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In vitro evaluation of antimicrobial efficacy of 2% chlorhexidine loaded electrospun nanofibers

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ABSTRACT

Introduction: Persistent endodontic infection might be due to retention of microorganisms in dentinal tubules of root canal. C. albicans and E. faecalis are commonly found micro organisms in root canals of previously treated teeth. These micro organisms have shown to be resistant to most of the anti microbial agents used during root canal treatment but were found to be sensitive to Chlorhexidine (CHX).

Aim: This *invitro* study was conducted to evaluate the efficacy of 2% Chlorhexidine loaded eletrospun nanofibres and compare them with Chlorhexidine solution and gel in a pour plate or traditional streak agar diffusion model.

Methodology: Standard holes in the cultivated Sabroaud's Dextrose and Muller Hinton agar plates were filled with one of the CHX preparations i.e 2% Chlorhexidine gluconate solution, 2% CHX gel, 2% CHX loaded electrospun nanofibre. The zones of microbial inhibition were measured after 24 and 72 hours of incubation at 37°C. Values obtained were subjected to statistical analysis.

Results: CHX Nanofibre showed values of zone of inhibition higher than solution ($p \leq 0.05$) and similar to that of gel.

Conclusion: CHX NF and gel had similar antimicrobial efficacy and CHX solution was least effective.

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1. Introduction

The main objective of root canal treatment is to eliminate microorganisms and prevent reinfection of root canal. Endodontic treatment success majorly depends on cleaning and shaping of root canals and intracanal medicament placement further enhances the reduction in number of microorganisms. Even after thorough mechanical instrumentation and effective irrigation few microorganisms have the ability to survive in adverse conditions. *Enterococcus faecalis* and *Candida albicans* are the most commonly found

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microorganisms in the therapy resistant and retreatment cases due to their altered virulence factors.[1] Hence, the intracanal medicament placed should be available for longer time periods and reach the microorganisms present in the inaccessible and distant areas of root canal. Various studies have reported susceptibility of *E. faecalis* and *C. albicans* to antimicrobial activity of 2% chlorhexidine (CHX). CHX is a cationic bis-biguanide with broad spectrum antimicrobial activity and substantivity.

In endodontics, CHX is used in solution and gel form. The positively charged molecules of CHX can adsorb onto the dentin, bind with the negatively charged molecules of cell wall and lead to damage of cell contents thus preventing microbial colonization on the dentin surface. But the activity of CHX solution is for a limited period of time and CHX gel cannot be completely removed from the canal walls.[2,3] Hence, there is requirement of methods that could provide substantive antimicrobial activity. Over the past few years drug-loaded nanopharmaceuticals have been used in dentistry. Various natural (collagen, chitosan, chitosan composites, silk fibroin) and synthetic (polylactic glycolic acid, polyvinyl alcohol (PVA), polydioxanone) compounds were used as scaffold to provide extended drug delivery.[4]

In our study, PVA polymer, a white water-soluble synthetic polymer, was used as it has advantages like it degrades faster, cost effective, inert and lack of leachable impurities.[5] PVA was used to prepare nanofibers (NFs) using electro-spinning technique. This technique allows fabrication of porous nanofibrous webs with specific characteristics like three-dimensional morphology, large surface area to volume ratio, higher colloidal stability, dispersibility and controlled drug delivery.[6] In the present study, CHX-loaded electrospun PVA NFs were compared with CHX solution and gel.

2. Methods

2.1. Different vehicles of 2% CHX

Commercially available 2% (v/v) CHX gluconate solution (V Consept, Kayvee Pharmaceuticals, India) and 2% (w/v) CHX gluconate gel (Consepsis V, Ultradent, USA) were used.

2.1.1. Preparation of CHX loaded electrospun NFs

PVA (Sigma–Aldrich, St Louis, MO) was dissolved at 800 mg (8 wt%) concentration with 10 ml of triple-distilled water and stirred for 8 h. CHX powder (Sigma–Aldrich) was directly added to the PVA polymer solution to obtain 2% concentration. The solution was mixed together thoroughly under vigorous stirring for overnight. The CHX containing polymer solution was loaded into a 5 ml plastic syringe (Becton-Dickinson Biosciences, NJ) fitted with a metallic 26-gauge needle, and the fibers were obtained on an aluminum foil using a laboratory based electrospinning apparatus (PICO ESPIN, India). The electrospinning parameters for fibers synthesis were optimized at 12–15 kV potential, 15 cm tip to target distance and at a flow rate of 0.4 ml/h. Thus, formed fibers were stored in desiccator at 4 °C until used. The fibers formed were sterilized under UV light chamber. The electrospun fiber morphology was investigated under a scanning electron microscope (SEM, EVO 18, Zeiss). The diameter of the fiber was measured using Image-J software (National Institute of Health, Bethesda, MD).

