Cytotoxicity evaluation of newly developed bi-functional, oligomer-based sealers and a methacrylate resin-based root canal sealer

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Abstract

Objectives: The purpose is to compare cell cytotoxicity of new oligomer-based root canal sealers to a methacrylate-based sealer (RealSeal SE), using MTT assay.

Materials and methods: L929 cells were sub-cultured into 96-well plates with 100 µl (1 x 10^4 cells) per well. Four formulas of the oligomer-based sealer (namely F68, F71, F72 and F75) and RealSeal SE sealer were prepared. Sealer rod (0.2 g) was produced in a polyethylene tube and set in a self-curing mode at room temperature for 7 days. Elute of a sealer rod was prepared and diluted with MEM medium at 1 (no dilution), 10, 100 and 1,000 times. Next, 100 µl of elute was added into a well, 16 wells of each dilution. MEM medium at 100 µl, without any elute, was used as a blank control. After 24-h incubation, MTT solution at 50 µl was added into each well and then incubated for 2 h. Optical density was read at 570-nm wavelength, and cell viability was determined as a percentage of the blank control value.

Results: Without dilution, F68 sealer showed the significantly highest cell viability (at 79%) among the experimental sealers. Other tested sealers had unacceptable cytotoxicity (cell viability < 30%) unless a dilution was made. For the undiluted condition, RealSeal SE sealer showed the lowest cell viability at 13% that was significantly different from the oligomer-based sealers. When elute of each sealer was sequentially diluted, cell viability significantly increased (> 80%) and became acceptable. In conclusion, experimental F68 oligomer-based sealer is highly biocompatible to L929 fibroblast cells.

Keywords: cytotoxicity, L929 cells, MTT assay, oligomer, RealSeal SE, root canal sealer

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Introduction

Long-term success of endodontic treatment is highly influenced by apical sealing from root canal obturation and coronal sealing from permanent restoration[1]. In root canal obturation, it is not only a root canal obturation material but also a root canal sealer that plays an important role in providing a good apical sealing inside root canal. Root canal sealer fills either remaining canal irregularities or spaces between master/accessory cones of the obturation material.

Conventionally, zinc oxide eugenol-based root canal sealer has been used in a root canal obturation for decades. Clinical trial shows a promising result of using this root canal sealer in endodontic treatment with a high success rate[2]. However, the eugenol-based sealer possesses some drawbacks, which the most importance is inability to bond with root canal dentin wall or the obturation material. Thus, apical sealing inside root canal is unlikely to be optimally achieved, which microbial leakage can occur within days, weeks or months[3].

Currently, the mostly used root canal obturation material is gutta percha (GP) that mainly consists of zinc oxide and thermoplastic polymer. Nevertheless, the era of GP has been challenged by an introducing of a polymer-based root canal obturation material, i.e. - Resilon/Epiphany. Resilon is a polymer-based (polycaprolactone) material that is used in combination with a methacrylate resin-based root canal sealer and a self-etching primer or, more recently, with a self-adhesive methacrylate sealer without the primer[4]. Since the Resilon may bond to root canal wall by using the methacrylate-based sealer, a good adaptation between the material, sealer and root canal wall may be anticipated. Consequently, sealing ability of root canal obturation with the Resilon system could be improved.

However, recent laboratory studies reported controversial results in sealing ability of the Resilon system in comparison to the traditional GP with conventional sealers[5-8]. Thus, the benefits of the Relison system has been questioned. In addition, cell cytotoxicity of freshly-mixed Resilon sealer is highly moderate even it is lower than that of epoxy resin-based sealer[9]. However, both sealers are eventually non-toxic to the cell cultures at 24 h after setting.

Optimal adhesion to root canal dentin of the Resilon system has not been achieved due to the difficulty and complexity in bonding within root canal[10]. Moreover, the bond between the Resilon cone and its methacrylate-based sealer has been questioned. The amount of methacrylate resin on the Resilon cone may not be enough to create a sufficient chemical bond to the sealer[11]. In addition, a degradation of the polymer matrix might occur to the Resilon cone when there is a presence of peroxidase enzymes released from the pathogenic bacteria[12-14]. Hence, a further development of new root canal filling material and sealer is necessary.

