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ORIGINAL ARTICLE

Histopathological Assessment of Tricalcium Aluminate-free Mineral Trioxide Aggregate and Two Antibacterial Enhanced Mineral Trioxide Aggregates As Pulpotomy Agents in Rat Model

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ABSTRACT

Objective: To evaluate the effect of a newly developed MTA-based material and two antibacterial-enhanced MTAs as pulp capping materials in immature permanent dental elements underwent full pulpotomy.

Methods: The present animal study included 20 Wistar albino rats that, after full pulpotomy, were randomly divided into 4 groups receiving different MTA formulations as pulp capping materials: conventional MTA, Tricalcium aluminate (TCA)- free MTA, and MTA enhanced with metronidazole or doxycycline. Histopathological assessments were carried out at 7- and 28-days post-treatment to evaluate dentinal bridge formation, inflammatory reactions, pulp tissue necrosis and internal resorption.

Results: Seven days post-treatment, all groups exhibited inflammation and pulp necrosis, that were minimal in Groups III and IV than Group I. Group II showed a statistically significant difference only in terms of pulp necrosis ($p<0.001$). At 28-days all Groups showed slight inflammation and pulp necrosis, mainly in Groups I. Dentinal bridge formation was appreciated in all samples belonging to Groups II, III and IV and in 7/10 specimens of Group I, resulting in a statistically significant difference ($p\leq0.001$).

Conclusion: TCA-free MTA and antibiotic-enhanced MTAs showed superior performances in dentinal bridge formation and exhibited minimal pulpal necrosis than conventional MTA. The inclusion of antibiotics might contribute to create a more sterile environment that would improve the outcomes, favoring deposition of a mineralized matrix. However, further studies are needed to support these preliminary results.

Keywords: Animal model, dentinal bridge, doxycycline, metronidazole, MTA, tricalcium aluminate, vital pulp therapy

HIGHLIGHTS

- TCA-free MTA enriched with antibiotics performed better than conventional MTA in terms of dentinal bridge formation.
- The presence of antibiotics should help to obtain a more sterile environment, improving the materials properties.
- Newly developed formulations of MTA might be helpful in the long-term outcomes of vital pulp therapy.

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INTRODUCTION

The dentine-pulp complex exhibits a notable capacity of repair through the deposition of reparative dentine in response to injuries, as dental caries or irritation stimuli, by newly differentiated odontoblast-like cells (1, 2). The ability of an inflamed pulp to heal plays a crucial role in the possibility to maintain tooth vitality over time, avoiding or delaying more invasive procedures as root canal treatment in case of pulpitis (3, 4). This rationale can be applied to treat not only dental elements affected by reversible pulpitis, but also in case of irreversible inflammation of pulp tissue (5–7). Vital pulp therapy (VTP) of immature permanent teeth have demonstrated successful and promising outcomes in the short and middle terms (8), allowing root development and apical closure (9). Full pulpotomy consists of amputation of inflamed coronal pulp and treatment of remaining radicular tissue with agents able to encourage healing and preserve vitality, ensuring tooth function and absence of pathological symptoms (10).

The materials used for pulp capping after pulpotomy had varied among years, and recent evidence showed that Mineral trioxide aggregate (MTA) and other hydraulic cements, as Biodentine, are most efficient than calcium-hydroxide-based agents in terms of success over time (11, 12). Despite its usefulness in various dental procedures and its standing as "gold standard material" in VPT (13, 14), MTA has notable limitations that hinder its optimal application in clinical practice. These include challenging handling characteristics due to its granular texture (15), prolonged setting time (16), discoloration (17) and limited effectiveness against *Enterococcus faecalis* (18), coupled with concerns on its potential cytotoxicity due to tricalcium aluminate (19). Therefore, new cement formulation that excludes tricalcium aluminate and incorporates calcium fluoride, metronidazole or doxycycline and calcium carbonate have been developed to improve setting and handling properties as well as antimicrobial characteristics, maintaining the same positive physical features (20, 21). Evaluation of this novel material during apexification has demonstrated enhanced healing and formation of a calcific barrier in an animal model (22). However, its efficacy as a pulpotomy agent remains unknown, highlighting the need for further investigation concerning the response of pulp tissue. Therefore, the current animal trial aimed to evaluate the effect of the newly developed MTA-based material and two antibacterial-enhanced MTAs as pulp capping materials in immature permanent dental elements underwent full pulpotomy.

