Toxicity of root canal sealers in vitro

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Abstract
Objective: To comparatively evaluate the cytotoxicity of EndoREZ (urethane dimethacrylate based), RoekoSEAL (silicone based) and Kalsogen Plus (ZOE based) sealers on HEK-293 (human embryonic kidney epithelial cells) and Vero (African green monkey kidney epithelial cells) cell lines in vitro.

Materials and methods: Sealers EndoREZ, RoekoSEAL and Kalsogen Plus were mixed according to manufacturer's instructions. Extract of these sealers were then treated with cultured cell (HEK-293 and Vero), cytotoxicity evaluated using MTT assay. Cytotoxicity between fresh and set specimens at 0h, 24h, 48h, 72h and 1wk time-interval were calculated. Statistical analysis was done using SPSS Version 15.0 (SPSS, Inc., Chicago, IL, USA) statistical software package (p<0.05).

Results: Extracts of Kalsogen Plus were more toxic, as compared to all other groups (p<0.05) on both cell lines. The mean value of EndoREZ was significantly lower as compared to all other groups on both cell lines. The mean value of RoekoSEAL was significantly higher as compared to EndoREZ but significantly lower as compared to Kalsogen Plus.

Conclusion: Kalsogen Plus and RoekoSEAL were slightly cytotoxic while EndoREZ was non-cytotoxic on both cell lines. The cytotoxic response decreased in the order of Kalsogen Plus>RoekoSEAL>EndoREZ overtime.

Keywords: Toxicity, root canal sealers, MTT assay, inhibition

Introduction
Root canal sealers are used for assisting in formation of hermetic seal during obturation of root canal [1]. Endodontic sealer should be selected on the basis of various physical as well as biological parameters, such as local and systemic biocompatibility [2]. Biocompatibility of these materials is important as root canal sealers frequently come in contact with soft and hard tissues, if extruded during root canal treatment [3]. Ideally a sealer should not hinder tissue repair, but aid or stimulate the reorganization of injured structures [1,4]. The success of root canal treatment is influenced by these materials [5]. Therefore, the biocompatibility of these materials is of utmost importance [6].

It is evident that the biological risks of endodontic sealers is relatively high, as the components of various root canal sealers may induce potential tissue toxicity, leading to apical periodontal tissue damage and inflammatory responses [7,8]. Unfortunately these materials might remain in close contact with periapical tissues for long periods. Even in cases where the sealer does not reach directly the periapical region, there is always the possibility of elutable substances leaching through the dentinal tubules, lateral and accessory canals or apical foramina [9]. Perforation of root also creates communication between the root canal system and the periodontal ligament [10]. However, Ørstavik in 2005 concluded that primary infection or infection secondary to root filling procedures is the principle cause of apical periodontitis and endodontic failure [11].

Nasseri et al., performed a study to evaluate the apical and periapical tissue responses after root canal obturation with two calcium hydroxide based sealers in dog's teeth, they concluded that regardless of the sealer used, there were observed necrosis in a few of delta apical's ramifications and inflammation of both apical and periodontal tissues [12]. Therefore, it is required that effect of sealers on apical and periodontal tissues should be well known before their use.

Different classes of root canal sealers have shown varying amount of cytotoxicity [13-16]. Resin based sealer are most commonly used now because of their better adhesion to dentinal wall, radiopacity and uniform sealing ability [17,18]. EndoREZ (Ultradent Products, Inc., South Jordan,UT, USA) is a single methacrylate based sealer which contains UDMA (Urethane Dimethacrylate), maximum working time of 12-15 minutes and it will begin to polymerize in the canal at that time [19]. It has...
hydrophilic characteristics providing excellent penetration into dentinal tubules [20]. This allows for improved sealing properties combined with ease of placement and removal. The seal has shown biocompatibility to periapical tissues in subhuman primates [21] and to rat cells and bone tissue [22].

The silicone-based endodontic sealer, RoekoSeal (Colte’ne Whaledent, Langenau, Germany), has been reported to be noncytotoxic [23] or slightly cytotoxic [24]. RoekoSeal is a polydimethylsiloxane based root canal sealer. It has chemical and physical properties that exhibit extremely high sealing ability and biocompatibility, has a setting time of 45-50 minutes and appears to be less cytotoxic than sealers based on methacrylate, zinc oxide-eugenol and epoxy resin [24]. However, another study rated the silicone based sealer equal to an epoxy resin-based sealer in terms of cytotoxicity [25].