Thermoplastic, elastomeric-based (TPE) root canal filling material has been recently invented to obtain a newly developed material that would be superior to the Resilon. TPE is a mixture of thermoplastic and elastomer, which is currently used in many products. From the preliminary laboratory studies, TPE contains desirable properties that makes it is possible to be developed as a root filling material. To obtain an optimal adhesion between this innovative material and root canal dentin, a new root canal sealer has been also developed. The sealer mainly consists of a bi-functional, oligomer-based material that contains an oligomer backbone with acrylate and/or methacrylate side chains (manufacturer's data). The sealer is set by a polymerization reaction from either light curing or chemical curing. The manufacturer claims that this oligomer-based sealer is possible to bond, mechanically and chemically, with either TPE cone or root canal dentin.

Extrusion of root canal sealer beyond the root apex into periapical area is possible. In laboratory studies, the resin-based root canal sealer has a considerable cytotoxicity at early stage[9, 15]. For an animal study, the extrusion of resin-based sealer is able to induce mild to moderate chronic inflammation at the periapical areas for a long period[16]. Freshly mixed sealer apparently has a higher cytotoxicity to the tested cells, which may be due to the initial release of some toxic components[9]. However, the resin-based sealer becomes less toxic or non-toxic over period after mixing[9, 17, 18]. Basically, the biocompatibility of a new root canal sealer should be extensively evaluated.

MTT assay is a simple and reliable method to evaluate cell cytotoxicity of endodontic materials in several studies[19-21]. Main principle of the assay is to detect enzymatic activity of mitochondria in living cells[22]. L929, laboratory-cultured fibroblast cells (in monolayer), are frequently used since the homogeneity of cells and the simplicity of culturing procedure are superior to the cultured PDL cells obtained from extracted tooth. For this assay, dehydrogenase enzyme from mitochondria converts the water-soluble tetrasolium salt (yellowish color) in the culture media into formazan crystals (dark blue), which are kept within living cells[22]. The formazan crystals are dissolved from the cells into the solution with dimethyl sulfoxide or isopropanol. After that, optical density of formazan-containing solution is read at a wavelength of 540 or 570 nm using the Elisa reader[22]. If a root canal sealer is cytotoxic, cell viability is reduced, which is directly related to optical density value. In comparison to the control without any allocated material, the percentages of cell viability can be also calculated.

Therefore, the purpose of this *in vitro* study is to investigate the cytotoxicity of newly developed bi-functional, oligomer-based root canal sealers in comparison to a methacrylate resin-based sealer, RealSeal SE, using the MTT assay method.

Materials and methods

The protocols for preparation of sealer specimens and testing cell cytotoxicity are based on ISO no. 10993-12/2009 and 10993-5/2009, respectively.

L929 cell culture

In a Biohazard Safety Cabinet (*Model 1356, Thermo Fisher Scientific, Marietta, OH, USA)*, L929 cell line (*mouse fibroblast cells, ATCC, Manassas, VA, USA*) was fed in a culture media- MEM (*Gibco® Minimum Essential Medium, Life Technologies, Grand Island, NY, USA*) in a culture flask (*Corning Flask, Corning Incorporated, Corning, NY, USA*). Culture media was supplemented with 10% fetal bovine serum (*HyClone®, HyClone UK Ltd., Northumberland, UK*; 10 ml/100 ml of MEM) and antibiotic-antimycotic solution, consisting of Penicillin, Streptomycin and Amphotericin B (*Gibco®, Life Technologies*; 1 ml/100 ml of MEM).

L929 cells were cultured in an incubator (*Thermo Scientific, Thermo Fisher Scientific*) at the conditions of 37 °C, 5% CO_2 and 100% humidity. Culture media was changed every two days until a monolayer cell was obtained, which was checked by using an inverted optical microscope (*Nikon Eclipse TS100, Nikon Instrument Inc., Melville, NY, USA*). Cultured cells were transferred and sub-cultured using 5 ml of 0.25% trypsin-EDTA (*Gibco®, Life Technologies*) with a concentration of 1 x 10^5 cells per 1 ml of MEM. Next, L929 cells were dispensed into flat-bottom 96-well plates no. 3959 for tissue culture (*Corning Incorporated*) at 100 µl per well, which was a concentration of 1 x 10^4 cells. Culture plates were placed into the incubator for 24 h.

