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Original Article

Antibacterial Efficacy and Biocompatibility of Calcium Hydroxide, Camphorated Parachlorophenol, and A Combination of Calcium Hydroxide and Camphorated Parachlorophenol as Root Canal Dressing against Enterococcus Faecalis in Single Rooted Mandibular Premolars : (A Comparative In-Vitro Study)

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Abstract

Aim: This in-vitro study aimed to assess and compare the antibacterial efficacy and biocompatibility of Ca $(OH)_2$, camphorated parachlorophenol (CPCP) and a combination of Ca $(OH)_2$ and CPCP as root canal dressings against E. faecalis in single rooted mandibular premolars compared to teeth with no intracanal medications.

Subjects and methods: The antimicrobial test: Fifty human extracted single-canalled teeth were collected and prepared. The teeth were sterilized and each root canal was infected with 20 µ of *E. faecalis* bacteria, which were then randomly categorized into five equal groups based on the intracanal medicament G1, the canals were medicated with CPCP using a cotton pellet placed into the pulp chamber. G2, the canals were medicated with CPCP using paper point till the apical 1/3. G3, the canals were medicated with Ca $(OH)_2$ till the apical 1/3. G4, the canals were medicated with a combination of Ca $(OH)_2$ and CPCP till the apical 1/3. G5, the canals were kept empty without any medications (control). By counting the number of colonies forming units per milliliter of each dilution on the agar medium, the resulting growth was visually quantified.The biocompatibility test: The BHK normal fibroblast cell line was collected and then cultured (96-well plates for all groups). Three different intracanal medications (Ca $(OH)_2$, CPCP, a combination of Ca $(OH)_2$ and CPCP) were added to the cultured cells. Fresh media were used to culture the cells in the Control Group (CG). Cytotoxicity was evaluated by MTT reduction assay. The significance level was set at $P \leq 0.05$.

Results: G4; intracanal medication with a combination of Ca $(OH)_2$ and CPCP showed the highest anti-bacterial effect as it showed the least post intracanal medication bacterial count followed by G3; intracanal medication with Ca $(OH)_2$. G2; intracanal medication with CPCP using paper point, G1; medication with CPCP inserted into the pulp chamber on a cotton pellet, respectively with no significant differences between them ($p>0.05$). Control group (G5); root canals kept with no intracanal medications showed significantly higher post-intracanal medication bacterial count than all other groups ($p<0.001$). According to the biocompatibility, The highst mean and standard deviation values of viability were 65.74±5 in Ca $(OH)_2$, and the low value was 28.33±3 in combination at a concentration of 400. There was a highy significant differences between CPCP and combination compared to Ca $(OH)_2$, while non significant difference between CPCP and combination.

Conclusion: Combining Ca $(OH)_2$ and CPCP shows high antibacterial efficacy compared to each medication alone. Ca $(OH)_2$ was biocompatible on the tissue. Thereafter, comes a mixture of CPCP and Ca $(OH)_2$ then finally CPCP.

Keywords: Calcium hydroxide; Intracanal medication; Intracanal dressings; Cytotoxicity; Camphorated Parachlorophenol.

I. INTRODUCTION

The goal of the root canal treatment is getting rid of the bacteria and its byproducts as well as preventing and stopping the microorganisms from coming back into the root canal system **(Li et al., 2023).** The root canal shaping and biomechanical preparation techniques successfully lower microbiota, but they do not eradicate bacteria from the apical deltas, isthmi, or lateral and accessory root canals **(de Paz and Ordinola Zapata, 2019).**

Enterococcus faecalis (*E. faecalis*) is the most prevalent bacterial species in resistant or recurrent infections because it may penetrate deeply into the dentinal layers that are challenging to eradicate, enhancing the possibility of endodontic treatment failures **(Gomes et al., 2021).**

Thus, it is advised to administer intracanal medication between appointments in order to further reduce pathogens within the root canal system **(Kumar et al., 2019).**

Since its introduction by Hermann in 1920 as a pulp-capping agent, calcium hydroxide ($Ca(OH)_2$), has been widely utilized in endodontics. Ca $(OH)_2$ is poorly soluble and diffusible, it may be challenging to raise the pH quickly enough to eradicate microorganisms found in tissue remnants, biofilms, dentinal tubules, and anatomic variations. Furthermore, the antimicrobial efficacy of Ca $(OH)_2$ decreased by the capability of serum and dentin to act as buffers **(Thienngern et al., 2022).**

