A novel petasin-modified zinc oxide eugenol sealer

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Abstract

Objective: Zinc oxide eugenol (ZOE) is one of the most commonly used root canal sealer. However, it has few drawbacks such as cytotoxicity, solubility, and irritation to periapical tissues. The scope of this study was to investigate the setting time, solubility, cytotoxic effects, and anti-inflammatory action of ZOE sealer with the modification of its liquid component by the addition of petasin extract in the ratios 1:1, 5:1, and 10:1.

Materials and Methods: Setting time was evaluated using the Vicat’s apparatus. For testing solubility, the American Dental Association’s specification #8 was adopted with certain modifications. Protein denaturation assay and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay with L929 mouse fibroblast cell lines were used to evaluate the anti-inflammatory property and cytotoxicity, respectively.

Results: ZOE sealer with petasin extract in the ratio of 5:1 showed the least initial and the final setting times. There was no statistically significant difference in the amount of solubility for all the groups at the various time intervals. The cytotoxicity of the control group was significantly greater than all the experimental groups, whereas the anti-inflammatory effect of the former was statistically lower.

Conclusions: The combination of ZOE with petasin extract in the ratio of 5:1 showed lower setting time, cytotoxicity, and better anti-inflammatory property.

Keywords: Cell culture; cytotoxicity; petasin; protein denaturation; Vicat’s apparatus; zinc oxide eugenol sealer

INTRODUCTION

Sealers play an important role in providing a fluid tight seal along with the core material in the obturation of the root canal system. Although many sealers have been introduced over the years with successful results, zinc oxide eugenol (ZOE) sealer is still being used as a preferred sealer in the many parts of the world. It, however, has many inherent disadvantages, which includes hydrolysis, disintegration, and release of toxic substances. When it contacts the periapical tissue, adverse tissue reactions, inflammation, and necrosis occur. There has been a constant effort to enhance the required properties of ZOE as a sealer. This includes the introduction of noneugenol-based sealers, nonstaining ZOE sealer, and medicated sealers.

Butterbur (Petasites hybridus) is a perennial shrub found in Europe, Asia, and North America. It is marketed in the United States as a dietary supplement. The use of butterbur for the prevention of allergic rhinitis and treatment of migraines has been supported by scientifically evidenced human studies. Petasin extract from the butterbur plant has been reported to have a wide range of anti-inflammatory and analgesic properties. This study is a novel attempt to add the petasin extract to ZOE sealer and evaluate its anti-inflammatory effect. The purpose of this in vitro study was to investigate the setting time, solubility, cytotoxic effects, and anti-inflammatory action of ZOE with and without incorporation of 15% petasin extract in different ratios. The null hypothesis was that the addition of petasin extract to ZOE sealer has no effect on the above-mentioned properties of the sealer.

MATERIALS AND METHODS

The Institutional Review Board of our institution (IRB/2014/ MDS/No. 305) approved the study protocol.

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Petasin extract was obtained from the petadolex capsules (Enzymatic Therapy, Germany). The liquid for the corresponding experimental groups was made by mixing eugenol and 15% petasin in ratios of 10:1, 5:1, and 1:1. This liquid was then mixed with zinc oxide powder. The study consisted of four groups: Group I being conventional ZOE, Groups II to IV consisting of ZOE, and eugenol liquid-containing petasin herbal extract in the ratios of 10:1 (ZEP10), 5:1 (ZEP5) and 1:1 (ZEP1), respectively. In all the groups, powder and liquid ratio was kept constant at 1:1 volume.

**Setting time**
The samples of each group were mixed and placed in a customized vicat mold (circular with 2.5 cm diameter and 3 cm height). The setting time of each sample was tested at 37°C room temperature using a Vicat’s needle apparatus. A needle with a diameter of 1 mm was carefully lowered vertically onto the flat surface of the mixed material. The initial setting time was recorded when the needle penetrated until 25 mm. The final setting time was recorded when the needle indentations ceased to be visible. The test was repeated five times. The results were tabulated and statistically analyzed.

**Solubility**
The test materials were loaded in a cylindrical mold (1 cm in diameter and height) made of siloxane soft-putty impression material (DPI, Mumbai, India) and were allowed to set. After being stored at 37°C for 24 h, the discs were weighed (W0) on a precision weight scale (Schimand Zu, Mumbai, India). Immediately, after weighing, the discs were stored in 100 ml of distilled water at 37°C until subjected to solubility testing. Each Whatman filter paper (No: 1) was taken and weighed (W1). Specimens were taken out from the distilled water at regular intervals (1 h, 6 h, 12 h, 24 h, 1 week, 2 weeks, 1, 2, and 3 months) and filtered with the filter paper. The specimen along with the filter paper was dried in an oven at 50°C for 1 h and weighed in a digital balance (W2). The loss of material was obtained from the difference between the initial and the final dry mass of each disc.