2.2. Preparation of E. faecalis and C. albicans inoculum for culture

A loop full of freeze dried *E. faecalis* and *C. albicans* spores, commercially procured from MTCC 439 and 7315 respectively were mixed in 5 ml of distilled water to convert the inactive into active form for stock culture. *E. faecalis* and *C. albicans* inoculum were prepared by picking a loop full of colonies from the stock plate and adding into brain heart infusion broth and Sabouraud's dextrose broth respectively. These prepared broths were incubated at 37 ± 2 °C for 24 h and McFarland adjustment to 0.5 standards was carried out for preparation of microbroth.

2.3. Preparation of culture media for tests

Mueller Hinton agar (MHA) and Sabouraud's dextrose agar (SDA) were prepared for *E. faecalis* and *C. albicans* culture respectively according to manufacturer's instructions by completely dissolving the media powder in distilled water and sterilized by autoclaving at 15 lb pressure and 121 °C for 15 min. After sometime when the media attained room temperature, 0.5 ml of fungal inhibitory concentration of clotrimazole in MHA and bacterial inhibitory concentration of gentamycin in SDA was added and poured in the sterile petridishes of 2 mm thickness and allowed to solidify.

2.4. Determination of antimicrobial efficacy

Agar well diffusion method: 24 h inoculum of both organisms, cultured on respective agar, were adjusted in sterile saline to the McFarland 0.5 standards to yield a cell density of 1.5×10^8 CFU/ml and plated using a lawn culture technique on respective agar medium. On the agar plate, wells were cut using a heat sterilized 3 mm borer and 20 μl of each test solution was dispensed into the wells. Positive growth controls and negative controls of sterile distilled water and methanol were also incubated along with the test plates at 37 ± 2 °C for 24 h and 72 h. Following incubation the antimicrobial activity was evaluated by measuring the mean diameter of zones of inhibition using a vernier caliper. The zone of inhibition around each sample was calculated by measuring its total size and then subtracting the sample size.

3. Results

In the present study, the formed fiber was characterized by SEM analysis; the PVA NFs containing CHX were composed of randomly arranged fibrous structures and diameter of fiber measured was observed to be ranging from 203 to 277 nm (Fig. 3).

Antimicrobial efficacy of 2% CHX in different vehicles was evaluated by recording the mean diameter of the zone of inhibition obtained on days 1 and 3 (Figs. 1 and 2). The results were recorded and analyzed statistically by paired 't' test and compared by post-hoc Tukey HSD test.

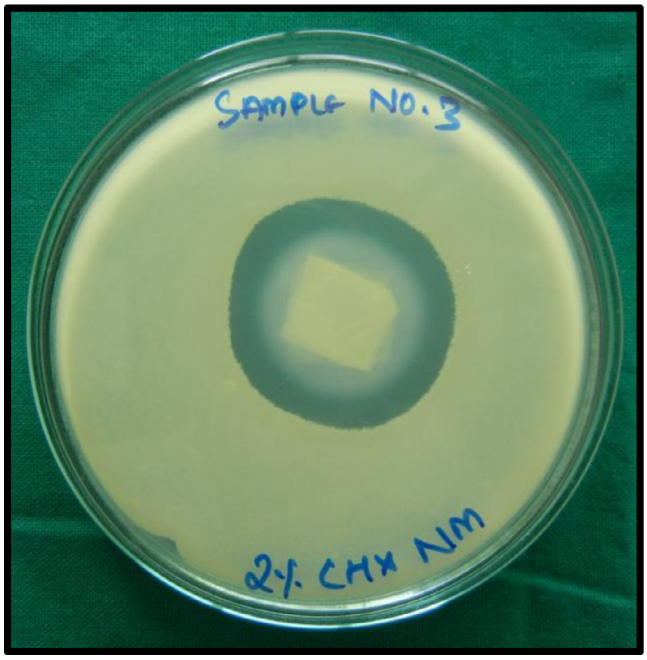


Fig. 1 – Zone of inhibition in *C. albicans* lawn culture.

