'To evaluate the influence of different irrigant's agitation methods on penetration depth of 2% Chlorhexidine Digluconate and Chitosan into radicular dentinal tubules using Conefocal Laser Scanning Microscope (CLSM) - An in-vitro study.'

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Abstract

Objective: 'To evaluate the influence of different irrigant's agitation methods on penetration depth of 2% Chlorhexidine digluconate and Chitosan into radicular dentinal tubules using Confocal Laser Scanning Microscope.

Methods: Total 88 single canal maxillary anteriors were selected for this study. The coronal portion of each tooth was sectioned using diamond disks to standardize the root length at 15 mm. The working length of all the samples were determined 1mm short of the apical foramen. Then cleaning and shaping was done using crown down technique with Rotary Protaper Gold system up to file no. F_4 corresponding to size no.40. The canals were irrigated with 1 ml of 5.25% NaOCl between each instrumentation. After that, canals were rinsed with 3 ml of 17 % EDTA for 1 minute and finally rinsed with saline for 1 min. A solution of about 0.1 g of Rhodamine b dye was mixed individualy with 500 ml of both 2 % CHX solution and Chitosan solution which is further reffered as 2 % CHX + R and 2 % chitosan+R, which were the final irrigants respectively. Viscosity and surface tension of 2 % CHX + R and 2% Chitosan + R were checked using Viscometer and Staglomometer. According to two irrigants (Chlorhexidine and Chitosan) 88 samples were divided into two experimental groups:-

1. Group I :- 2% CHX+R solution **(N=44)**

2. Group II :- 2% Chitosan+R solution **(N=44)**

These two groups further divided into four subgroups on the basis of different agitation methods (conventional needle irrigation, manual dynamic agitation, sonic irrigation, passive ultrasonic irrigation) containing N=11 in each subgroup.

After agitation procedures the samples were cross sectioned at 3 mm (apical), 6mm (middle) and 12mm (coronal) levels using diamond discs to obtain a dentin section of 1 mm thickness. The section were viewed under Conefocal Laser Scanning Microscope to evaluate the penetration depth of irrigants in radicular dentin and the results obtained were subjected to statistical analysis by one way ANOVA and Tukey Post Hoc test.

Results: Comparing the penetration depth of 2% chlorhexidine and chitosan within subgroups at different levels, there was statistically significant difference in depth of penetration at coronal, middle and apical 3rd levels. Overall comparing all the groups 2% Chlorhexidine digluconate shows maximum depth of penetration than the Chitosan.

Conclusions: With passive ultrasonic irrigation Chlorhexidine and Chitosan were able to penetrate more in the apical third of root canal as compared to other devices and the difference was statistically significant.

Key words: 2% Chlorhexidine Digluconate, Chitosan, Agitation Methods, Surface Tension, Viscosity.

INTRODUCTION

Bacteria play an essential role in the inception, progression and persistence of apical periodontitis and pathology.[1] Therefore, endodontic therapy aims to eradicate bacteria from the infected root canal and prevent infection. Cleaning through irrigating & shaping of the root canal greatly reduces the number of bacteria but doesn't completely eliminate them. Concern exists not only to the consequence of bacteria left in the root canal but also to those micro organisms that remain in the dentinal tubules.[2,3] Tubule diameter are larger near the pulpal cavity (3 to 4µm) and smaller at their outer end (1µm).[4]

