An *in vitro* Evaluation of Antimicrobial Efficacy of Primary Root Canal Filling Materials

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Objective: *The present study aimed to evaluate and compare six different materials commonly used for filling the root canals of primary teeth for antimicrobial efficacy against some of the microorganisms commonly found in infected root canals.* ***Study design:*** *In this experimental in vitro study six root canal filling materials were tested for antimicrobial efficacy against eight microbial strains using the agar diffusion method.* ***Results:*** *Zinc oxide eugenol paste exhibited the strongest antimicrobial potential followed by Endoflas™, zinc oxide-calcium hydroxide-sodium fluoride mixture, zinc oxide-calcium hydroxide mixture and calcium hydroxide paste (Apexcal™). The addition of sodium fluoride to the zinc oxide-calcium hydroxide mixture enhanced the antimicrobial efficacy. Metapex™ demonstrated minimal inhibition and Vaseline™ was non-inhibitory.* ***Conclusions:*** *All the test filling materials demonstrated varying antimicrobial activity against the microorganisms tested. Zinc oxide eugenol paste and materials containing zinc oxide were found to be more effective against the microorganisms compared to materials without zinc oxide.*

Keywords: *antimicrobial efficacy, root filling materials, primary teeth*
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INTRODUCTION

The success of endodontic therapy depends on elimination of microorganisms from the root canal system and prevention of subsequent re-infection, achieved by biomechanical cleaning and shaping, and followed by three-dimensional filling of the root canal space.[1] Conventional canal debridement procedures, however, may not be able to completely eliminate the bacteria from the root canal system, especially in primary teeth owing to their complex anatomy.[1] Studies[2,3] have shown that mechanical instrumentation with antibacterial irrigation will only render 50-70% of infected canals free of microorganisms, depending on which irrigants are used. The use of root canal filling materials with broad antimicrobial activity would enhance the chances of success in root canal therapy.

An ideal root canal filling material should be bactericidal or at least not encourage microbial growth. The antimicrobial activity of filling materials is considered important for various reasons: to avoid contamination during the manipulative phase, to complete the antimicrobial effects of the intracanal medicaments, and to inhibit the growth of microorganisms and thus prevent failure of root canal therapy.[4] In the search for an ideal filling material for primary teeth, over the years a number of materials have been tried with varying degrees of success.[5-12] These materials include zinc oxide eugenol alone[6,9,10,12] and in combination with formocresol,[6,9] chloramphenicol and tetracycline,[10] camphorated phenol,[6,9] chlorhexidine dihydrochloride[6] and zinc acetate,[13] KRI paste,[6,12] calcium hydroxide with sterile water,[6,9,10] camphorated parachlorophenol[6] and iodoform (Metapex, Vitapex),[6,9,12] iodoform paste,[12] Maisto's paste,[12] Guedes-Pinto paste,[10] Endoflas[14] and a mixture of zinc oxide and calcium hydroxide with and without sodium fluoride.[7,8]

The current study is an attempt to evaluate and compare six different root canal filling materials for antimicrobial efficacy against some of the microorganisms commonly isolated from infected root canals.

MATERIALS AND METHOD

In this experimental *in vitro* study, six materials - zinc oxide and eugenol paste (ZOE, Neelkanth, Jodhpur, India), a mixture of zinc oxide powder and calcium hydroxide paste in distilled water ($ZO+Ca(OH)_2$), a mixture of zinc oxide powder and calcium hydroxide paste in 10% sodium fluoride ($ZO+Ca(OH)_2+NaF$), calcium hydroxide paste (ApexCal™, Ivoclair-Vivadent), calcium hydroxide with iodoform (Metapex™, Meta Dental Co., Korea), and a mixture of iodoform, calcium hydroxide and zinc oxide (Endoflas™, Sanlor

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Laboratory, Florida) were tested for antimicrobial efficacy against eight microbial strains. Petroleum jelly (Vaseline™, Hindustan Unilever Ltd., India) was used as the control. These root canal filling materials were selected as they have been successfully used for filling the primary tooth canals, are easy to manipulate and readily available.[5-14]

The test materials were either obtained in a premixed form (ApexCal™, Metapex™, Endoflas™) or mixed using standardized powder-liquid ratios (ZOE,[6] ZO+ $Ca(OH)_2$[7], ZO+$Ca(OH)_2$+NaF[8]). Mixing was done on a pre-sterilized glass slab using a cement spatula at room temperature and the materials loaded into sterile 2 ml syringes just prior to filling the agar wells.

