Original Article

An *in vitro* assessment of cytotoxicity and genotoxicity of root repair materials

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Abstract

Background: The success of the root-end procedure depends on the regeneration of the functional periodontal attachment system, including the cementum on the resected root-end surface, periodontal ligament (PDL), and alveolar bone. As root end filling materials remain in close contact with live periapical tissues, they may influence the endodontic treatment outcome.

Aim: To assess and compare the cytotoxicity and genotoxicity of three root repair materials, mineral trioxide aggregate (MTA), endosequence, and geristore in human-cultured periodontal ligament fibroblasts.

Materials and Methods: Cultured human periodontal ligament fibroblasts of the third passage were used in the study. They were placed in contact with the root repair materials. The cytotoxic effect on PDL fibroblasts was determined by (3-(4,5-dimethylthiazol-2-yl)-2,5-tetrazolium bromide) assay after 24 hours and 48 hours intervals. Cell viability was determined using an inverted phase contrast microscope. The genotoxic effect on the periodontal fibroblast cells was determined by comet assay using imaging software.

Statistical Analysis Used: Data were analyzed using Tukey's multiple comparison test and Dunnett's multiple test.

Results: All the test materials showed higher cytotoxicity and genotoxicity at the 48th hour interval with a statistically significant difference from the control group ($P < 0.05$). MTA was shown to be least cytotoxic and genotoxic to PDL fibroblasts, followed by endosequence root repair material and geristore at 24 hour and 48 hour intervals.

Conclusion: The cytotoxicity and genotoxicity of MTA were the least compared to endosequence and geristore on human-cultured PDL fibroblasts.

Keywords: Cytotoxicity, endosequence, genotoxicity, geristore, mineral trioxide aggregate, root repair materials

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INTRODUCTION

An annual report of endodontic treatments reveals that about 5.5% of all surgical procedures performed include apical root-end surgery and perforation repair.[1] Periapical surgery is indicated either in case of persistent periradicular pathosis or when orthograde endodontic retreatment is

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contraindicated.[2] The success of the root-end procedure depends on the regeneration of the functional periodontal attachment system, including the cementum on the resected root-end surface, periodontal ligament (PDL), and alveolar bone. To achieve this goal, a root-end filling material should provide adequate seal against the ingress of microorganisms or their by-products and aid in development of a normal periodontium across its surface.[3] Additionally, it should be capable of adhering to radicular dentin, insoluble in tissue fluids, dimensionally stable, non-resorbable, radiopaque, easy to manipulate, and compatible with human tissues.[4,5]

Cytotoxicity of root filling materials can affect the pulp and periradicular region, leading to their lysis. Cytotoxicity of a material is determined using colorimetric assay based on MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-tetrazolium bromide). This assay measures the activity of the enzyme, which reduces MTT to formazan dyes.[6] Genotoxicity damages the genome of the cells, decreasing their capacity to self-repair, or may cause neoplasia in future.[5] A variety of genotoxicity assays, such as chromosome metaphase aberrations, micronuclei, and chromatid sister exchange and assessment of DNA breakage, can be employed to determine the genotoxicity of a material. The single cell gel (comet) assay is a rapid, easy, and effective biochemical technique for assessment of DNA damage in mammalian cells.

Over the years, various restorative materials have been proposed as root-end filling materials, namely, silver amalgam, zinc oxide eugenol (flat or reinforced), ethoxy benzoic acid (EBA) and super EBA cement, polycarboxylate cement, glass ionomer cement (GIC), gutta-percha (GP, burnt or injectable), composite resin, cyanoacrylate, teflon, cavit, and so on. Newer bioactive materials have been introduced to overcome toxicity, poor sealing ability, and solubility of the above-mentioned materials.[7] Calcium silicate-based cements such as mineral trioxide aggregate (MTA) have excellent marginal adaptation, seal, and bioactivity, making them a favorable option as a retrograde filling material. However, it is difficult to manipulate and has a long setting time. Endosequence root repair material, calcium phosphate silicate cement, is available in the form of putty, which can be easily compressed into the defective areas and has a shorter setting time. Geristore is a dual-cure, hydrophilic, non-aqueous polyacid-modified composite resin with increased adhesion to tooth structures.[8] The biocompatibility of root repair materials is imperative in the successful outcome of surgical treatment as they come in direct contact with the periradicular tissues. There is limited literature regarding the biocompatibility of geristore and endosequence in comparison to MTA. Therefore, this study aimed to assess and compare the cytotoxic and genotoxic effects of three commercially available root repair materials, MTA, endosequence root repair material, and geristore, using periodontal ligament fibroblast cells.