MATERIALS AND METHODS

Study Design

The present *in-vivo* animal trial was reported according to Preferred Reporting Items for Animal studies in Endodontology (PRIASE) 2021 guidelines (23). The study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Animal Ethical Committee (IAEC) of Saveetha Dental College, Saveetha Institute of Medical and Technical Sciences (protocol number: BRULAC/SDCH/SIMATS/IAEC/09-2023/02).

Sample Size

The sample size was determined according to a previous research (24), targeting a study power of 80% and a confidence level of 95%. A minimum sample size of 10 teeth per group (n=40 samples), was obtained with an effect size of 0.45 and a desired power of 0.95.

Experimental Materials

The materials tested in the present study were: 1) novel Tricalcium aluminate (TCA)-free MTA, 2) Metronidazole-MTA and 3) Doxycycline-MTA. The core materials of these cement were tricalcium and dicalcium silicate, that were manufactured by the research analytics lab of our dental institute. All components used to developed the tested materials were purchased from TCI Chemicals Pvt. Ltd, India. Briefly, tricalcium silicate powder was obtained dissolving 0.3 M calcium nitrate tetrahydrate in a mixture of 70% ethanol, 5% polyethylene glycol (molecular weight 10,000), 1% 1 M hydrochloric acid, and 0.1 M tetraethyl orthosilicate (TEOS). At a temperature of 60°C, the solution was agitated for 3 hours. After keeping the solution at 70°C for 24 hours, the gelation process was induced by drying at 120°C. Initially, the material was calcined at 500°C for 1 hour, and then at 1200°C for 3 hours. Following the pulverization of the final product, it was submitted to heat treatment at a temperature of 1450°C for a period of 8 and 10 hours, respectively. After being ground, the finished product was sieved through a mesh size of 45 µm. The precursor solution utilized for the synthesis of tricalcium silicate was modified by the addition of 0.2 M calcium nitrate tetrahydrate to ease the production of dicalcium silicate powder. Except for the heat treatment, which was carried out at a temperature of 1000° C, the developed technique was the same. All the components were accurately weighed using a digital balance (SBA 31, Scaltec, Germany) and mixed using mortar and pestle to obtain a uniform mix. To obtain 10% calcium chloride solution, 1g of calcium chloride powder was mixed with 10 mL of distilled water using a magnetic stirrer (IKA-Werke, Staufen, Germany). Complete details of tested materials' compositions are provided within Table 1.

Animals

Twenty Wistar albino rats (Rattus norvegicus), weighing between 200 to 250gr and approximately 8 to 9 weeks old, were obtained from the animal testing center at Biomedical Research Unit and Lab Animal Centre of Saveetha Dental College, Saveetha Institute of Medical and Technical Sciences. The rats were accommodated in chambers at a temperature of 27°C and underwent a 12-hour light/12-hour dark cycle. Animals were provided with unrestricted access to rat pellet feed (Biogen Animal Health product, Bangalore) and water.

Treatment Protocol

A single trained veterinary surgeon, blinded regarding experimental groups and under authors' supervision, managed the rats, provided dental treatments and performed euthanasia. Briefly, animals were anesthetized with intraperitoneal injections of ketamine (50 mg/kg) and xylazine (10 mg/kg). Oral antisepsis was obtained by rinsing with 0.2% Chlorhexidine digluconate solution for 60 seconds. Treatment procedures followed a similar protocol according to Prabhakar et

TABLE 1. Powder composition of TCA-free MTA and two antibacterial-enhanced MTAs used in the current study