To enhance its bacterial action, combining Ca $(OH)_2$ with other antimicrobial agents, such as camphorated parachlorophenol (CPCP) and chlorhexidine (CHX), has been suggested **(Silva et al., 2023).**

A phenolic compound called camphorated parachlorophenol used as intracanal medicament. CPCP has bactericidal properties via disintegrating the cytoplasmic membrane of bacteria, denatureing proteins, and deactivating enzymes like dehydrogenases and oxidases **(Zhang et al., 2021).**

This in-vitro study aimed to evaluate the antibacterial efficacy and biocompatibility of Ca $(OH)_2$, CPCP and a combination of Ca $(OH)_2$ and CPCP as root canal dressings against E. faecalis in single-rooted mandibular premolars compared to teeth with no intracanal medications.

The null hypothesis is that there is no difference between the Ca $(OH)_2$, CPCP and a combination of Ca $(OH)_2$ and CPCP as root canal dressings when compared to teeth with no intracanal medications regarding antibacterial effect against *E. faecalis* and biocompatibility in single-rooted mandibular premolars.

II. SUBJECTS AND METHODS

Randomization:

A. Sequence generation

Following the collection of the 50 single-canalled mandibular premolars, a random sequence was generated using the random sequence generator website (http//: www.random.org). According to the tested intracanal medicament, teeth were randomly distributed into five equal groups with 10 samples in each group (n= 10).

B. Allocation concealment

Allocation concealment was done by inserting each sample into a separate opaque sealed envelope, shuffling the envelopes then giving each envelope a number; each sample took a number from one to hundred and four.

C. Implementation

Random allocation sequence and allocation concealment were done by the co-supervisor, and the technical procedures of the research method were carried out by the investigator.

Blinding

The trial was a triple-blinded study, where the outcomes assessors and statistician were blinded during outcome evaluation.

Sample size calculation

Sample size was calculated using the (PS software). Regarding the primary outcome (antibacterial effect) it was found that 7 teeth per group will be appropriate sample size for the study with a total sample size of 35 teeth (5 groups) the power is 80% and α error probability =0.05. The sample size was increased to 10 per group with total sample size of 50 teeth to compensate for anticipated missing data 30%. The magnitude of the effect to be detected was estimated as the mean and standard deviation of the variable of interest and obtained from the scientific literature **(Pavaskar et al., 2012).**

Selection of the samples

Fifty human permanent single-canalled mandibular premolars extracted due to orthodontic, periodontal or prosthodontics reasons with inclusion criteria being: Permanent teeth with single canals, straight roots and completely formed roots.

Samples preparation

Using an ultrasonic scaler, the teeth were freed out of calculus and soft-tissue debris. All teeth were decoronated using a high-speed hand piece at the level of cemento-enamel junction in addition to standardizing the root lengths at 15 mm. Stst hand file #10 K-type was used to establish patency, the working length (WL) was determined to be 1 mm less than the apical foramen. Preparation of the root canals was done using the Protaper Next rotary system (ProTaper Next®, DENTSPLY International, Inc., Johnson, Switzerlan) till size X4. After each instrument, 2 ml of 5.25% NaOCl solution was used to irrigate canals using side vented needle. Five ml of 5.25% NaOCl was used then 5'ml of 17% EDTA for 1 min. 5 ml of saline was used to inactivate the EDTA. Five ml of 5.25% NaOCl was used as a final irrigant.

For sterilization process, all the teeth were sterilized in autoclave at 121°C for 30 minutes. E. faecalis (ATCC 29212) strain was used and aerobically cultivated on Brain Heart agar at 35°C for 2 days, A sterile brain heart infusion BHI broth was used to prepare the inoculum, and the turbidity was adjusted to 0.5 McFarland. Twenty microliters of bacteria were placed inside every root canal and transferred to tube filled with BHI and was kept at 37°C for 14 days. At this time BHI was changed every three days, then the samples were divided in random manner into 5 groups according to the intracanal medication: G1, the canals were medicated with CPCP using a cotton pellet inserted into the pulp chamber. G2, the canals were medicated with CPCP1using paper point1 till the apical 1/3. G3, the canals were medicated1 with Ca $(OH)_2$ till the apical1/3. G4, the canals were medicated with a combination of Ca $(OH)_2$ and CPCP till the apical11/3. G5, the canals were kept empty without any medications (control).