Invitro studies have shown that bacteria are able to penetrate dentinal tubules of the root upto 800µm, when the cementum is removed from the root surface and the smear layer from the root canal wall.[5,6] Sen et al[7] found bacteria and yeasts in the dentinal tubules of extracted teeth in a range from 10 to 150µm using scanning electron microscopy. It has been hypothesized that these viable bacteria within the root canal system and dentin tubules can be a source of reinfection or continued periapical inflammation.[8] To prevent reinfection of a treated root canal, it is important to disinfect the pulp space & dentinal tubules thoroughly with an endodontic irrigant. Important requirements of an endodontic irrigant include properties such as antimicrobial activity, tissue dissolving property and nontoxicity to the periapical tissues.[9,10] There have been many studies performed to determine which root canal medicines and irrigants are most effective. Among most of the microorganisms *E.faecalis* plays a major role in root canal failures as it can invade into the dentinal tubules which is inaccessible for any chemomechanical disinfection or host defence mechanism.[11] Several studies have reported that sodium hypochlorite, Ethylene diamine tetra acetic acid, Calcium hydroxide are ineffective on *E.faecalis*.[8] It has been shown that, at present 2% Chlorhexidine is effective in the elimination of E.faecalis from dentinal tubules.[12] Apart from the positive antimicrobial efficacy of chlorhexidine, it has got substantivity. chlorhexidine can bind to the hydroxyapatite of the dentine[13] and a gradual release of this bound chlorhexidine may protect the canal against microbial colonization beyond the actual medication period. CHX was used because invitro studies reported that chlorhexidine completely inhibited the growth of *E.faecalis*.[14]

Other new root canal irrigants used in endodontics are Chitosan , MTAD, Tetraclean, Electrochemically Activated solutions, Ozonated water, Photon-Activated

Disinfection, Herbal irrigants etc. The irrigant's flow and depth of penetration are primarily affected by its viscosity and surface tension. Since the wettability of such solutions is crucial to their ability to penetrate the main and lateral canals and predentin dentinal tubules, lowering the surface tension of an endodontic irrigants might increase its efficacy. [15] Chitin is the second-most abundant natural polysaccharide composed of β- (1, 4) -linked N-acetyl glucosamine units. Partial deacetylation of chitin results in the production of chitosan. It has shown a large number of pharmaceutical applications like in drug delivery, peptide delivery, as an absorption enhancer and in gene delivery.[15]

Even Activation of irrigants increase the efficacy of irrigants to a great extent.[16] Various irrigant activation systems used are sonic and ultrasonic agitation, manual activation with gutta-percha cones and agitation with brushes and conventional needle irrigation, Endovac system etc. The performance of activation systems varies because of complex anatomy of the root canal systems.[17]so the study has been designed to evaluate the penetration depth (coronal, middle, apical) of CHX and Chitosan.

MATERIALS AND METHODS

Ethical Reference

The protocol of this study was scientifically approved by the Institutional Ethical Community of Surendera Dental College and Research Institute, Rajasthan, India at clearance number (IEC reference no. SDCRI/IEC/2020/011).

Sample Selection:

Total 88 single canal maxillary anteriors were selected for this study. All teeth were intact, straight mature roots, without caries lesion and completely formed root apex were selected and seen under stereomicroscope. Teeth with more than one root canal, root caries, resorption and apical curvature and teeth with previous endodontic treatment were excluded from the study. Teeth were carefully cleaned with curettes to remove any calculus or soft tissue debris and were stored in stored in 0.1% thymol solution until ready for use.

Endodontic Procedure:

Total 88 samples were decoronated with the help of a diamond disk to standardized root length to 15mm. Access cavities were modified cervically and working length was determined by inserting a size #10k file (Dentsply Maillefer, Ballaigus, Switzerland) till it was just visible at the apical foramen and 1mm was deducted from it. The coronal portion of canal was enlarged by Sx protaper gold (**Dentsply Maillefer, Ballaigues)** then cleaning and shaping was done using crown down technique with Rotary Protaper Gold system up to file no, F_4 corresponding to size no.40. The canals were irrigated with 1 ml of 5.25% NaOCl (**V Dentcare, Gujrat, India)** between each instrumentation. After that, canals were rinsed with 3 ml of 17 % EDTA **(Prevest, Jammu**, **India**I) for 1 minute and finally rinsed with saline for 1 min. The canals were dried using absorbent point.

Preparation of dye

A solution of about 0.1 g of Rhodamine b dye (**Ases Chemical works)** was mixed individualy with 500 ml of both 2 % CHX solution **(V Dentcare , Gujrat, India)** and Chitosan solution (Everest Biotech ,Benguluru, Karnataka) which is further reffered as 2 % CHX + R and 2 % chitosan+R,which was the final irrigant.

