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ORIGINAL RESEARCH

Comparative Evaluation of Cytotoxicity of Root Canal Sealers on Cultured Human Periodontal Fibroblasts: *In vitro* Study

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ABSTRACT

Aim: To evaluate and compare the cytotoxic effects of different types of root canal.

Materials and methods: The sealers were eluted with culture medium for 1 hour, 7 days, and 14 days. Cell viability was estimated by 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay and trypan blue exclusion method on human periodontal ligament (PDL) fibroblast cells. Sealers used are mineral trioxide aggregate (MTA)-based sealer (MTA Fillapex, Angelus), calcium hydroxide-based sealer (Apexit Plus, Ivoclar Vivadent), resin-based sealer (AH Plus, Dentsply), and zinc oxide eugenol-based sealer (Tubli Seal, SybronEndo).

Results: The order of cytotoxicity through MTT assay, at the end of the second week, was observed as MTA Fillapex> Tubli Seal> Apexit Plus > AH Plus. The percentage cell viability obtained after trypan blue exclusion method decreased in the order of Apexit Plus> Tubli Seal> AH Plus> MTA Fillapex, which was similar to the reported cytotoxicity from the MTT assay after 1 hour.

Conclusion: Each type of sealer showed moderate-to-severe cytotoxic response when compared with the control. The MTA Fillapex was found to be the most cytotoxic sealer. Use of resin-based material as a root canal sealer may result in a more favorable response to PDL fibroblasts.

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Clinical significance: Having knowledge of the cytotoxicity of various sealers will help in increasing patient's comfort.

Keywords: 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide assay, Cytotoxic, Periodontal ligament fibroblasts, Root canal sealer.

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INTRODUCTION

Endodontic therapy involves cleaning and shaping of the root canal system and filling with an inert, biocompatible, and dimensionally stable material.[1] Sealer cements are the substances used for root canal sealing along with endodontic treatment procedures. During the last two centuries, substances used for root canal sealing have improved considerably.[2]

An ideal root canal sealer must have the properties of promoting healing and achieving an effective seal.[3] Root canal sealers come into direct contact with hard and soft tissues of the periapical area; therefore, they should have the property of biocompatibility and should not potentially damage periapical tissue. Thus, it becomes necessary to test the biocompatibility of the sealers on the tissue concerned. Biocompatibility of the root canal sealers can be evaluated using an *in vitro* model for cellular response.[4] Established cell lines including human PDL fibroblasts, gingival fibroblasts, murine granulocyte–macrophage progenitor cells, primary human osteoblast, etc, are currently preferred for the evaluation of the cytotoxicity of sealers.[5-7]

A large variety of root canal filling materials with different formulations, such as epoxy resin, calcium hydroxide, zinc oxide–eugenol, and methacrylate are used in current practice. These sealers, besides being studied for the required sealing ability, have also been reported for their cytotoxicities in various studies. Zinc oxide–eugenol-based sealers, despite *in vitro* cytotoxicity, have been widely used for many decades.[8] Epoxy resin-based sealers also have been considered as cytotoxic due to their constituent bisphenol (diglycidyl ether), which has been known for its mutagenic nature.[9] Mineral trioxide aggregate-based sealers, assessed for *in vitro* toxicity on cultured human PDL fibroblasts, also displayed cytotoxicity in a study.[10] A study assessing the calcium hydroxide-based sealer showed slight toxicity up to almost the 7th day, after which the toxicity reduced drastically by the 14th day.[11] Epiphany and metaseal, which are types of methacrylate-based sealers, have shown high cytotoxic on Balb C 3T3 fibroblastcells.[12] The purpose of this present *in vitro* study was to evaluate and compare the cytotoxic effects of different types of root canal sealers like MTA-based sealer (MTA Fillapex, Angelus), calcium hydroxide-based sealer (Apexit Plus, Ivoclar Vivadent), resin-based sealer (AH Plus, Dentsply), and zinc oxide–eugenol-based sealer (Tubli Seal, SybronEndo).