MATERIALS AND METHODS

Cell culture preparation

Periodontal ligament (PDL) fibroblasts were obtained from healthy premolars extracted for the orthodontic purpose only. Premolars with fracture, caries, or restoration were excluded from the study. Collected samples were carried in phosphate buffer saline solution. Cell pellets from the sample were collected by centrifuging, and the supernatant was obtained. Cell cultures were grown in 60 mm culture dish containing the culture medium (DMEM medium, Invitrogen Life Technologies, Grand Island, NY) at 37°C in a humidified atmosphere of 5% carbon dioxide in the air. Experiments were conducted using third passage cells. Exponentially growing cells were seeded at a density of total 5×10^3 cells per well.

Test material preparation

All test materials, namely, MTA, endosequence, and geristore, were mixed according to the manufacturer's instructions, and discs were prepared (2 mm in diameter and 2 mm in length) using sterile Teflon moulds. All test specimens were allowed to set and were kept in laminar air flow for 15 to 20 minutes for sterilization. The individual disc was treated in 96-well plates in Dulbecco's modified Eagle's medium (DMEM) and incubated for 24 hours and 48 hours.

A total of 80 samples were included in the study divided into four groups of 20 samples each.

Group I: MTA (ProRoot White MTA; Dentsply Tulsa Dental, Tulsa, OK), 20 discs.

Group II: Endosequence (Brasseler, Savannah, GA), 20 discs.

Group III: Geristore (DEN-MAT Corporation, Santa Maria, CA) 20 discs.

Group IV: Control group (containing only PDL cells).

Cytotoxicity evaluation

The cytotoxic effect of these three materials on the viability of PDL fibroblasts was determined by MTT assay at 24 hours and 48 hours time intervals. Absorbance was recorded with a 570 nm using a micro plate reader.

Percentage of cell viability was obtained by using the formula

$$\text{Surviving cells (\%)} = \frac{\text{Mean optical density of test compound} \times 100}{\text{Mean optical density of control}}$$

Genotoxicity evaluation

The genotoxic effect of these three materials on the PDL fibroblasts was determined by Comet assay using imaging software system at 24 hours and 48 hours time intervals. For visualization of DNA damage, observations were made of ettrium bromide-stained DNA using a 40x objective on a fluorescent microscope. Ten comets per slide were calculated. Calculations were performed by the imaging software system.

Two parameters were estimated, namely, tail intensity, that is, percentage of DNA in the tail, and tail length, that is, the distance of DNA migration from the body of the nuclear core recorded as the distance from the perimeter of the comet head to the last visible point in the tail.

Statistical analysis

Data of cell viability percentage were statistically analysed using Tukey's multiple comparison test to compare the mean values of each experimental group to those of control groups and to compare mean values of groups to those of other groups. A *P* value <0.05 was considered statistically significant.

Data of tail DNA percentage and tail length were statistically analysed using Dunnett's multiple comparison test to compare the mean values of each experimental group to those of control groups and to compare mean values of groups to those of other groups. A *P* value <0.05 was considered statistically significant.

RESULTS

Cytotoxicity using MTT assay

After 24 hours time period, group I (MTA), group II (endosequence), group III (geristore), and group IV (control) resulted in cell viabilities of 91%, 81.44%, 73.83%, and 100% respectively [Table 1 and Figure 1]. After 48 hours time period, group I (MTA), group II (endosequence), group III (geristore), and group IV (control) resulted in cell viabilities of 86.56%, 63.80%, 58.83%, and 100%, respectively [Table 1 and Figure 2].

Genotoxicity using comet assay

After a 24-hour time period, MTA, endosequence, geristore, and control groups resulted in tail DNA percentages of 8.462 ± 6.37, 14.47 ± 6.326, 22.303 ± 8.318, and 3.04 ± 1.467, respectively. After a 48-hour time period, MTA, endosequence, geristore, and control groups resulted in tail DNA percentages of 21.819 ± 4.756, 31.453 ± 9.7, 40.157 ± 13.368, and 2.842 ± 1.419, respectively [Table 2 and Figures 3 and 4]. There was an increased tail DNA percentage for all the groups at the 48th hour compared to the 24th hour interval.

After a 24-hour time period, MTA, endosequence, geristore, and control groups resulted in mean tail lengths of 4.256 µm, 14.306 µm, 18.433 µm, and 3.689 µm, respectively. After a 48-hour time period, MTA, endosequence, geristore, and control groups resulted in mean tail lengths of 18.463 µm, 27.830 µm, 37.750 µm, and 2.610 µm, respectively [Table 3]. There was an increased tail length for all the groups at the 48th hour compared to the 24th hour interval.