Kalsogen® Plus is a polymer reinforced fast setting zinc oxide eugenol cement in a light-yellow shade corresponding to Vita® B2 and Biodent 15. Kalsogen® Plus complies with the specification of ISO 3107, Type III, Class I, for temporary restorative materials and base materials. Working time is 2 mins from end of mix at room temperature of 23°C and 50% relative humidity. Zinc oxide–eugenol-based endodontic sealers have been used for many years but release potentially cytotoxic concentrations of eugenol [26,27]. Eugenol is found to leak from zinc oxide-eugenol sealers [28]. Both zinc oxide-eugenol and eugenol induce a toxic effect in cell culture models [29] and reduce the transmission in nerve cells [30]. The effects are persistent, also after setting of the material.

Materials and methods

Biological cell lines used in the study

The human embryonic kidney cells (HEK-293) were obtained from a healthy aborted foetus and originally cultured by Vander Eb of Holland.

The Vero lineage was isolated from kidney epithelial cells extracted from an African green monkey (Cercopithecus aethiops). The lineage was developed in 1962, by Yasumura and Kawakita at the Chiba University in Chiba, Japan.

The standardized cell culture methods and the frequently used MTT assay, employing target cells, are well recognized methods for assessing dental materials nonspecific cytotoxicity. MTT [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide)] is dye which on oxidation converted in to purple coloured formazan crystals of substrate. Live cell mitochondria contains cytochrome oxidase enzyme which converts the MTT dye in to its coloured substrate. Thus percentage of live cell in the population can be quantified by measuring the absorbance at 540nm (OD) of treated versus untreated cells.

The present study was performed in vitro. Samples prepared were divided in to two main groups - untreated control group and experimental group (sealers used in the study). Control group is DMEM (Dulbecco's Modified Eagle's Medium) alone. DMEM is a modification of Basal Medium Eagle that contains four fold concentrations of the amino acids and vitamins.

Experimental groups are depicted in (Table 1).

<table>
<thead>
<tr>
<th>Groups</th>
<th>Materials used</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Group</td>
<td>DMEM media alone</td>
<td>Dulbecco's Modified Eagle's Medium</td>
</tr>
<tr>
<td>Experimental Groups</td>
<td>Sealers used in study</td>
<td>dual-curing contains zinc oxide, barium sulfate, resins, and pigments in a matrix of urethane dimethacrylate.</td>
</tr>
<tr>
<td>gp-I</td>
<td>ER (EndoREZ)</td>
<td>Polydimethylsiloxane, Silicone oil, Paraffin-base oil, Platinum catalyst, Zirconium dioxide</td>
</tr>
<tr>
<td>gp-II</td>
<td>RS (RoekoSEAL)</td>
<td>Powder contains zinc oxide liquid contains eugenol</td>
</tr>
<tr>
<td>gp-III</td>
<td>ZOE(Kalsogen Plus)</td>
<td></td>
</tr>
</tbody>
</table>

Cell culture and preparation of extract

All the experimental sealers (Endorez, RoekoSeal and Kalsogen Plus) were mixed according to manufacturers’ instructions under aseptic environment. Extracts were prepared aseptically under laminar flow hood. DMEM (phenol free) is used for preparing the root canal sealer’s extract. The materials were then placed in non-reactive 12 well plates and allowed to set according to the manufacturers’ instructions at 37°C. Fresh extracts (FE) were collected immediately after mixing by adding 2ml DMEM and then kept for 5minutes. After that extract was collected; syringe filtered and then stored at -20°C till further use. 0 hr extracts (OE) were collected after setting of mixed root canal sealers in the same way as freshly mixed extract. 24 hr extracts (24E) were collected 24 hr after setting of mixed root canal sealers. After setting of mix 2ml of DMEM was added and allowed to incubate at 4°C. Extracts were then collected after 24 hr. Similarly, 48 hr(48E), 72 hr(72E) and 1 week(1Wk) extracts were collected.

Cell lines HEK-293 and Vero were propagated in minimum essential medium, supplemented with 5% foetal bovine serum (Invitrogen, USA), 2mmol/L L-glutamine, 100 U/mL penicillin (Gibco-BRL, USA) and 100 μg/mL streptomycin at 37°C in air atmosphere containing 5% CO2 and 95% relative humidity. Cells were passaged by treatment with trypsin-EDTA in phosphate-buffered saline solution.

