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Evaluation of the cytotoxicity of different root canal sealers on L929 cell line by MTT assay

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The purpose of this study was to evaluate the cytotoxic effects of commercially available root canal sealers [Sealite Ultra (SU), Tubli-Seal (TS), Tubli-Seal EWT (TS-EWT), Pulp Canal Sealer (PCS), Pulp Canal Sealer EWT (PCS-EWT), Endomethasone N (En N), and Apexit Plus (AP)] on L929 cells by using MTT assay. After incubation with each sealer's extract at 37°C in a humidified air atmosphere containing 5% CO_2 for 24 h, MTT (5 mg/mL) in saline was added into each well and further incubated at 37°C for 4 h. Formazan precipitate was dissolved in a buffer containing 23% sodium dodecyl sulfate and 50% *N*,*N*-dimethylformamide (pH 4.7). Optical densities of dissolved formazan were read using a microplate spectrophotometer. AP, TS, and TS-EWT showed no cytotoxicity at any dilution tested. Other sealers exhibited some degree of cytotoxicity at the 1/4 and 1/2 dilutions. PCS-EWT and SU exerted more potent cytotoxicity at 1/2 dilution than the other sealers.

Keywords: Cytotoxicity, MTT assay, Root canal sealer

INTRODUCTION

In endodontic therapy, cleaning and shaping of the root canal system is complemented with a three-dimensional fluid-tight filling with a chemically inert, biologically compatible, and dimensionally stable material[1)]. Root canal filling materials are classified as core filling materials and sealer cements. Regardless of the core filling material, a sealer is essential to every obturation technique employed and helps achieve a fluid-tight seal of the root canal system[2)]. A root canal sealer fills the gaps between gutta-percha points and the walls of the root canal. It also fills the voids between individual gutta-percha points applied during obturation of the root canal system[3)].

Biocompatibility of root canal sealers is of primary importance, but it varies considerably[4)]. It was reported that root canal sealers may cause adverse local and/or systemic effects on periradicular tissues and alveolar bone due to the release of extractable monomers and/or other inorganic and organic ingredients[4)]. Therefore, root canal sealers could cause not only degeneration of the tissue lying underneath the endodontic sealer but also delay wound healing. Since root canal fillings should be biocompatible and well tolerated by periradicular tissues, the cytotoxicity of new root canal sealers should be subjected to stringent preclinical screening before recommended for commercial use.

A plethora of root canal sealers with considerably different formulations are currently available in the market: zinc oxide-eugenol-based sealers, calcium hydroxide-based sealers, glass ionomer-based sealers, and resin-based sealers[2)]. Zinc oxide-eugenol-based sealers are widely used for many decades although they have been shown to exhibit *in vitro* cytotoxicity because of the release of eugenol[5)]. However, the eugenol and formaldehyde ingredients in zinc oxide-eugenol-based sealers account for their well-favored antimicrobial activity. For clinicians that use the thermoplastic compaction technique, their preferred zinc oxide-eugenol-based sealers are Pulp Canal Sealer (Kerr Italia Srl, Salerno, Italy) and Pulp Canal Sealer™ EWT (Extended Working Time) (Kerr Italia Srl, Salerno, Italy). For easy mixing, Tubli-Seal (SybronEndo, Glendora, CA, USA) is a catalyst/base zinc oxide-eugenol sealer which boasts of this convenience, but it has a faster setting time when compared to liquid/powder sealers. For an extended working time, the alternative is Tubli-Seal EWT (Extended Working Time) (SybronEndo, Glendora, CA, USA).

Endomethasone (Septodont, Cedex, France), another zinc oxide-eugenol based sealer, contains therapeutic agents which may cause serious neurotoxic complications. To ameliorate the neurotoxic effect, Endomethasone N (Septodont, Cedex, France) is manufactured without paraformaldehyde. With increasing concern about cytotoxicity, Sealite™ Ultra (Pierre Rolland, Merignac Cedex, France) is a zinc oxide-eugenol sealer with neutral pH and which reportedly contains no harmful components to prevent cytotoxic reactions.

Calcium hydroxide-based sealers were introduced for their potential therapeutic benefits, and they reportedly cause mild to moderate tissue-irritating activities[6,7)]. Apexit Plus (Ivoclar Vivadent AG, Schaan, Liechtenstein) is a calcium hydroxide-based sealer which comprises an activator (disalicylate, bismuth hydroxide/bismuth carbonate, and filler) and a base (calcium hydroxide, hydrated colophonium, and fillers).