\[ \text{Solubility (W)} = \text{W0} - (\text{W2} - \text{W1}). \]

**Cytotoxicity**

**Cell culture**
L929 mouse fibroblast cells (National Center for Cell Science, Pune, India) were cultured in Dulbecco modified Eagle’s medium (DMEM), supplemented with 25 ml culture flasks containing 10% fetal bovine serum (Sigma-Aldrich, St Louis, MO), 2 mmol/L-glutamine, 100 mg/ml streptomycin, and 100 mg/ml penicillin (Life Technologies, Mumbai, India). The cultures were maintained in an incubator at 37°C and in an air atmosphere of 5% CO₂. The cells were further trypsinized, and the harvested cells were used for cytotoxicity experiments.

**Root canal sealer preparation**
The sealers were freshly mixed according to the manufacturer’s instructions. A uniform mix of 1 ml of DMEM and 1 ml of sealer was obtained by mixing in a cyclomixer.

**Cytotoxicity testing (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide assay)**
L929 mouse fibroblast cells were seeded into 96-well plates (Costar, Rochester, NY) (1 × 10⁴ cells/well) and were incubated for 24 h in a CO₂ incubator at 37°C with the prepared experimental sealer of each group after overnight. 50 µL 0.5% 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) dye (Life Technologies, Mumbai, India) was added to each well. The plates were then incubated in a CO₂ incubator for approximately 2 h. The MTT dye was removed, and after the removal of formazan crystals by adding 100 µL isopropanol, the plates were shaken, and the viability of the cultured cells was determined at a wavelength of 570 nm using ultraviolet spectrophotometer (V-5100; Haida Equipment, Mainland, China).

The percentage of cell viability was calculated after 0 h, 24 h, and 48 h as follows:

\[ \frac{\text{Absorbance of treated cells in 570 nm} \times 100\%}{\text{Absorbance of control cells in 570 nm}} \]

**Anti-inflammatory activity**
5 ml of reaction mixture (0.2 ml of egg albumin, 2.8 ml of phosphate-buffered saline [PH 7.4], and 2 ml of experimental samples) was incubated in an incubator at 37°C for 15 min and then heated for 5 min at 70°C. Their absorbance was measured at 660 nm using the ultraviolet spectrophotometer after cooling. Diclofenac sodium was used as reference drug at the final concentration and absorbance was determined similarly.

The percentage inhibition of protein was calculated using the following formula:

\[ 100 \times \left( \frac{\text{Vt}}{\text{Vc}} - 1 \right) \]

Vt - absorbance of the test sample
Vc - absorbance of the control.

The percentage inhibition of protein was also calculated after 0 h, 24 h, and 48 h.

**Data and statistical analysis**
The variables (solubility and setting time) do not follow normal distribution according to the Normality tests Kolmogorov–Smirnov and Shapiro–Wilks test results. Thus,
nonparametric methods such as Kruskal–Wallis test to compare between the groups (destructive samples) followed by Bonferroni adjusted Mann–Whitney post hoc test for the multiple pairwise comparisons was performed. To analyze the other variables (cytotoxicity and anti-inflammatory effect), one-way ANOVA, post hoc tests, and Tukey’s honest significant difference tests were applied. The SPSS software Version 22.0 (Statistical Product and Service Solutions, SPSS, Chicago, USA) was used to analyze the data with a significance level was fixed as 5% (α = 0.05).

RESULTS

Setting time
The initial and final setting time of conventional ZOE was 463.0 and 698.0, respectively [Figure 1]. The addition of petasin extract in the ratio of 5:1 (ZEP5) showed decrease in both, compared to all the other groups [Figure 1]. The final setting time of ZEP1 and 10 increased when compared to the control group. However, none of the values were significant.

Solubility
The control and experimental groups did not show any solubility at 1 and 6 h [Figure 2]. The solubility increased gradually from 12 h to 3 months [Figure 2]. However, there was no statistically significant difference in the amount of solubility for all the groups at various time intervals.

Cytotoxicity
According to the results of the study, all the experimental groups showed 0% cell viability at 1100 μl dilution and 1200 μl dilution at 48 h [Figure 3a] and 24 h [Figure 3b], respectively. Although there is no statistically significant difference between the experimental groups, the cytotoxic effect of the control group was significantly greater than the experimental groups.

Anti-inflammatory effect
All the experimental groups showed no protein denaturation until 800 μl, after which it gradually increased with increase in the dilution [Figure 4a and b]. There was statistically significant difference between the control and the experimental groups. There was no significance among the experimental groups.