Freeze-dried, pure strains of some of the microorganisms which are reported to inhabit nonvital root canals of primary teeth[9,11,15,16] were employed in this inhibition experiment. These microbial strains were obtained from the Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology, Chandigarh, India, and are listed below with their MTCC reference numbers and corresponding American-Type Culture Collection (ATCC) numbers. These strains were further grouped into aerobic gram-positive, aerobic gram-negative and fungi.

Group I: Aerobic gram-positive

Staphylococcus aureus (MTCC 737, ATCC 6538), *Staphylococcus epidermis* (MTCC 435), *Streptococcus mutans* (MTCC 497, ATCC 25175), *Bacillus subtilis* (MTCC 441, ATCC 6633), *Enterococcus faecalis* (MTCC 439, ATCC 29212)

Group II: Aerobic gram-negative

Escherichia coli (MTCC 443, ATCC 25922), *Pseudomonas aeruginosa* (MTCC 424, ATCC 27853)

Group III: Fungi

Candida albicans (MTCC 227, ATCC 10231)

These microbial strains were selected because they are commonly isolated from the infected root canals of primary teeth.[11,15,16] Microorganisms such as *E coli*, *E faecalis*, *B subtilis* and *S aureus* also serve as a reference in quality control procedures used in antimicrobial sensitivity tests.[17]

The pure strains of the microorganisms were inoculated in nutrient broth to prepare a suspension which was then inoculated on MacConkey agar and incubated at 37°C for 18-24 hours to obtain microbial growth. The purity of each test strain was confirmed by Gram's stain and colony morphology.[18]

Agar diffusion assay

Sensitivity testing was done by the standard agar diffusion method.[18] At least 3 to 5 well-isolated colonies of the same morphological type were selected. The top of each colony was touched with the wire loop and transferred into a tube containing normal saline and mixed well. Turbidity of the suspension was adjusted to match that of 0.5 McFarland turbidity standard using normal saline and a suspension containing approximately 1-2 CFU/ ml of microorganisms was obtained. Eight suspensions of the eight microbial strains used in the study were prepared using this method.

Muller-Hinton agar plates were pre-dried in an incubator for 30 minutes and inoculated by lawn culture.[17] A sterile cotton swab was dipped into the microbial suspension obtained earlier, rotated several times and pressed firmly on the inside wall of the test tube above the fluid level to remove excess inoculum. The swab was then streaked two or more times over the entire surface of a Muller-Hinton agar plate rotating the plate approximately 60° each time to ensure an even distribution of inoculum.

Three wells, 3mm in depth and in diameter, were made in each of eight agar plates with a sterile agar puncher. Four wells of similar dimensions were made in each of another eight agar plates. The seven wells were completely filled with the test and control materials. The plates were pre-incubated for 1 hour at room temperature to allow diffusion of the materials through the agar and then incubated at 37° C for 16-24 hours.[9,19]

The diameters of the zones of microbial inhibition around each test material were measured in millimeters (HiAntibiotic Zone Scale, HIMEDIA). The experiment was repeated twice for each strain and one observer measured the zones. The mean zone of inhibition for each material-microbial strain combination was then calculated.

Statistical analysis

Statistical analysis was carried out by one-way ANOVA using software SPSS (Statistical Package for Social Sciences) version 12.0 with post-hoc (Scheffe's) test to compare the statistical difference between antimicrobial effects of materials tested. Also, a non-parametric Kruskal Wallis test was used to compare the data from the growth inhibition zones using the same software. A p-value of less than 0.05 was considered statistically significant.

RESULTS

Measurements of inhibitory zones were ranked arbitrarily into four categories as strong, medium, weak and non-inhibitory according to the proportional distribution of the data set (Table 1). The zones of inhibition produced by the test materials against the selected microorganisms together with the ranking of the inhibitory potential are presented in Table 2.