Preparations of root canal sealers

Four formulas of oligomer-based root canal sealer- F68, F71, F72 and F75, which contains different types and amounts of oligomer and side chains, were supplied from the manufacturer in a form of two separate pastes. The two pastes were mixed thoroughly before use. RealSeal SE sealer was provided and mixed following the manufacturer's instruction. The two components of sealer were dispensed from the dispenser and mixed thoroughly. Sealer rods (0.2 g of each) were produced in polyethylene tubes with a diameter of 5 mm and leaved to set in a self-curing mode at room temperature in a dark container for 7 days. Sealer rods were sterile, immersed into 1 ml MEM culture media and incubated at 37 °C for 24 h to obtain the elute of sealer. Elutes of the sealers were diluted with MEM at 1, 10, 100 and 1,000 times for cell viability test.

MTT assay cytotoxicity test

Elute of sealer (100 µl) at various dilutions was added into a well containing L929 cells, 16 wells of each sealer (in two culture plates, 8 wells/plate) (Figure 1). Polyurethane elution (polyurethane film containing 0.1% zinc diethyldithiocarbamate (ZDEC), *Hatano Research Institute, Kanagawa, Japan*) at 100 µl was used as a positive control, while polyethylene elution (*Thremanox® plastic cover slip, Nunc, Rochester, NY, USA*) at 100 µl was used as a negative control. In addition, 100 µl MEM without any elute of sealer is used as a blank control. Culture plates were incubated for 24 h before elute of root canal sealers was removed. L929 cell characteristics were checked using an inverted optical microscope at 10x magnification. MTT solution (*Sigma Aldrich, St Louis, MO, USA*; 1 mg/ml) at 50 µl was added into each well and further incubated for 2 h, which formazan crystals were formed within the living cells. Isopropanol at 100 µl was added into each well to dissolve formazan crystals out of the cells. Optical density (OD) of the solution was read at 570-nm wavelength (*Epoch, BIO-TEK Instrument Inc., Winooski, VT, USA*).

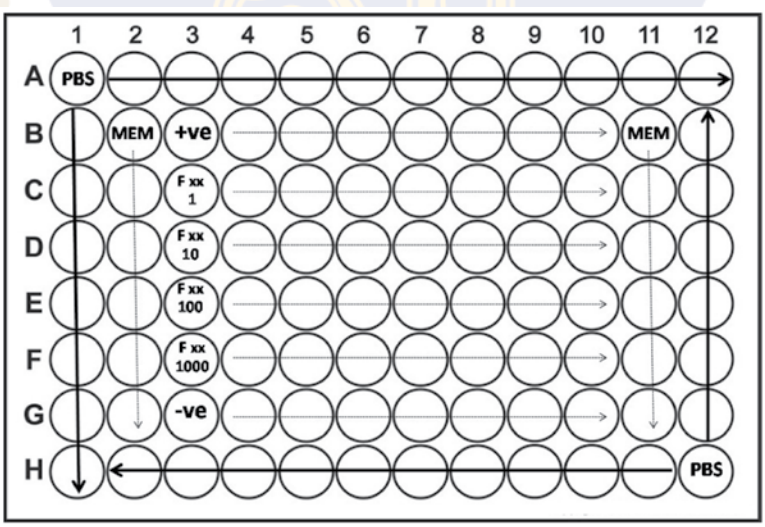

Figure 1 Illustration of assignments of the experimental sealers at 1, 10, 100 and 1,000 times dilutions in a 96-well culture plate (n = 8 of each dilution/plate; two plates were used with total n = 16). (F xx = experimental sealers F68, F71, F72, F75 or RealSeal SE; +ve= polyurethane, a positive control, -ve= polyethylene, a negative control, MEM= MEM blank control)

Optical density of blank control (MEM) was set as 100% cell viability. Cell viability of the tested sealers was determined as a percentage of control value. Means and standard deviations (SD) of cell viability in percentages were then calculated.

Percentages of cell viability = OD of sealer group / OD of control group x 100

Shapiro-Wilk test and Levene's test were used to check normal distribution and equal of variance, respectively. Taking into consideration each independent variable (sealers and concentrations), one-way ANOVA was used with multiple comparisons using Tukey's test.

