Original Article

Comparative evaluation of calcium ion release, pH change, and dentinal tubule penetration of four different formulations of calcium hydroxide-based intracanal medicaments – An *in vitro* study

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Abstract

Aim: The aim of the study was to evaluate and compare the release of calcium ion, pH change, and dentinal penetration depth of four different formulations of calcium hydroxide-based intracanal medicaments.

Materials and Methods: Eighty mandibular single-rooted premolar teeth were divided into four groups (n = 20): Group 1 – calcium hydroxide (CH) + distilled water (DW), Group 2 – nanocalcium hydroxide + DW, Group 3 – calcium hydroxide + chitosan, and Group 4 – calcium hydroxide + 2% chlorhexidine gluconate. Biomechanical preparation was done till the F2 rotary ProTaper system and intracanal medicaments were placed. Calcium ions and pH were assessed at 24 h, 7 days, 15 days, and 30 days using an ultraviolet spectrophotometer and pH meter, respectively. The evaluation of tubule penetration was scanned under a field emission scanning electron microscope.

Results: A significant difference was seen in calcium ion release and pH change among the four groups at 24 h, 7 days, 15 days, and 30 days as well as depth of dentinal penetration.

Conclusion: Calcium hydroxide mixed with 2% chlorhexidine gel showed alkaline pH and the highest calcium ion release as well as significant dentinal tubule penetration among all the four groups under observation. Both combinations can enhance antimicrobial effectiveness as intracanal medicaments. Further clinical study should be carried out to optimize its use as an alternative treatment modality.

Keywords: Calcium hydroxide; calcium ion release; chitosan; chlorhexidine; dentinal tubule penetration; nanocalcium hydroxide

INTRODUCTION

The main focus of root canal therapy is the elimination of microorganisms from the root canal space and rendering a hermetic seal preventing further reinfection and enhancing complete periradicular healing.[1] However, many a time, as a result of the complex anatomy of root canals and the tortuous nature of dentinal tubules, total disinfection is difficult to accomplish. Therefore, varied canal shaping techniques and irrigation protocols have been advocated as well as placement of intracanal medicaments to reduce microbial flora.[2-6]

Calcium hydroxide $(CaOH)_2$ as an intracanal medicament was introduced to endodontics by Hermann in

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Date of submission : 15.06.2023
Review completed : 10.08.2023
Date of acceptance : 18.09.2023
Published : 22.11.2023

Access this article online

Quick Response Code:

Website: https://journals.lww.com/jcde

DOI: 10.4103/JCDE.JCDE_39_23

How to cite this article: Thonai S, Kataki R, Das L, Begum F, Deka A, Borah DK. Comparative evaluation of calcium ion release, pH change, and dentinal tubule penetration of four different formulations of calcium hydroxide-based intracanal medicaments – An *in vitro* study. J Conserv Dent Endod 2023;26:657-62.

1920.[7] The mechanism of action is due to the release of hydroxyl ions, which increases the pH of the medium, thereby promoting the inactivation of bacterial lipopolysaccharides which are found in the external membrane of Gram-negative bacteria.[8] Hence, their efficacy will depend on the amount of hydroxyl ions released and its capacity to diffuse through the dentinal walls where sufficient levels are required to produce a toxic effect.

The association of calcium hydroxide with other antimicrobial agents such as chlorhexidine and chitosan (CT) or incorporation of nanosized particles has been added to improve antibacterial action, radiopacity, flow, and consistency as many endodontic failures related to *Enterococcus faecalis* have shown resistance to calcium hydroxide application.[9]

Chlorhexidine, a bisbiguanide, has both antibacterial and antifungal actions.[10] It is used in endodontics as an irrigant and intracanal medicament and is found effective against *E. faecalis* and *Candida* species.[11-13] Moreover, its property of substantivity is equally important to dictate whether the use of chlorhexidine would behave differently in terms of affecting the pH and calcium release when combined with calcium hydroxide.