It was observed that ZOE showed strong inhibitory activity against *S aureus*, *S epidermis*, *B subtilis*, *P aeruginosa* and *C albicans,* and medium inhibition of the rest of the

Table 1. Arbitrary ranking of inhibitory zone measurements

Rank	Range of zone diameters (mm)	% of data set represented*	Frequency (N = 56)
No	0	44.6	24
Weak (W)	0.1 – 6.1	8.9	05
Medium (M)	– 18.1	37.5	21
Strong (S)	>18.1	10.7	06

* Data consisted of mean zone measurements of 8 test microorganisms and 7 materials

Table 2. Mean zones of inhibition (mm) with ranking of test materials against microbial strains

Microorganis	Material						
	ZOE	ZO+$Ca(OH)_2$	ZO+$Ca(OH)_2$+NaF	$Ca(OH)_2$	Metapex™	Endoflas™	Vaseline™
S aureus	19 ± 2.82 (S)	0 (No)	7 ± 0 (M)	3 ± 0 (W)	0 (No)	11.5 ± 3.53 (M)	0 (No)
S epidermis	19 ± 2.82 (S)	6 ± 1.41(W)	12 ± 5.65 (M)	9 ± 2.82 (M)	0 (No)	9 ± 2.82 (M)	0 (No)
S mutans	17 ± 2.82 (M)	11 ± 1.41 (M)	13.5 ± 0.70 (M)	5 ± 2.82 (W)	0 (No)	13.5 ± 3.53 (M)	0 (No)
B subtilis	20.5 ± 0.70 (S)	9.5 ± 0.70 (M)	7 ± 2.82 (M)	9.5 ± 2.12 (M)	10 ± 4.24 (M)	17 ± 1.41 (M)	0 (No)
E faecalis	17 ± 2.82 (M)	0 (No)	4 ± 1.41(W)	0 (No)	0 (No)	10 ± 1.41(M)	0 (No)
E coli	16 ± 4.24 (M)	6.5 ± 1.12 (M)	0 (No)	0 (No)	0 (No)	12.5 ± 3.53 (M)	0 (No).
P aeruginosa	18.5 ± 2.12 (S)	0 (No)	0 (No)	6 ± 1.41(W)	0 (No)	8.5 ± 0.70 (M)	0 (No)
C albicans	26 ± 1.41 (S)	0 (No)	0 (No)	12 ± 1.41 (M)	0 (No)	26 ± 1.41 (S)	0 (No)

W = weak, M = medium, S = strong
Kruskal-Wallis Test

microorganisms. ZO+$Ca(OH)_2$ exhibited no inhibition of *S aureus*, *E faecalis*, *P aeruginosa* and *C albicans*, weak inhibition of *S. epidermis* and medium inhibition of the remaining four microorganisms. ZO+$Ca(OH)_2$+NaF showed medium inhibitory activity against *S. epidermis*, *S mutans*, *S aureus* and *B subtilis*, weak inhibition of *E faecalis* and no inhibition of the rest. ApexCal™ displayed medium inhibitory potential against *C albicans*, *S. epidermis* and *B subtilis*, and weak inhibitory potential against *S aureus*, *S mutans* and *P aeruginosa*. It did not, however, show any inhibition of *E. coli* and *E faecalis*. Metapex™ did not inhibit most of the microorganisms, with the exception of *B subtilis*, against which it was found to have medium inhibitory potential. Endoflas™ showed strong inhibition of *C.albicans* and medium inhibition of the rest of the microorganisms. Vaseline™, which was used as the control, was the only material to show no inhibition of any of the eight microorganisms (Table 2).

An inter-material comparison of antimicrobial efficacy showed that the inhibitory potential of ZOE was comparable to that of Endoflas™ and significantly more ($p < 0.001$) than that of all the other materials tested. Endoflas™ demonstrated significantly more inhibitory activity than the remaining four test materials ($p < 0.05$) (Table 3).

