ORIGINAL RESEARCH

Biocompatibility of *Ajwain* Oil Combined with Eugenol and Zinc Oxide as a Deciduous Root Canal Obturating Material: An *In Vivo* Study

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Abstract

Aim and objectives: The present study was intended to assess the biocompatibility of newly formulated materials such as zinc oxide (ZO) admixed with ajwain oil (A) as well as ajwain eugenol (E) (1:1 ratio) against ZOE through an animal model as root canal obturating materials in deciduous teeth.

Materials and methods: The study involved randomly selected 24 albino rats, which were divided into three groups based on test materials. Two polyethylene tubes (PETT) (8 mm long × 1 mm internal diameter) were inserted into connective tissues of the dorsal side on either side of each rat *viz* empty tube (negative control) and another containing test material (test group). Animals were sacrificed at the end of the 7th and 21st days. PETT with surrounding connective tissues were excised. Histopathological evaluations of the material's biocompatibility were done by determining inflammatory tissue responses. Non-parametric tests such as Kruskal–Wallis and Mann–Whitney *U* were used for statistical analysis ($p < 0.05$).

Results: Histopathological examination on the 7th day showed increased polymorphonuclear cells for all test materials compared to the negative control ($p = 0.92$), suggesting acute inflammation. The inflammation subsided gradually after 21 days ($p = 0.48$). The lymphocytes increased after 21 days for all the materials indicating chronic inflammation ($p = 0.79$), as well as fibroblasts ($p = 0.34$) and capillaries ($p = 0.35$), indicating healing and repair.

Conclusion: The newly formulated obturating materials were found to be biocompatible compared to ZOE.

Keywords: Biocompatible materials, Connective tissues, Inflammation, Root canal filling materials, Tooth deciduous.

International Journal of Clinical Pediatric Dentistry (2023): 10.5005/jp-journals-10005-2531

Introduction

The exact diagnosis, viable cleaning, complete decontamination, and appropriate obturation of root canals are the significant elements to achieving predictable endodontic therapy results.[1] An ideal obturating material needs to be biocompatible and endurable by the periradicular tissues. The most frequently utilized material for obturating primary teeth to date is ZOE cement.[2] ZOE demonstrates antimicrobial, anti-inflammatory, and analgesic properties with good availability but sets hard[3], leaving behind traces of unresorbed material, which slows down deciduous root resorption. It is also reported to deflect the eruption path of permanent successor due to the presence of residual material in tissues.[4]

Various other obturating materials advocated in the primary teeth are calcium hydroxide and its modifications, iodoform-based materials like Maisto's paste, key risk indicator (KRI) paste, Guedes-Pinto paste, endoflas, metapex, etc. Calcium hydroxide with ZO, iodoform, and sodium fluoride, when utilized for deciduous root obturation, has been demonstrated to create a "hollow tube" effect with a compromised hermetic seal.[5] Various iodoform-based obturating materials like calcium hydroxide iodoform paste (vitapex/metapex), KRI paste, Maisto paste, Walkhoff paste, and endoflas have demonstrated variable antibacterial, clinical, and radiographic success but with fewer disadvantages like faster resorption than deciduous roots, tooth discoloration, irritation to periapical tissues, and cemental necrosis.[6,7] To overcome such pitfalls, the quest still continues through preclinical and clinical trials to find out a suitable biocompatible obturating material utilizing various new combinations of organic or inorganic, as well as chemical/herbal agents worldwide. The great interest has been focused on herbal agents in the form of essential oils such as tea tree oil, thyme oil, and peppermint oil, which were tried previously in combination with ZO (inorganic agent) with predictable antibacterial efficacy.[8]

Ajwain oil (A), derived from seeds of a medicinal herb, has been advocated in ancient ayurvedic science for numerous benefits, such as antimicrobial, antioxidant, analgesic, anti-inflammatory, and hepatoprotective activity.[9] Dhore et al. demonstrated the antibacterial effect of A against multiple bacterial species responsible for caries and endodontic infections.[10] A couple of studies cited in the literature[11] investigated the anti-inflammatory

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How to cite this article: Dahake PT, Joshi SS, Kale YJ, *et al.* Biocompatibility of *Ajwain* Oil Combined with Eugenol and Zinc Oxide as a Deciduous Root Canal Obturating Material: An *In Vivo* Study. Int J Clin Pediatr Dent 2023;16(3):431–436.