CT [poly-(b-1/4)-2-amino-2-deoxy-D-glucopyranose] is a collective name for a group of partially and fully deacetylated chitin compounds, which is extracted mostly from crustacean shells. It has a wide spectrum of antimicrobial activity against Gram-positive and Gram-negative bacteria with almost no toxicity toward mammalian cells.[14,15]

Nanoparticles have been an area of interest in dentistry due to their unique characteristics, where particle size possesses external dimensions in the range of 1–100 nm. These nanosized particles exhibit properties such as higher surface area-to-volume ratio, increased depth of penetration, and better action against oral microorganisms.[16]

Since, the antibacterial efficacy of calcium hydroxide-based intracanal medicament depends on many factors such as hydroxyl ions release, high pH maintenance, as well as depth of penetration into the dentinal tubules. Therefore, this research was executed to evaluate the concentration of calcium ions and depth of penetration and measure the pH values over 30 days.

The comparative study was carried out for conventional calcium hydroxide, nanocalcium hydroxide (NCH), a mixture of calcium hydroxide with CT, and a combination of calcium hydroxide with 2% chlorhexidine gel.

MATERIALS AND METHODS

Specimen selection and preparation

The study followed the modified consort guidelines for *in vitro* research, and ethical clearance was acquired from the Institutional Ethical Committee Board before conducting the study. A total sample size of 80 teeth was determined using the following statistical formula, $n = ([Z_{a/2} \times s]/E)$, where $Z_{a/2} = 1.96$ (at 95% confidence level), s is the estimated population standard deviation (0.5), and E is the desired error (10%) of the estimated population mean. The crowns of all teeth were decoronated to achieve a standardized root length of 15 mm using a carborundum diamond disc. A conventional cleaning and shaping procedure was carried out with a rotary filing instrument till F2 ProTaper Gold (Dentsply Sirona, Switzerland), and irrigation was done with 2 ml each of 2.5% NaOCl and 17% ethylenediaminetetraacetic acid for 1 min followed by 5 ml of saline.[17]

The samples (80) were split into four groups ($n = 20$) each on the basis of intracanal medicaments used. Out of which, 50% were taken to evaluate calcium and pH change and the remaining for dentinal tubule penetration.

- Group I ($n = 20$): calcium hydroxide powder (Prevest DenPro Ltd. Jammu, India) with distilled water (DW)
  It was prepared by mixing $Ca(OH)_2$ powder and DW in the ratio of 6:4.[18]
- Group II ($n = 20$): NCH with DW
  It was prepared with 100 mg of NCH powder manufactured by chemical precipitation method[19] (Nano Research Elements, Haryana India) dissolved in 0.5 mL of DW, DW was added till its final volume was 1 mL, and a dough-like consistency was obtained.
- Group III ($n = 20$): calcium hydroxide with CT
  It was prepared by soaking CT (Nano Research Elements, Haryana, India) in 2% acetic acid forming a gel base, to which calcium hydroxide (1% w/v) was added.
- Group IV ($n = 20$): calcium hydroxide with 2% chlorhexidine gel (GLUCO-CHeX 2% Gel CERKAMED, Kwiatkowskiego, Stalowa Wola, Poland)[20]
  A total of 0.20 g (1:1) of each medication was weighted and prepared using a glass plate and spatula.

Estimation of calcium ion release and pH values

Calcium ion liberation was measured by a colorimetric method (o-cresolphthalein complex-calcium reaction, Medsource Ozone Biomedicals India), which was then calculated using a reference calibration curve formulated with water. The experimental formulations were condensed with a 21-mm #25 size Lentulo Spiral (Mani Paste Carriers, Japan), and the orifice of all root canals was sealed with glass ionomer cement (Fuji II-GC Corporation, Tokyo,

Japan). All prepared samples were suspended individually over 10 ml DW in a glass vial with modeling wax, where only an apical third of the root was submerged [Figure 1a]. Solutions of 10 µL were withdrawn with micropipette at predetermined time intervals at 24 h, 7 days, 15 days, and 30 days, and calcium ion concentrations were analyzed using an ultraviolet spectrophotometer (Spectrascan UV 2600 Chemito Instruments Pvt. Ltd, India) at 570 nm.

Measurement of pH

Changes in pH were determined using a pH meter (Labtech Digital pH Meter, India), and standard buffers were taken in separate beakers. The process of calibration was done with buffers of pH 7, 4, 9, and 10. The electrode was dipped into the test solution, and pH reading was noted. After every reading, the electrode was cleansed with DW and wiped with sterile tissue paper to eliminate calcium hydroxide residues that might interfere with subsequent readings. pH is measured of the test solutions at 24 h, 7 days, 15 days, and 30 days.

