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Research Protocol

Dentistry Section

Comparative Evaluation of Antimicrobial Efficacy of Different Root Canal Filling Materials Combined with Theobromine in Primary Teeth: A Research Protocol of an In-vitro Study

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ABSTRACT

Introduction: Preserving the health and structure of primary teeth is crucial for a child's growth and development. Premature extraction of primary teeth can lead to unfavourable changes in the eruption pattern and alignment of permanent teeth. To ensure the success of endodontic therapy in primary teeth, it is essential to use a biocompatible obturating material with effective antimicrobial properties. An ideal root canal filling material for primary teeth should not harm the periapical tissues, should promote the normal development of the permanent successor tooth and should undergo resorption simultaneously with root resorption. Additionally, it should be easy to place, adhere to the root canal walls, resorb if extruded beyond the apex, appear radiopaque on radiographs and not cause tooth discolouration. Zinc oxide Eugenol (ZnOE), iodoform-based pastes and calcium hydroxide are commonly used obturating materials for primary teeth. However, ZnOE has several disadvantages, including slow resorption, potential tissue irritation, bone and cementum necrosis and interference with the eruption of permanent teeth. Theobromine is a crystalline, water-insoluble alkaloid found in cacao plants. It has been shown to strengthen tooth enamel and exhibit antibacterial activity against microorganisms such as *Lactobacillus acidophilus* and *Enterococcus faecalis*.

Need of the study: There is a need to identify root canal filling materials suitable for primary teeth that combine effective antimicrobial activity with biocompatibility and appropriate resorbability. Existing materials, such as zinc oxide eugenol, possess limitations that may compromise treatment outcomes. Therefore, investigating formulations enhanced with theobromine may represent a potential advancement in paediatric endodontics.

Aim: To assess and compare the antimicrobial effectiveness of root canal filling materials, including zinc oxide eugenol and calcium hydroxide, combined with theobromine in primary teeth.

Materials and Methods: An in-vitro study will be conducted at the Department of Paediatric and Preventive Dentistry at Sharad Pawar Dental College and Hospital, in collaboration with the Department of Microbiology, Jawaharlal Nehru Medical College, Datta Meghe Institute of Higher Education and Research (DMIHER), Sawangi, Wardha, Maharashtra, India, involving 25 patients aged 4-8 years. Microbial samples will be collected from infected primary molar teeth. The antimicrobial efficacy of four different root canal filling materials—zinc oxide mixed with theobromine, zinc oxide eugenol, calcium hydroxide mixed with theobromine and calcium hydroxide mixed with saline—will be evaluated. Vaseline will serve as the negative control. The mean zone of inhibition will be considered the primary outcome measure. Data will be tested for normality using the Shapiro–Wilk test. If the data are normally distributed, one-way Analysis of Variance (ANOVA) followed by Tukey's post hoc test will be applied. If the data are not normally distributed, the Kruskal–Wallis test followed by the Mann-Whitney U test with Bonferroni correction will be used. A p-value of <0.05 will be considered statistically significant.

Keywords: Biocompatability, Calcium hydroxide, Zinc oxide eugenol

INTRODUCTION

For normal growth and development in children, it is essential to preserve the health and structure of primary teeth until their natural exfoliation. This also supports normal occlusion, complete development of skeletal and facial structures and improved aesthetic outcomes. Premature extraction of primary teeth can result in unfavourable changes in the eruption and alignment of permanent teeth; therefore, timely treatment of primary teeth with pulpal or periapical pathology is essential until exfoliation occurs [1].

Complete elimination of infection-causing microorganisms is critical for the success of endodontic therapy in primary teeth. Pulpal infections in primary teeth are typically polymicrobial due to complex root canal anatomy that can harbour a variety of pathogens. Moreover, thorough debridement through chemomechanical preparation is often challenging because of the curved and intricate morphology of primary root canals. This highlights the importance of using an antimicrobial root canal filling material that can inhibit residual microorganisms and enhance treatment success [2].

An ideal root canal filling material for deciduous teeth should be antibacterial, resorb at a rate similar to root resorption, promote the development of the permanent successor tooth and not damage periapical tissues. Additionally, it should be easy to insert, adhere to canal walls, resorb if extruded beyond the apex, be radiopaque on radiographs and not cause tooth discolouration [3].

