Genotoxicity of three endodontic sealers by single cell gel-electrophoresis/comet assay

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Received 12 June 2014; revised 25 June 2014; accepted 26 June 2014
Available online 27 September 2014

Abstract

Introduction: The aim of this study was to compare the genotoxicity of two MTA-based sealers (MTA Fillapex and MTA Plus) and a RealSeal self etch (SE) sealer.

Methods: Twenty discs of each sealer were constructed, dissolved in cell culture media and sealer extracts were diluted into two different concentrations 100% and 50%. Thereafter Baby Hamster kidney fibroblast cell cultures were treated with each concentration of tested sealers for two exposure periods 24 h and 5 days. Comet assay was used to evaluate DNA damage by measuring tail length and intensity, and the results were statistically analyzed by Kruskal–Wallis and Mann–Whitney U tests ($P \leq 0.05$).

Results: After 24 h, 100% medium extracts of RealSeal SE showed significantly the highest DNA damage followed by MTA Fillapex. After 5 days, MTA plus induced the least DNA damage at both tested concentrations. The DNA damage of both RealSeal SE and MTA Fillapex was significantly dose and time dependant.

Conclusions: MTA Plus was the least genotoxic sealer in this study and its genotoxicity is not significantly affected by exposure time or its concentration.

Keywords: Genotoxicity; MTA-based sealers; RealSeal SE sealer; Comet assay

1. Introduction

Root filling materials usually remain in close contact with living periapical tissues over a long period of time via the apical foramen and occasional lateral foramina. The tissue's response to these materials is important and may influence the outcome of endodontic treatment [1]; therefore, an ideal endodontic material should be biocompatible with the peri-radicular tissues [2].

Genotoxicity is one of the important factors influencing biocompatibility. Genotoxic damage will not...
necessarily lead to cell death; it may damage the cell genome that may significantly diminish the tissue's self-repairing potential or in the long term cause the development of neoplasia [1]. A variety of genotoxicity assays assess DNA breakage such as metaphase chromosomal aberrations, micronuclei, and sister chromatid exchange. Over the past decade, the single cell gel (comet) assay was developed as a rapid, simple, and reliable biochemical technique for evaluating DNA damage in mammalian cells [3].

Mineral trioxide aggregate (MTA) is a cement composed of tricalcium silicate, dicalcium silicate, tricalcium aluminate, calcium sulfate, bismuth oxide, and small amounts of other mineral oxides that modify its chemical and physical properties. It is widely used as a root-end filling material [4], in vital pulp therapy [5] and as an apical barrier in immature teeth with necrotic pulps [6]. Lately, it has been successfully used in regenerative endodontic procedures in immature teeth with apical periodontitis [7]. MTA is biocompatible [8], noncytotoxic [9], nonmutagenic, neither genotoxic nor carcinogenic [10] and has excellent sealing properties [8].

Based on these favorable characteristics and for the purpose of improving the drawbacks of the conventional MTA as long setting time and the difficulty in handling, a new formulation of MTA-based sealer has been introduced. MTA Fillapex is a resin sealer based on MTA in its composition in addition to salicylate resin, natural and diluting resins, nano-particulated resin, bismuth trioxide, nano-particulated silica, and pigments. It has low solubility, and easy handling [11], however the results related to its biological response are conflicting. Several researches revealed that this material showed high cytotoxicity and genotoxicity [12] even after 90 days [13]. Despite others showed that the cytotoxicity of MTA Fillapex decreases by time [14].

MTA Plus material has been introduced in the market that has basically the same composition of the original MTA formulation. It has a finer particle size than other commercially available versions of MTA (50% of the particles finer than 1 mm). The material being proposed would bond to tooth structure thus providing a hermetic seal [15].

Moreover, RealSeal self-etch (SE) is the simplified dual-cured version of RealSeal methacrylate resin-based sealers [16]. It has hydrophilic characteristics that enable them to wet canal wall, penetrate dentinal tubules [17], bond to radicular dentin [18], and to root-filling materials [19]. In spite of the cytotoxic effect of RealSeal SE sealer that was clarified by some studies [20–22], it has been approved for endodontic use [23–24].

Genotoxicity of RealSeal SE and MTA Fillapex sealers is scarcely studied and yet for MTA Plus. However studying the genotoxicity of some dental materials it was concluded that there was an evidence of dose-dependent response [1]. Consequently, this study was conducted to evaluate the genotoxicity of MTA Fillapex, MTA Plus, and RealSeal SE sealers using Comet assay to detect genomic damage expressed in two parameters including tail length and tail intensity at two concentrations (100% and 50%) after 24 h and 5 days exposure periods.

2. Materials and methods

2.1. Sample preparation

Twenty discs of each tested material; MTA Plus,1 MTA Fillapex2, and RealSeal SE3 sealers representing groups I, II, and III respectively were fabricated according to the manufacturer’s instruction under aseptic conditions in sterile cylindrical Teflon blocks, 5 mm in diameter and 2 mm in height. Samples of MTA Plus and MTA Fillapex were allowed to set at 37 °C for the time given by the manufacturer. RealSeal SE samples were light cured using the Elipar TriLight halogen curing unit4. Immediately after setting, excess flash material was removed with a sterile scalpel and the hardened discs were sterilized with ethylene oxide after exposure to ultraviolet light for 2 h.