Group II: TCA-free MTA		Group III: Metronidazole-MTA		Group IV: Doxycycline-MTA	
POWDER	**Weight % for every 100 mg of powder**	**POWDER**	**Weight % for every 100 mg of powder**	**POWDER**	**Weight % for every 100 mg of powder**
Tricalcium silicate	55 wt %	Tricalcium silicate	60 wt %	Tricalcium silicate	60 wt %
Dicalcium silicate	30 wt %	Dicalcium silicate	20 wt %	Dicalcium silicate	20 wt %
Calcium Fluoride	5 wt %	Calcium Fluoride	5 wt %	Calcium Fluoride	5 wt %
Calcium Sulphate	5 wt %	Calcium Sulphate	5 wt %	Calcium Sulphate	5 wt %
Calcium Carbonate	5 wt %	Calcium Carbonate	4 wt %	Calcium Carbonate	4 wt %
Zirconium oxide	1 wt %	Zirconium oxide	1 wt %	Zirconium oxide	1 wt %
-	-	Metronidazole	5 wt %	Doxycycline	5 wt %

TCA: Tricalcium aluminate, MTA: Mineral trioxide aggregate

al. (24). The rats were placed in dorsal decubitus on a surgical table and immobilized with a holding device to keep mouths open. Animals presented permanent teeth with incomplete root formation. Access openings were obtained in the right and left mandibular first molars using a ¼ carbide round bur, ensuring consistent pulp exposure in all treated molars. The coronal pulp was removed with a small spoon excavator, and bleeding was controlled with a sterile saline-soaked cotton pellet. After achieving hemostasis, different tested materials were placed following random allocation.

Ten rats received treatments with conventional MTA (MTA Angelus, Londrina, PR, Brazil) in right mandibular molars (Group I, n=10) and a novel fast-set TCA-free MTA in left mandibular molars (Group II, n=10). Another set of 10 rats was treated with fast-set Metronidazole-MTA in right mandibular molars (Group III, n=10) and with fast- set Doxycycline-MTA in left mandibular molars (Group IV, n=10).

In the experimental groups (Groups II, III, IV), 100 mg of the specified powder was combined with 40 µl of 10% calcium chloride on a mixing pad. After achieving full hydration, the mixture was wholly homogenized to a uniform, moldable consistency. Conventional MTA (Group 1) was prepared following the manufacturer's guidelines, using a 3:1 powder-to-liquid ratio. The powder was incrementally added to the liquid and mixed until a thick, moldable consistency. The final mixture was applied to the pulp stumps using a plastic instrument, and coronal seal was obtained with resin modified glass ionomer cement (GC Corporation, Tokyo, Japan).

The animals were placed back in their chambers with unrestricted access to their regular diet and water. Pain management involved administration of meloxicam (1 mg/kg body weight) twice a day for the initial 2 days. On the third day, additional pain relief was provided only if rats showed signs of lack of appetite, suggesting heightened discomfort during chewing.

Samples Processing

Ten animals, 5 from each group, were euthanized after 7 days, whereas the remaining 10, underwent euthanasia 28 days post-treatment using carbon dioxide gas inhalation. The retrieved jaws were collected, fixed in 10% formalin for 24 hours, and decalcified with a 17% ethylenediaminetetraacetic acid solution. After processing and paraffin embedding, the samples were trimmed to expose apical foramen. Transverse sections - 6 µm thick - were stained with hematoxylin and eosin and examined under light microscopy (Eclipse E600; Nikon, Tokyo, Japan) to evaluate the periodontal ligament, cementum, and dentine. Digital images were taken at a fixed magnification of 40X using a Nikon DX-m1200C (Nikon, Tokyo, Japan) camera and analyzed for dentinal bridge formation, inflammatory reactions, pulp necrosis and internal resorption. The latter was identified through the presence of reversal lines and resorption concavities with osteoclasts along the entire radicular pulp tissue.

Statistical Analysis

Statistical Package for the Social Sciences software (SPSS) version 21.0 (IBM Corporation, NY) was applied to compare the histologic data. Analysis of the data was obtained using non-parametric Kruskal-Wallis test followed by Mann-Whitney U test post hoc. A p-value of less than 0.05 was considered statistically significant.

RESULTS

All animals exhibited good tolerance to the operative procedures, with no changes in behavior or eating habits. No animals or teeth were lost throughout the follow-up periods.