MATERIALS AND METHODS

Four different types of sealers were used for the study. The details of various materials along with sealers used in the study are detailed in Table 1. The cell culture of human PDL fibroblasts and informed consent were obtained from patients prior to the procedure. Periodontally healthy premolar indicated for extraction for orthodontic treatment was extracted and stored in a high media-storage container containing 30 mL of Dulbecco's Modified Eagle's medium. Fibroblast colonies were observed under a phase contrast microscope after 7 to 8 days. Cultured medium was removed; confluent cells were detached with 0.25% trypsin and 0.05% ethylene diaminetetraacetic acid , and incubated for 5 minutes.

Cultured medium was added to the flask, and aliquots of separated cells were subcultured in 25 Petri dishes. The medium was changed every other day until the cells were confluent. Five Petri dishes were randomly selected for each group.

Test Sealer Sample Fabrication and Elution

Five separate disks of each of the sealers mixed according to manufacturer's instructions were made under sterile, aseptic conditions. Cylindrical Teflon blocks of 3 mm diameter and 2 mm height were used for this purpose. Excess material, if any, was reduced using sterile scalpels. After 1 hour, the sealers were carefully removed from the Teflon blocks. Each specimen went through a specified process in which after initial setting, specimens were placed in 10 mL of fresh culture medium and subsequently transferred into fresh media at 1-hour, 7-day, and 14-day intervals. Cytotoxicity was determined after each elution period and the elutes were incubated for 24 hours.

Control samples containing only culture medium were treated similarly.

Table 1: Composition and manufacturer of the tested sealers and chemicals used in the study

Material	*Composition*	*Manufacturer*
Root canal sealer		
1. MTA Fillapex	Salicylate Resin, Diluting Resin, Natural Resin, Bismuth Trioxide, Nanoparticulated Silica, MTA, Pigments	Angelus
2. Apexit plus	Base: Calcium hydroxide Hydrated Collophonium, Fillers and others auxiliary materials (Highly Dispersed Silicon Dioxide, Phosphoric Acid Alkyl Ester) Accelerator: Disalicylate, Bismuth Hydroxide, Fillers and others auxiliary materials (Highly Dispersed Silicon Dioxide, Phosphoric Acid Alkyl Ester)	Ivoclar Vivadent
3. AH plus	Paste A: Epoxy Resins, Calcium Tungstate, Zirconium Oxide, Silica, Iron Oxide Pigments, Aerosil Paste B: Adamantane amine, N,N-Dibenzyl-5-oxanonane, TCD-Diamine, Calcium Tungstate, Zirconium Oxide, Aerosil	Dentsply
4. Tubli Seal	Base: Zinc oxide, Oleoresins Bismuth trioxide, Thymol iodide, Oils and waxes Accelerator: Barium sulfate, Eugenol, Polymerized resin, Annidolin	SybronEndo
Others		
1. Dulbecco's modified Eagle Medium (DMEM)	10% foetal bovine serum,100 µg/ml streptomycin, 100 units/ml penicillin, 250 µg/ml gentamycin sulphate, 5 µg/ml Amphotericine B, 100 µg/ml streptomycin, 1.16 g/L glutamine	
2. MTT Formazan	1-(4,5-Dimethylthiazol-2-yl)-3,5-diphenylformazan, Thiazolyl blue formazan	Sigma-Aldrich
3. Trypan blue dye	Dye content, ~40%	Sigma-Aldrich

Cytotoxic Assay

The viability of the cells was measured after exposure of the PDL fibroblast cell lines to the sealer extract using MTT assay (Mosmann[13]) and trypan blue dye exclusion method (Correa et al[14]). All the tests were performed in triplicate, and cytotoxicity was evaluated for each sample at 1 hour, 7 days, and 14 days.