Viscosity and surface tension of 2 % CHX **(V Dentcare , Gujrat, India)** and CHX + R and 2% chitosan **(Everest Biotech ,Benguluru, Karnataka)** and Chitosan + R was checked using Viscometer **(ABG 398)** and Staglomometer **(S.P. Crystal Glass).**

Grouping of the samples:

According to final irrigants (Chlorhexidine and Chitosan) total 88 samples divided into two experimental groups:- Group I :- 2% CHX+R solution and Group II :- 2% Chitosan+R solution, containing 44 samples in each group. Then these groups were further subdivided into 4 ubgroups according to different agitation methods (manual dynamic irrigation, conventional needle irrigation, sonic & ultrasonic irrigation) containing 11 teeth each.

In conventional needle irrigation A 30 gauge side vented needle was used for irrigation.Total 3ml of CHX + R solution was used for 3 min. The tip of the needle was placed 1 mm short of working length and moved up and down with 2mm amplitude during irrigation. The irrigant was delivered at a rate of 1ml /min.

In Mannual dynamic agitation 3ml of CHX+ R solution was agitated using 20 no. gutta purcha cone to the working length and moved in a corono-apical direction using back and forth strokes for 3 minutes. This sequence of irrigation was repeated for three times.

In Sonic irrigation, the root canals was agitated using a flexible tip of super endo sonic irrigator of size 20 , 2 % taper will be inserted in the root canal, 1 mm short of the working length. The solution was sonic activated for 1 minute; this sequence of irrigation was repeated for three times.

In Passive Ultrasonic Irrigation, the root canal was agitated using a U file of size 20 , 2 % taper was inserted into the canal, at 1mm short of working length. The irrigant was ultrasonically activated for 1 minute at a power setting of 3 with a ultrasonic unit. This sequence of irrigation was repeated thrice, the time was 3 minutes and a total irrigation volume of 3 ml was taken.

In Group II (2% Chitosan+R) was taken as a final irrigant solution and then solution was agitated with different methods as done in Group I.

Confocal laser scanning microscopy and Statistical Analysis:

Finally the samples were cross sectioned at 3 mm (apical), 6mm (middle) and 12mm (coronal) levels using diamond discs to obtain a dentin section of 1 mm thickness. The section was viewed under CLSM at 2x magnification to evaluate the penetration depth of 2 % CHX+ R and 2% Chitosan+R in radicular dentin and the mean and standard deviation values of dye penetration of different groups were taken and data was then subjected to statistical analysis using a one way ANOVA and Post Hoc Tukey's HSD comparison test. The significance level used was $P<0.05$.