In vitro assays for assessing the cytotoxicity of endodontic materials have been reviewed and

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evaluated in terms of their relevance, advantages, and limitations[8-10]. Assays which provide useful information on cellular functions include the reduction of tetrazolium salt (MTT), the uptake of neutral red dye (NRU), and the total nucleic acid content (NAC)[11,12]. Widely used for cell viability and cytotoxicity evaluations, the dimethylthiazol diphenyltetrazolium bromide (MTT) reduction assay is an *ex vivo* biocompatibility test which assesses cell survival rate by measuring cellular metabolic activity[13,14].

In this study, the cytotoxicity of different commercially available root canal sealers [Sealite Ultra (SU), Tubli-Seal (TS), Tubli-Seal EWT (TS-EWT), Pulp Canal Sealer (PCS), Pulp Canal Sealer EWT (PCS-EWT), Endomethasone N (En N), and Apexit Plus (AP)] was evaluated by using MTT assay to assess the survival rates of L929 cells.

MATERIALS AND METHODS

Cell cultures

L929 mouse fibroblast cells (ATCC) were cultured in 25-cm^2 culture flasks containing RPMI-1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine (Sigma Chemical Co., St. Louis, MO, USA), 100 μg/mL of streptomycin, and 100 mg/mL of penicillin (Sebak, Biologische Fordchungs, Germany). Cultures were maintained at 37°C in a humidified incubator under ambient pressure air atmosphere containing 5% CO_2. Confluent cell monolayers were trypsinized, and harvested cells were used for cytotoxicity experiments.

Root canal sealers and extract dilutions

Root canal sealers evaluated in this study were obtained from their respective manufacturers. Compositions of the root canal sealers' components (liquid and powder, activator and base) are listed in Table 1.

Root canal sealers were prepared according to manufacturers' instructions and placed in 96-well U-bottom plates (Costar, Cambridge, MA, USA). Sealers filled one-third of the wells, and three wells were prepared for each root canal sealer. Plates were kept for 1 h in a humidified incubator at 37°C with 5% CO_2. Extraction media of three wells containing the same sealer were collected into one sterile tube. Cytotoxicity assay was performed using various final dilutions (1/2, 1/4, 1/8, 1/16, 1/32) of these extraction media.

MTT assay

The MTT assay is a simple colorimetric assay developed by Mosmann[15] to measure cell proliferation and survival. A modified method of the colorimetric MTT assay[16] was used in this study for cytotoxicity testing.

After seeding 2×10^4 cells in 50 μL of culture medium per well in flat-bottom microplates, 50 μL of exraction medium was added into each well. Cells in 50 μL of culture medium alone served as a control for cell viability. The assay was run in quadruplicate so that control and dilution values were obtained as the mean values of four identical wells.

After 24-h incubation at 37°C in a humidified air atmosphere containing 5% CO_2, 25 μL of 5 mg/mL of MTT in saline was added into each well and further incubated at 37°C for 4 h. To dissolve formazan precipitate, 80 μL of a buffer containing 23% sodium dodecyl sulfate

Table 1 Compositions of root canal sealers evaluated in this study

Root canal sealer	Composition	
Sealite Ultra (SU)	Powder: 1% Enoxolone, diiodothymol, zinc oxide, *radio-opacifier*: *silver* powder	Liquid: Eugenol
Apexit Plus (AP)	Base: Calcium hydroxide/Calcium oxide, hydrated collophonium, silicon dioxide, phosphoric acid alkyl ester	Activator: Disalicylate, bismuth hydroxide/bismuth carbonate, silicon dioxide, phosphoric acid alkyl ester
Tubli-Seal (TS)	40% Zinc oxide, 2.75% barium sulphate, 25% oleo resins, 7,5% thymol iodide, 22.75% eugenol, 2% modifiers	
Tubli-Seal EWT (TS-EWT)	Mineral oil, barium sulfate, zinc oxide, lecithin, cornstarch, eugenol	
Pulp Canal Sealer (PCS)	Powder: Zinc oxide, staybelite resin, bismuth subcarbonate, barium sulfate, sodium borate, anhydrate	Liquid: Eugenol
Pulp Canal Sealer EWT (PCS-EWT)	Powder: Silver powder, zinc oxide, thymol iodide, dimeric acid resin	Liquid: Clove oil, Canada balsam, eugenol
Endomethasone N (En N)	Powder: Hydrocortisone acetate, thymol iodide, barium sulfate, zinc oxide, magnesium stearate	Liquid: Eugenol

(Sigma Chemical Co.) and 50% *N*,*N*-dimethylformamide (pH 4.7) was added into each well. Further incubation was performed overnight at 37°C. Optical densities (OD) of dissolved formazan were read at 570 nm using a microplate spectrophotometer (SpectraMax, USA). Viability ratio (%) at each dilution was determined using this formula: [Mean OD of treated cells/Mean OD of control cells]×100%.