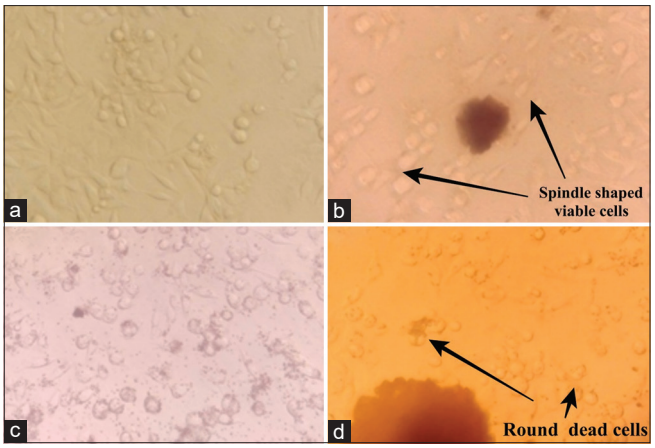

Figure 1: Microscopic images of samples after MTT assay at 24th hour interval: (a) Control group (intense spindle-shaped viable cells seen), (b) MTA (marked number of viable cells seen) (c) endosequence (moderate number of viable cells seen), and (d) geristore (faint number of viable cells seen)

Table 1: Mean cell viability of control group and other test materials at 24th h and 48th h intervals

Groups	PDL			
	Control	I	II	III
24 h				
Mean cell viability	100±4.47	91.00±3.52	81.44±4.45	73.83±5.12
48 h				
Mean cell viability	100±2.35	86.56±3.35	63.80±3.85	58.73±5.31

DISCUSSION

Elimination of microbial agents and their by-products and subsequent three-dimensional obturation of the root canal system are the main objectives of non-surgical endodontic therapy (NSET). The inherent complexities of the root canal system pose a major challenge in achieving these goals.[9] Orthograde endodontic retreatment is indicated

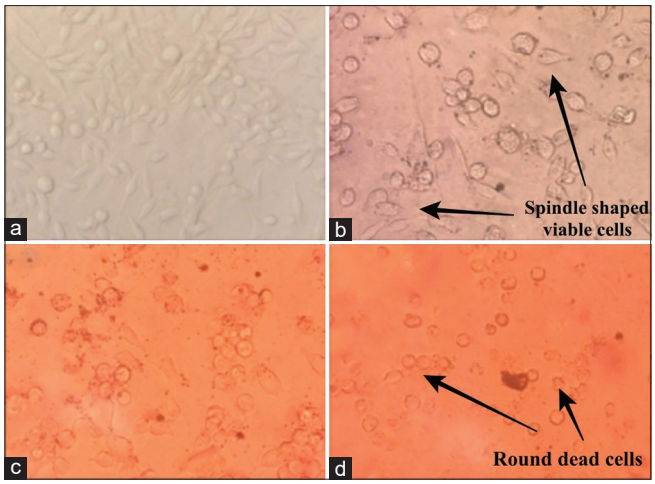

Figure 2: Microscopic pictures of samples after MTT assay at the 48th hour interval: (a) Control group (intense spindle-shaped viable attached cells seen), (b) MTA (marked number of viable cells seen), (c) endosequence (moderate number of viable cells seen), and (d) geristore (faint number of viable cells seen)

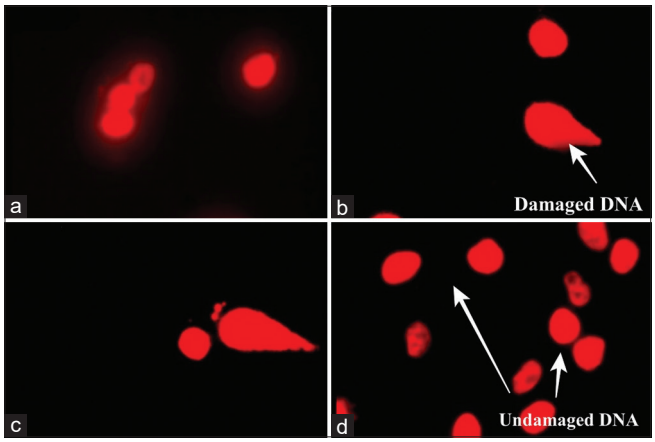

Figure 3: Comet assay results at the 24th hour interval: (a) MTA, (b) endosequence, (c) geristore, and (d) control group