Intervention for intracanal medicament preparation

For G1: Sterile paper points were used to dry the intracanal BHI broth, then the canals were medicated with CPCP (Camphorated parachlorophenol, Prevest DenPro, Bari Brahmana, India) using a cotton pellet inserted into the pulp chamber in order to assess the antibacterial efficacy. The pulp chamber was closed with a temporary filling to simulate the clinical situation.

For G2: Sterile paper points were used to dry the intracanal BHI broth, then the canals were medicated with CPCP using paper point till the apical 1/3 to evaluate the antibacterial efficacy. The pulp chamber was closed with a temporary filling to simulate the clinical situation.

For G3:Sterile paper points were used to dry the intracanal BHI broth, then the canals were medicated with Ca $(OH)_2$ (Calcium hydroxide, Ultradent Products, Inc, Hauppauge, USA) Paste till the apical 1/3 to evaluate the antibacterial efficacy. The pulp chamber was closed with a temporary filling to simulate the clinical situation.

For G4: Sterile paper points were used to dry the intracanal BHI broth, then the canals were medicated with a combination of Ca $(OH)_2$ and CPCP (ratio 1:1) till the apical 1/3 to evaluate the antibacterial efficacy. The pulp chamber was

closed with a temporary filling to simulate the clinical situation.

For G5: Sterile paper points were used to dry the intracanal BHI broth, but the canals were kept empty without any medications (control). The pulp chamber was closed with a temporary filling to simulate the clinical situation.

Outcome assessment for the antibacterial test:

It was achieved by using culture technique. A first sample (S1) was collected from every canal after the 2 weeks incubation period before implementing the tested intracanal medication. Bacterial biofilm on the canal walls was loosened by H-file and collected using 3 paper points, then put in Wasserman's tube filled with 1 ml broth of BHI. The vortex was utilized for dispersion of the samples for 1 min. Fifty microliters of the samples after dilution were placed over BHI agar plates and cultured aseptically, then incubated at 37°C for 1 day **(Gajan et al., 2009).** The number of (CFUs/ml) was counted with the naked eye on the agar medium **(Molander et al., 1998; Martinho et al., 2015).** The number of CFUs of second sample (S2) after implementing the intracanal medicament was calculated as explained in the first.

The Biocompatibility test:

Experimental groups:

CPCP Group: Cells were grown in medium conditioned by CPCP.

Ca $(OH)_2$ Group: Cells were grown in medium conditioned by Ca $(OH)_2$.

Combination Group: Cells were grown in medium conditioned by a combination of Ca $(OH)_2$ and CPCP.

Control Group (CG): Cells were grown in a fresh medium.

Cell culture

Normal fibroblast (BHK) cell lines were collected from Egypt's National Cancer Institute. In a sterile flask, 10% of the culture medium used to grow this cell line contained bovine serum. Depending on the cell metabolism, the medium was changed every two to three days (96-well plates for all groups) **(Hussein et al., 2021).**

Conditioned medium

Intracanal1medicaments were prepared in accordance with the manufacturer's1instructions. The culture1media were conditioned1by dispensing 1.4 grams of each intracanal medicament into a 15 mL sterile1centrifuge1tube, distributing the intracanal medicament on the bottom1surface. The intracanal medicament was covered with 7 mL of culture media (ratio of 0.2g of intracanal medicament per 1 mL of media) and centrifuged for one minute at 1,000 rpm, then filtered through 0.2 µm syringe filters to eliminate any solid particles **(Chen et al., 2009).**