On days 1 and 3 for *C. albicans* the mean value of zone of inhibition of CHX NF was higher compared to CHX solution and lower than CHX gel. On intergroup comparison CHX solution and gel showed statistical significance with p value ≤0.05 (Tables 1 and 2). For *E. faecalis* on days 1 and 3, the mean values of zone of inhibition obtained for CHX NF was higher compared to CHX solution and lower than CHX gel. On intergroup comparison CHX solution, gel and NF showed statistical significance with p value ≤0.05 (Tables 3 and 4).

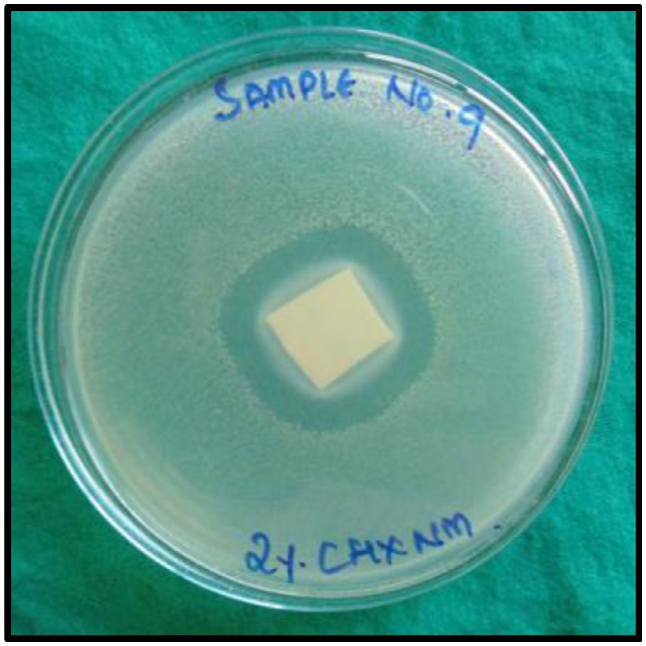


Fig. 2 – Zone of inhibition in *E. faecalis* lawn culture.

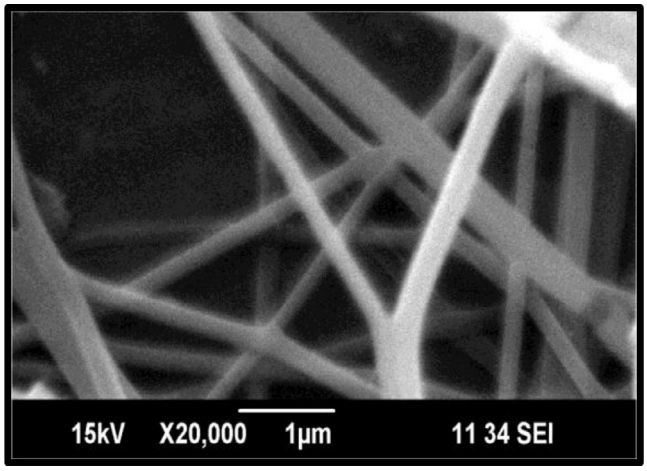


Fig. 3 – SEM image of CHX-loaded PVA NF.

Table 1 – Intragroup comparison of mean values of zone of inhibition in *C. albicans*.

	C. albicans		
Groups	Mean values (mm)		p value ($p \leq 0.05$)
	Day 1	Day 3	
CHX sol	51.33	50.73	0.144
CHX gel	59.86	57.60	0.001
CHX NF	57.40	55.20	0.001

Statistical analysis: Paired 't' test, statistically significant if $p < 0.05$.

Table 2 – Intergroup comparison of mean differences at both experimental periods.

	Candida albicans		
Inter-group comparisons	Experimental periods	Mean differences	p value ($p \leq 0.05$)
CHX sol vs. gel	Day 1	8.533	0.006
	Day 3	6.866	0.032
CHX sol vs. NF	Day 1	6.066	0.065
	Day 3	4.466	0.216
CHX gel vs. NF	Day 1	2.466	0.619
	Day 3	2.622	0.634

Statistical analysis: Post-hoc Tukey HSD, statistically significant if $p < 0.05$.

Table 3 – Intragroup comparison of mean values of zone of inhibition in *E. faecalis*.

	Enterococcus faecalis		
Groups	Mean values (mm)		p value ($p \leq 0.05$)
	Day 1	Day 3	
CHX sol	42.26	40.13	0.001
CHX gel	56.00	54.86	0.001
CHX NF	54.00	53.00	0.001

Statistical analysis: Paired 't' test, statistically significant if $p < 0.05$.

Table 4 – Intergroup comparison of mean differences at both experimental periods.