Fig 1: Decoronated permanent single rooted teeth

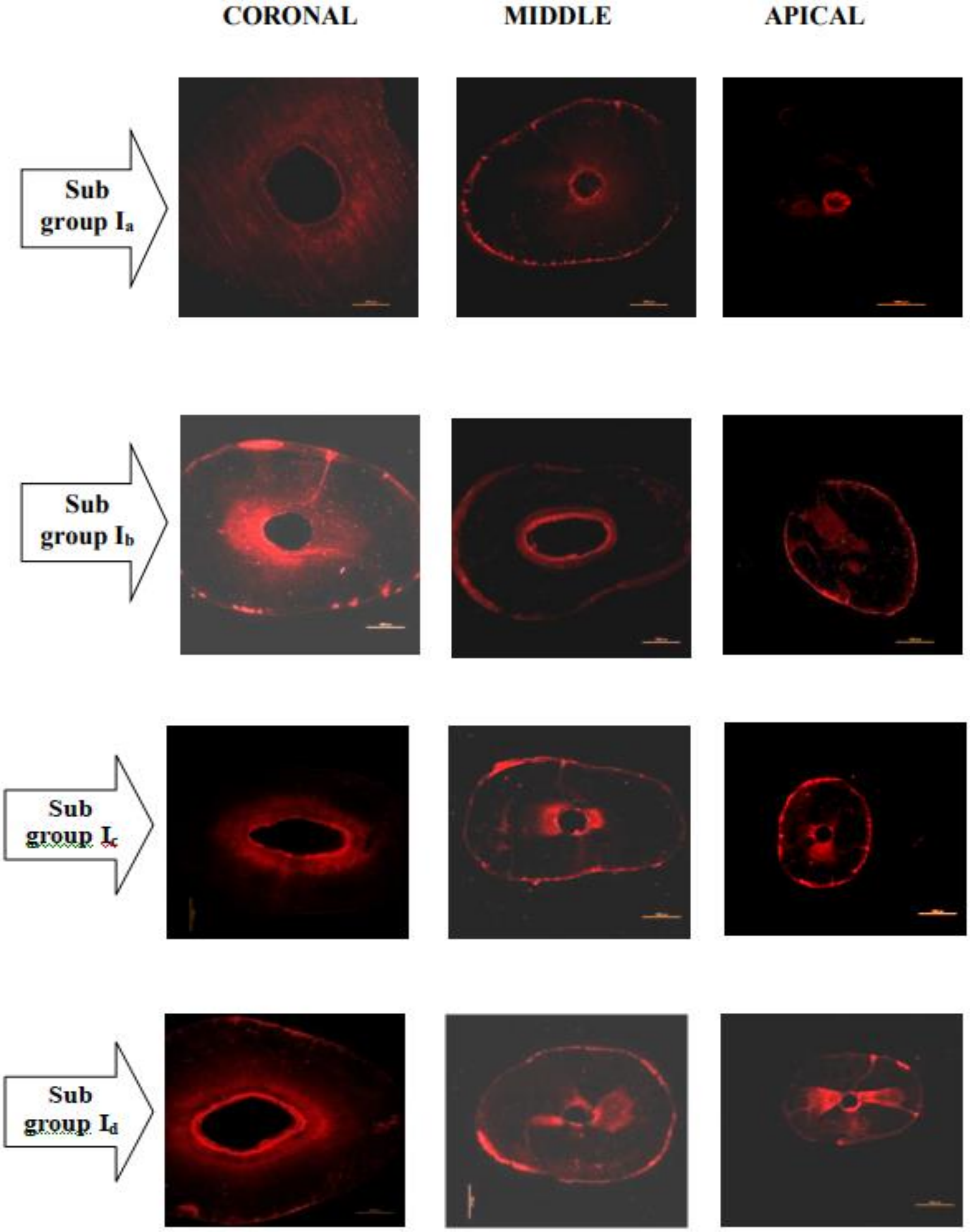


Fig 2: Images of penetration depth of (CHX+R) at different levels.