Outcome assessment for biocompatibility

Ninety-six well tissue culture plates were inoculated with 1 X 105 cells/ml (100µl / well) and incubated for 24 hours at 37°C to form a confluent monolayer sheet. Once a confluent sheet of cells had formed, the growth media was decanted from the 96-well microtiter plates. Wash media was used twice to wash the cell monolayer. After the samples were seeded into different wells, 24 wells were left as controls and only received the maintenance medium. Plates were incubated at 37°C and examined. Cytotoxicity was evaluated after 24 hrs by methyl thiazolyl tetrazolium assay MTT reduction assay. MTT solution was prepared (0.5mg/ml in PBS). To effectively combine MTT with the media, 50 µL of MTT solution was added to each well, and the wells were shaken at 150 rpm for 5 minutes. To enable the metabolism of the MTT, incubation was done for three to four hours at 37°C with 5% CO2. MTT metabolic product being dispensed in 50 µl of DMSO. Spinning at 150 rpm for 5 minutes to effectively combine the formazan and solvent. Using an ELIZA reader (ELIZA reader, Huawei Diatek Instrument Co., Wuxi, China), The spectrophotometric absorbance was measured at 570 nm.

Cell viability percentage = (optical density of treated cells / optical density of untreated cells) X 100 (Chen et al., 2009).

IC50 of test venoms were determined using Master –plex-2010 program. The 50% inhibition concentration (IC50 value) was calculated.

Primary outcome: Antibacterial efficacy against E. faecalis. Assessment method: Bacterial count **(de Bretas et al., 2017).** Unit of measurement: Colony Forming Units/ml (CFU /ml). Timing: After 24 hrs.

Secondary outcome: Biocompatibility. Assessment method: MTT reduction assay **(Soekanto et al., 1996; Hussein et al., 2021).** Unit of measurement: Numerical (Ratio). Timing: After 24hrs.

Statistical analysis: Data of post-intracanal medication bacterial counts were presented as mean, standard deviation, median, minimum, and maximum values. ANOVA test was used for comparison between groups followed by Fisher post hoc test for pairwise comparisons. Shapiro-Wilk and Kolmogorov-Smirnov tests investigated the normality of the data. The distribution of the data was parametric (normal). A significance level of $p < 0.05$ was established. SPSS software was used to conduct the statistical analysis.

III. RESULTS:

In every group, the difference between (S1) and (S2) was statistically significant where ($p<0.001$). (S1) groups had highest mean value. while the lowest mean value was found in (S2) groups.

Relation between groups according to Postoperative bacterial count (S2):

The highest mean value was found in control group, while the least mean value was found in group 4 (G4) **(Figure 1) (Table 1).**

Fisher's LSD test revealed that G4 showed the highest anti-bacterial effect as it showed the least post intracanal' medication bacterial count followed by G3. G2 and G1, respectively with no significant difference between them ($p>0.05$). Control group: showed significantly higher post-intracanal medication bacterial count than all other groups ($p<0.001$).

The biocompatibility test

The highest mean and standard deviation values of viability were 65.74±5 in $Ca(OH)_2$, and the low value was 28.33±3 in combination at a concentration of 400. There was a highy significant differences between CPCP and combination compared to $Ca(OH)_2$, while non significant difference between CPCP and combination.

Fisher's LSD test revealed that CPCP group showed the highest cytotoxic effect followed by the combination group and $Ca(OH)_2$ group, respectively **(Table 2).**

