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Original Article

Comparative Evaluation of pH and *In Vitro* Cytotoxicity of Zinc Oxide–Ozonated Eugenol and Conventional Zinc Oxide Eugenol as Endodontic Sealers

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ABSTRACT

Background: Eugenol released from zinc oxide eugenol (ZOE)–based sealants may cause irritation to the periapical tissues and has cytotoxic potential. Ozone therapy has numerous clinical applications with humans because of its bactericidal action, detoxifying effect, stimulation of angiogenesis, and wound-healing capacity. Therefore ozone can be incorporated in ZOE sealer to exploit these properties. **Materials and Methods:** Eugenol was ozonated using ozonator machine and the samples were divided into two groups: Group I: zinc oxide eugenol (n = 10) and Group II: zinc oxide–ozonated eugenol (OZOE; n = 10). The pH of the fresh sealer samples and the set samples was measured using calibrated pH meter after predetermined time intervals. Cytotoxicity of the set sealer was evaluated on mouse L929 fibroblasts using cellular metabolic assay. **Results:** pH of the samples in Group II was higher when compared to Group I. Group II showed higher cell viability than the Group I. **Conclusion:** OZOE sealers can be used as an alternative to the conventional ZOE sealers.

Keywords: *Cytotoxicity, eugenol, ozone, sealer, zinc oxide*

Introduction

The challenge to the prophecy of endodontic therapy lies on the consistency of root canal

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filling. Endodontic sealers are used to create a fluid-tight seal throughout the canal irregularities in the obturation of the root canal system.[1] Excellent sealing, dimensional stability, biocompatibility, and ability to resolve periapical lesions should be provided by an ideal root canal sealer when set.[2] In 1936, Grossman introduced zinc oxide eugenol (ZOE)–based sealers to endodontics. These sealers have been used successfully over extended period of time and it has both anesthetic and antimicrobial properties.[3] Albeit these advantages, it is a known irritant to periapical tissues. Continued researches are being executed for chemical compounds with better physical properties and biocompatibility to be used as an endodontic sealer.

Ozone therapy has a long history of research and clinical uses for humans in search of other treatment modalities due to its impressive biological properties. In 1840, Schonbein introduced the word ozone when he subjected oxygen to electric discharge and noted the odor of electric matter.[4] The modes of applications of ozone can be in gaseous form, aqueous solution, or in the form of ozonated oils. In dental practice, ozonated oil applied to the infection of herpes labialis and osteomyelitis has shown better healing rate. That has also been used in treating jaw avascular osteonecrosis.[5] Zinc oxide-ozonated sesame oil has shown good success rate when used as root-filling material. In restorative dentistry, gaseous ozone has been used in the treatment of incipient lesions because it causes reduction in the number of microorganisms.[6] In endodontics, when used as an irrigant, ozonated water had almost the same antimicrobial activity as 2.5% NaOCl with fewer side effects.[7]

Eugenol is a member of phenylpropanoids, a yellow oily liquid extracted especially from clove oil (80%–85%), which can be ozonated to overcome its cytotoxic potential on periapical tissues. The research, therefore, aims at determining and comparing the physical properties and biocompatibility of ozonated ZOE and conventional ZOE for being used as an endodontic sealer.

Materials and Methods

Eugenol (Dental Products of India, Mumbai, India; [Figure 1]) was ozonated using ozonator machine (Ozonator; Eltech Ozone, Mumbai, India; [Figure 2]) with an output of 400 mg/h. In a glass beaker, 100 mL of eugenol is taken and subjected to ozonation for a period of 30 min. Ozonation is an active process that results in the splitting of an oxygen molecule into singlet oxygen. This singlet oxygen then interacts with diatomic oxygen to form ozone. Zinc oxide powder (Dental Products of India) and eugenol were weighed before manipulation using electronic balancer (Mettler AE 260 Delta range Analytical Balance, Mumbai, India) with an accuracy of 0.0001 g. Pre-weighed quantities of zinc oxide powder were mixed with ozonated and non-ozonated eugenol in the ratio 6:1 by weight (P:L ratio). Manipulation was performed as per manufacturer's instructions and the samples were divided into two groups: zinc oxide eugenol (Group I [n = 10]) and zinc oxide–ozonated eugenol (Group II [n = 10]).