MTT Assay: The cells were gently washed with 1.0 mL of phosphate buffered saline after the culture medium was removed from each well. The wash was replaced with MTT–succinate solution for 4 hours. Cell monolayers were washed with distilled water after the aspiration of solution. Formazan crystals produced within the cells by succinate dehydrogenase reduction of MTT were dissolved using destaining solution (isopropanol—10% NP40–0.4 N HCl). Aliquots (20 µL) of the solution were then transferred to each well to a 96-well plate, and the absorbance was measured at 490 nm using a microplate reader.

Trypan blue dye exclusion test: One sample from each sealer group was placed into one cultured plate from each group.

The tested material and medium were removed after 1 hour, and 1 mL of trypsin was added to remove the cells from the bottom of the wells.

Then, 15 mL of the cell suspension was transferred to the test tube. Two droplets (10 µL) of trypan blue were placed on parafilm. Dilution of 4× was prepared by mixing 10 µL of cell with 10 µL of trypan blue. About 10 µL of trypan blue–cell suspension was transfer to a hemocytometer, and viable and non viable cells were counted.

The total number of viable cells per mL of aliquot was obtained by multiplying the total number of viable cells by 4 (the dilution factor for trypan blue).The total number of cells per mL of aliquot was obtained by adding up the total number of viable and nonviable cells and multiply by 4. The percentage of viable cells was obtained by the following formula:

$$\text{Viable cell} = \frac{\text{Total number of viable cells per mL of aliquot}}{\text{Total number of cells per mL of aliquot}} \times 100$$

Data Analysis

The statistical analysis was done using GraphPad prism software version 5.01 from GraphPad Software, Inc. The difference between the control and experimental groups was analyzed statistically by one-way analysis of variance (ANOVA) combined with the Bonferroni test. All values were expressed as mean ± standard deviation, and differences were considered significant at $p < 0.05$.

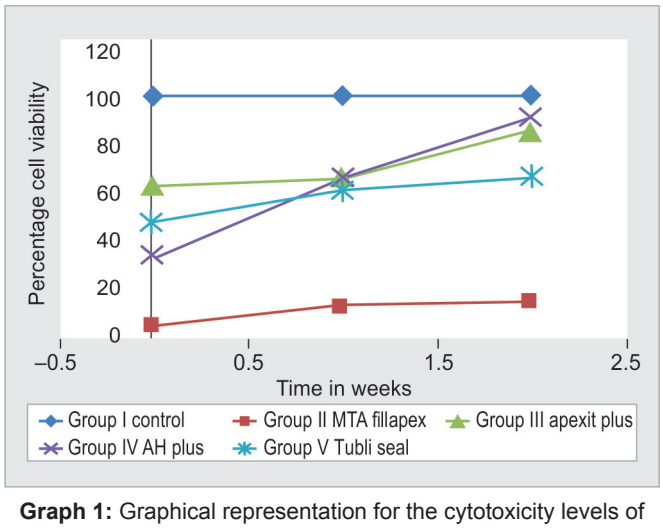
RESULTS

MTT Assay: At 1 hour of incubation, a statistically significant viability reduction in comparison with control was observed for all the four sealer extracts ($p < 0.001$). Cytotoxicity was induced by all the groups of tested sealers (Graph 1). No statistical significant difference was observed when test groups were compared with each other. At week 1, all groups showed statistically significant differences (<0.001) when compared with the untreated control group. Comparison of each test group among each other also showed statistically significant difference, except for the Apexit Plus *vs* AH Plus comparison, where the difference observed was nonsignificant. At the end of week 2, all the four groups still showed statistically significant difference (<0.001) when compared with the untreated control group. However, when compared among the sealers, no statistically significant difference was observed. The overall mean percentage viability values from the MTT assay, regardless of the time of analysis, was observed in the order of MTA Fillapex> Tubli Seal> Apexit Plus >AH Plus.

The results were obtained by one-way ANOVA followed by Bonferroni correction as *post hoc* test are shown in Table 2.