DISCUSSION

Sealer-induced cytotoxicity results in the induction of pro-inflammatory cytokines such as interleukin-1 β and tumor necrosis factor-α leading to inflammation and bone resorption at the periapical region.[7] The matrix metalloproteinases, which can be activated by the endodontic sealers, play a vital role in the pathogenesis of sealer-induced periapical inflammation.[8]

According to Brodin et al., the strongest neurotoxic effect was found in sealers based on ZOE, leading to pain, tenderness, and swelling of the affected area.[9] Hence, the properties of the endodontic sealers and their diffusible components have to be critically evaluated for the development of new products. This study was a novel attempt at one such modification, by the addition of petasin, an extract from the perennial shrub, butterbur, that has been used in the medicine for various ailments.[10-13] Petasin has been evidenced to have a range of anti-inflammatory and analgesic properties.[14,15]

The butterbur root extract (Petadolex) is a standardized special lipophilic extract of the rhizome of P. hybridus with a minimum content of 15% petasin.[10] Petadolex consists of essential oils (0.4%), sequiterpenes (petasine and isopetasine), and related sequiterpene lactones (bakkenolides and eremopetasitenins and the pyrrolizidine alkaloids, for example, senecionine).[5,16] The extract is procured by standardized and patented high-pressure liquid carbon dioxide extraction procedure...
that removes pyrrolizidine alkaloids and hence suggested as safe.\textsuperscript{[5,17]}

One of the major drawbacks of ZOE is its prolonged setting time, which is longer compared to the other sealers.\textsuperscript{[18]} A sealer that is unset, or only partially set, may allow more rapid penetration of irritants and their byproducts.\textsuperscript{[18]} Thus, a decrease in the setting time favors better clinical applications. Among the different methods used to evaluate the setting time of sealers, Vicat’s apparatus was used, since it is one of the standard, reliable, and simple methods for evaluating the setting time of dental cements.\textsuperscript{[19,20]} In the present study, we used molds of 3 cm depth as per the Vicat’s testing requirements. The results of the study infer that petasin may be added in 5:1 ratio to ensure faster setting.

The solubility of sealers influences both their rate of degradation and their biocompatibility. It also plays an important role in leaching out of the components into the periapical tissues. American Dental Association’s specification #8 with a few minor modifications was adopted to evaluate the solubility of sealers as it is an economical and simple procedure giving reliable results.\textsuperscript{[19]} Our results show that there was no statistically significant difference in the amount of solubility for all the groups at various time intervals. This might be due to the identical physical nature (oil based) of eugenol and petasin.\textsuperscript{[20,21]} Further studies are required to know the actual products that are leached out and their action on the surrounding environment.

Toxic effects such as the inflammation of periapical tissues and bone resorption associated with ZOE based sealers have been studied extensively.\textsuperscript{[22-25]} The presence of free eugenol remaining in the mixed cement and its release due to the hydrolysis of the mixed cement may be related to the toxicity of ZOE sealer.\textsuperscript{[17,26-28]} MTT assay used in this study is one of the most commonly used methods because of its simplicity, processing speed, reliability, and precision. The mitochondrial dehydrogenases in viable cells clear the methyl-tetrazolium ring and forms formazan that can be measured with a spectrophotometer.\textsuperscript{[28]} The presence of formazan is directly related to the total number of viable cells. L929 mouse fibroblast cell lines are commonly employed in biocompatibility studies due to its longer life span and are easy to prepare and provide more reproducible results.\textsuperscript{[29]} There was a statistically significant difference between the control and experimental groups in all the concentrations. It can be inferred that the presence of petasin in the sealer has enhanced the cell-viability, thereby reducing the cytotoxicity of the ZOE sealer.
compared with the controls. It has been stated that there is the inhibition of leukotriene synthesis by petasin and isopetasine, and the vasodilatory effect may contribute to the antiinflammatory effect of petasin.[30] The literature shows considerable variation in studies testing the cytotoxicity, anti-inflammatory, setting time, and solubility of root canal sealers. This may be related to the evaluation of sealers at various stages, at different dilutions, different cell culture systems, and variation at different intervals of time. Vigilance should be implemented while deducing the results of the in vitro studies to the clinical scenario.

**CONCLUSIONS**

The results of the present study showed that the addition of petasin in the ratio of 5:1 significantly decreased the setting time of ZOE sealer, though it did not considerably alter the solubility of the sealer. The same ratio also effectively reduces the inflammatory and cytotoxic effects of ZOE sealer. Further in vivo studies are required before it could be brought to the clinical use. Within the limitations of this study, the combination of ZOE with petasin extract in the ratio of 5:1 seems to have beneficial effects in reducing the cytotoxicity and inflammatory effects of ZOE-based sealers.

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**Conflicts of interest**

There are no conflicts of interest.

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