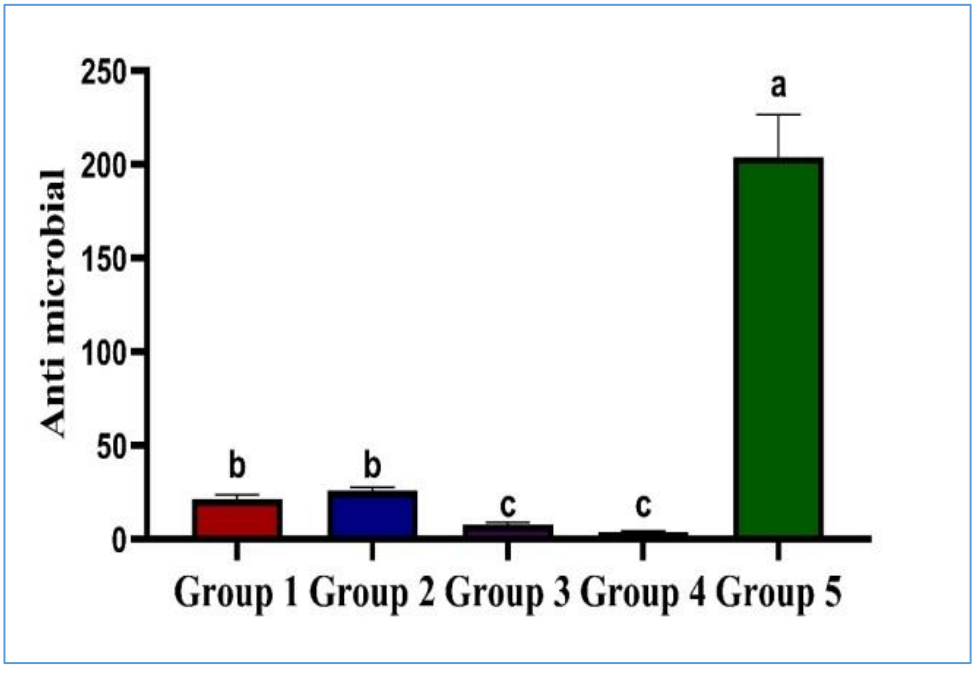

Figure (1): A column chart representing the mean post-intracanal medication bacterial count in S2 groups

Table (1): Mean± SD values of bacterial counts in S1 and S2 groups

Variables	Antibacterial efficacy		
	S1	S2	*P-value*
	Mean± SD	Mean± SD	
G1	204±22.48 [a]	21.5±2.09 [b]	<0.001*
G2	204±22.48 [a]	26±1.59 [b]	<0.001*
G3	204±22.48 [a]	7.8±1.22 [c]	<0.001*
G4	204±22.48 [a]	4.1±0.28 [c]	<0.001*
G5	204±22.48 [a]	204±22.48 [a]	<0.001*
P-value	--	<0.001*	

Table (2): The tissue viability of Ca $(OH)_2$, CPCP, and their combination at concentrations ranging from 800 to 0.390625 µg/mL was assessed using the MTT assay after a 24-hour period.

Concentration µg/ml	Ca $(OH)_2$	CPCP	Combination	P value
800	34.81±7 [a]	27.04±3 [a]	28.15±5 [a]	0.227 [ns]
400	65.74±5 [a]	28.7±5 [b]	28.33±3 [b]	<0.001 [HS]
200	95.37±8 [a]	31.85±2 [c]	49.07±7 [b]	<0.001 [HS]
100	96.67±9 [a]	38.15±4 [c]	74.63±5 [b]	<0.001 [HS]
50	97.96±12 [a]	49.26±5 [b]	99.44±5 [a]	<0.001 [HS]
25	97.96±10 [a]	63.89±3 [b]	99.81±4 [a]	<0.001 [HS]
12.5	98.33±8 [a]	76.3±5 [b]	100.19±7 [a]	<0.001 [HS]
6.25	100±12 [a]	78.33±7 [b]	100.19±9 [a]	<0.001 [HS]
3.125	100.19±7 [a]	83.7±5 [b]	100.37±4 [a]	<0.001 [HS]
1.5625	100.19±15 [a]	85.74±8 [b]	100.56±6 [a]	<0.001 [HS]
0.78125	100.74±6 [a]	94.07±10 [a]	100.74±8 [a]	0.547 [ns]
0.390625	101.67±19 [a]	101.3±5 [a]	100.93±5 [a]	0.997 [ns]

IV. DISCUSSION

Intracanal medicamentsn are recommended to supplement the chemomechanical preparation to enhance disinfection. Ca $(OH)_2$ can disintegrate organic tissue, inactivate bacterial endotoxins, and exhibit biological and antibacterial activity. Thus, it has shown a widespread application. Ca $(OH)_2$ based pastes work by allowing hydroxyl ions (OH) to diffuse at concentrations that are sufficient to achieve appropriate pH levels **(Ballal et al., 2007)**. It has been recommended that Ca $(OH)_2$ be combined with other antimicrobial agents, such as camphorated monochlorophenol (CMCP) and chlorhexidine (CHX), to enhance its antibacterial activity **(Silva et al., 2023).**

Therefore, this in-vitro study was designed to1assess the antibacterial efficacy and biocompatibility of Ca $(OH)_2$, camphorated parachlorophenol (CPCP) and a combination of Ca $(OH)_2$ and CPCP as root canal dressings against *E. faecalis* in single rooted mandibular premolars compared to teeth with no intracanal medications.