Source of support: Nil

Conflict of interest: None

potential of A in rat models to target acute and subacute inflammation with predictable results. Gilani et al. explored the analgesic and healing properties of A used in cream through an *in vivo* model on rats by incorporating A in topical paste.[12] Though the multi-functional role of this essential oil has been investigated extensively in other fraternities of medicine, its utilization in the field of dentistry in general and pediatric endodontics in specific has not been investigated and given justice due to a lack of research with the same. From previous *in vitro* experimental studies, harnessing the various therapeutic properties, the present experimental study was planned to evaluate the biocompatibility and tissue tolerance of A, E, and their combination (1:1 ratio) with ZO as deciduous root obturating materials through an animal model.

Hypothesis

The newly formulated materials, ZO-ajwain oil (ZOA) and ZO-ajwain E (ZOAE), show better biocompatibility than conventionally used ZOE.

Materials and Methods

The present animal study was initiated after getting approval from the Institutional Animal Research Ethics Committee (Reg. No. 731/PO/Re/S/2002/CPCSEA). It was conducted in the Department of Pediatric and Preventive Dentistry in association with the Department of Pharmaceutics.

Selection of Animals for Experiment

A total of 24 white Wistar rats (12 males and 12 females) of age 2.5–3 months, weighing between 200 and 250 gm, were randomly selected from the animal house.[13] The animals were housed in a temperature-controlled environment with the provision of food and water ad-libitum.[14] The animals were taken care of, following the protocols given by the guidelines of the "Animal Ethical Committee" and "Committee For the Purpose of Control and Supervision on Experiments on Animals."

Test Materials Used in the Study were

- Zinc oxide (ZO) powder (Prime Dental Products Pvt Ltd, Bhiwandi, Maharashtra, India)
- Eugenol (E) (Deepti Products, Ratnagiri, Maharashtra, India)
- Ajwain oil (A) (Healthvit DF Pharmacy Gota, Ahmedabad, Gujarat, India)

The animals were divided randomly into three groups (N = 8) based on the type of test materials used, and each group was further divided into two subgroups ($n = 4$) based on postoperative response, as mentioned in Table 1.

Preparation of Materials for Experiment

A total of 48 PETT having 1.0 mm inner diameter and 8.0 mm long were prepared by cutting scalp vein (Medicon Health Care, Vashi, Navi Mumbai, MH India). The tubes were disinfected with 5% sodium hypochlorite (Organo Biotech Laboratories Private Limited, Delhi, India) and detoxified with normal saline (Facmed Pharmaceuticals Private Limited Dwarka, Delhi, India) for 15 minutes, followed by drying with a sterile gauge.[15]

Zinc oxide (ZO) (200 mg) powder was dispensed on a clean, dry glass slab and mixed with E (7 drops or 0.07cc) with a sterile stainless-steel spatula to a creamy consistency as per the formula given by Tchaou et al.[16] As there was no standardization for other two test materials, they were mixed as per ZOE paste. All the test materials were prepared just prior to the procedure and filled in designated PETT.[15]

Preparation of Animals for Experiment

The rats were anesthetized by injecting 10% ketamine hydrochloride (50 mg/kg) (Neon Laboratories Andheri (East) Mumbai, Maharashtra, India) and 2% xylazine hydrochloride (10 mg/kg) (Brilliant Bio Pharma Private Limited Bollaram Sangareddy, Telangana, India) intraperitoneally.[13] The skin on the dorsal area of rats was shaved to remove all the hair without inducing any iatrogenic injury, followed by its disinfection with 5% tincture iodine (Swastik Pharmaceuticals, Vijayawada, Andhra Pradesh, India).[15] Two parallel incisions of approximately 10 mm length were made with no 15 Bard parker scalpel blade[14,17] followed by blunt dissection to create subcutaneous pockets on either side of incisions to embed PETT tubes with test material in one pocket and empty tube (negative control) in another pocket cauda-cranially. The incisions were sutured back to close the wounds using a 4-0 black braided silk suture (Johnson and Johnson company) with eyeless reverse cutting needle (¾ inches half round Mani Japan).[18,19] Analgesics were given postoperatively to reduce pain with proper postoperative care.