Histopathological Analysis on 7th Day

At the 7th day, no samples showed dentinal bridge formation or internal resorption. All groups exhibited inflammatory reactions, with inflammatory infiltrate observed in approximately 90% of samples in Group I, compared to 50% in Group II. On the contrary, about 70% of samples in Groups III and IV showed no inflammatory response. Pulp necrosis was observed in 50% of Group I samples, whereas the majority of samples in Groups II, III, and IV did not show pulp necrosis (60% in Group II and 70% in Groups III and IV, respectively). Intergroup analysis demonstrated statistically significant differences, showing minimal inflammatory exudates in Groups III and IV (<0.001) and minimal pulp necrosis in Groups II, III, and IV compared to Group I (<0.001) (Table 2).

TABLE 2. Histopathological analysis of samples 7 days post-treatment

Day 7	Group I - Conventional MTA		Group II - TCA-free MTA		Group III - Metronidazole-MTA		Group IV - Doxycycline-MTA		p
	n	%	n	%	n	%	n	%	
Inflammatory reaction									
Present	9	90	5	50	3	30	3	30	<0.001*
Absent	1	10	5	50	7	70	7	70	
Pulp necrosis									
Present	5	50	4	40	3	30	3	30	<0.001*
Absent	5	50	6	60	7	70	7	70	
Dentinal bridge									
Present	0	0	0	0	0	0	0	0	-
Absent	10	100	10	100	10	100	10	100	
Internal resorption									
Present	0	0	0	0	0	0	0	0	-
Absent	10	100	10	100	10	100	10	100	

*: Statistically significant difference for intergroup comparisons (p<0.05). TCA: Tricalcium aluminate, MTA: Mineral trioxide aggregate

TABLE 3. Histopathological analysis of samples 28 days post-treatment

Day 28	Group I - conventional MTA		Group II - TCA-free MTA		Group III - Metronidazole-MTA		Group IV - Doxycycline-MTA		p
	n	%	n	%	n	%	n	%	
Inflammatory reaction									
Present	2	20	2	20	2	20	2	20	-
Absent	8	80	8	80	8	80	8	80	
Pulp necrosis									
Present	2	20	1	10	1	10	1	10	-
Absent	8	80	9	90	9	90	9	90	
Dentinal bridge									
Present	7	70	10	100	10	100	10	100	<0.001*
Absent	3	30	0	0	0	0	0	0	
Internal resorption									
Present	1	10	0	0	0	0	0	0	-
Absent	9	90	10	100	10	100	10	100	

*: Statistically significant for intergroup comparisons (p<0.05). TCA: Tricalcium aluminate, MTA: Mineral trioxide aggregate

The inflammatory reaction consisted of inflammatory cells infiltration in the pulp cavity below the necrotic region. The inflammatory cells were higher in Group I compared to Group II, III and IV. Pulpal tissue necrosis was mainly noticed below the tested material in Group I than other groups, as shown in Figure 1.

Histopathological Analysis on 28th Day

At 28 days post-treatment, all samples exhibited slight inflammation, whereas none of the specimens from Groups II, III, and IV showed internal resorption. Pulpal necrosis was minimal across all groups, as 20% in Group I and 10% in Groups II, III, and IV. Except for 3 samples in Group I (30%), all specimens showed dentinal bridge formation. Within Group I, a small percentage of samples displayed inflammatory infiltrate (20%) and internal resorption (10%). Intergroup analysis revealed statistically significant differences in terms of dentinal bridge formation (<0.001) (Table 3, Fig. 2).

DISCUSSION

This study investigated the histopathological changes in pulpal tissues following pulpotomy in rat immature permanent molars, utilizing modified and antibiotic-enhanced MTAs over 7 and 28 days. While conventional MTA caused some tissue necrosis and inflammatory reactions by the 7th day, TCA-free MTA and antibiotic-enhanced MTAs showed better results for the same observed parameters. Accordingly, 28-day post-treatment, the modified MTA formulations resulted in enhanced dentine bridge formation and exhibited minimal necrosis and inflammatory response. Moreover, almost all tested materials did not show any signs of internal resorption.