An evaluation of the inhibitory potential of the test materials against microbial groups, i.e., gram-positive, gram-negative and fungi, revealed that ZOE showed strong inhibition of fungi and gram-positive and medium inhibition of gram-negative microorganisms. ZO+$Ca(OH)_2$ did not inhibit fungi but showed weak inhibition of the remaining two groups. ZO+$Ca(OH)_2$+NaF and Metapex™ exhibited medium inhibitory potential against the gram-positive microorganisms, and none against fungi and gram-negative microorganisms. Apexcal™ weakly inhibited the gram-positive and gram-negative microorganisms, while against fungi it displayed medium inhibitory potential. Endoflas™ showed medium inhibition of the gram-negative and gram-positive

Table 3. Inter-material comparison of antimicrobial efficacy

Material	Material	Mean Difference	p-value
ZOE	ZO+$Ca(OH)_2$	15.00	0.000**
	ZO+$Ca(OH)_2$+ NaF	14.31	0.000**
	ApexCal™	13.56	0.000**
	Metapex™	17.88	0.000**
	Endoflas™	05.63	0.115
	Vaseline™	19.12	0.000**
ZO+$Ca(OH)_2$	ZO+$Ca(OH)_2$+ NaF	-0.68	1.000
	ApexCal™	-1.44	0.992
	Metapex™	02.88	0.804
	Endoflas™	-9.38	0.001*
	Vaseline™	4.13	0.431
ZO+$Ca(OH)_2$+NaF	ApexCal™	-0.75	1.000
	Metapex™	03.56	0.604
	Endoflas™	-8.69	0.002*
	Vaseline™	04.81	0.25
Apexical™	Metapex™	04.31	0.377
	Endoflas™	-7.94	0.006*
	Vaseline™	05.56	0.122
Metapex™	Endoflas™	-12.25	0.000**
	Vaseline™	01.25	0.996
Endoflas™	Vaseline™	13.50	0.000**

Post-Hoc (Scheffe's) test
* Significant; ** Highly Significant

Table 4. Mean zone (mm) and rank of microbial-group inhibition by test materials

Microbial group	ZOE	ZO+ $Ca(OH)_2$	ZO+ $Ca(OH)_2$ +NaF	ApexCal™	Metapex™	Endoflas™	Vaseline™
Gram-positive	18.5 (S)	5.3 (W)	8.7 (M)	5.3 (W)	2 (W)	12.2 (M)	0 (No)
Gram-negative	17.3 (M)	3.3 (W)	0 (No)	3 (W)	0 (No)	10.5 (M)	0 (No)
Fungi	26 (S)	0 (No)	0 (No)	12 (M)	0 (No)	26 (S)	0 (No)

W = weak, M = medium, S = strong

microorganisms and a strong inhibition of the fungi. Vaseline™, which was used as control, was found to be non-inhibitory (Table 4).

No differences of statistical significance were observed in microbial resistance of the three microbial groups against the test materials ($p > 0.05$). Similarly, a comparison of the antibacterial efficacy of each material against each microbial group did not reveal any statistically significant difference ($p > 0.05$).

DISCUSSION

Debate over what may be an ideal root canal filling material for primary teeth has existed for several decades. Numerous materials have been proposed[5-12] but no single material has been found to meet all requirements. Considering the particularity of primary teeth, the complete sanitization process requires the use of root canal filling materials with properties such as antimicrobial potential and biocompatibility.[10] Observing the importance of this aspect of pulp therapy for the primary dentition, several authors have evaluated the antimicrobial action of materials used in the endodontic treatment of primary teeth; however, the results obtained show significant divergence.[5-12,15,20]

The present study evaluated the antimicrobial potential of some materials commonly used for filling root canals of primary teeth, such as, zinc oxide eugenol paste, calcium hydroxide paste and Metapex™, and a few newer materials that have been successfully used in primary tooth obturation like Endoflas™, a mixture of calcium hydroxide paste and zinc oxide powder and a mixture of calcium hydroxide paste and zinc oxide powder with sodium fluoride.[5-14]