To evaluate the biocompatibility and tissue tolerance of the materials, the animals were sacrificed with an anesthetic overdose at predetermined durations, that is, 7 and 21 days. The skin overlying the implanted tubes was resected carefully to retrieve them with surrounding connective tissues.[13] All the tissue samples were fixed in 10% buffered formalin solution (0.1 M/L) for 48 hours,[15] embedded in paraffin wax, and cut into 5 µm thick tissue sections for histopathological evaluation.[15] The tissue sections were mounted on glass slides and stained with hematoxylin and eosin for visualization and histopathological reporting.[20,21] The same procedure was followed at the end of 21 days. During the evaluation of tissue sections under an optical microscope, the tissue reaction at the open ends of tubes was evaluated, while the connective tissue responsible for the outer surface of tubes was not recorded intentionally as independent negative control (empty tubes) was inserted separately on the contralateral side.[14,20,21]

In the present study, the tissue inflammatory response was evaluated by using Coutinho-Filho modified criteria.[22] The various inflammatory cells, such as neutrophils, plasma cells, lymphocytes, and eosinophils, were identified. The number of fibroblasts and capillaries in the form of neovascularization was recorded. The following grading system was used to determine inflammatory response (Table 2):

Evaluation of vascular changes was done according to Onay et al.[23]

Table 1: Grouping of animals based on test materials and response duration

Group (N = 8)	*Test material*	*Subgroup (n = 4)*	*Response noted*
I (positive control)	ZOE	A	at 7 days
		B	at 21 days
II (test material 1)	ZOA	A	at 7 days
		B	at 21 days
III (test material 2)	ZOAE	A	at 7 days
		B	at 21 days

ZOE, zinc oxide eugenol; ZOA, zinc oxide ajwain; ZOAE, zinc oxide ajwain eugenol

- Grade 0: (No reaction), no significant vascular proliferation,
- Grade 1: (Mild reaction), the number of vascular structures in one high-power field (40×) <25,
- Grade 2: (Moderate reaction), the number of vascular structures in one high-power field (40×) between 25 and 50,
- Grade 3: (Severe reaction), the number of vascular structures in one high-power field (40×) >50.

Table 2: Grading system to determine inflammatory response

Grade	*Inflammation*	*Histological analysis*
0	None	No inflammatory cells
1	Mild	Inflammatory cells present in small numbers or in small groups
2	Moderate	Inflammatory cells are present in large numbers, yet not predominantly in the microscopic field
3	Intense	present as infiltrated, predominantly in the microscopic field
4	Necrosis	Loss of tissues with voids and no/minimal cells in the field

Histopathological evaluation was performed using microscope (Olympus CX21, Tokyo, and Japan) under 40 and 100× magnifications. The differences in the number of inflammatory cells, fibroblasts, and capillaries between the groups and among the two experimental periods were evaluated using non-parametric tests such as Kruskal–Wallis and Mann–Whitney *U* tests. The level of significance was set at 0.05.

Results

The intensity of the inflammatory response of the connective tissues at both ends of PETT was analyzed by two blinded observers. The histopathological evaluation showed that all the materials showed an intense to moderate inflammatory reaction after seven days which was decreased after 21 days when compared with negative control. There was an increase in the fibrous tissues and a number of capillaries with all the materials after 21 days indicating healing and repair of the periapical tissues.

The distribution of inflammatory cells, fibroblasts, and the number of capillaries related to all three materials during the experimental periods of the 7th and 21st days have been presented in Table 3.

Table 3: Mean scores and standard deviation attributed to all test materials after an experimental period of 7 and 21 days for seven events