Results

Table 1 presents the percentages of cell viability from experimental oligomer-based and RealSeal SE sealers. Figures 2-6 show the appearances of L929 cells in response to blank control and tested sealers. The results of cell viability are consistent with the cell characteristics observed under the light microscope.

Without dilution, F68 sealer showed the significantly highest cell viability among the experimental root canal sealers ($p < .05$). L929 cells were slightly changed in shape and reduced in number (Figure 2, no dilution) in comparison to the control (Figure 2, control). Other oligomer-based sealers, F71, F72 and F75, had unacceptable cytotoxicity with cell viability lower than 30%, unless a dilution was made. L929 cells were changed to round-shape and highly reduced in number (Figure 3-5, no dilution) in comparison to the control (Figure 3-5, control). Without dilution, RealSeal SE sealer showed the lowest cell viability that was significantly different from the oligomer-based sealers ($p < .05$). In comparison to the control (Figure 6, control), the cultured cells were severely reduced in number and turned to be round-shape (Figure 6 no dilution).

Table 1 Cell viability (%, mean ± SD) of experimental root canal sealers at different dilutions

Concentrations	Sealer F68	Sealer F71	Sealer F72	Sealer F75	Sealer RSE
No dilution	78.8 ± 7.9 $^{A, a}$	28.1 ± 16.3 $^{B, c}$	23.9 ± 3.5 $^{B, e}$	22.0 ± 5.7 $^{B, g}$	12.6 ± 2.1 $^{C, i}$
Dilution 10x	84.5 ± 6.6 $^{D, a, b}$	86.2 ± 4.7 $^{D, d}$	81.3 ± 8.5 $^{D, E, f}$	92.8 ± 5.6 $^{F, h}$	80.3 ± 7.2 $^{E, j}$
Dilution 100x	88.5 ± 8.3 $^{G, b}$	87.1 ± 7.3 $^{G, d}$	85.6 ± 6.8 $^{G, f}$	94.5 ± 5.7 $^{H, h}$	85.2 ± 8.5 $^{G, k}$
Dilution 1,000x	90.6 ± 8.9 $^{I, J, b}$	89.4 ± 5.7 $^{I, d}$	88.0 ± 8.9 $^{I, f}$	96.9 ± 4.1 $^{J, h}$	91.9 ± 7.4 $^{I, J, m}$

Capital letter- significant differences in row, *Small* letter- significant differences in column

Figure 2 L929 cells appearances in F68 experimental sealer groups.

Figure 3 L929 cells appearances in F71 experimental sealer groups.

Figure 4 L929 cells appearances in F72 experimental sealer groups.

Figure 5 L929 cells appearances in F75 experimental sealer groups.

Figure 6 L929 cells appearances in RealSeal SE sealer groups.

When elute of each sealer was diluted at 10, 100 or 1,000 times, cell viability significantly increased and became acceptable (> 80%). Cell viability of the four oligomer-based sealers was not significantly different between each other, regardless of the dilution stages (10-1,000 times). Characteristics of L929 cells were similar to those observed in the controls (Figure 2-5, 10x to 1,000x dilutions). In RealSeal SE groups, cell viability significantly increased after dilutions with the acceptable level above 80% cell viability. Nevertheless, the amount of cultured cells were slightly reduced, in comparison to the control (Figure 6, 10x to 1,000x dilutions).

Discussion

Based on the ISO standard 10993-5/2009, a tested material will be considered as cytotoxic if percentages of cell viability is less than 70%. Without dilution, only experimental, oligomer-based F68 root canal sealer had an acceptable biocompatibility to the cultured cells with cell viability at 78.8%. Other experimental and RealSeal SE sealers were predominantly cytotoxic, which percentages of cell viability were only 12-28%. Clinically, extrusion of root canal sealer beyond root apex into periapical area is possible. Extruded sealer is likely to be fully concentrated, which might induce inflammation and delay healing at periapical area[16]. Thus, root canal sealer must be biocompatible as much as possible. In this study, only F68 sealer has been biologically accepted in the undiluted condition and will be selected for further development.