The superior outcomes observed with the investigated materials might be attributed to their specific constituents. Tricalcium aluminate, known for its potential cytotoxic activity (25), was excluded from the tested formulations, probably

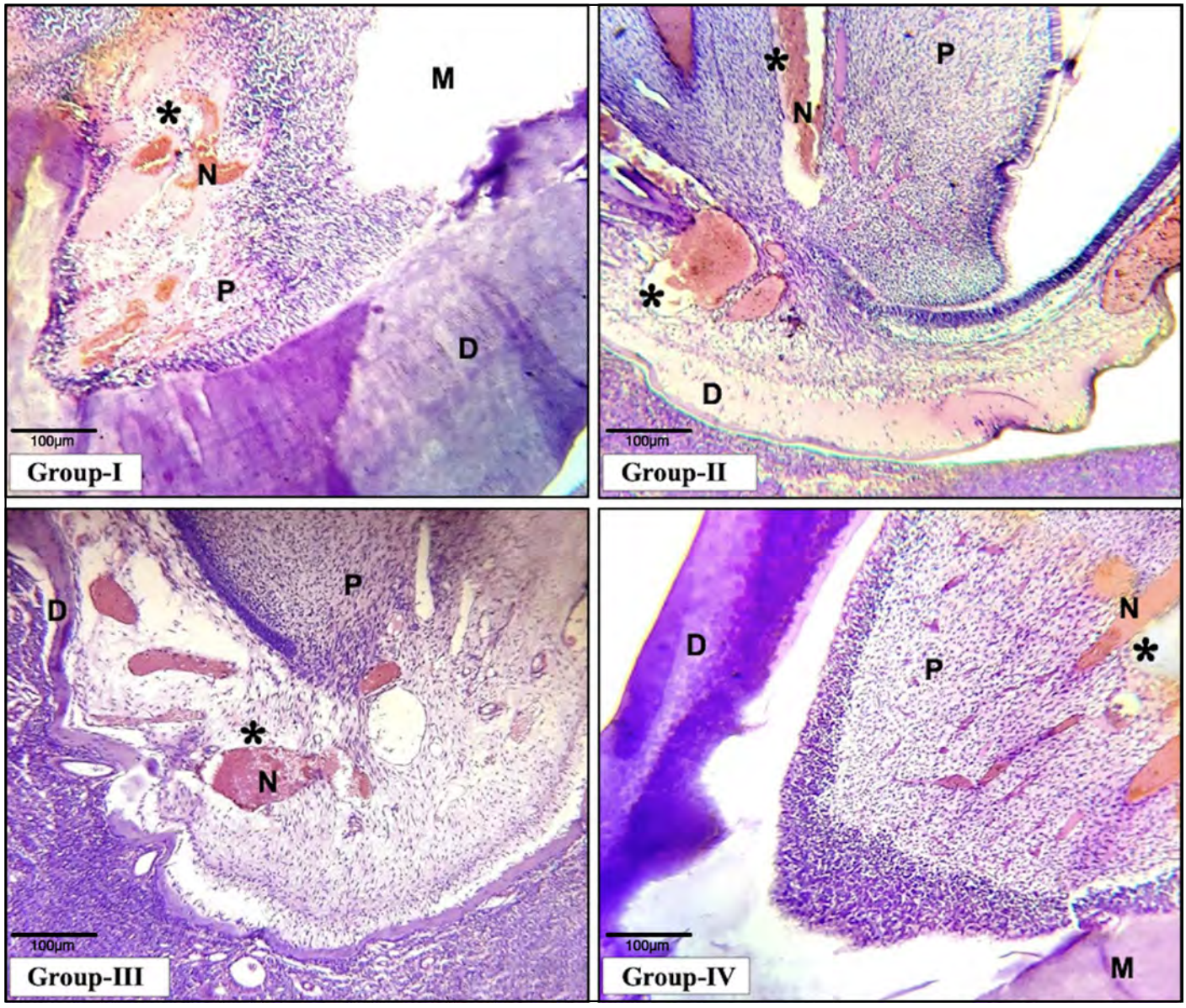

Figure 1. Histopathological images of samples of each group 7 days post-treatment (* : Inflammatory cells, N: Necrotic pulp, D: Dentine, P: Pulp, M: Material). Staining: hematoxylin and eosin; magnification 40X

reducing the pulp necrosis occurrence. The inclusion of calcium fluoride, which has been documented to promote dental pulp healing, might contribute to the improved results (26). Additionally, according to Lu et al. (27), calcium carbonate should prevent pulp necrosis and cause only mild to moderate inflammation. Indeed, the contact of calcium carbonate with pulp stumps would stimulate odontoblastic activity, leading to dentine bridge formation as that observed among Groups II, III and IV (28).

