International Journal Dental and Medical Sciences Research
Volume 4, Issue 2, Mar-Apr 2022 pp 566-572 www.ijdmsrjournal.com ISSN: 2582-6018

Evaluation of the Cytotoxicity of Four Different Root Canal Sealers on Human Periodontal Ligament Fibroblast Cells

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Submitted: 15-04-2022 Accepted: 28-04-2022

ABSTRACT
Aims of study: This study aims to evaluate and compare sealer´s cytotoxicity of four different root canal sealers (BioRoot, GuttaFlow Bioseal, EasySeal and Endofill) by MTT assay, also to evaluate and compare the effect of time on the cytotoxicity of these sealers.
Materials and method: Samples of BioRoot™ RCS, GuttaFlow Bioseal, EasySeal and Endofill were fabricated in rubber molds of 5 mm diameter and 2 mm thickness. Human periodontal ligament fibroblasts cells (hPDLFCs) were exposed to the extracts of these materials in afreshly mix and set condition after (1 day, 3 days and 7 days) at 37C° with 5% CO_2. Cell viability was evaluated by the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay.The obtained datawere analyzed by One-Way ANOVA and post hoc Duncan's tests at ($p \leq 0.001$).
Results: Based on the results ofOne-way analysis of variance (ANOVA) and Post hoc Duncan's multiple range tests there was a significant difference in the sealers cytotoxicityat ($p \leq 0.001$) between the four tested sealers, the BioRoot™ RCS showing the highest mean value for the cell viability followed by GuttaFlow Bioseal, EasySeal and Endofill respectively. Also showed that the cell viability increased over the time.
Conclusion: : According todifferent types of the sealers used in this study for assessing and comparing the sealer cytotoxicity at different incubation periods. BioRoot™ RCS had highest cell viability while Endofill had the lowest cell viability. Cell viability increased with the time.
Keywords: Root canal sealer, Biocompatibility,Cell viability, Cytotoxicity,Human Periodontal Ligament Fibroblast Cells.

I. INTRODUCTION

Endodontic therapy refers to any treatment that aims to keep the pulp healthy in its entirety or in part. The goal of treatment when the pulp is diseased or injured is to keep the periradicular tissues healthy or restore them. Treatment aimed at restoring periradicular tissues to normal when pulpal disease has spread to them. Root canal therapy or endodontic surgery are frequently used to accomplish this(Gulabivala and Ng, 2014).

Obturation is one of the important steps of root canal therapy, the goals of obturation are to fill root canal system with an impervious biocompatible and dimensionally stable seal (Orstavic. 2005). A root canal sealer is an important component in root canal obturation, they are applied to fill the space between the core material (i.e., gutta-percha) and the root canal inner wall during the canal filling process to seal the root canal system, trap the remaining microbes, and pack irregularities in the root canal (Jung et al.,2019).

Sealers also may pass into periapical tissues due to different reasons such as anatomy of the canal or tooth, over filling during condensation of filling material together with root canal sealers might lead to treatment failure after a long time of exposure to endodontic sealers in periapical areas (Małgorzata et al.,2015).

The interaction of eluents from the sealer with the periradicular tissue through apical foramen, dentinal tubules, accessory foramina and lateral canals has concentration and time-dependent effects on cell metabolism and regeneration (Costa et al.,2016). As a result, the root canal sealant must be biocompatible. Biocompatibility is defined as the ability of a material to achieve a proper and advantageous host response in specific applications . In other words, a material is said to be biocompatible when the material coming into contact with the tissue fails to trigger an adverse reaction, such as toxicity, irritation, inflammation, allergy, or carcinogenicity.