Statistical analysis of cytotoxicity measurements

Potential cytotoxicity of root canal sealers was evaluated using linear regression analysis. Dilutions which caused 50% cell death (CD_{50}) were calculated using Instat software (GraphPad, San Diego, CA, USA). These CD_{50} values expressed the cytotoxicity potentials of the evaluated root canal sealers, and they were used for comparison against the corresponding correlation coefficient values (r^2).

RESULTS

Cell viability at different dilutions of root canal sealers

Table 2 shows the viability ratios (%) of root canal sealers at different dilutions. At dilutions ranging from 1/32 to 1/8, no sealers exhibited cytotoxicity. At 1/8 dilution, a slight decrease in cell viability was observed but did not exceed 40% cell death. At 1/4 dilution, some sealers exhibited a marked increase in cytotoxicity. At 1/2 dilution, three sealers still showed high viability ratios with AP showing no cytotoxic effect even at this dilution.

With TS, cell viability decreased at 1/2 dilution but decline did not exceed 50%. With TS-EWT, 80% cell viability was observed at 1/4 dilution but *circa* 50% decrease in viability was observed at 1/2 dilution. With SU, PCS, and En N, cytotoxic effect was exhibited at 1/4 dilution and confirmed at 1/2 dilution. For PCS EWT, it exerted a pronounced cytotoxic effect on L929 cells at 1/2 dilution.

CD_{50} dilutions of root canal sealers

Table 3 lists the CD_{50} dilutions for root canal sealers evaluated in this study. TS yielded the highest CD_{50} value at 0.84. SU, PCS, and En N had similar CD_{50} dilution values, which were in good agreement with the severe toxic effect exhibited at 1/2 dilution (Table 2). For AP, its CD_{50} value could not be determined.

DISCUSSION

Ideally, the roles of root canal filling materials after root canal treatment are to eliminate or minimize the presence of bacteria and their byproducts, as well as promote the healing of periapical tissues[17]. Therefore,

Table 2 Viability ratios (%) at various dilutions of root canal sealers

Root canal sealer	Control	1/32	1/16	1/8	1/4	1/2
Sealite Ultra (SU)	100	93.01	80.14	64.53	36.65	9.96
Apexit Plus (AP)	100	93.93	90.03	100	100	100
Tubli-Seal (TS)	100	91.07	87.67	87.92	82.08	66.29
Tubli-Seal EWT (TS-EWT)	100	95.40	93.16	89.49	83.19	52.58
Pulp Canal Sealer (PCS)	100	90.39	81.96	63.69	18.48	13.73
Pulp Canal Sealer EWT (PCS-EWT)	100	92.14	87.58	72.01	57.29	9.86
Endomethasone N (En N)	100	87.46	82.45	67.11	37.25	11.54

Table 3 CD_{50} dilutions for root canal sealers evaluated in this study

Root canal sealer	CD_{50}	r^2	p
Sealite Ultra (SU)	0.233736	0.9380	<0.0001
Apexit Plus (AP)	—	0.5004	=0.0005
Tubli-Seal (TS)	0.839107	0.8263	<0.0001
Tubli-Seal EWT (TS-EWT)	0.557220	0.9355	<0.0001
Pulp Canal Sealer (PCS)	0.215358	0.8100	<0.0001
Pulp Canal Sealer EWT (PCS-EWT)	0.273261	0.9754	<0.0001
Endomethasone N (En N)	0.237082	0.9518	<0.0001

the biocompatibility of a root canal sealer is critical to its efficacy in fulfilling these roles in endodontic therapy. In this study, we evaluated the cytotoxicity of six zinc oxide-eugenol-based root canal sealers and one calcium hydroxide-based sealer at five different dilutions.

Cell culture techniques are widely used to evaluate the biocompatibility of root canal sealers. In this study, the cytotoxicities of root canal sealers at different dilutions were assessed using L929 cell cultures *via* the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide MTT assay. This method was employed for *ex vivo* biocompatibility evaluation because it provides information on cellular viability based on the chemical reduction of soluble tetrazolium salt by viable/living cells into an insoluble colored formazan compound[18]. Results of several studies have confirmed non-radioactive MTT reduction assay to be a safe, simple, fast, and reliable method to quantify cell viability[3].