MTA is widely used as a pulpotomy agent due to its role as hydraulic material allowing the release of calcium and hydroxyl ions upon hydration, which help to increase pH and foster the deposition of detin-like mineralized tissue (29). Moreover, the release of specific chemical signals seems to favor periapical tissue repair (30). Efforts have focused on enhancing MTA by decreasing its toxicity and improving its handling, sealing, and biocompatibility (16, 29). MTA's capacity to promote dentinal bridge formation is invaluable (2), however, the cytotoxicity of tricalcium aluminate, a component of traditional MTA, highlights the necessity for long-term evaluations to ensure biocompatibility over time (25). Therefore, it is essential to modify MTA to balance antimicrobial properties with preservation of physical characteristics (19).

Antibiotic-combined MTAs have shown better performance than the conventional and TCA-free MTA in terms of pulp necrosis and inflammatory activity. It can be speculated that the inclusion of antibacterial agents significantly hastened the healing of pulp stumps (31), demonstrating the advantages of metronidazole and doxycycline enhancement within tested materials (32). Indeed, antibiotics are crucial for disinfection in several dental fields and are successfully applied as irrigants, intracanal medicaments and additives to dental cements. Specifically, nitroimidazole antibiotics (i.e. metronidazole) and tetracycline antibiotics (i.e. minocycline and doxycycline) are