Evaluation of dentinal tubule penetration depth

The test samples for the evaluation of tubule penetration were incubated at 37°C and 100% relative humidity for 2 weeks. Longitudinal grooves were made on opposite external surfaces of each root sample using a diamond disc and without perforating the canal space. The roots were then split into two halves with a chisel after which the specimens were mounted on labeled stub and sputter coated with gold [Figure 1b]. The measurements (in µm) for the depth of penetration were then scanned by field emission scanning electron microscope (GeminiSEM 500, ZEISS) at ×170 magnification in the coronal, middle, and apical thirds of each sample [Figure 2].

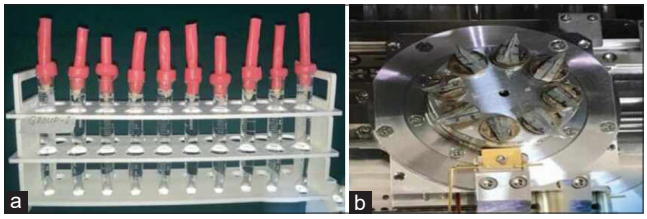

Figure 1: Samples suspended in a glass vial containing 10 ml distilled water with the help of molding wax for calcium and pH assessment (a), Gold-sputtered samples placed in an FESEM chamber for the analysis of dentinal penetration depth (b)

Statistical analysis

Statistical analysis was carried out using the IBM SPSS Statistics for Windows, version 20 (IBM Corp., Armonk, N.Y., USA) software version 20 (IBM SPSS Corporation Released 2011). The comparison of mean was determined by the analysis of variance and paired sample *t*-test. $P < 0.05$ was treated as statistically significant.

RESULTS

There were statistically significant differences in the mean calcium ion release among the four groups ($P < 0.001$), with Group 4 showing the highest mean, followed by Group 2, Group 3, and Group 1 at 24 h, 7 days, 15 days, and 30 days, respectively [Table 1 and Figure 3].

For the pH measurement at different time intervals of 24 h, 7 days, 15 days, and 30 days, significant differences were observed. The mean pH level is the highest for Group 4 at 7 days as well as toward the end of 30 days [Table 2 and Figure 4].

In all three root levels – coronal, middle, and apical third, the mean dentinal penetration depth is found to be the highest in Group 4 and the lowest in Group 1. The differences in mean among the root thirds are found to be statistically significant [Table 3 and Figure 5].

Table 1: Comparison of the mean calcium ion release among the four different formulations of calcium hydroxide intracanal medicaments at different time intervals (24 h, 7 days, 15 days, and 30 days)

Follow-up periods	Groups	Mean calcium ion release	SD	ANOVA (*P*)
24 h	Group 1	8.72	0.81	0.120, NS
	Group 2	9.07	0.71	
	Group 3	8.08	0.83	
	Group 4	8.87	1.30	
7 days	Group 1	10.55	0.72	<0.001,
	Group 2	12.84	1.35	significant
	Group 3	11.98	0.89	
	Group 4	15.54	1.94	
15 days	Group 1	12.86	0.61	0.022,
	Group 2	13.58	0.56	significant
	Group 3	13.29	0.54	
	Group 4	12.69	0.92	
30 days	Group 1	8.83	0.45	<0.001,
	Group 2	9.58	0.37	significant
	Group 3	8.86	0.58	
	Group 4	10.16	0.55	

NS: Nonsignificant, SD: Standard deviation

Figure 2: Representative FESEM image showing penetration depth at the coronal (a), middle (b), and apical third (c) of the root

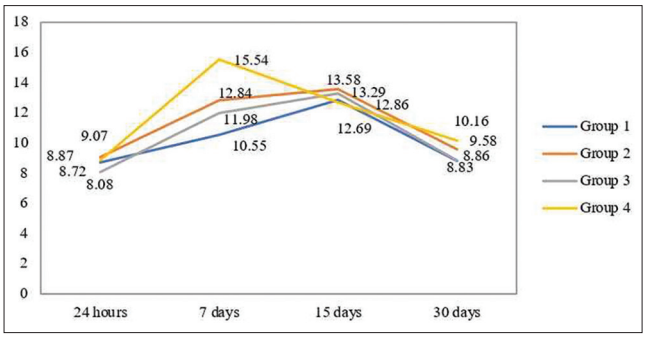

Figure 3: A graph showing the mean calcium release from various formulations tested at different time intervals