Several methods have been described to evaluate the biological effects of the endodontic sealers in vitro (Jung et al., 2019). The cytotoxicity is performed commonly using Methyl Thiazol Tetrazolium (MTT) Assaywhich is a colorimetric assay. It is a sensitive indicator of the cellular metabolic activity and due to its simplicity, reliability, accuracy and time-saving attributes, it is preferred over other methods (Rodriguez et al., 2017). The MTT assay was introduced by Mosmann in 1980 (Bajrami et al., 2014).It is based on the ability of a mitochondrial dehydrogenase enzyme from viable

International Journal Dental and Medical Sciences Research
Volume 4, Issue 2, Mar-Apr 2022 pp 566-572 www.ijdmsrjournal.com ISSN: 2582-6018

cells to cleave the tetrazolium rings of the pale-yellow MTT solution and form dark-blue formazan crystals that are generally impermeable to cell membranes, thus appear in crystal accumulation in healthy cells (Asadet al., 2017).

Most studies that assessed the cytotoxicity of sealers used mouse and human fibroblast cells or human periodontal ligament cells (PDLCs) (Taraslia et al.,2018; Jung et al.,2019). Clinically, sealers are inserted into root canals before setting; thus, it is possible that toxic components are released into the tissue . Leachable toxic substances could also be released after setting. For this reason, the cytotoxicity of sealers needs to be evaluated both before and after setting (Jung et al.,2019).

So the aims of the current study are to evaluate and compare sealer´s cytotoxicity of the four different root canal sealers (BioRoot, GuttaFlow Bioseal, EasySeal and Endofill) by MTT assay, also to evaluate and compare the effect of time on the cytotoxicity of these sealers.

II. MATERIALS AND METHODS

Four different root canal sealers (Bioroot™ RCS, GuttaFlow Bioseal, EasySeal and Endofill)were used in compliance with the manufacturer's instructions.

Table (1): Details of the root canal sealers used in the study

Endodontic sealer	Manufacturer	Composition	Setting time
BioRoot™ RCS	Septodont, Saint-Maur-des Fosses, France	Powder: tricalcium silicate, zirconium oxide, povidone Liquid: aqueous solution of calcium chloride and polycarboxylate	4 Hours
Endofill	Dentsply, Petrópolis Ind. e Com. Ltda, Riode Janeiro, Brazil	Zinc oxide, hydrogenated resin, bismuth subcarbonate, barium sulfate, sodium borate. Eugenol and oil of sweet almonds	2 Hours
EasySeal	Komet Dental -Gebr. Brasseler, Lemgo, Germany	Paste 1: 4-[-2-(4-hydroxyphenyl) propan-2-yl] phenol epichlorohydrine resin, alkylglycidyl ether, barium sulfate, tricalcium phosphate, diphenylolpropane-diglycidyl ether; Paste2: Polyalkoxyalkylamine copolymer,5-amino-1,3,3-trimethylcyclohexanmethylamine, aqua, barium sulfate, tricalcium phosphate, nanodispers silicone dioxide, polyhexamethylene biguanides-hydrochloride	15 Minutes
GuttaFlow Bioseal	Coltene/Whaledent Inc. Switzerland	Gutta-percha powder particles, polydimethylsiloxane, platinum catalyst,zirconium dioxide, calcium salicylate, Nano-silver particles, paraffin, coloring, bioactive glass ceramic	12-16 Minutes

2.1 Sample and Extract Preparation

The four sealers were prepared according to the manufacturers' instructions and placed in rubber molds (5 mm in diameter, 2 mm in height) under aseptic conditions.Excess material was scraped away with a sterile scalpel, and the sealers were gently removed from the molds after one hr. One group of samples was tested immediately after mixing (fresh specimens) by prepearing it´s extract. Another group of the samples was placed in a humidified 5% CO_2 (CO_2 is needed as part of the media buffering system to regulate the pH), 95% air atmosphere for 24 hrs. at 37C° (set specimens) (Grzegorz et al., 2018).