Zinc oxide eugenol, iodoform-based pastes and calcium hydroxide are commonly used obturating materials for primary teeth. Zinc oxide eugenol was first discovered by Bonastre in 1837 and introduced into dentistry by Chisholm in 1876. In 1930, Sweet reported its use as a root canal filling material. Until 2008, ZnOE was recommended by the American Academy of Paediatric Dentistry (AAPD) for obturating primary teeth. However, ZnOE has several disadvantages, including slow resorption, potential irritation of periapical tissues, risk of bone and cementum necrosis and possible disruption of permanent tooth eruption [4].

Calcium hydroxide was first used as a root canal filling material for deciduous teeth by Hermann in 1920. Since then, it has been used

either alone or in combination with iodoform, as in formulations such as Vitapex and Metapex. Calcium hydroxide is known for its favourable resorption properties, radiopacity and ability to remain in a non-hardened state. It is easy to place and remove from root canals, does not adversely affect developing permanent tooth germs and resorbs from the apex within a few weeks [3,5].

Theobromine is a crystalline, water-insoluble alkaloid found in cacao plants and is also present in tea, chocolate and other food products. It strengthens tooth enamel by increasing resistance to demineralisation. In calcium- and phosphate-rich environments, theobromine promotes the formation of larger hydroxyapatite crystallites, making enamel less susceptible to acid attack. Additionally, theobromine exhibits antibacterial activity against *Lactobacillus acidophilus* and *Enterococcus faecalis* and has been shown to inhibit *Streptococcus mutans* [6,7].

The purpose of this study is to evaluate the antimicrobial effectiveness of four root canal filling materials: zinc oxide eugenol mixed with theobromine, zinc oxide eugenol alone, calcium hydroxide mixed with theobromine and calcium hydroxide mixed with saline, using vaseline as a control, against microorganisms isolated from the root canals of infected primary teeth.

REVIEW OF LITERATURE

Amorim LDFGD et al., (2006) employed two experimental methods, namely the Direct Exposure Test and the Agar Diffusion Test, to compare the antimicrobial activity of various root canal filling pastes used in paediatric dentistry. The materials evaluated included Guedes-Pinto paste, zinc oxide eugenol paste, calcium hydroxide paste, Chloramphenicol + Tetracycline + Zinc oxide eugenol Paste (CTZP) and Vitapex®. In the Direct Exposure Test, microbial growth was assessed after exposing different microbial strains to the pastes for varying time periods. Within one to twenty-four hours, all materials demonstrated antimicrobial activity. However, Vitapex® did not produce any inhibition zone. Except for Vitapex®, which showed no antimicrobial effect under the test conditions, all other pastes exhibited effective antimicrobial action during the initial 24 hours [8].

Reddy S and Ramakrishna Y (2007) conducted a study evaluating the antimicrobial efficacy of calcium hydroxide with sterile water ($CaOH+H_2O$), Zinc Oxide Eugenol (ZOE), Zinc Oxide Eugenol with Formocresol (ZOE+FC), Zinc Oxide with Camphorated Phenol (ZO+CP) and calcium hydroxide with iodoform (Metapex). Vaseline was used as the control. Using the agar diffusion technique, the greatest antibacterial effect against the tested microorganisms was observed with ZOE+FC, while Vaseline and Metapex showed no inhibitory activity. Statistical analysis revealed that ZOE+FC was significantly more effective than ZOE, ZO+CP and $CaOH+H_2O$ [1].

Kriplani R et al., (2013) evaluated the antimicrobial activity of various root canal filling materials against 18 bacterial strains isolated from infected primary molars, both with and without aloe vera. The materials tested included aloe vera with sterile water, ZOE, ZOE mixed with aloe vera, calcium hydroxide with sterile water, calcium hydroxide mixed with aloe vera, calcium hydroxide with iodoform (Metapex) and Vaseline as a control. The agar diffusion method was used to determine the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of aloe vera. Aloe vera mixed with sterile water demonstrated the maximum antibacterial activity, followed by aloe vera combined with calcium hydroxide and ZOE. Vaseline showed no inhibitory effect, while ZOE, calcium hydroxide and Metapex exhibited comparatively reduced activity. The study concluded that aloe vera enhances the antibacterial efficacy of root canal filling materials [9].