The pH of the fresh samples (immediately after mixing) and the set samples (stored in the incubator at 37°C, >95% relative humidity for 12 and 24 h) were measured for both the groups. For set samples, stainless steel rings of inner diameter 10 mm and height 1.5 mm were used as molds and allowed to set. In a flask containing 10 mL of distilled water, each set sample was placed and stored at 37°C to simulate oral environment throughout the research. For fresh samples, the same volume as the set samples of mixed sealer was placed in a flat bottom container containing 10 mL of distilled



Figure 1: Zinc oxide powder, eugenol, glass slab, stainless steel spatula, and stainless steel rings



Figure 2: Ozonator machine (output: 400 mg/h; Ozonator; Eltech Ozone, Mumbai, India)

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water at 37°C. After predetermined periods of 12 and 24 h, the solutions' pH were measured using a digital pH-meter calibrated (pH Tester, Eutech Instrument, Mumbai, India).

Cell viability of the samples was evaluated using the MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) tetrazolium reduction assay. Nine samples from each group were tested for cytotoxicity and the experiment was performed thrice in triplicate for reproducibility. The mouse L929 fibroblasts have been propagated in the Dulbecco's Modified Eagle Medium added with antimycotic, antibacterial agents, and 5% vol/vol fetal bovine serum. The culture was maintained in an atmosphere of 5% CO_2/95% air for 24 h at 37°C. The tetrazolium salt, MTT, was dissolved at a concentration of 0.2–0.5 mg/mL in phosphate-buffered saline solution (PBS, pH = 7.4) and stored at 4°C immediately prior to use. When the cellular monolayer was attained, the culture medium was removed, rinsed with PBS, and set samples were placed in pre-labeled wells directly over the cellular monolayer. Cells with medium alone served as negative control (NC). Cells with 100 µL distilled water served as positive control. The culture plate was incubated for 24 h at 37°C in an atmosphere of 5% CO_2. NC was used as reagent blank.

Morphological alteration was assessed by microscopic examination. The samples were removed and the cells were washed with PBS. To each well, 50 µL of MTT solution was added, and the culture plate was incubated for 3 h in a dark environment at 37°C in an atmosphere of 5% CO_2. The MTT solution was aspirated, adding 100 µL of dimethyl sulfoxide to each well, and swayed rhythmically to dissolve the blue formazan crystals formed for 30 min. Subsequently, the culture plate was transferred to a microplate reader, usually a UV-visible spectrophotometer equipped with a 570-nm filter, to read the absorbance (reference wavelength of 650 nm). The equation, cell viability % = (OD570e/OD570b) × 100 was used to calculate the cell viability. OD570e and OD570b denote mean values of the optical density (OD) calculated in the 100% of the test sample extracts and blanks, respectively. The lower the viability% value, the higher the cytotoxic potential of the samples. The amount of formazan (presumably directly proportional to the number of viable cells) is measured using a plate-reading spectrophotometer to record changes in the OD at 570 nm.

Results

The data obtained were evaluated statistically using unpaired *P* value *t* test significant at 5% level ($P < 0.05$). Table 1 shows the mean and standard deviation of groups concerning the pH values at different time intervals. The mean pH values of the freshly mixed samples in Group I and Group II were 6.5667 and 7.5667, respectively with statistically significant difference between them ($P = 0.013$). After 12 h, the mean pH values of the samples in Group I and Group II were 6.1000 and 6.7667, respectively with statistically significant difference between them ($P = 0.007$). After 24 h, the mean pH values of the samples in Group I and Group II were 5.7667 and 6.2667, respectively with statistically insignificant difference between them ($P = 0.123$).

Table 2 shows the mean and standard deviation of cell viability %. The mean cell viability % of the samples in Group I and Group II was 52.6000 and 68.4333, respectively. There was statistically significant difference between the groups with *P* value set at level 5% after 24 h ($P = 0.002$).

Discussion

ZOE sealers have a proven history of successful use over long period. Despite its advantages, gutta-percha and conventional ZOE sealer combination still has its own drawbacks and also the eugenol released from

Table 1: Mean and standard deviation of pH values

Time of testing	Group	Mean	Standard deviation	*P* Value
Freshly mixed	I	6.5667	0.20817	$P = 0.013$ (significant)
	II	7.5667	0.35119	
After 12 h	I	6.1000	0.17321	$P = 0.007$ (significant)
	II	6.7667	0.15275	
After 24 h	I	5.7667	0.30551	$P = 0.123$
	II	6.2667	0.32146	

Table 2: Mean and standard deviation of cell viability %

Group	Mean	Standard deviation	*P* Value
I	52.6000	1.00167	$P = 0.002$ (significant)
II	68.4333	1.15326	

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ZOE is reported to be an irritant to periapical tissues and has cytotoxic properties with limited antimicrobial activity.[8] In this study, ozone is incorporated into eugenol to overcome those disadvantages by bubbling ozone gas through eugenol.