Extracts of the materials were prepared in 24-well dishes by immersing them in Dulbecco's Modified Eagle's Medium (DMEM) cell culture

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media [supplemented with 10% fetal bovine serum (FBS)(which has a high content of embryonic growth promoting factors like hormones, carrier proteins, and macromolecular proteins. It also has low levels of antibodies), 100 µg/mL penicillin, and 100 µg/mL streptomycin (antibiotics are often used to control the growth of bacterial and fungal contaminants)] using the surface area-to-volume ratio of approximately 150mm^2 /ml between the surface of the samples and the volume of medium (Elgendy and Hassan. 2021) and incubated in the dark at 37C ° for 1day, 3 days and 7 days.

Pure DMEM medium was used as negative control, whereas the positive control was cells without extracts. The sealer discs were removed from the medium using a long precision tweezer at the end of each immersion time period, leaving the culture media containing the sealers extracts (Hohenbildet al., 2020). Undiluted extracts were used in this study.

2.2 Cell Culture Procedure

Cytotoxicity of the sealers was evaluated on cultured human periodontal ligament fibroblast cell line (hPLFCs). These cells were obtained from the Centre for Natural Products Research and Drug Discovery, University of Malaya, Malaysia.

The hPDLFCs were cultured in (DMEM) with 10% (FBS) and penicillin/streptomycin. The culture was incubated at 37°C in a humidified atmosphere, 95% air, and 5% CO_2. Every other day, the medium was changed. When the cells achieved maturity, they were detached using a 0.2% (w/v) Trypsin-EDTA solution and moved to new culture flasks (Vajrabhaya and Korsuwannawong, 2018).

After sufficient growth for experimentation, the cells were trypsinized (Trypsin enzyme is used to detach the adherent cultured cells from the plate) and plated in 96-cluster well culture plates at a concentration of 1×10^4 cells/well. Each well contained 100 µl of cell suspension. After 24hrs. of incubation at 37°C under 5% CO_2, the cells established a confluent monolayer on the base plate of the culture well.A phase-contrast microscope was used to examine the adhesion of the cells.The research only included wells with a cell layer that was uniformly distributed over the bottom of the well (Vajrabhaya and Korsuwannawong, 2018).

After overnight attachment, cells were exposed to the extracts of the different tested sealers (200µL) into each well (6 well for each group of each sealer). Cytotoxicity testing was done immediately after mixing (for extract of fresh mix), and for extract of 1 day, 3 days, 7 days according to immersion time to study the cytotoxicity of the sealers. The cells were exposed to sealer extracts at 37 °C, 5% CO_2 and 95% humidity for 24 hrs.

2.3 The MTT Assay Procedure

Cell survival was determined using the 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

The used MTT assay kit as represented in(Figure 1)is composed of the following :

1. MTT solution 3-(4,5-dimethylthiazol-2yl) 2,5diphenyl tetrazolium bromide (Mw=414) 1mL x 10vials.
2. Solubilization solution (dimethyl sulfoxide) 50 mL x 2 bottles.

Figure (1): MTT assay kit: a- MTT solution b- Solubilization solution

After the 24hrs, the extract containing materials were removed, and 1 mL of MTT solution (yellowish) at a concentration of 0.5 mg/ mL of medium was added to the plates, which were then incubated for 4 hrs in the darkat 37 C°, 5% CO_2 and 95% humidity. The fluid was then aspirated from the culture. After carefully rinsing the residue with 1 mL of Phosphate Buffered Saline (PBS), 200 µL of dimethyl sulfoxide was poured into each well to lyse the cells and elute their intracellular formazan salt.Then the plates were shaken by the plateshaker at room temperature for 10 min. to dissolve the crystals. Finally, the absorbance (i.e., optical density) of the purple formazan-stained dimethyl sulfoxide was measured using (ELISA) reader (spectrophotometer) at a wavelength of 595 nm (Grzegorz et al., 2018).

