-value (Inter-Group)	0.001***		0.001***		0.001***		0.001***		0.001***	

P-value (Inter-Group) Comparisons by paired t test. P-value<0.05 is considered to be statistically significant. ***P-value<0.001, SD- Standard deviation.

Table 2: Intra-group comparison of mean optical density.

		Group A (n=20) [NAOCL]		Group B (n=20) [EDTA]		Group C (n=20) [CHITOSAN]		Group D (n=20) [CHX]		Group E (n=20) [SALINE]	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Difference	Group I (n=10)	0.37	0.05	0.24	0.04	0.31	0.05	0.35	0.05	0.15	0.02
	Group II (n=10)	0.46	0.09	0.33	0.07	0.41	0.06	0.43	0.08	0.24	0.04
P-value (Intra-Group)	Group I vs Group II	0.120NS		0.999NS		0.012*		0.126NS		0.001***	

P-values by ANOVA with Post-Hoc Bonferroni's correction for multiple group comparisons. P-value<0.05 is considered to be statistically significant. *P-value<0.05, **P-value<0.01, ***P-value<0.001, NS-Statistically non-significant, SD-Standard deviation.

Conclusion

Within the limitations of this study its can be concluded that - Chitosan exhibited antimicrobial effect similar to Chlorhexidine and Sodium Hypochlorite.

Most of the groups exhibited higher antimicrobial efficacy with the intracanal heating as compared to that of room temperature of the irrigant.

Conflict of Interest

Not available

Financial Support

Not available

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