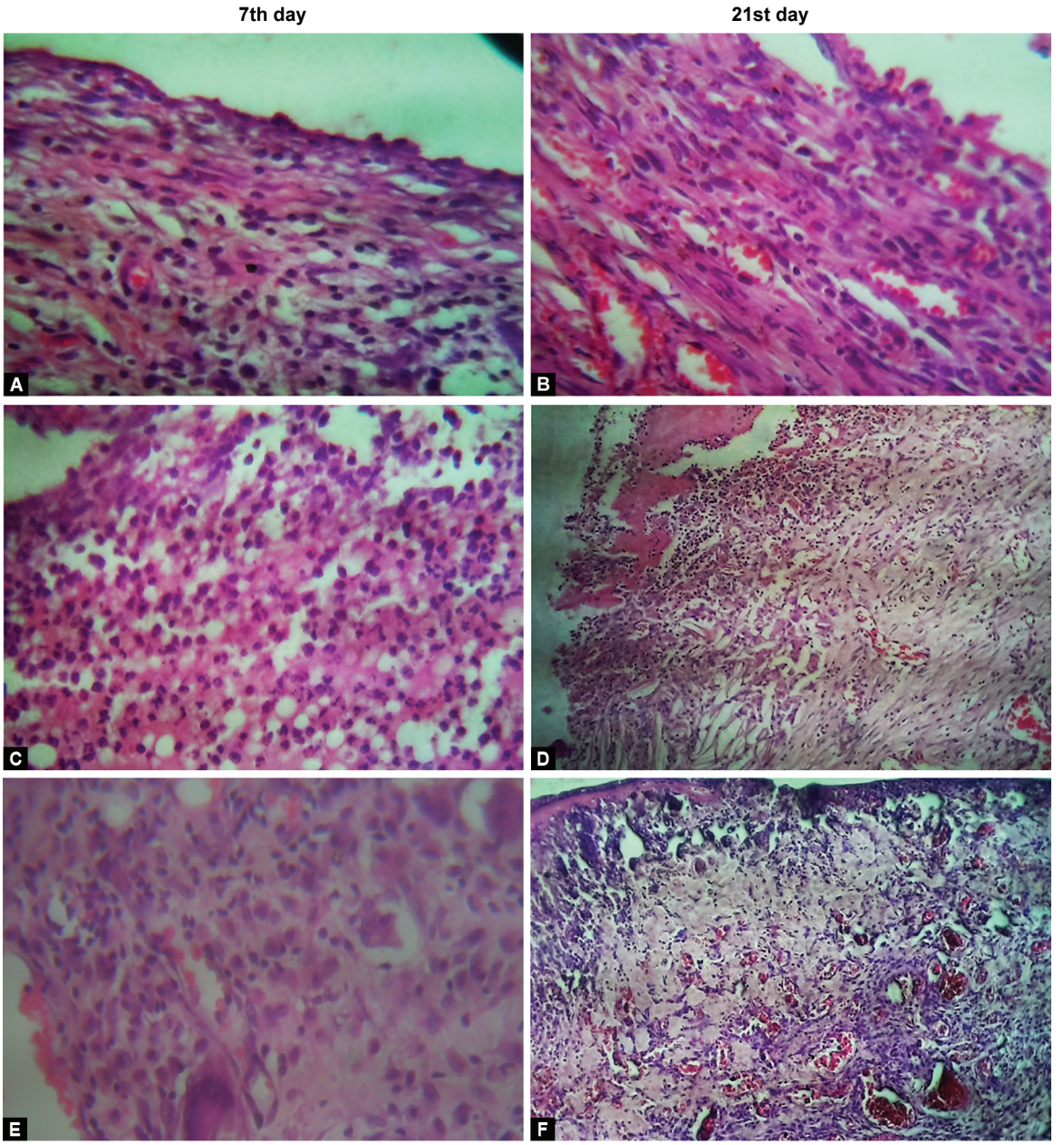
	Group	*Result after 7 days*			*Result after 21 days*		
Cells		*Mean ± SD*	*F*	*p*	*Mean ± SD*	*F*	*p*
PMN	T1	2000.00 ± 753.88	0.77	0.92 NS	812.50 ± 225.00	0.84	0.48 NS
	T2	2187.50 ± 804.54			847.50 ± 231.71		
	T3	1937.50 ± 1199.57			477.50 ± 100.12		
	Total	2041.87 ± 858.07			579.17 ± 192.47		
LYMPHO	T1	880.00 ± 834.09	0.890	0.52 NS	1882.50 ± 724.28	0.240	0.79 NS
	T2	925.00 ± 1037.22			1957.50 ± 550.05		
	T3	327.50 ± 283.71			1847.50 ± 891.15		
	Total	837.50 ± 700.14			1829.17 ± 812.28		
Plasma	T1	222.50 ± 89.58	1.085	0.25 NS	237.50 ± 243.38	0.70	0.93 NS
	T2	140.00 ± 27.08			280.00 ± 104.58		
	T3	132.50 ± 99.48			242.50 ± 149.08		
	Total	185.00 ± 83.07			253.33 ± 159.98		
EOSINO	T1	77.50 ± 22.17	0.883	0.45 NS	192.50 ± 151.30	0.310	0.73 NS
	T2	115.00 ± 58.02			137.50 ± 35.00		
	T3	75.00 ± 54.47			172.50 ± 71.82		
	Total	89.17 ± 47.19			187.50 ± 92.45		
FIBRO	T1	115.00 ± 125.83	0.792	0.48 NS	375.00 ± 184.84	1.190	0.34 NS
	T2	190 ± 158.53			220 ± 87.82		
	T3	80 ± 82.87			297.50 ± 144.10		
	Total	128.33 ± 123.88			297.50 ± 144.10		
Total cells	T1	3075.00 ± 1530.52	0.237	0.79 NS	3087.50 ± 1049.11	0.120	0.88 NS
	T2	3300.00 ± 1949.38			3137.50 ± 923.20		
	T3	2500.00 ± 1578.92			2800.00 ± 1122.50		
	Total	2958.33 ± 1573.91			3008.33 ± 948.88		
CAPILL	T1	141.25 ± 21.75	3.089	0.09 S	192.50 ± 34.03	5.280	0.03 S
	T2	101.25 ± 8.54			275.00 ± 50.00		
	T3	110.00 ± 34.40			282.50 ± 45.00		
	Total	117.50 ± 28.18			250.00 ± 58.00		