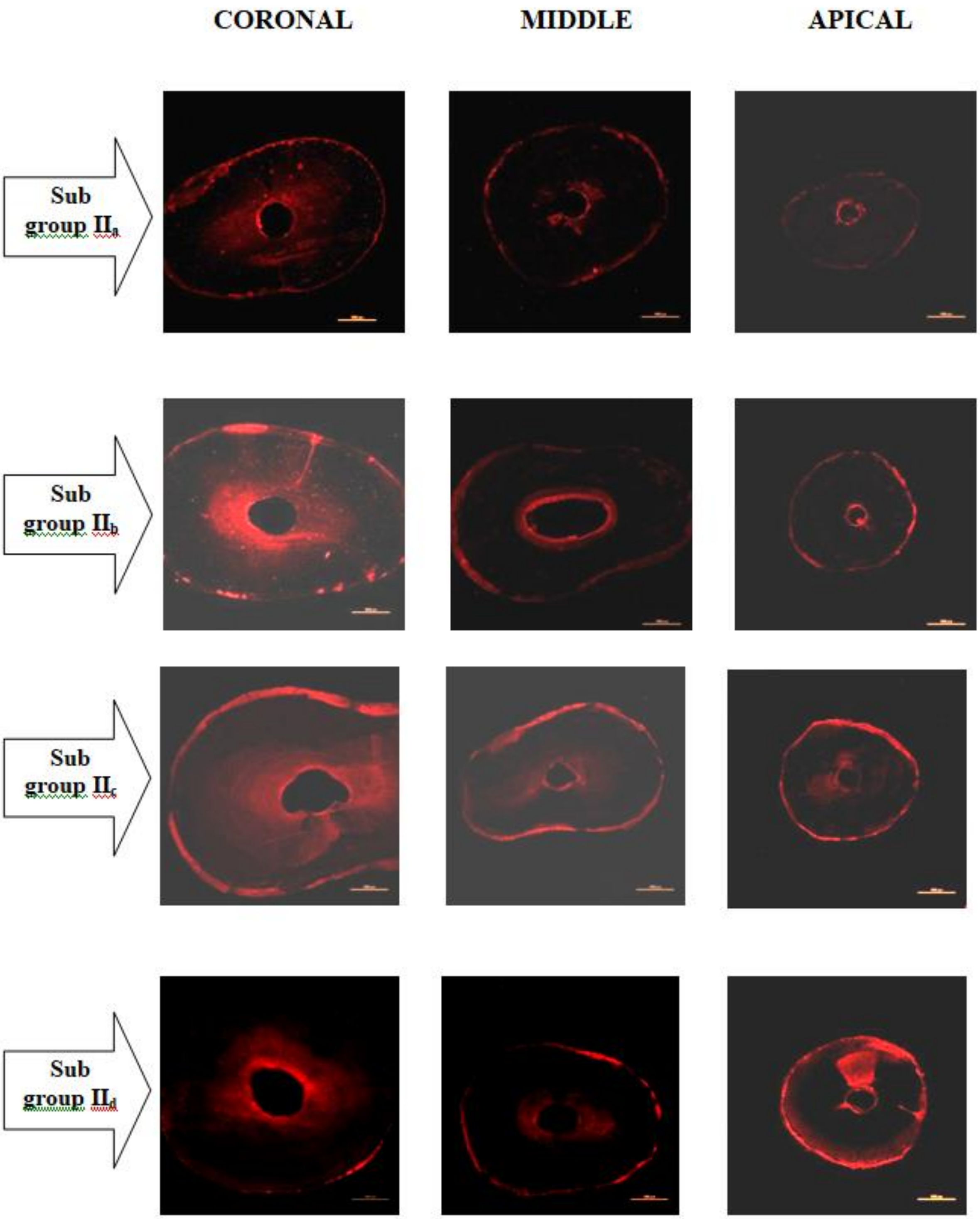

CORONAL
MIDDLE
APICAL
Sub group IIa
Sub group IIb
Sub group IIc
Sub group IId

Fig. 3:- Images of penetration depth of (CHITOSAN+R) at different levels

RESULT:

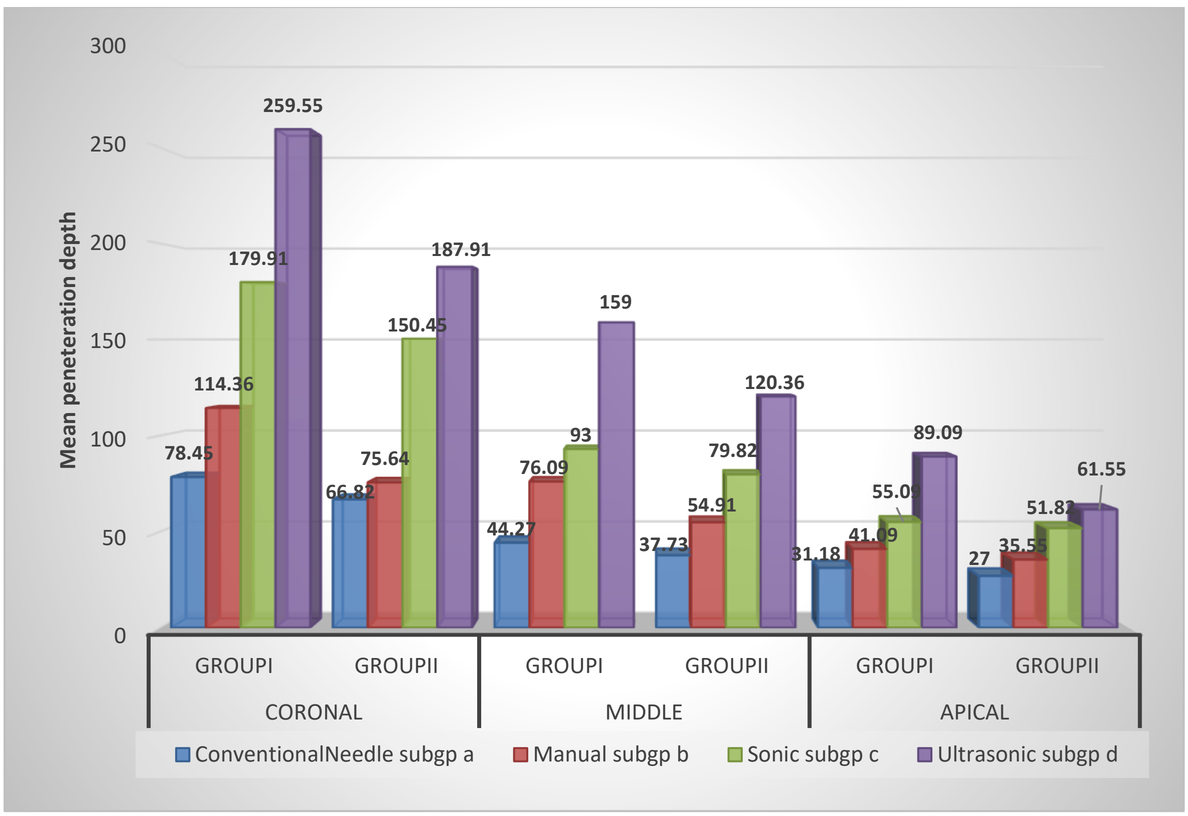
According to the maximum penetration depth of Chlorhexidine and Chitosan into the dentinal tubules, subgroups were ranked in the order of ultrasonic > sonic > manual dynamic > conventional needle irrigation. A statistically significant result was seen at coronal, middle and apical levels with respect to all the subgroups ($p < 0.01$).

The maximum penetration depth of disinfectants 2% Chlrohexidine digluconate and Chitosan showed at coronal , middle and apical level with ultrasonic and sonic irrigation system and least penetration with manual dynamic and conventional needle irrigation delivery system.

Intergroup comparison showed, that when we compared both disinfectants, there was non significant difference coronal level **(**p value = 0.098) and apical level **(**p value = 0.062) **.**But significant difference was seen at middle level **(**p value = 0.003***) with conventional irrigation delivery system. By **Manual Irrigation** method both groups showed a significant difference at coronal, middle & apical **(**p value <0.01*). By **Sonic Irrigation** method there was a significant differences at coronal level & middle & apical level **(**p value = 0.03***).** By **Ultrasonic Irrigation** method, there was a significant differences at coronal, Middle and apical level **(**p value <0.01***).** It is depicted in graph and table.

Section	**Coronal**		**Middle**		**Apical**	
Groups → **Subgroups ↓**	**Group I**	**Group II**	**Group I**	**Group II**	**Group I**	**Group II**
Conventional Needle subgp a	78.45±9.69	66.82±19.98	44.27±4.05	37.73±4.96	31.18±4.24	27.00±5.57
t test value	1.738		3.389		1.982	
P value	0.098		0.003*		0.062	
Manual subgp b	114.36±13.58	75.64±19.24	76.09±5.84	54.91±12.52	41.09±4.64	35.55±6.27
t test value	5.455		5.086		2.359	
P value	<0.01*		<0.01*		0.029*	
Sonic subgp c	179.91±13.63	150.45±25.94	93±11.28	79.82±6.11	55.09±7.29	51.82±5.12
t test value	3.334		3.408		1.219	
P value	0.03*		0.03*		0.237*	
Ultrasonic subgp d	259.55±37.72	187.91±20.41	159.00±28.49	120.36±14.65	89.09±6.11	61.55±20.09
t test value	5.54		4.00		4.351	
P value	<0.01*		0.01*		<0.01*	

Table 1: Demonstrated Inter group comparison of penetration depth of Disinfectant 2% Chlrohexidine digluconate and Disinfectant 2% Chitosan by different agitation methods at Coronal, Middle & Apical level.