The formazan content of each well (six replicate readings) computed as a percent of the control group (untreated cells). The amount of formazan is directly proportional to the number of viable cells in the cultureas represented in (Figure 2).For reduced cell survival, little enzymatic activity is detected, resulting in a small amount of purple formazanand lower absorbance values (Camps et al., 2015; Rodriguez-Lozanoet al., 2017)

Cell viability was calculated using the following formula (Kamiloglu et al., 2020):

(Test sample absorbance / Control sample absorbance) × 100%

The viability of hPDLFCs was used to assess the cytotoxicity of root canal sealers. Cytotoxicity responses were rated as severe (≤30%), moderate (30-60%), mild (60-90%) or non-cytotoxic (≥90%) (Catunda et al., 2017).

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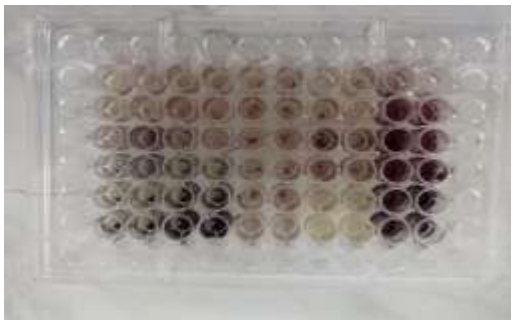

Figure (3.13): 96-wellcell culture plates after production of formazan.

Statistical Analysis

The data were analyzed using the Statistical Package for the Social Sciences (IBM.SPSS) software, version 25. The level of significance was chosen at p≤ 0.001.Following normality testing, the following tests have been carried out:

1. One-way analysis of variance (ANOVA) test was used to find if there is a significant difference in the cell viability between the different tested root canal sealers at (p≤0.001).
2. The means were compared using Post hoc Duncan's multiple range test to determine which groups gave the highest cell viability.

III. RESULTS

The cytotoxicity of root canal sealers was obtained from the assessment of the viability of human periodontal ligament fibroblast cells (hPDLFCs).

One Way Analysis of Variance and Duncan's Multiple Range Tests "$P \leq 0.001$" utilized for determination of the variations in cell viabilityof the tested root canal sealers.

The results of analysis of variance (One-Way ANOVA) for the cell viability of different tested sealers showed that there is a significant difference in cell viability of the sealers at different times intervals,also showed a significant difference in the cell viability of the tested sealers within same time.

Post hoc Duncan's multiple range test for cell viability demonstrated that significantly the highest cell viability was for BioRoot followed by GuttaFlow Bioseal, EasySeal and Endofill respectively at all tested times (vertical analysis which referred in the table with small letters).

Also demonstrated that at fresh mixed state, only BioRoot was non-cytotoxic while GuttaFlow Bioseal showed mild cytotoxicity and moderate cytotoxicity for Endofill followed by EasySeal.

The cytotoxicity of all tested sealers tends to decrease over time, to be at 7 days, more non-cytotoxic for BioRoot followed by GuttaFlow Bioseal and mild cytotoxicity for Endofill followed by EasySeal (horizontal analysis which referred in table with capital letters) (Table 1, Figure 3).

Table (1): Duncan's Multiple Range Test of the cell viability of different types of the tested sealers at different time interval.

Material	Metric	0 fresh			1 day			3 days			7 days		
BioRoot	Mean (%)	91.931	*D	**a	94.158	C	a	96.451	B	a	97.878	A	a
	***N	6			6			6			6		
	Std. Deviation	.80928			.85896			.61730			.75470		
GuttaFlow Bioseal	Mean	87.836	D	b	93.623	C	a	94.850	B	b	96.340	A	b
	N	6			6			6			6		
	Std. Deviation	.51239			.66102			.50963			.45303		
EasySeal	Mean	41.073	D	c	44.333	C	b	64.353	B	c	78.545	A	c
	N	6			6			6			6		
	Std. Deviation	.81148			.69945			.56156			.37835		
Endofill	Mean	37.660	D	d	39.675	C	c	61.325	B	d	73.768	A	d
	N	6			6			6			6		

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Volume 4, Issue 2, Mar-Apr 2022 pp 566-572 www.ijdmsrjournal.com ISSN: 2582-6018

	Std. Deviation	.75470	.45303	.83849	.41335

*Capital letters (horizontal analysis) indicate cell viability of the tested endodontic sealer at different time intervals.
**Small letters (vertical analysis) indicate cell viability of different tested endodontic sealers within same time.different letters mean there is a significant difference.
***N represents the number of samples.