Trypan Blue Exclusion Assay Result

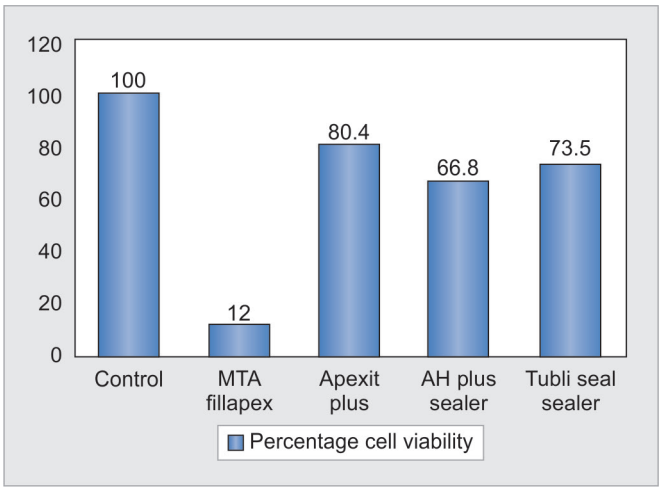
The effect of trypan blue on the viability of human periodontal fibroblast cells is shown in Graph 2. The percentage of viable cell was reduced significantly by all four test sealers when compared with the control group. In this study, Apexit Plus sealer was seen to have minimum cytotoxicity showing about 80.4% cell viability. Extracts of Tubli Seal sealer showed 73.5% of cell viability and extracts of AH Plus sealer showed 66.8% cell viability. The maximum cytotoxicity was seen with MTA Fillapex sealer, which showed 12% cell viability.



Graph 1: Graphical representation for the cytotoxicity levels of various sealers during the complete study period

Table 2: The results were obtained by one-way ANOVA followed by Bonferroni correction as *post hoc* test

Time	*Groups*	*Mean*	*SD (±)*
Cell viability at 1 hours	Control	99.932***	0.068
	MTA Fillapex	5.456***	0.26
	Apexit plus	63.241***	0.75
	AH plus	33.793***	0.21
	Tubli Seal	48.165***	0.40
Cell viability at 1 week	Control	99.95***	0.057
	MTA Fillapex	13.815***	0.47
	Apexit plus	66.333***	0.96
	AH plus	66.430***	1.1
	Tubli Seal	60.936***	1.3
Cell viability at 2 week	Control	99.973***	0.052
	MTA Fillapex	15.491***	0.25
	Apexit plus	86.014***	0.80
	AH plus	91.184***	0.38
	Tubli Seal	66.430***	1.1



Graph 2: Effect of the test sealers on human periodontal fibroblast cell by trypan blue dye exclusion assay expressed as percentage of viable cells in control and test groups

DISCUSSION

In vitro tests are considered as screening tests to assess any biological and cytotoxic risks due to any medical material like root canal sealants.[15] The main advantages of *in vitro* tests include faster assessment, cost-effectiveness, and long-term predictivity. However, the sole major disadvantage of *in vitro* studies is that they do not simulate *in vivo* conditions and results may not be exactly relevant to clinical settings.[16]

Human PDL fibroblast cells represent an appropriate model for testing cytotoxicity of endodontic filling materials. Moreover, PDL fibroblasts cells *in vitro* could simulate responses like human PDL *in vivo*, owing to which PDL fibroblast cells were used in this study instead of other established cell lines.[17]

According to the present results, MTA Fillapex showed a severe cytotoxicity when cells were exposed to fresh elute of the sealer during the first hour. This toxicity did not decrease during the first and second weeks. The findings of the present study are in agreement with a previous study, which has shown strongly affected cell viability by MTA Fillapex, using several methodologies.[18] The same result in regard to MTA was observed using MTT assay for the evaluation of cytotoxicity in one of a recent study showing long-term cytotoxic effect of sealers.[19]

The results suggest the correlations between the components, such as salicylate resin, diluting resin, and silica with the cytotoxic effects. The higher cytotoxicity of MTA Fillapex is majorly attributed to high pH of 12.5.