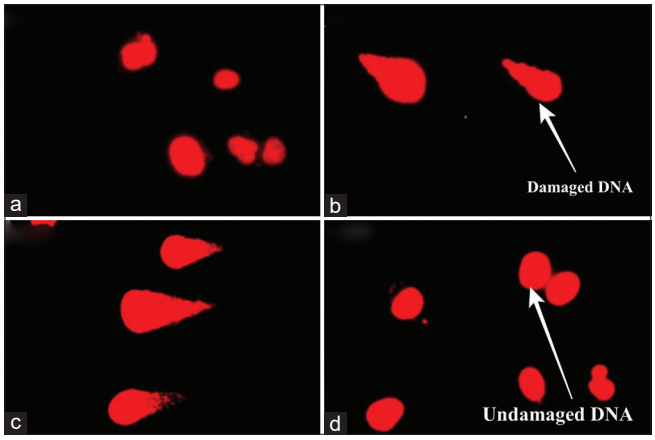

Figure 4: Comet assay results at the 48th hour interval: (a) MTA, (b) endosequence, (c) geristore, and (d) control group

in case of endodontic failure. However, if orthograde endodontic retreatment is contraindicated, root-end resection, followed by retrograde filling, is an alternative treatment strategy to extraction of such teeth.[10]

The main objective of root-end filling material is to provide 'physical seal' preventing microleakage into the root canal system.[11] Newer root repair materials have been indicated for retrograde filling, such as MTA, biodentine, geristore, endosequence, bioaggregate, and so on.[12] Their biocompatibility is considered imperative as the release of leached components from the set cements determines their biological effects on the surrounding tissues.[10] MTA is known to be bioactive in nature as it has both hard tissue conductive and inductive properties. MTA is known for its outstanding biocompatibility and has become the gold standard to which new root-end filling materials are being compared.[10,13] However, some clinicians subjectively report difficulties in handling MTA due to its consistency and long setting time. Several new bioceramic materials with similar biological components have been developed to address the drawbacks of MTA.[6] EndoSequence root repair material (ERRM; Brasseler, Savannah, GA) is one such bio-ceramic material which contains calcium silicates, zirconium oxide, tantalum pentoxide, calcium phosphate monobasic, and filler agents. It is available as premixed putty or as a syringe-able paste with a uniform consistency.[6]

Geristore is a hydrophilic, non-aqueous, polyacid-modified composite resin with fluoride-releasing glass in an organic polymerizable matrix paired with a photoinitiator. The reported advantages of resin ionomers are increased adhesion to tooth structures, dual-cure potential, low polymerization shrinkage, radiopacity, release of fluoride, and biocompatibility.[8,14] Histological studies of geristore have showed strong attachment and cells spread through relatively normal morphological tissues.[10] There is limited literature regarding comparison of biocompatibility of geristore and endosequence. In this study, cytotoxic and gentoxic effects of three root filling materials, MTA, endosequence root repair material, and geristore, on periodontal ligament fibroblasts at the 24th hour and 48th hour intervals were assessed using MTT assay and comet assay.

For precise sensitivity testing, the main tissue-derived cell lines are needed. In the present study, human PDL fibroblasts were selected to simulate the clinical environment.[8] Viability of the cells was calculated using the colorimetric assay based on MTT to monitor cell response in culture. The results of this study showed that MTA was most biocompatible compared to other tested materials at both 24th hour and 48th hour intervals. These findings are in correspondence to several *in vitro* studies showing good biocompatibility when tested for cell viability, apoptosis,

Table 2: Mean tail DNA percentage of control group and other test materials at 24th h and 48th h intervals

Groups	PDL			
	Control	I	II	III
24 h				
Tail DNA percentage	3.047±1.467	8.462±6.373	14.470±6.326	22.303±8.318
48 h				
Tail DNA percentage	2.842±1.419	21.819±4.756	31.243±9.779	40.157±13.368

Table 3: Mean tail length of control group and other test materials at 24th h and 48th h intervals

Time (hr)	C		I		II		III	
	Mean	Std dev	Mean	Std dev	Mean	Std dev	Mean	Std dev
24 h	2.610	2.267	4.256	2.636	14.306	7.227	18.433	7.983
48 h	3.689	1.590	18.463	6.930	27.830	9.670	37.750	12.173

and mitochondrial dehydrogenase activity with human PDL fibroblast cells.[15-17] An *in vitro* study evaluated the cytotoxic effect of three endodontic materials (MTA, endosequence, biodentine) on PDL fibroblasts, which showed MTA being least cytotoxic, similar to the findings of the present study.[1]