Eight microorganisms which are commonly isolated from infected root canals of primary teeth[11,15,16] - *S mutans*, *C albicans*, *P aeruginosa*, *B subtilis*, *E coli*, *E faecalis*, *S epidermis* and *S aureus* - were selected for the current study. *E faecalis* can be found in the oral cavity of children through contamination, such as from a pacifier, and is part of the microbiota of infected canals of primary teeth.[11] Though *B subtilis* presents some resistance to sterilization, chemical and physical processes, it does not participate in the oral pathogenic flora. It was only included in this study as a quality parameter for the sensitivity test of an antimicrobial agent, having exhibited an excellent action. Additionally, microorganisms such as *E coli*, *E faecalis* and *S aureus* serve as a reference in quality control procedures used in antimicrobial sensitivity tests.[17]

The agar diffusion method, which was used in the present study, is one of the most commonly employed to evaluate antimicrobial activity using pure cultures of oral bacteria[6] because it allows direct comparison of the materials against the microorganisms, indicating which material has the potential to eliminate bacteria in the local micro-environment of the root canal system.

The strongest antimicrobial effect was exhibited by ZOE, a finding that is in accordance with that of Bonow *et al.*[20] ZOE showed medium inhibition of *S mutans*, *E coli* and *E faecalis*, and a strong inhibition of the rest of the microorganisms. These findings corroborate those of several studies[10,12,21,22] which have shown that ZOE inhibits all these microorganisms with the exception of *S mutans*. However, contradictory results have been reported from some studies where ZOE did not inhibit *E coli,*[13] *P aeruginosa*[9] and *E faecalis.*[9] ZOE showed strong inhibition of gram-positive microorganisms and fungi, and medium inhibition of the gram-negative microorganisms. This is partly in accordance with a study by Cox *et al* [13] which showed that gram-positive microorganisms were sensitive to ZOE but not the gram-negative microorganisms. Cox *et al* [13] found that when zinc oxide is used alone there is no inhibitory effect on any of the test organisms and concluded that the activity of ZOE may be attributable to the free eugenol released from the set materials.[23] On the contrary, Spencer and co-workers[24] have found that zinc oxide does have an antimicrobial action even when used without eugenol, which was evidenced in the present study by the fact that materials containing zinc oxide were found to be more effective than materials without zinc oxide.

In the present study, ZO+$Ca(OH)_2$ exhibited no inhibition of *S aureus*, *E faecalis*, *P aeruginosa* and *C albicans*, weak inhibition of *S epidermis* and medium inhibition of the remaining four microorganisms. ZO+$Ca(OH)_2$ did not inhibit fungi but showed weak inhibition of the remaining two groups. This material has been tried as a root canal filling material for primary teeth[7]; however, its antimicrobial potential has not been tested. Its weaker action, as observed in this study, may be explained by the absence of eugenol.[13,23]

A mixture of zinc oxide powder and calcium hydroxide paste in sodium fluoride displayed moderate inhibitory activity against *S epidermis*, *S mutans*, *S aureus* and *B subtilis*, weakly inhibited *E faecalis* but not any of the remain-

ing microorganisms. This mixture moderately inhibited only the gram-positive microorganisms. The improved efficacy of this mixture could be attributed to the addition of fluoride, whose antimicrobial effect is well-established.[25]

Calcium hydroxide paste - ApexCal™, when used alone, displayed moderate inhibitory potential against *C albicans*, *S epidermis* and *B subtilis*, and weak inhibition of *S aureus*, *S mutans* and *P. aeruginosa*. These findings are in accordance with those of several studies[10,16] and contradictory to those of some other studies.[26,27] ApexCal™ did not inhibit *E coli* and *E faecalis* which supports the findings of several studies.[9,26,28] The failure of calcium hydroxide to eliminate enterococci effectively may be explained by their tolerance of high pH values, varying from 9 to 11.[19] Some studies,[10,28] however, have reported a weak inhibition of *E faecalis* by calcium hydroxide.