New oligomer-based sealer mainly consists of low molecular weight polymers and bi-functional, oligomer(s) (*manufacturer's data*). Other compositions are light- and self-curing initiators, catalyst, radiopaque material, plasticizer and fillers. For the four experimental sealers, the major different composition is bi-functional oligomer(s). Bi-functional oligomer has an A-B-A molecular structure, which A is a side-chain functional group- *acrylate* and/or *methacrylate*, while B is one or two central functional groups- *urethane, ester* and/or *amide*. It has been claimed by the manufacturer that reactivity, viscosity and cytotoxicity of the sealer are primarily affected by types and amounts of bi-functional oligomers in the compositions. Complete details of bi-functional oligomers in each formulation of experimental sealer are still confidential and undisclosed by the manufacturer. However, it seems that F68 sealer which contains *methacrylate-based* oligomer

was significantly less cytotoxic than the other experimental sealers containing *acrylate-based* oligomer.

RealSeal SE sealer mainly consists of *methacrylate* monomers, which this component may be more or less to the methacrylate functional group in the experimental oligomer-based sealers. However, cell cytotoxicity of RealSeal SE was considerably higher than those of the oligomer-based sealers. This may be explained by the difference characteristic between monomer and oligomer structures. Due to the larger molecular structure of the oligomer, its release into the culture media may be less than that of the monomer, which might cause lower cytotoxicity to the cultured L929 cells.

Basically, a freshly-mixed sealer has higher cytotoxicity to cultured cells, which might be due to initial release of toxic components at early stage[18]. Laboratory studies reported that resin-based sealer becomes less toxic or even non-toxic over time period after mixing[18-21]. Using the Millipore filter assay, cell cytotoxicity of freshly-mixed Resilon sealer is moderate, but lower than that of epoxy resin-based sealer (*AH Plus, Dentsply, USA*)[18]. However, both sealers eventually become non-toxic to the cultured cells at 24 h after setting. From a laboratory study using a root model, diffusion of the methacrylate-based root canal sealer (*Epiphany, Pentron, USA*) has considerably high cytotoxicity during 1-2 days after setting[18, 21]. From the same study, direct contact of the diluted Epiphany sealer caused higher cell cytotoxicity than those of the root model, which cell cytotoxicity is still detected in a low level at 7 days after setting. In our study, the sealers were tested at 7 days after setting since the specimens of oligomer-based sealers were prepared and sent by the manufacturer. In this study, elution of either experimental or RealSeal SE sealer was highly toxic to L929 cells unless it was diluted. In contrast, the other studies reported that the methacrylate root canal sealer (*Epiphany*) was not toxic or just minimally toxic at 7 days after setting[18, 21]. The different result may be explained by the self-curing setting under partial aerobic condition of sealer specimens in this study. In the previously mentioned studies, the specimens were set by light curing under anaerobic condition. In comparison to the light-curing mode, self-cured sealer might leave higher amount of unset monomers/oligomers that are possible to release into the culture media and cause cell cytotoxicity. Nevertheless, the sealer specimens in our study were designed to set in a self-curing mode only in order to simulate the clinical condition that root canal sealer at apical area is set without light-curing.

Resilon sealer is completely set much slower in aerobic condition, which the setting period could be extended from 30 min to one week[22]. In our study, sealer specimens were set in a plastic tube, covered both ends with glass slides and kept in a dark plastic box. Thus, the amount of oxygen that contacts with the sealer specimens would be limited. Incompletely cured, oxygen-inhibited layer on the surface of sealer rods would be minimally present. This should be comparable to a clinical situation when sealer is set within root canal at apical area.

Biocompatibility of a new root canal sealer should be evaluated at either freshly mixed or after setting period. In this preliminary study, the experimental sealers were only tested at 7 days after setting. Extruded unset sealer tends to immediately irritate periapical area due to its released toxic components. Experimental oligomer-based root canal sealers will be further tested at different setting periods to confirm the biocompatibility.

In conclusion, the experimental oligomer-based F68 root canal sealer had the lowest cytotoxicity to L929 fibroblast cells, which the cell viability was significantly higher than that of RealSeal SE methacrylate-based sealer. F68

sealer will be further tested and developed to obtain a final product with other desirable properties.

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