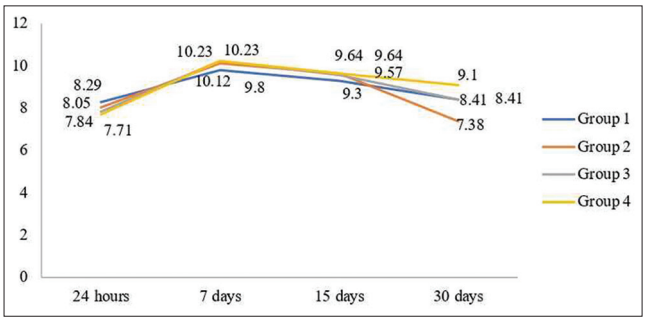

Figure 4: A graph showing the mean pH values from various formulations tested at different time intervals

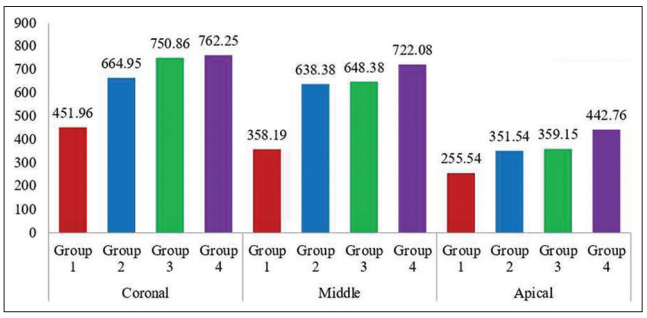

Figure 5: The mean dentinal penetration depth in the coronal, middle, and apical third of the root

DISCUSSION

The significance of the alkalinizing effects of calcium hydroxide due to hydroxyl ions released when it comes into contact with an aqueous medium has been extensively reported.[21] Nerwich *et al*. also measured pH changes in root dentin over 30 days, which is considered a practical time interval to demonstrate effective therapeutic benefits from calcium hydroxide-based intracanal medicaments.[22]

Numerous studies on calcium ion release and pH changes from calcium hydroxide-based medicaments have followed different methodologies, which include placement of testing samples either in saline or DW completely or partially submerged.[23] In some earlier *in vitro* studies, they have employed cylindrical glass tubes instead of extracted teeth.[24] However, in this research study, extracted human teeth were acquired where only the apical root third was submerged in DW so as to emulate the clinical scenario and also prevent probable dispersion of medicaments from the root canal orifice. Ultraviolet spectrophotometer was adopted due to its rapid analysis and cost-effectiveness compared to other techniques.

Table 2: Comparison of the mean pH values among the four different formulations of calcium hydroxide intracanal medicaments at different time intervals (24 h, 7 days, 15 days, and 30 days)

Follow-up period	Group	Mean pH level	SD	ANOVA (*P*)
24 h	Group 1	8.29	0.23	<0.001, significant
	Group 2	8.05	0.08	
	Group 3	7.84	0.10	
	Group 4	7.71	0.08	
7 days	Group 1	9.80	0.25	<0.001, significant
	Group 2	10.12	0.13	
	Group 3	10.23	0.07	
	Group 4	10.23	0.16	
15 days	Group 1	9.30	0.18	<0.001, significant
	Group 2	9.64	0.06	
	Group 3	9.57	0.02	
	Group 4	9.64	0.06	
30 days	Group 1	8.41	0.19	<0.001, significant
	Group 2	7.38	0.21	
	Group 3	8.41	0.17	
	Group 4	9.10	0.06	

SD: Standard deviation

According to the results obtained, calcium ion release is observed to significantly increase from 24 h to 15 days ($P < 0.001$). This was in accordance with findings by Sonali *et al*.,[25] Grover and Shetty,[18] Carvalho *et al*.,[26] and Ballal *et al*.,[27] where they found a rapid increase in calcium ion release till 15 days, which gradually decreased with time after 1 month.