Firstly, the high pH of MTA Fillapex may neutralize the acids secreted by osteoclasts and help prevent destruction of mineralized tissue.[20] Secondly, the high pH and initial cytotoxicity may prove to be advantageous as high pH usually has a destructive effect on bacterial cell membranes and protein structure, which seems interesting, especially, knowing that microorganisms can remain in the ramifications of the root canal system after chemo-mechanical preparation and intracanal dressing. Thus, the sealers with antimicrobial activity can reduce the microbial load and provide a better chance for a successful root canal treatment.[21]

Apexit Plus fresh was moderately cytotoxic and mildly cytotoxic after 1 week and became noncytotoxic after 2 weeks. In our study Apexit Plus sealer, a calcium hydroxide-based sealer, showed minimal cytotoxicity. The results suggesting minimal cytotoxicity were in conflict with studies showing highest cytotoxicity with freshly mixed calcium hydroxide-based root canal sealer,[18,22] while the results were in accordance with studies that claimed calcium hydroxide-based sealers to be mildly or moderately toxic[23] with some studies even claiming that these sealers exhibited good or excellent biocompatibility.[18,23] The initial high pH of calcium hydroxide-based sealers may be the reason for the above-mentioned discrepancy.[22]

The alkaline pH of calcium hydroxide-based sealers can be sufficiently irritating to cause severe inflammatory responses, thereby, proving to be toxic.[25] AH Plus also showed high cytotoxicity initially, but the cytotoxicity reduced at the end of first week and was further reduced at the end of the second week. It has been said that the root canal sealers of epoxy resin-based class, due to the formaldehyde released after the reactions, is responsible for cytotoxicity, and it has recently been reported that epoxy-*bis*-phenol resin content also contributes to the cytotoxicity.[26] AH Plus is a relatively new member in the epoxy resin-based sealer class, its manufacturer emphasizing on that that the cured

resin will not release formaldehyde and is thus, more biocompatible.

However, a previous laboratory study reported that this new formulation could also release a minimal amount of formaldehyde. In our experiment too, an initial cytotoxicity was seen. Hence, it can be said that AH Plus is not perfectly biocompatible. This result has also been found to be in agreement with the result stated by Huang et al. The AH Plus sealer releases numerous substances into the adjacent tissue in the oral cavity, which suggests that the nature of the AH Plus sealer depends on the chemical composition.

The Tubli Seal sealer showed consistently a moderate viability rate. The cytotoxicity of Tubli Seal increased moderately from the first hour to week 2, and was found to be most cytotoxic after MTA Fillapex. Tubli Seal sealer is a zinc oxide-based sealer.

The toxic effects of zinc oxide–eugenol-based sealers have been extensively studied. Most zinc oxide–eugenol-based sealers show high antibacterial activity because they contain formaldehyde, which is both cytotoxic and mutagenic.[4,27] Similarly, eugenol, which is known to be an antioxidant and anti-inflammatory agent, was shown to have high toxic potency.[28,29]

The order of the degree of cytotoxicity of different root canal sealers in the trypan blue assay was found to be similar to the order of degree of cytotoxicity seen with the MTT assay at hour 1 of the study. Various studies of the similar kind have been performed earlier, and different studies have led to conflicting results. The conflicting results regarding the biocompatibility may be due to usage of differing cell lines/types as well as due to the cell viability parameter assayed. Moreover, the evaluation of different cell viability parameters like mitochondrial dehydrogenase activity and membrane integrity may more accurately identify any possible cytotoxic effects of endodontic sealers, with primary human cells intimately related to the *in vivo* tissue response to endodontic sealers.

CONCLUSION

This study was concerned to show the extent of cytotoxicity possessed by commercially available root canal sealers: MTA Fillapex Apexit Plus, AH Plus, and Tubli Seal on human PDL fibroblast cells. Our result confirms that these sealers possess severe-to-moderate cytotoxicity upon the cells. The MTA Fillapex was found to be the most cytotoxic. The order of cytotoxicity at the end of second week was observed as MTA Fillapex> Tubli Seal> Apexit Plus > AH Plus. Still more detailed *in vivo* research and long-term clinical assessments are needed to be able to judge the biocompatibility of these root canal sealers.

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