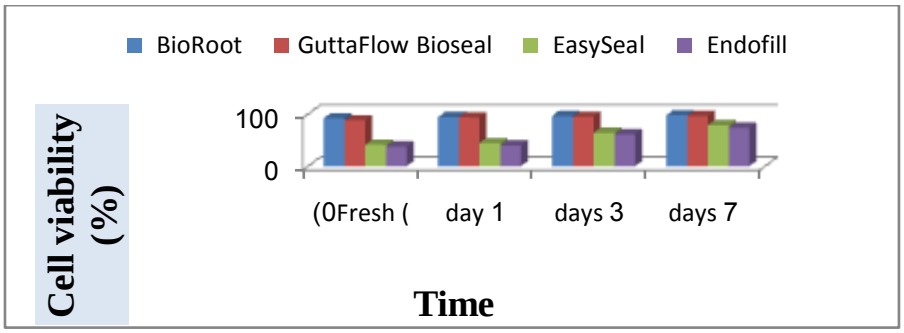

Figure (4): Histogram for the cell viability of tested root canal sealers.

IV. DISCUSSION

Root canal sealers may come into contact with the periapical tissues for an extended period of time, so they must be biocompatible. Direct contact with sealers, as well as their breakdown over time, may cause cytotoxic damages to cells and tissues, compromising the root canal treatment's result (López-Lópezet al., 2012).

One of the main prerequisites for a successful endodontic treatment and periodontium healing is the biocompatibility of an endodontic sealer. Physical characteristics and biocompatibility are therefore important factors to consider when selecting an appropriate root canal sealer, but, despite the fact that endodontic sealers have the possibilities to irritate periapical tissues, endodontists should evaluate the benefits and drawbacks of sealer extrusion because the unsealed remaining areas in the apical region could serve as microorganism niches, causing or prolonging endodontic failure (Silva et al., 2013).

Part of this study wasdesigned to determine the cytotoxicity behavior of fourdifferent bases endodontic sealers on hPDLFCs. Although cytotoxicity testing of freshly mixed sealers is important because they are placed in the root canal system in a freshly mixed and incompletely polymerized state, it is also important to evaluate sealers over extended periods after setting because it is likely that changes in cytotoxicity levels will be observed after diffusion of toxic components from the materials into the surrounding environment during some time after clinical application (Camargo et al., 2014), therefor in this study the cytotoxicity was evaluated at different times intervals.

Most studies assessing the cytotoxicity of sealers used mouse and human fibroblast cells or hPDLFCs (Eldenizet al., 2016; Tarasliaet al.,2018). Furthermore, fibroblasts are the most prominent constituents of the connective tissue, the predominant cell type of the periodontal ligament, and are the most important collagen producers in this tissue (Smith et al., 2019). For these reasons, hPDLFCs were used in the present study.

Regarding the results of cytotoxicity of the tested sealers, BioRoot™ RCS was the least cytotoxic sealer and Endofill had the highest value of cytotoxicity, while GuttaFlow Bioseal and EasySeal ranging between them with the higher value for EasySeal.

BioRoot™ RCSwas the least cytotoxic sealer tested in the present study in both fresh and set conditions. In the presence of set BioRoot™ RCS, hPDLFCs showed a high degree of proliferation. This is due to the mechanism of action ofBioRoot™ RCS, which is very similar to that of original calcium silicate cements: an aqueous matrix that promotes the $Ca(OH)_2$production and Ca^{2+}leaching during the hydration process (Camilleri,2015). Thus, the ability to release ions and the formation of (CaPs) layer might potentially explain the high in vitro biocompatibility ofBioRoot™ RCS(Dimitrova-Nakov et al.,2015).