Navit S et al., (2016) evaluated the antimicrobial activity of various obturating materials used in paediatric pulpectomy. The materials tested included Endoflas, ZOE, calcium hydroxide mixed with chlorhexidine, calcium hydroxide mixed with iodoform, Metapex and saline as a control, against *Enterococcus faecalis* (ATCC 29212) using the agar diffusion technique. Endoflas demonstrated significantly greater antimicrobial activity compared to all other materials, except ZOE. Intergroup comparison using Tukey's test at 24 hours showed statistically significant differences in antibacterial efficacy among the materials. The ranking of effectiveness was as follows: Endoflas > ZOE > calcium hydroxide + chlorhexidine > calcium hydroxide + iodoform = Metapex > saline. This study emphasised the importance of selecting obturating materials with strong antibacterial properties to ensure the success of pulpectomy in primary teeth [10].

Lakshmi A et al., (2019) investigated the antimicrobial potential of theobromine and two commercially available fluoride toothpastes for children. The aim of the study was to compare the efficacy of Kidodent and Colgate Kids fluoride toothpastes with a cocoa-based non-fluoride toothpaste containing theobromine in inhibiting oral pathogens. Toothpaste containing theobromine demonstrated greater antimicrobial potency compared to fluoride toothpastes. While theobromine showed neutral activity against *Candida albicans*, it produced larger zones of inhibition against *Streptococcus mutans*, *Lactobacillus acidophilus* and *Enterococcus faecalis*. These findings suggest that theobromine may serve as a suitable alternative for preventing paediatric oral infections [6].

Konde S et al., (2021) assessed the antibacterial activity of theobromine in combination with neem extract, tulsi extract, Gow-Arka and Sodium Hypochlorite (NaOCl) as root canal irrigants against *Enterococcus faecalis*. Antibacterial efficacy was evaluated using the pour plate method and agar well diffusion test. A combination of theobromine and neem extract exhibited the highest antibacterial activity against *E. faecalis*, with results comparable to NaOCl and no statistically significant difference between them. The findings suggest that theobromine, particularly when combined with neem, may be used as a natural alternative to sodium hypochlorite for root canal irrigation, offering reduced toxicity and improved biofilm removal [11].

Rafiq IH et al., (2024), in a pilot study, assessed the antimicrobial activity of theobromine, sodium fluoride and their combination against the caries-causing bacteria *Streptococcus mutans* and *Actinomyces naeslundii*. Antimicrobial susceptibility was evaluated using microbial suspensions grown on selective media and assessed by the broth microdilution method. The results demonstrated that bacterial density was significantly lower under all experimental conditions—namely theobromine, fluoride and their combination—compared to the control group. However, no statistically significant differences in viable bacterial counts were observed among the treatment groups ($p>0.05$). The findings indicated that the antimicrobial activity of theobromine against *S. mutans* and *A. naeslundii* was comparable to that of fluoride alone or in combination. Further studies were suggested to explore the potential of theobromine as an alternative anticaries agent [12].

The present study aims to assess and compare the antimicrobial effectiveness of different root canal filling materials combined with theobromine in primary teeth.

Objectives

Primary objective:

- To compare the antibacterial effectiveness of zinc oxide eugenol and calcium hydroxide, with and without the addition of theobromine.

Secondary objective:

- To evaluate the antibacterial effectiveness of zinc oxide + theobromine and calcium hydroxide + theobromine against bacteria isolated from the root canals of infected primary teeth.
- To assess the antibacterial effectiveness of zinc oxide eugenol and calcium hydroxide combined with saline against bacteria isolated from the root canals of infected primary teeth.

Null hypothesis: There will be no statistically significant difference in the antimicrobial efficacy of the tested root canal filling materials—

zinc oxide eugenol, zinc oxide eugenol + theobromine, calcium hydroxide + theobromine, calcium hydroxide + saline and Vaseline (control)—against aerobic and anaerobic microbial isolates obtained from infected primary molars.

MATERIALS AND METHODS

An in-vitro study will be conducted in the Department of Paediatric and Preventive Dentistry at Sharad Pawar Dental College and Hospital, in collaboration with the Department of Microbiology, Jawaharlal Nehru Medical College, Datta Meghe Institute of Higher Education and Research (DMIHER), Sawangi, Wardha, Maharashtra, India. The study will include a total of 25 patients.

Before data collection, all patients and their parents/guardians will be adequately informed about the study, and written informed consent will be obtained. Ethical clearance has been obtained from the Institutional Ethics Committee, and the registered IEC approval number is DMIHER(DU)/IEC/2025/558.