A study conducted using mouse fibroblasts demonstrated that the cell viability of the endosequence root repair is comparable to that of ProRoot MTA.[18] However, in our study, endosequence root repair material showed lesser cell viability compared to MTA. This could be attributed to the filtration procedure of ERRM, where a certain amount of particulate matter was present. This could be attributed to composition of the material or possibly the problems arrived while achieving a complete set of the material. Although the material seemed to be completely set, some amount of it remained unset internally, thus leaching a significantly greater amount into the culture medium.[6]

In the present study, geristore demonstrated the highest cytotoxicity of 73.83% and 58.83% at 24th hour and 48th hour intervals, respectively. A study evaluating the healing ability of root-end materials showed geristore being the least positive for healing compared to others.[19] It has low pH following setting, which can explain the production of slightly more inflammation than other groups.[20] In contrast, after 24 and 48 hours time intervals, SK Gupta. *et al*. showed geristore to be less toxic to human PDL fibroblasts.[19] Geristore has shown a more favourable result, possibly due to its surface topography. It revealed certain soft-edged granulations along the grooves on the surface, which could promote better scaffolding. Also, geristore eludes fewer toxic materials into the medium as it is a resin ionomer.[9,19] Nevertheless, the precise component variations are not understood at present.

In our study, all the test samples showed higher cytotoxicity and genotoxicity at the 48th hour than at the 24th hour. This may be due to the production of calcium hydroxide on hydration, which is in accordance with previous studies.[21-23]

Genotoxicity testing can be characterized as *in vitro* and *in vivo* tests for substances that cause genetic damage, including DNA damage, gene mutation, chromosomal breakage, altered DNA repair capability, and cellular transformation caused by the by-products or compounds over an extended period of time, which may further lead to cancer.[24]

Cells suppress genotoxic mutation production through either a repair of DNA or an apoptosis. Furthermore, it is not always possible to repair the damage that leads to mutagenesis. Several sophisticated techniques to assess the DNA damage have been developed, such as Ames assay, toxicology studies *in vitro* and *in situ*, and Comet assay.[25] The current study used an alkaline version of the comet test, which can determine the type of DNA damage and specify partially repaired areas. Tailed DNA percentage is calculated by a computerized image analysis system that determines the actual intensity and frequency of tails and tail length (length of DNA migration). This parameter is one of the most efficient measures to determine the induced DNA damage.[26]

In our study, MTA presented the lowest genotoxicity of 8.462 ± 6.37 and 21.819 ± 4.756 at the 24th hour and 48th hour intervals, respectively. MTA has been rated consistently in different cell lines and test systems as non-toxic root canal cement.[27] In contrast, N Naghavi *et al*. reported MTA to be more toxic compared to calcium-enriched mixtures at higher concentrations.[4] This can be attributed to the introduction of strong arsenic levels in the medium supplying MTA. Nevertheless, it has been stated that MTA demonstrated a poor arsenic release rate, thereby proving no contraindication with respect to this chemical element for its clinical application.[27]

In the present study, endosequence showed a higher genotoxicity of 14.47 ± 6.326 and 31.243 ± 9.779 at 24th hour and 48th hour intervals, respectively, than MTA. However, this was lower in comparison to geristore. This could be due to release of a considerably higher amount of material into the culture medium, leading to toxic effects on fibroblasts.[7] Previous studies have reported that geristore is less biocompatible than grey MTA, consistent with the results

of the present study.[20,28] Geristore releases five monomers, Bis-GMA, Bis-DMA, TEGDMA, UDMA, and Bisphenol A. Furthermore, geristore releases calcium and aluminium ions and fluoride. Resin monomers have been found to show cytotoxicity and might be capable of tumour initiation at relatively low concentrations.[29]

The findings of this study demonstrated that MTA was the most biocompatible retrograde filling material, followed by endosequence on human cultured fibroblasts at the 24th hour and 48th hour intervals. Geristore demonstrated higher cytotoxicity and genotoxicity at both the time intervals. Future studies should adapt advanced methods like quantitative real-time polymerase chain reaction, necrosis, and apoptosis assay by FACS, aiding in better assessment of cell viability and damage.

CONCLUSION

Within the limitations of the study, the following can be concluded:

1. All the test materials, MTA, endosequence root repair material, and geristore showed significantly different cytotoxic and genotoxic effects on human periodontal fibroblast cells ($P < 0.05$).
2. MTA proved to be least cytotoxic compared to endosequence root repair material, followed by geristore on human periodontal fibroblast cells at both 24th hour and 48th hour intervals.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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