Graph 1: Demonstrated Inter group comparison of penetration depth of Disinfectant 2% Chlrohexidine digluconate and Disinfectant 2% Chitosan by different agitation methods at Coronal, Middle & Apical level.

DISCUSSION

The primary goal of chemomechanical preparation of root canal systems is eradication**,** of pulpal tissue, organic and inorganic debris, bacteria, and their toxic by-products through the use of such instruments and irrigation devices.[18]

Irrigant penetration in the root canal system depends upon the root canal anatomy, irrigant application techniques, solution volume, root canal instrumentation and irrigants physic-chemical characteristics.[19]

The course of the dentinal tubules follows a gentle curve in the crown, less so in the root, where it resembles a gentle S (sigmoid curve) in shape. The tubules are longer than the dentin thickness because they curve through dentin. The dentin thickness ranges from 3-10mm or more.[4]

There are various new root canal irrigants like 2% Chlorhexidine digluconate, MTAD, Tetraclean, Electrochemically Activated Solutions, Ozonated water, Photon-Activated disinfection, Herbal Irrigants like Neem Leaf Extract, Triphala Extract, Chitosan etc. Currently, a variety of irrigation techniques and devices were being used to improve the disinfection of root canal systems.

In the present study two irrigants 2% chlorhexidine and Chitosan were used. CHX is a broad-spectrum synthestic cationic bis-guanide antimicrobial agent, which also presents an additional benefit that is its adsorption to the dentine and slow-release posterior due to a phenomenon known as substantivity that extends its antimicrobial activity. Even invitro studies reported that chlorhexidine completely inhibited the growth of *E.faecalis*. Furthermore it has proved to be effective even at low concentrations against the microorganisms which are most frequently present in infected canals, anaerobic bacteria and *candida albicans*.[14]

Another irrigant Chitosan which is a production of chitin is also used as an root canal irrigant. Chitin is the second-most abundant natural polysaccharide composed of β- (1, 4) -linked N-acetyl glucosamine units. Partial deacetylation of chitin results in the production of chitosan. It is a naturally occurring polysaccharide comprising copolymers of glucosamine and N-acetyl glucosamine. Chitosan has shown a large number of pharmaceutical applications. Even it has been used in drug delivery, peptide delivery, as an absorption enhancer and in gene delivery.[15]

Although owning a broad spectrum of antimicrobial activity, chitosan exhibits differing inhibitory efficiency against different fungi, Gram-positive and Gram-negative bacteria. Even Chitosan exerts an antifungal effect by suppressing sporulation and spore germination (Hernandez-Lauzardo et al. 2008).

So in the present study we evaluated the influence of different irrigant's agitation methods on penetration depth of 2% Chlorhexidine Digluconate and Chitosan into radicular dentinal tubules using ConFocal Laser Scanning Microscope (CLSM) and the factors which influenced the results of this study were surface tension and viscosity of irrigants, agitation methods, diameter of the dentinal tubules at coronal , middle and apical levels. The Surface tension can be defined as "the force between molecules that produces a tendency for the surface area of a liquid to decrease." This force tends to limit the ability of a liquid to penetrate a capillary tube.[15] Surface tension of CHX and Chitosan were measured with stagnometer and values received were CHX+R (46.65 dyne/ cm) and Chitosan+ R (65.22 dyne/ cm). Viscosity is the ability of a liquid to flow. A liquid with reduced viscosity tends to have a higher penetration into the dentinal tubules than a highly viscous liquid.[17]The viscosity of CHX+ R and Chitosan+ R were measured with viscometer and values received for CHX+ R (0.67cp) and Chitosan+ R (0.74cp) respectively. Rhodamine b dye did not modified surface tension of the irrigants because .01% volume of dye was added to it which was mandatory to it seen under conefocal laser scanning microscope. This microscope controls depth of field, eliminates or reduces background information away from the focal plane, and collects serial optical sections even from thick specimens. Furthermore, the use of confocal laser scanning microscope is a non-destructive approach and has less potential to cause technique artifacts.