CAPILL, capillaries; EOSINO, eosinophils; F, Fisher test; FIBRO, fibroblasts; LYMPHO, lymphocytes; NS, nonsignificant; PMN, polymorphonuclear cells; SD, standard deviation; S, significant; T1, test material 1—ZOE; T2, test material 2—ZOA; T3, test material 3—ZOAE; *p*-value < 0.05

After 7 days, no statistically significant difference in inflammatory cell infiltrates, vascular reactivity, and the number of fibroblasts was found between the test and control groups and among the test materials. All three test materials showed grade 2 inflammation which showed an increase in the number of polymorphonuclear cells ($p \pm 0.92$) while a minor increase in the number of other inflammatory cells, such as lymphocytes ($p \pm 0.52$), plasma cells ($p \pm 0.25$), and eosinophils ($p \pm 0.45$). The number of fibroblasts ($p \pm 0.48$) and capillaries ($p \pm 0.09$) also increased. The total number of cell infiltrates increased with the formation of connective tissues.

After 21 days, a grade 1 inflammatory response was observed with all three test materials. There was a decrease in inflammatory cell infiltration and an increase in fibrous tissues and capillaries with all three materials. There was a moderate decrease in the number of polymorphonuclear cells ($p = 0.48$) while a slight increase in the number of other inflammatory cells such as lymphocytes ($p = 0.79$), plasma cells ($p = 0.93$), and eosinophils ($p = 0.73$) indicating chronic inflammation. The number of fibroblasts ($p = 0.34$) was increased. A statistically significant increase in the number of capillaries ($p = 0.03$) was observed. The total number of cell infiltrates increased with the organization of tissues, fibrous condensation, and increased vascularity.

The overall inflammatory tissue response developed by all three materials was decreased, while the vascular reaction was increased with time (Fig. 1).



Figs 1A to F: Inflammatory response of materials (ZOE, ZOA, and ZOAE) after 7 and 21 days. (A) ZOE group—primary infiltration; (B) ZOE group—Increase in number of capillaries after 21 days; (C) ZOA group—infiltration of inflammatory cells; (D) ZOE group—Increase in number of fibers and capillaries; (E) ZOAE group—presence of inflammatory cells; (F) Several collagen fibers and large number of capillaries

Discussion

The root canal obturating materials remain in contact with periapical tissues. These materials or their by-products may evoke tissue reactions or may cause irritation or degeneration of the surrounding tissues if they get extruded into periapical tissues.[15] So, whenever new materials are introduced, their biocompatibility has to be evaluated, and results must be compared with other conventional materials.[24] The most suitable method to evaluate the local effects and their biocompatibility is implanting these new materials in rat subcutaneous tissues.[22,24–26] Wistar rats used in this study showed less susceptibility to infection after surgery which are easily available, cost-effective, and acts as an acceptable model for evaluation of the biocompatibility of materials.[13] The PETT used in the study are neutral/inert, which do not evoke any immune response or inflammatory reaction when coming in contact with connective tissues and effectively place the materials in contact with the surrounding connective tissues.[13] The average length of root canals in primary teeth is 10 mm. that's why the length of PETT tubes used in this study was kept at 8 mm. As the diameter of an apical foramen in primary teeth is 1 mm, PETT tubes of 1 mm inner diameter were used to place the materials.[25] An empty tube placed on one side acts as negative control while a tube filled with material acts as positive control or test material. This bilateral placement of tubes in the same rat is helpful in evaluating the tissue response.[14] Tissue sections at both ends of tubes were used for evaluation as material at the ends of tubes came in contact with tissues provoking the response, while tissues at the ends of empty tubes act as a negative control.[14]