Sample size calculation: Sample size was calculated by using formula-

$n_1=[(\sigma_1^2+\sigma_2^2/\kappa)*(z_{1-\alpha}/2+z_{1-\beta})^2]/\Delta^2$

The notation for the formulae are:

n_1 - sample size of Group 1

n_2 - sample size of Group 2

σ_1 - standard deviation of Group 1

σ_2 - standard deviation of Group 2

Δ - difference in group means

κ = ratio = n_2/n_1

$Z_{1-\alpha}/2$ = two-sided Z value (e.g., Z=1.96 for 95% confidence interval)

$Z_{1-\beta}$ = power

Mean Zone of Inhibition in group II=3.80

Mean Zone of Inhibition in group IV=4.53

σ1= SD of Zone of Inhibition in group II=0.82

σ2= SD of Zone of Inhibition in group IV=1.02

For detecting mean difference of 0.73 i.e. Δ =4.53-3.80=0.73

K =1

$$N = \frac{(0.82*0.82+1.02*1.02)(1.96+0.84)^2}{0.73}$$

= 25.19 = 25 patient samples needed in study

Inclusion criteria:

- Children aged 4-8 years;
- Infected primary molars with at least one unresorbed root;
- Presence of an abscess, fistula, or interradicular (furcation) radiolucency;
- Medically healthy children.

Exclusion criteria:

- Patients who received antibiotics within 4 weeks prior to sampling;
- Teeth with resorbed roots;
- Teeth with fractured or broken crowns;
- Medically compromised children.

Study Procedure

The microbial specimens will be obtained from the root canals of infected primary molar teeth of paediatric patients aged 4-8 years. Patients presenting with pulpal or periapical pathology will be selected for sample collection. Isolation will be achieved using a rubber dam and samples will be collected using sterile absorbent paper points, which will then be transferred to Robertson's cooked meat medium for microbiological analysis. After 24 hours of incubation, subcultures will be made on pre-reduced blood agar for anaerobic organisms and on agar plates such as Mueller–Hinton agar for aerobic organisms.

The bacterial isolates obtained from infected primary molar root canals will be classified into two groups:

Group 1: Aerobic microorganisms

Group 2: Anaerobic microorganisms

The antimicrobial efficacy of four different root canal filling materials, along with Vaseline as a negative control, will be assessed against both groups of microorganisms using the agar diffusion assay.

Test materials:

1. Zinc oxide eugenol mixed with theobromine;
2. Zinc oxide eugenol;
3. Calcium hydroxide mixed with theobromine;
4. Calcium hydroxide mixed with saline;
5. Vaseline (control)

Actively growing microbial cultures will be inoculated onto agar plates and wells will be filled with the respective test materials. The zones of inhibition surrounding the test materials will be measured after 16-24 hours of incubation. The results will then be statistically analysed to compare the antimicrobial efficacy of the materials.

Preparation of test materials: Zinc oxide powder will be mixed with theobromine powder in a 1:1 (w/w) ratio and homogenised to ensure uniform dispersion. Eugenol will then be added incrementally until a clinically usable paste consistency is achieved.

Similarly, calcium hydroxide powder will be blended with theobromine powder in a 1:1 (w/w) ratio, after which sterile saline will be added dropwise until a paste-like consistency is obtained.

Outcome Measures

The primary outcome of the study will be the mean zone of inhibition (in millimeters) produced by the tested root canal filling materials against aerobic and anaerobic microbial isolates.

The secondary outcomes will include comparative antimicrobial effectiveness between groups (pair-wise differences in inhibition zones) and the distribution of antibacterial activity between aerobic and anaerobic isolates.

STATISTICAL ANALYSIS

Data will be entered into Statistical Package for Social Sciences (SPSS) version 17.0 and checked for accuracy and completeness. Descriptive statistics, including mean, standard deviation, median and interquartile range, will be calculated. The Shapiro-Wilk test will be used to assess the normality of data distribution within each group. If the data are normally distributed, one-way ANOVA will be used to compare mean zones of inhibition across groups, followed by Tukey's post-hoc test for pair-wise comparisons. If the data do not satisfy normality assumptions, the Kruskal-Wallis test will be applied as a non-parametric alternative, followed by the Mann-Whitney U test with Bonferroni correction for pair-wise comparisons. A p-value<0.05 will be considered statistically significant.

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