E. faecalis was selected being one of the most commonly found bacteria, which are responsible for the persistent periapical infections. It has1been used in previously conducted studies **(Delgado et al. 2010).**

The antibacterial efficacy of the medications in the present study was calculated by using the bacterial count reduction used by **de Bretas et al., 2017.**

The present study suggests the use of combinations rather than using Ca $(OH)_2$ alone. This finding coincided with **Teja et al., 2023.**

Also, the laboratory evaluation in **Barbosa et al., 1997** study showed that, camphorated paramonochlorophenol showed the largest zones of bacterial inhibition against all bacterial strains tested. However, there was no statistically significant difference among the medicaments tested. Unlike them our study had a significant results.

In the present study, when the concentration of the dressing substance was increased, its antibacterial efficacy increased as well, this fact was in coincidence with **Blanscet et al., 2008.**

As for the biocompatibility, it was assessed by using methyl thiazolyl tetrazolium assay (MTT) reduction assay as determined by **Hussein et al., 2021 and Soekanto et al., 1996.**

Normal fibroblast cells (BHK) was chosen to represent the clinical condition as these cells are responsible for normal maintenance and the regeneration of the peridontium **(Hauman et al., 2003).**

The viability test of Ca $(OH)_2$, CPCP, and their1combination were measured at the concentrations ranged from 800 to 0.390625 μg/mL obtained by the MTT assay after 24 hours.

The highest viability with Ca $(OH)_2$ was 101.67±19 in 0.390625 concentration, and the lowest value was 34.81±7 at concentration 800. The highest viability with CPCP was 101.3±5 in 0.390625 concentration, and the lowest value was 27.04±3 at concentration 800. The highest viability with the combination was 100.93±5 in 0.390625 concentration, and the lowest value was 28.15±5 at concentration 800.

A highly statistically significant divergence existed between the different concentrations in $Ca(OH)_2$ group, CPCP group, and1combination group. Therefore, increasing1the concentration of Ca $(OH)_2$ or CPCP lead to increase in their cytotoxic effect in single-canalled mandibular premolars.

The components of CPCP are camphor and chlorophenol, which are toxic. The inflammatory reaction was milder to that of tricresol formalin and formocresol which is in agreement with Engstrom and Spangberg 1967 who stated that the tricresol formalin is more toxic than camphorated parachlorophenol by 64 times.

On the other hand, it was thought that camphor is a vehicle, and it lowers the toxicity of phenol and parachlorophenol. However, our results revealed that CPCP has the highest cytotoxic effect on the tissue which was in line with Soekanto et al., 1996 who stated that the camphor demonstrated cytotoxicity on its own, and when phenol and parachlorophenol were added, reconfirming the cytotoxicity of these conventional antiseptics.

The antibacterial and biocompatibility results of CPCP in the current study may be clarified as that, parachlorophenol is derived from phenol. Phenol works as an antibacterial by breaking down and penetrating bacterial cell walls, which precipitates protoplasmic protein. At lower concentrations, it also deactivates essential enzyme systems that result in cell death. The low biocompatibility of CPCP is attributed to the fact that camphor is a vehicle, and it is believed that it reduces the toxicity of phenol and parachlorophenol. However, camphor itself demonstrated cytotoxicity, and the addition of camphor enhanced the cytotoxicity of phenol and parachlorophenol, proving that these conventional antiseptics are cytotoxic. CPCP triggers an inflammatory reaction by binding to the protein and lipids of cell membranes causing their disruption (de Bretas et al., 2017).

V. CONCLUSION :

Combining Ca $(OH)_2$ and CPCP shows high antibacterial efficacy compared to each medication alone. Ca $(OH)_2$ is biocompatible on the tissue followed by a combination of Ca $(OH)_2$ and CPCP then CPCP. Increasing the 'concentration of the 'medication reduces the viability of tissue, so lower concentrations of dressing substances are recommended to be less irritant on the tissue.

Conflict of Interest:

The authors declare no conflict of interest.

Funding:

Every aspect of the study was self-funded.

Ethics:

This study protocol was approved by the ethical committee of the faculty of dentistry-Cairo university on: 28/2/2023.

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