Zinc oxide E (ZOE) is the oldest and most commonly used obturating material in pediatric endodontics.[13] E is an extract of clove oil having a chemical formula of 4-allyl-2-methoxyphenol. ZOE may develop periapical inflammation due to E, which leaches out through it.[4] But the release of corticosteroids, such as dexamethasone and hydrocortisone, causes the neutralization of E. This leads to a reduction in inflammation.[13]

Various studies verified that ZOE shows moderate to severe inflammatory response.[27] Several studies have compared cytotoxicity and tissue reactions of ZOE-based obturating materials with others.[28,29] Hume, in his research on ZOE, stated that water availability was the prime determinant of the release of E, which produces toxicity.[30] E oil, even at a low concentration of 0.06 µM, showed toxicity on human dental pulp fibroblasts in primary teeth, affects the expression of genes causing apoptosis and inflammatory processes, causes damage to Deoxyribonucleic acid restricting its use.[31]

Several modifications have been made to this obturating material to counteract the disadvantages and improve its biological properties. Combination of calcium hydroxide, ZO powder, and sodium fluoride as obturating material showed good results.[32] Chitra HAP-Fil is an obturating material in which hydroxyapatite nanoparticle gel was added in iodoform.[33] A combination of ozonated oil and ZO was used as an obturating material in primary teeth, with a high success rate of 93.3%.[34] ZO mixed with aloe vera paste as obturating material was proved to be clinically and radiographically successful.[35] ZO with propolis has been used with 82.5 % success.[28] Thus, researchers worldwide are trying to use herbal compounds that are readily available and traditionally used as medicines. The combinations of ZO mixed with essential oils such as thyme oil, peppermint oil, and tea tree oil as obturating materials in primary teeth showed excellent antibacterial properties.[8]

Ajwain oil (A) is an essential oil obtained from *Trachyspermum ammi* fruit; it contains thymol (35.80%) and other non-thymol compounds like para-cymene, α-terpinene, α- and β- pinenes, dipentene, and carvacrol while camphene, myrcene, and α-3-carene present in minute quantities.[13] Presence of thymol in A is responsible for its analgesic effect.[36] The alcoholic and aqueous extracts of ajwain seeds had anti-inflammatory potentials when evaluated for acute and subacute infections in rat models.[11,37] Antimicrobial activity of A against various gram-positive and gram-negative bacterial strains and selected fungi has been evaluated by Khan et al., which was mainly due to the diethyl ether fraction of ajwain.[38] The A showed excellent antibacterial properties, and it can be used as an anti-plaque agent.[10]

But a combination of ZO with A and a mixture of ajwain and E oil have not been tried as obturating materials in primary teeth. In the present study, ZO admixed with ajwain and E showed better anti-inflammatory action than ZOE and ZOA. While the anti-inflammatory action of ZO admixed with A is comparable to that of ZOE.

Ajwain oil (A) demonstrates biphasic inhibition/reduction in inflammation. Initially, there was a release of histamine and serotonin followed by prostaglandin, bradykinin, and lysozyme. Then increase in the number of fibroblasts leads to the synthesis of collagen fibers and mucopolysaccharides, which are responsible for healing and repair.[11] Phytochemical investigation discovered the presence of terpenes, sterols, and polar constituents, such as flavonoids and glycosides, in A, demonstrating its anti-inflammatory effects. The inhibition of prostaglandin synthetase in acute and subacute inflammation in the presence of A indicates its anti-inflammatory action.[11] An increase in the number of fibroblasts and capillaries indicating repair of the wound by using A in a cream formulation was evaluated.[12]

Thus, the additive anti-inflammatory effect of both E oil and A may be responsible for better biocompatibility of ZO admixed with E and A. Also, the concentration of E used was half as used in ZOE, leading to decreased cytotoxicity.

Conclusion

An inflammation induced by newly formulated materials—ZOA and ZOAE is comparable to conventionally used ZOE, which may be attributed to the anti-inflammatory action of A. The results of the histopathological examination of the study demonstrated a reduction in inflammation and an increase in vascularity, indicating good tissue tolerance by all the materials. Thus, the present study revealed that ZO mixed with a mixture of ajwain, E (ratio of 1:1), and A could be used as obturating materials in the deciduous teeth. But further clinical studies and investigations are needed.

Compliance with Ethical Standards

Ethical Approval

The present study was done on animals. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. Ethical approval of the institutional animal ethical community was taken. It was approved by IAEC Protocol Number CPCSEA/CBPL/AH-36/2017-2018.

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