Ozone (O_3) is an energized form of oxygen and dissociates readily back to oxygen, liberating a singlet oxygen that is responsible for its so-called oxidizing potential.[9] The concentration of ozone in eugenol was checked using ozone analyzing strips and was kept under 0.05 ppm (safety dose of ozone as per OSHA: less than 0.1 ppm). In Group II, the pH of freshly mixed samples was relatively higher than the samples in Group I. This may be attributed to the formation of hydroxyl ions readily due to the unstable nature of ozone. The transient radical anion readily gets protonated, liberating hydrogen trioxide (HO_3) which in turn disintegrates into the hydroxyl radical (OH), an even more powerful oxidant. However, there was no statistically significant difference between the set samples after 24 h, which may be due to the depletion of singlet oxygen with time.

In Group II, there was higher percentage of viability when compared to conventional eugenol with statistically significant difference between them ($P < 0.05$). This may be attributed to the fact that ozone stimulates the release of interleukins, leukotriene, and prostaglandins as a response to inflammatory processes. It also activates protein synthesis mechanisms and increases the quantity of ribosomes and mitochondria in cells.[10] Such cellular modifications justify the elevation of functional activity and the capacity for tissue regeneration, which may account for the increased percentage of viability in mouse fibroblasts when treated with ozonated eugenol. Ozone gas has a high oxidizing potential, and when used as an antimicrobial agent against bacteria, viruses, fungi, and protozoa, it is 1.5 times more efficient than chloride.[11] These findings were similar to the results of Ebensberger *et al.*[12] in which a high level of biocompatibility of aqueous ozone has been found on human oral epithelial cells, gingival fibroblast cells, and periodontal cells. When the cells were treated with ozonated water, the metabolic activity of L-929 mouse fibroblasts was high when compared to that of 2.5% NaOCl. It also stimulates aerobic processes such as glycolysis and the cellular-level Krebs cycle to promote blood circulation; thus ozone is also used to treat circulatory disorders.[13]

In 1930, Dr. E. A. Fisch, a German dentist, treated Austrian surgeon Ernst Payr for a gangrenous pulpite using ozone and he was then inspired to start a new line of research into the use of ozone in healthcare. However, ozone looks likely to have disappeared from dental practice until 2001, when the first scientific study examining the biomolecules in dental caries was published before and after ozone treatment. In the era of minimally invasive treatment, the trend in dental practice has shifted and it was important to tackle the new challenges of handling and treating an elderly population. This has been of particular concern in treating root surface caries with increased sensitivity to exposed root surfaces in older patients.[14]

Estrela *et al.*[15] used ozonated oils to disinfect the root canal systems and to remove the necrotic debris by virtue of ozone's bactericidal and effervescent properties. Ozone oil when used as an irrigant, the canal sterilization is quicker and more efficient than conventional irrigation by the combination of sodium hypochlorite and sodium peroxide. Chandra *et al.*[16] conducted a study to assess the success rate of the ozonated sesame oil and zinc oxide mixture as the primary teeth root-filling material. They concluded that after 12 months of follow-up, ozonated sesame oil–ZnO demonstrated good clinical and radiographic success, and can be considered as an alternative obturating material in infected primary teeth.

The results of this *in vitro* research may not be directly and completely applied to *in vivo* conditions. However, considering *in vitro* researches as a simple means of evaluation and with confounding factors eliminated, they are often regarded as sources of proof in assessing the cytotoxicity of endodontic sealers. Limitations in the simulation of *in vivo* environment should be taken into consideration while generalizing the results of *in vitro* experiments to clinical practice.[17] Though incorporation of ozone into eugenol improves its antibacterial properties and the percentage of cell viability, other physical properties such as solubility, hardness, dimensional stability, and sealing ability have to be evaluated and further researches are needed in this field to be used as an endodontic sealer *in vivo*.

Conclusion

Within the limitations of the study, it can be concluded that ozonating eugenol increased the pH and cell viability when compared to the conventional ZOE.

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Conflicts of interest

There are no conflicts of interest.

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