At 30 days, differences in mean calcium ion release among the four groups are found to be statistically significant ($P < 0.001$) with Group 4 showing the highest mean. This high alkaline pH might be due to the gel base of chlorhexidine, Natrosol, which is a methyl cellulose classified as an aqueous vehicle.

In the results obtained for pH level [Table 2], Group 4 exhibits the highest mean values, followed by Group 3, Group 1, and the least in Group 2 gradually increasing till 15 days and slightly declining at a 30-day period, which was in conjunction with a study by Duarte *et al*.[28] and Carvalho *et al*.[26]

Few studies have focused on the effects of chlorhexidine on the pH of calcium hydroxide. According to a study by Freire *et al*.,[29] they have observed that the combination of chlorhexidine did not significantly change the pH of calcium hydroxide.

Table 3: Comparison of the mean penetration depth into dentinal tubules at the coronal, middle, and apical third of the root

Group	Root level	Mean dentinal penetration depth	SD	Comparisons	*P*
Group 1	Coronal	451.96	28.95	Coronal versus middle	<0.001, significant
	Middle	358.19	29.71	Middle versus apical	<0.001, significant
	Apical	255.54	28.51	Coronal versus apical	<0.001, significant
Group 2	Coronal	664.95	32.34	Coronal versus middle	0.040, significant
	Middle	638.38	29.05	Middle versus apical	<0.001, significant
	Apical	351.54	31.26	Coronal versus apical	<0.001, significant
Group 3	Coronal	750.86	25.48	Coronal versus middle	<0.001, significant
	Middle	648.38	22.93	Middle versus apical	<0.001, significant
	Apical	359.15	32.75	Coronal versus apical	<0.001, significant
Group 4	Coronal	750.86	25.48	Coronal versus middle	<0.001, significant
	Middle	648.38	22.93	Middle versus apical	<0.001, significant
	Apical	359.15	32.75	Coronal versus apical	<0.001, significant

SD: Standard deviation

Evaluation of calcium hydroxide and their nanocalcium counterparts with different vehicles has observed elevated readings after 24 h even as several similar studies dictate the initial spurt of hydroxyl ions release on exposure to aqueous media.[30-32]

The assessment of dentinal tubule penetration depth exhibits higher values at the coronal third of the root, followed by the middle third and lowest in the apical third of root length. According to research done by Dianat *et al*.,[33] where they evaluated the mean depth of tubule penetration between calcium hydroxide and its nanocalcium counterparts, they reported significant differences ($P < 0.05$) which might be attributed to their nanoscale variations, thereby enhancing penetrability into deeper layers of dentin when compared with conventional $Ca(OH)_2$.

Furthermore, the credibility of chemomechanical preparation and removal of the smear layer decrease in the apical third of the root,[34] as well as tubular density decreases as we move from the coronal toward the apical, which explains why penetration depth reduces at the root apex. Among the experimental groups, Group 4 has the highest penetration depth, which might be attributed to chlorhexidine's high adsorption power, explained by electrostatic interaction. Furthermore, due to its cationic character, chlorhexidine has a strong affinity for anions like the phosphate ions present in the dentin.[35]

Our results should be considered within the experimental conditions used, facing natural limitations of comparing *in vitro* and *in vivo* studies as well as considering the adverse effects of prolonged use of $Ca(OH)_2$.[36] Definitive inference must not be drawn, and further *in vivo* investigations are desirable. Nevertheless, it was possible to verify the amount of calcium ion release and the variation in pH level over 1 month among the four experimental groups, as well as dentinal penetration depth at different levels of the root.[35]

CONCLUSION

Within the limitations of this study, it can be concluded as follows:

- Calcium ion release, pH change, as well as dentinal penetration depth are the highest for the combination of calcium hydroxide with 2% chlorhexidine when evaluated over 1 month
- The mean dentinal penetration depth is the highest at the coronal third, followed by the middle and apical third of the root.

Acknowledgment

The authors would like to thank the "Department of Biochemistry, Guwahati Medical College and Hospital Guwahati Assam" and "North East Center for Biological Sciences and Health-care Engineering (NECBH), IIT Guwahati, and Department of Biotechnology (DBT), Government of India, for Project No. BT/COE/34/SP28408/2018 for the FESEM Instrumentation Facility."

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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