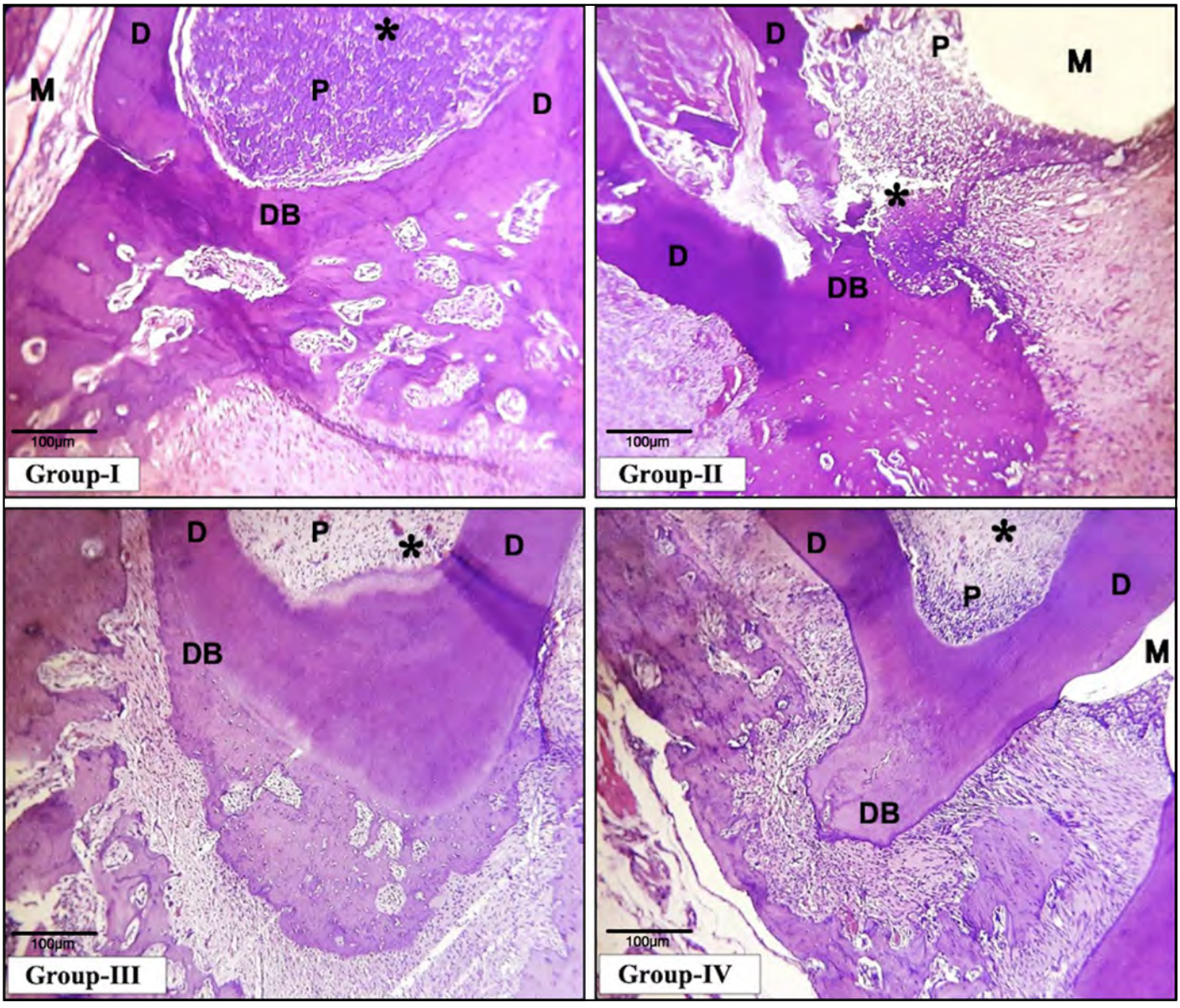

Figure 2. Histopathological images of samples of each group 28 days post-treatment (* : Inflammatory cells, DB: Dentinal bridge, D: Dentine, P: Pulp; M: Material). Staining: hematoxylin and eosin; magnification 40X

effective against *E. faecalis*, a stubborn endodontic pathogen, through various mechanisms (33). On the other hand, few information are available concerning the side effects of antibiotics -as metronidazole and doxycycline- in direct contact with pulp tissue. The reported drawbacks as tooth discoloration, bacteria resistance and cytotoxicity are mainly due to mixed pastes of antibiotics in case of pulpectomy of primary teeth or intracanal medicaments of immature permanent teeth before revitalization procedure (34, 35). Thus, the potential negative impact of antibiotics used as pulp capping materials and their limitations are very crucial issues that should be further developed in future studies to support their safe application.

All samples treated with modified MTA cements, both TCA-free and antibiotic-enhanced, showed dentinal bridge formation. This aspect might be caused by the inclusion of calcium sulfates and carbonates, which increased calcium ion release and improved the calcification occurrence (22). The new formulation might also have stimulated the odontoblastic potential of nearby stem cells, further promoting calcification (36). Therefore, the combination of calcium additives should facilitate mineralization, while the antimicrobial agents should maintaine a sterile, non-inflammatory environment conducive to osteoblastic activity (19, 37).

To the best of our knowledge, this is the first study that evaluates the *in-vivo* response of pulp tissue following pulpotomy with antibiotic combined MTAs, hence, comparative assessment with other similar studies could not be done. However, a similar composition of experimental MTA had been used for apexification in rat incisors, demonstrating enhanced calcific bridge formation with improved antimicrobial activity (22). On the other hand, the present study presents some limitations that need to be acknowledged. A longer follow-up could provide more insight into the nature of the formed dentinal bridge. Additionally, using advanced radiographic techniques

might have offered a close view of the quality of dentinal tissue. Further analyses are required to understand the precise mechanism of calcific bridge deposition, including studies on cell lines and on the material's effects on odontoblasts, osteoblasts, cementoblasts and periapical stem cells.

CONCLUSION

Within the limitation of the present animal study, it could be concluded that TCA-free MTA and antibiotic-enhanced MTAs outperformed conventional MTA regarding dentinal bridge formation and exhibited minimal pulpal necrosis. The inclusion of antibiotics such as metronidazole or doxycycline helped to obtain a more sterile environment, that might contribute to improve outcomes. Further long-term *in-vivo* studies are necessary to support these findings before considering clinical trials in humans.

Disclosures

Ethics Committee Approval: The study was approved by the Saveetha Dental College, Saveetha Institute of Medical and Technical Sciences Institutional Animal Ethical Committee (no: BRULAC/SDCH/SIMATS/IAEC/09-2023/02, date: 02/09/2023).

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