Because of BioRoot RCS's solubility, it's possible that it's not only non-cytotoxic (biocompatible), but it also releases certain components into the surrounding tissue that might promote tissue repair (bioactivity), this could explain the increase of cell viability for an extended period of time (Junget al., 2018).

The results of this study are in a line with findings of Collado-Gonza´lez et al.,(2017) and Gaudin et al., (2020), which shown that BioRoot™ RCS was non-cytotoxic in both fresh and set conditions, with no effect on cell viability or morphology.

While the finding of Poggio et al., (2017), showed decreased cell viability of BioRoot™ RCS over 48, 72 hrs. To be mild cytotoxic,this disagrees with the findings of the present study. The type of cell line used to assess biocompatibility had a significant impact on the results, which led to this disagreement. Also, the difference might possibly be due to changes in experimental conditions.

According to the results of the present study, GuttaFlow Bioseal was regarded as a cytocompatible material in a set condition. Despite its low calcium release values, GuttaFlow Bioseal has a high pH, which might be related to its ability to form hydroxyapatites over time due to the presence of bioactive ceramic glass. As a result, the decrease in cytotoxicity might be attributed to the production of hydroxyapatite. The alkalinity found in this material may contribute to its osteogenic potential, biocompatibility, and antibacterial properties (Gandolfi et al., 2016).

The findings of the studies of Gandolfi et al., (2016)and Collado-Gonza´lezet al., (2017),which showed that GuttaFlow Bioseal in both fresh and set conditions was more cytocompatible than the epoxy resin and ZOE based endodontic sealers when tested on hPDLFCs, seem to agree with our results.

Concerning the cytotoxicity of EasySeal, fresh sealer was cytotoxic due to therelease of cytotoxic substances (4-[-2-(4-hydroxyphenyl) propan-2-yl] phenol epichlorohydrine resin).The cytotoxicity decreased after setting, which might be attributed to the diminished release of toxic compounds from the set sealer (Zhou et al., 2015; Silva et al.,2016).

Instead, the biocompatible and bioactive fillers added to resin sealers (such as tricalcium phosphate) may be enclosed in the resin matrix, which acts as a physical barrier and inhibits water diffusion. So due to lack of a hydration phase, the fillers stay relatively inert (Viapiana et al., 2014;Xuereb et al., 2015). The finding of the study ofPoggio et al., (2017) was in contrast with the results of the present study.

Concerning the biocompatibility of ZOE based (Endofill) root canal sealer, it had the highest cytotoxicity compared to other tested sealers. This type of sealer caused a significantly decrease of hPDLFCs proliferation.

The results ofSzczurkoet al., (2018) revealed that the ZOE containing sealer was significantly less toxic in the set form than immediately after mixing, Camps et al., (2015); Collado-Gonza´lezet al., (2017) are in accordance with the present results. ZOE sealers are irritating mainly because of the eugenol (Ahmed. 2018). Cytotoxicity and inflammation may have been exacerbated by the release of unreacted components such as zinc ions, benzyl alcohol, methyl salicylic acid, and rosin. Furthermore, free eugenol released from the freshly mixed paste may have interfered with the cytoplasmic membrane, impeding cell respiration and leading to cytotoxicity (Sharma et al., 2022).

The cytotoxicity of the tested sealer reduced in the older specimens, most likely due to reduced leaching of these cytotoxic components. This could be proved because all of the sealers tested in this study demonstrated varying degrees of cytotoxicity reduction following repeated testing over extended periods of time.

V. CONCLUSION

According to the results of this study with its limitations, BioRoot™ RCS had the highest cell viability rate and Endofill had the least solubility rate while GuttaFlow Bioseal and EasySeal ranged between them with the higher rate for GuttaFlow Bioseal. Also, there was an increase in cell viability of all tested sealers over the time.

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