Extract of each material was filtered using Millex-GS sterile filter (Millipore,USA) and used as the experimental material. A volume of 20μL of filtered extracts in 200μL medium (control) was added to each well containing cell lines used in the study along with DMEM media. Six replicates were used for each sample. Cells were incubated for 2 hr at 37°C and 5% CO2. The pH of the extracts was checked using pH indicator strips (Decibel instrument, India). The MTT assay was carried out according to Mossman T (1983) to evaluate cytotoxicity of samples [31].
Cells were taken after 80-90% confluency. Media was decanted and washed with PBS (6ml for 25cm² and 10 ml for 75cm²). Cells were trypsinized by using 0.25% trypsin and split accordingly. Cells were then stained with 0.5% of trypan blue and viable cells were counted with the help of a hemocytometer. 20μl of cell suspension was added to 80μl of 0.5% of Trypan blue stain and mixed thoroughly in a sterile 1.5ml micro centrifuge tube. There after 10μl of the mixture was filled in a hemocytometer for cell counting. Under a microscope, viable cells were observed and average no of cells were calculated according to the given formula:

\[
\text{NO of cells} = \text{Average No of cells} \times \text{Dilution Factor} \times \text{Counting Factor}
\]

Calculation of percentage of inhibition of cell growth/cytotoxicity was calculated with the formula given below –

\[
\% \text{ of Cell Growth Inhibition} = \left( \frac{\text{Mean O.D. of Control} - \text{Mean O.D. of the Treated}}{\text{Mean O.D. of Control}} \right) \times 100
\]

The formazan content of each well was computed as a percent of the control group (untreated cells). Cytotoxicity responses were rated as severe (>70%), moderate (40-70%), mild/slightly (10-40%) or noncytotoxic (<10%) [32].

The statistical analysis was done using SPSS Version 15.0 (SPSS, Inc., Chicago, IL, USA) statistical software package. p-values <0.05 were considered statistically significant. The values were represented in Number (%) and Mean±SD. Statistical values for each group of data were subsequently calculated with analysis of variance.

**Results**

As shown in (Tables 2 and 3), Kalsogen Plus showed highest mean value at 24hr on both cell lines. EndoREZ and RoekoSEAL had highest mean value at 48hr on both cell lines (p<0.05). Data also showed that in all the type (HEK-293 and VERO cells) and point of time (Fresh, 0 hr, 24 hr, 48 hr, 72 hr and 1wk), EndoREZ had the minimum cell inhibition and Kalsogen Plus had maximum (p<0.001) (Figures 1 and 2).

<table>
<thead>
<tr>
<th>Sealer</th>
<th>Fresh</th>
<th>0hr</th>
<th>24hr</th>
<th>48hr</th>
<th>72hr</th>
<th>1 week</th>
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<tbody>
<tr>
<td>ZOE</td>
<td>5.46±1.86a</td>
<td>11.67±1.64a</td>
<td>31.07±2.86a</td>
<td>23.39±2.00a</td>
<td>17.66±5.08a</td>
<td>17.07±5.02a</td>
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<tr>
<td>ER</td>
<td>2.63±1.43b</td>
<td>4.48±1.26b</td>
<td>6.17±0.87b</td>
<td>8.28±1.87b</td>
<td>6.86±2.80b</td>
<td>4.92±1.53b</td>
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<tr>
<td>RS</td>
<td>4.43±0.61c</td>
<td>8.73±1.41c</td>
<td>15.31±3.01c</td>
<td>16.56±4.82c</td>
<td>10.66±3.30c</td>
<td>7.03±3.51c</td>
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</table>

For each column, data with different letter superscripts denote significant difference (p<0.05). As compared to ZOE the mean value of all the other groups was found to be significantly (p=.000 versus ER, p=.000 versus RS) different. The mean value of ER was minimum and significantly different as compared to all the other groups. RS had significantly lower mean value as compared to ZOE but significantly higher mean value as compared to ER. None of the other comparisons was significant statistically. Overall the increasing order of toxicity on HEK-293 follows – ER<RS<ZOE.
The results of the present study is in agreement with several previous studies that showed that RoekoSeal was only slightly cytotoxic or completely non-cytotoxic even in fresh conditions [15,17,23,40]. It appears to be less cytotoxic than sealers based on methacrylate, zinc oxide-eugenol and epoxy resin [24]. However, another study rated the silicone-based sealer equal to an epoxy resin-based sealer in terms of cytotoxicity [25].