E. faecalis			
Inter-group comparisons	Experimental periods	Mean differences	p value ($p \leq 0.05$)
Group 1 vs. 2	Day 1	13.721	0.000
	Day 3	14.701	0.000
Group 1 vs. 3	Day 1	11.700	0.000
	Day 3	12.861	0.000
Group 2 vs. 3	Day 1	2.000	0.614
	Day 3	1.866	0.653

Statistical analysis: Post-hoc Tukey HSD, statistically significant if $p < 0.05$.

4. Discussion

The agar diffusion method is the most accepted procedure to test the antimicrobial susceptibility of intracanal medicaments in routine laboratory conditions. This method is most simple, standard and reproducible method for determining in-vitro sensitivity.[1] Thus, in the present study agar diffusion model was used. CHX NF was compared with CHX solution and CHX gel for its antimicrobial efficacy.

The CHX-loaded PVA NFs were smooth, continuous, linear without any bead formation. This indicates the various electrospinning parameters such as molecular weight, viscosity, conductivity, voltage potential, tip to target distance and flow rate were appropriate for synthesis. Kinetics of drug release from PVA occurs in two stages: diffusion and hydrolytic degradation. Diffusion of drug from the matrix occurs as a result of difference in concentration gradient on the matrix surface and core.[7] Thus, the initial burst release of drug from the matrix occurs. Hydrolytic degradation of the matrix core occurs due to break in linkage between the drug and the polymer.[8] In the present study, it was observed that CHX NF was as effective as gel, i.e. the initial burst release of drug from NF showed antimicrobial activity similar to gel. The drug release from the NF might have occurred due to diffusion and dispersion.

In the present study, it was observed that CHX NF still persisted even after the experimental time period of 1–3 days. This may be due to high molecular weight polymer used and limited availability of aqueous medium in in vitro conditions, thus limiting the hydrolytic degradation of the NFs and drug release. In a study by Samprasit et al. it was observed that high molecular weight fibers showed better stability upto 3 months compared to low molecular weight fibers when immersed in phosphate buffered saline.[9] Hence, in in vivo conditions where there will be more availability of aqueous medium better efficacy of drug loaded NFs due to hydrolytic degradation, and higher drug release can be expected. Further, in vitro studies viz. drug release kinetics, degradation are to be evaluated. In vivo studies for its clinical efficacy, and multidrug loaded NFs have to be tested.

5. Conclusion

Within the limitations of the study the results suggest that CHX-NF is as effective as CHX gel on both *E. faecalis* and *C. albicans*. PVA NFs can serve as an alternative to gel formulations for delivery of CHX.

Conflicts of interest

The authors have none to declare.

REFERENCES

1. Turk BT, Sen BH, Ozturk T. In vitro antimicrobial activity of calcium hydroxide mixed with different vehicles against *Enterococcus faecalis* and *Candida albicans*. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod*. 2009;108:297–301.
2. Basrani B, Santos M, Tja L, et al. Substantive antimicrobial activity in chlorhexidine-treated human root dentin. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod*. 2002;94:240–245.
3. Gomes PFA, Vianna ME, Zaia AA, Almeida JFA, Souza-Filho FJ, Ferraz CCR. Chlorhexidine in endodontics. *Braz Dent J*. 2013;24:89–101.
4. Virlan MJR, Mirisecsu D, Radulescu R, et al. Organic nanomaterials and their applications in the treatment of oral diseases. *Molecules*. 2016;21:207–230.
5. Srivastava A, Yadav T, Sharma S, Nayak A, Kumari A, Mishra N. Polymers in drug delivery. *J Biosci Med*. 2016;4:69–84.
6. Ashammakhi N, Wimpenny I, Nikkola L, Yang Y. Electrospinning: methods and development of biodegradable nanofibres for drug release. *J Biomed Nanotechnol*. 2009;5:1–9.
7. Fu Y, Kao WJ. Drug release kinetics and transport mechanisms of nondegradable and degradable polymeric delivery systems. *Expert Opin Drug Deliv*. 2010;7:429–444.
8. Fredenberg S, Wahlgren M, Reslow M, et al. The mechanisms of drug release in poly (lactic-co-glycolic acid)-based drug delivery systems – a review. *Int J Pharm*. 2011;415:34–52.
9. Samprasit W, Rojanarata T, Akkaramongkolporn P, Ngawhirunpat T, Kaomongkolgit R, Opanasopit P. Fabrication and in vitro/in vivo performance of mucoadhesive electrospun nanofiber mats containing α-mangostin. *AAPS Pharm Sci Tech*. 2015;16:1140–1152.