Long-term *in vivo* studies are a gold standard for determining clinical performance. However, such studies are uncommon because of two key hurdles: they are impractical because of the cost and time involved, and they may pose ethical limitations[19-21]. Alternatively, *in vitro* tests are used to screen and assess biological risks posed by medical materials such as root canal filling materials, because *in vitro* short-term cytotoxicity is predictive of the longer-term for many types of dental materials[22]. However, although *in vitro* tests are faster and less expensive than *in vivo* tests, they do not comprehensively simulate *in vivo* conditions and the results obtained may not be relevant for the clinical setting[23]. In the case of MTT assay, Bryan *et al.*[24] emphasized that it reduces undue discriminations as its test conditions clinically correspond to a classical filling.

At 1/32, 1/16, and 1/8 dilutions, no sealers evaluated in this study were found to be cytotoxic. For AP, TS, and TS-EWT, they did not show any cytotoxic effect at all dilutions ranging from 1/32 to 1/2. For the other sealers, they exhibited cytotoxicity at 1/4 and 1/2 dilutions. Huang *et al.*[14] had demonstrated that zinc oxide-eugenol-based and calcium hydroxide-based sealers induced a dose-dependent survival effect. Our present study confirmed that zinc oxide-eugenol-based sealers showed dose-dependent cytotoxicity.

Results of this study also showed that zinc oxide-eugenol-based sealers were more toxic than calcium hydroxide-based sealers. AP, a calcium hydroxide-based sealer which did not contain formaldehyde or pharmaceutical substances such as corticoids and antibiotics, did not exert any cytotoxic effect at any dilution tested in this study. The manufacturer claimed that because of excellent tissue tolerance to AP, biological balance is re-established around the tooth after root canal treatment.

However, our results concerning the non-cytotoxicity of calcium hydroxide-based sealers conflicted with the findings of Huang *et al.*[14] —who reported that highest cytotoxicity was found in freshly mixed calcium hydroxide-based root canal sealer, as well as with other studies which found calcium hydroxide-based sealers to be highly toxic[10]. On the other hand, our results found agreement in studies which claimed calcium hydroxide-based sealers to be mildly or moderately toxic[6,7], with some studies even claiming that these sealers exhibited good or excellent biocompatibility[25-27-34]. The initial high pH from calcium hydroxide in these sealers might account for the fore-mentioned discrepancy, and which had to be buffered by the cell culture medium to obtain favorable tissue response[28].

Besides the calcium hydroxide-based sealer AP, two zinc oxide-eugenol-based sealers TS and TS-EWT were also non-cytotoxic, while other zinc oxide-based sealers exhibited some degree of cytotoxicity especially at the 1/4 and 1/2 dilutions. Oxidants at low concentrations can induce cytostasis without affecting viability, or that they target cell lysis but do not induce apoptosis with the characteristic morphology changes and DNA fragmentation. Root canal sealers which contain ingredients such as poly-methylene, methyl salicylate, isobutyl salicylate, eugenol, and silicone oil may induce tissue toxicity, leading to apical periodontitis and transient or persistent inflammatory responses[29-31]. Owing to the eugenol content[32], zinc oxide-eugenol-based sealers were shown to be moderately to severely toxic[6]. Zinc oxide-eugenol was also shown to exhibit prominent cytotoxic and neurotoxic effects[33,34].

Most zinc oxide-eugenol-based sealers show high antibacterial activity because they contain formaldehyde. Paradoxically, formaldehyde has been shown to be both cytotoxic and mutagenic[35,36]. Similarly, eugenol —which is known to be an antioxidant and anti-inflammatory agent— was shown to have high toxic potency[25,34].

PCS-EWT and SU were found to be the most cytotoxic of the sealers tested in this study. For PCS-EWT, it had been shown to possess a marked cytotoxic and tissue-irritating potency[4,28] on the one hand, but was also reported to yield better tissue organization than AH Plus after subcutaneous implantation in rat connective tissue[37] on the other hand. For SU, it contained enoxolone, a non-steroidal anti-inflammatory agent claimed by its manufacturer to exhibit good local and systemic safety. However, information on the cytotoxicity of SU is scarce.

CONCLUSIONS

Commercially available zinc oxide-eugenol-based sealers were found to be severely cytotoxic because of the eugenol content. For newly developed root canal sealers, they should undergo careful and rigorous cytotoxicity testing before they are introduced for clinical use.

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