According to result of present study, both disinfectants could penetrate at all levels with different irrigation agitation systems. But 2% chlorhexidine digluconate showed maximum penetration depth at coronal & middle levels than apical third. The results were statistically significant ($p < 0.01$). The reason behind this could be the density and diameter of dentinal tubules that is more at coronal & middle levels than apical third level. The dentin present apically exihibit sclerosis[20]and amount of peritubular dentin is more, which could not allow penetration of disinfectants.

Another objective of the study was performance of different agitation system. So current study showed better penetration of both disinfectants into dentinal tubules with subgroups I_d & II_d (**Ultrasonic Irrigation)** than other subgroups, at all three levels. The finest outcomes from PUI can be attributed to a number of physical phenomena. Acoustic streaming is the first type of physical effect. Rapid fluid motion in the form of a circular or vortex formed by the vibrating file can draw debris & germs off the wall into the vortex. Another physical result is cavitation which is the behaviour, formation & collapse of bubbles. These bubbles can burst near walls, loosening the smear layer and improving cleaning by creating a high-velocity jet that is directed toward the wall. The best results obtained during passive ultrasonic irrigation could be explained that the range of frequencies used in the ultrasonic unit is between 25 and 40 kHz. We believe that the high-velocity jet directed towards the wall not only enhances its cleaning but also forces the irrigating solution to penetrate the tubules.[21]

Arathi G, Rajakumaran A, Kandaswamy D (2019)[15] examined the effect of ultrasonic irrigation on the penetration of chlorhexidine and chitosan into dentinal tubules. It was discovered that 2% chlorhexidine irrigation with ultrasonic agitation reached the deepest into dentinal tubules as compared to chitosan.

After ultrasonic irrigation, subgroups I_c & II_c (**Sonic irrigation)** showed maximum penetration depth of both disinfectants at all the levels of radicular dentin because It is a sonically-driven canal irrigation system that comprises a portable handpiece and 3 types of disposable flexible polymer tips of different sizes that do not cut root dentin. Its design allows for the safe activation of various intracanal reagents and could produce vigorous intracanal fluid agitation.[21]

Subgroups I_b & II_b (manual dynamic irrigation) & subgroups I_a & II_a (conventional needle irrigation) could not give better penetration depth of both disinfectants. But manual dynamic irrigation showed better results as compared to conventional needle irrigation. Because in CI method, the penetration depth of the irrigant was only 1-mm deeper than the needle tip. After the application of method, inaccessible canal irregularities are likely to harbour debris and bacteria; thus, disinfection of dentinal tubules is limited.[22]

The positive results of manual-dynamic irrigation are : the push-pull motion of the gutta-percha point probably acts by physically displacing, folding, and cutting of fluid under ''viscouslydominated flow'' in the root canal system. The latter probably allows better mixing of the fresh unreacted solution with the spent, reacted irrigant.[23,24]

Vadhana S et al. (2015)[16] PUI group showed better penetration of 2% CHX irrigant into dentinal tubules than conventional syringe irrigation group, at coronal, middle and apical third. The reason behind that PUI oscillates at an ultrasonic frequency of 25-30 KHz. Due to this oscillation, it has the potential to create acoustic streaming and aids in the deeper penetration of irrigants laterally.

Salas H, Castrejon A, Fuente D et al (2021)[25] evaluated the penetration of 2% CHX on dentinal tubules using Conventional Irrigation, Sonic Irrigation (EDDY) and Passive Ultrasonic Irrigation (PUI) techniques. The result of this study showed that the greatest penetration capacity of CHX seen with ultrasonic activation, because in this technique, the irrigant is agitated through two physical effects, acoustic microstreaming and cavitation, which improve the action of irrigants.

There is no literature or study evaluating the penetration depth of chitosan into the dentinal tubules. To the best of our knowledge this is the first study that has been done in -vitro using chitosan with all the mentioned irrigation systems together.

CONCLUSION

- Within the limitation of this study, it was concluded that 2% Chlorhexidine showed the maximum depth of penetration.
- With passive ultrasonic irrigation Chlorhexidine and Chitosan were penetrate more in the apical third of root canal as compared to other devices and this difference was statistically significant.

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