The antimicrobial activity of calcium hydroxide is related to its high pH and its effectiveness is linked to the diffusion of hydroxyl ions through the dentinal tubules and accessory canals into areas where bacteria and their byproducts may be harbored. In addition to acting as a physical barrier, the calcium hydroxide may both prevent root canal re-infection and interrupt the nutrient supply to the remaining bacteria. Its alkaline pH promotes a destructive effect on cell membranes and protein structure.[29]

A systematic review with meta-analysis by Sathorn *et al*[30] has reported that calcium hydroxide has limited effectiveness in eliminating bacteria from human root canals when assessed by culture techniques. Rezende *et al*[31] have reported a reduced in-vitro antimicrobial activity of calcium hydroxide paste against polymicrobial cultures using agar diffusion assay. This is surprising because the high pH of calcium hydroxide should inhibit bacteria. It is possible that the pH was neutralized by blood or buffers in culture media in the in-vitro experiments, a phenomenon that may also occur in vivo where blood and buffering systems are present. Another reason may be that the high pH of calcium hydroxide precipitates this medicament on agar, preventing its diffusion. These facts may explain its limited performance when the agar diffusion method is employed.[16,26,27]

As in several other studies,[6,9,12,26] in the present study Metapex™ demonstrated the least antimicrobial action. It did not inhibit most of the microorganisms except *B subtilis*, against which it was found to have medium inhibitory potential. This is in agreement with the findings of other studies where Metapex™ or another commercial product Vitapex™ having the same formulation was employed,[10,12,26] with the exception of a few studies which have reported inhibition of *S aureus*[9,32] and *S mutans*.[32] The weak activity may be partially explained by the fact that calcium hydroxide, an ingredient of Metapex™, has been demonstrated to interfere with the antiseptic capacity of dyadic combinations of endodontic medicaments.[33] However; many studies have reported high clinical success rates.[34,35]

In the present study, Endoflas™ moderately inhibited the gram-negative and gram-positive organisms and showed strong inhibition of *C albicans*. Neelakantan *et al* [1] reported that Endoflas™ inhibited *C albicans* and *E faecalis*. The high antimicrobial activity of Endoflas™ was probably due to the presence of iodoform and eugenol, both of which have antibacterial action. Eugenol acts by protein denaturation, while iodoform is an oxidizing agent. Even after the material sets, surface hydrolysis of the chelate (zinc eugenolate) results in release of eugenol, thus explaining the effective antibacterial activity of this substance even after 72 hours.[36]

It is difficult to draw conclusions based on in-vitro evaluation of antimicrobial activity with isolated bacteria. It is well-known that endodontic infections are mixed with complex floral interactions. The effect of test filling materials against a single strain may not be the same against a mixed variety of infection. The use of artificial media also plays an important role in determining the experiment results. The method utilized to determine the antimicrobial activity i.e., agar diffusion assay, has its own limitations. It is possible that different results might have been obtained had other methods of testing antimicrobial activity been employed. Moreover, the results of the agar diffusion method, as the other *in vitro* tests, depend upon the molecular size, solubility and diffusion of the materials through the aqueous agar medium, the sensitivity of the drug, bacterial source (wild strains or collection species), the number of bacteria inoculated, pH of the substrates in plates, agar viscosity, storage conditions of the agar plates, incubation time and the metabolic activity of the microorganisms. Therefore, the inhibition zones may be more related to the materials' solubility and diffusibility in agar than to their actual efficacy against the microorganisms.[37]

While the objective of the present study was to test bacteria that were more representative of endodontic microbiota, comparing our data with previous studies is difficult because of the different test strains, media and culture conditions involved. In addition, some of the test materials were prepared in the laboratory by the investigator; hence, the formulations may have differed slightly from similar products used in other investigations. Further, *in vivo* studies are required to state the specific antimicrobial activity and merits and demerits of any of the test filling materials.[9]

CONCLUSION

In conclusion, from the present in-vitro evaluation of antimicrobial activity with eight microbial strains commonly found in root canals of primary teeth, it may be acknowledged that zinc oxide eugenol has strong inhibitory properties followed by Endoflas.™ The addition of sodium fluoride to the zinc oxide-calcium hydroxide mixture enhanced the antimicrobial efficacy, but not above that of zinc oxide eugenol. Materials containing zinc oxide were found to be more effective against the microorganisms compared to materials without zinc oxide.

Although the antimicrobial efficacy of a root canal filling material may be vital to achieving success in endodontic therapy, it is not the only property desired of an ideal material. In fact, studies have demonstrated clinical success using materials which have exhibited limited in-vitro antimicro-

bial action. Also, endodontic infections are complex in terms of the microflora and their interactions. Further studies, especially in vivo, are required before any single material may be considered ideal.

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