In overall assessment, extracts of Kalsogen Plus were more toxic, as compared to all other groups (p<0.05) on both cell lines. Extracts of EndoREZ were least toxic and mean value of percentage inhibition was significant as compared to all the other groups on both cell lines. RoekoSeal had the minimum intergroup difference while the maximum difference was seen between Kalsogen Plus and EndoREZ.

Extracts of Kalsogen Plus and RoekoSeal were rated slightly cytotoxic, whereas extract of EndoREZ was noncytotoxic.

Discussion
The MTT Cell Proliferation Assay measures the cell proliferation rate and conversely, when metabolic events lead to apoptosis or necrosis, the reduction in cell viability. The number of assay steps has been minimized as much as possible to expedite sample processing. The MTT Reagent yields low background absorbance values in the absence of cells. For each cell type the linear relationship between cell number and signal produced is established, thus allowing an accurate quantification of changes in the rate of cell proliferation [31,33].

The popularity of resin-based sealers is increasing, despite their well-documented toxicity and mutagenicity [34,35]. In addition, leakage has been observed between sealer and dentinal wall as a result of contraction of the resin sealers during setting [36]. Thus, new resin formulations have been designed to improve the adhesion of the sealers to dentine (EndoREZ). A sealer containing a single methacrylate has been described as well tolerated by connective tissues [14,21]. The sealer has shown biocompatibility to periapical tissues in subhuman primates [37] and to rat cells and bone tissue [22]. The results of the present study is in agreement with several other studies [14,21-23], but not with that by Bouillaguet et al., [24], where EndoREZ was found to be more cytotoxic than RoekoSeal when tested by the MTT assay. Urethane dimethacrylate (UDMA) in the structure of this sealer could be responsible for the cytotoxic effect, as it has been previously shown that UDMA is a toxic agent [38].

RoekoSeal is a new silicone-based material, which is described as a biocompatible material [39]. The findings of present study is in agreement with previous studies that showed that RoekoSeal was only slightly cytotoxic or completely non-cytotoxic even in fresh conditions [15,17,23,40]. It appears to be less cytotoxic than sealers based on methacrylate, zinc oxide-eugenol and epoxy resin [24]. However, another study rated the silicone-based sealer equal to an epoxy resin-based sealer in terms of cytotoxicity [25].

The setting of zinc oxide eugenol cements is a chemical process combined with physical embedding of zinc oxide in a matrix of zinc eugenolate. Analyses of the release of eugenol from set ZnOE cement showed that the oil is available only as the result of surface hydrolysis of the chelate [41-43]. Samples of ZnOE placed into saline showed an immediate release of eugenol from the ZnOE surface with the highest rate of release in the first seconds after contact, the release rate declined exponentially thereafter. In contrast, the release rate through intervening dentine was found to be entirely different. The release of eugenol was found to be much slower and could only be detected after several hours, it peaked after about a day and then declined slowly over several weeks. A sustained release occurred with the establishment of a relatively stable concentration gradient across the dentine which persisted for several months [44]. In the dentine immediately beneath the ZnOE, the concentration of eugenol is sufficient to inhibit bacterial metabolism whilst the concentration in more remote dentine would be below the threshold for killing mammalian cells but at a sufficient level to exhibit pharmacological properties for which eugenol is renowned such as inhibition of nerve action potential [45] and prostaglandin synthesis [46]. If ZnOE contacts wetter tissue the release is more rapid, leading to the development of concentrations sufficient to kill cells. This explains the toxic effects of ZnOE when applied to wet tissues or to cells in culture.

The findings of the present study mostly corroborate with already reported toxicity profile of the sealers investigated. Whilst biocompatibility is a desirable quality, extrapolations to the clinical situation must be made with caution, as the results of such in vitro toxicity tests may not correlate with the in vivo response.
Conclusion
EndoREZ was non cytotoxic amongst the sealers used. Kalsogen Plus and RoekoSEAL were slightly cytotoxic. The order of cytotoxicity was ZOE>R5>EZ.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions

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<th>AA</th>
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<td>Data analysis and interpretation</td>
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