2.2. Preparation of extracts

Samples from each tested material were dissolved in cell culture Dulbecco's Modified Eagle medium5 (DMEM) (1 g/5 mL) and incubated at 37 °C. The obtained extracts were sterile filtered using Millex-GS sterile filter6. To observe a dose-response relationship, the extracts were diluted with DMEM to achieve 100% and 50% concentrations V/V.

2.3. Cell cultures

BHK-21; Baby Hamster kidney fibroblast cells (clone CCL-10) was supplied from cell culture

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1 Prevest-Denpro, Jammu City, India.
2 Angelus, Londrina, PR, Brazil.
3 SybronEndo, Orange, CA.
4 3M ESPE, St. Paul, MN, USA
5 Sigma–Aldrich, St Louis, MO, USA.
6 Millipore S.A.S., Molsheim, Cedex, France.
department (VACVSERA—EGYPT) and maintained in an incubator until use. Growth medium from mother bottle was decanted, cells were washed with Trypsin (0.25%) containing 0.1 mm ethylene diamine tetraacetic acid (EDTA), in phosphate buffered saline (PBS) for 5 min at 37 °C for cell dissociation. Washing trypsin was decanted and cells were kept in contact with the residual trypsin for 10 min at 37 °C with periodic observation under the inverted Nikon microscope till complete cell dissociation. Detached cells were dispensed in growth medium to maintain 2 × 10⁵ cell/mL. Cells were dispensed into tissue culture flasks, 25 ml TV with periodic examination under the inverted microscope till confluence sheet detected. Then cultures were treated with each concentration of tested material extract for 24 h and 5 days at 37 °C [25].

2.4. Slide preparation and comet assay

The comet assay was performed according to a standard protocol [26]. All the chemicals needed to perform the comet assay were obtained from Sigma. The culture medium was carefully removed, and 5 µL of the sedimented fibroblasts was suspended in 100 µL of 0.5% low melting agarose to obtain 10,000 of fibroblasts per slide. This agarose layer was sandwiched between a layer of 0.6% normal melting agarose and a top layer of 0.5% low melting agarose on fully frosted slides. The slides were coded and kept on ice during the polymerization of each gel-layer. After the solidification of the 0.5% agarose layer, the slides were immersed in a lysis solution [1% sodium sarcosinate, 2.5 mm sodium chloride (NaCl), 100 mm Na₂EDTA, 10 mm Tris-hydrochloric acid (HCl), 1% Triton X-100 and 10% dimethyl sulfoxide (DMSO)] at 4 °C. After 1 h, the slides were placed in an electrophoresis buffer [0.3 mm sodium hydroxide (NaOH), 1 mm Na₂EDTA, pH 13] at 0 °C for 20 min to unwind the DNA. The electrophoresis was performed at 300 mA and 1.0 V cm⁻¹ in a horizontal electrophoresis platform for 20 min.

The slides were neutralized with a Tris-HCl buffer (pH 7.5) and stained with 10% ethidium bromide for 10 min. Each slide was analyzed using an inverted Nikon fluorescence microscope. A hundred comets per slide were analyzed by the Comet assay V analysis system to determine DNA damage. Two parameters were estimated: tail length (the distance of DNA migration from the body of the nuclear core which recorded as the distance from the perimeter of the comet head to the last visible point in the tail) and tail intensity (percentage of DNA in the tail). During the analysis, the edges and eventually damaged parts of the gel as well as debris, superimposed comets, comets of uniform intensity and comets without a distinct head (‘clouds’, ‘hedgehogs’ or ‘ghost cells’) were excluded.

3. Statistical analysis

Data were presented as mean and standard deviation (SD) values and explored for normality using Kolmogorov–Smirnov and Shapiro–Wilk tests. Data showed non-parametric distribution; so Kruskal–Wallis test was used to compare the three materials. Mann–Whitney U test was used for pairwise comparisons between the materials when Kruskal–Wallis test is significant; to compare both tested concentrations (100% and 50%) as well as the two time periods of evaluation (24 h and 5 days). The significance level was set at P ≤ 0.05. Statistical analysis was performed using IBM® SPSS Statistics Version 20 for Windows.

4. Results

Comet assay results revealed that all tested sealers (MTA Plus, MTA Fillapex and RealSeal SE) recorded tail length and intensity measurements exhibiting a genotoxic effect at both tested exposure periods and concentrations as shown in Table 1.

4.1. Inter-material comparison (Table 1)

4.1.1. After 24 h exposure period

Considering the 100% medium extracts, the significantly highest mean values of DNA damage, tail length and intensity, were associated with group III (RealSeal SE) while the lowest were associated with group I (MTA Plus). There was no statistical significant difference between groups I and II regarding tail length, however group I showed statistically lower mean value of tail intensity compared to group II (Fig. 1). Alternatively, no significant differences were recorded among tested materials for both tail length and tail intensity at 50% medium extracts.
4.1.2. After 5 days exposure period

At 100% concentrations there were significant differences among all tested groups regarding the tail length with the highest mean value for group III and the lowest for group I. While regarding the tail intensity, group I recorded the significantly lowest mean value versus both groups II and III.

Also, at 50% medium extracts, group III recorded the highest significant mean value of tail length compared to groups I and II. Regarding tail intensity, there was no statistical significant difference among the three tested materials ($P = 0.603$).

4.2. Intra-material comparison (Table 2)

4.2.1. Comparison between concentrations of sealer extract

The concentrations of the studied sealers had a significant effect on DNA damage, tail length and intensity, at both 24 h and 5 days exposure periods for groups II and III. The significantly higher mean values were noted at 100% concentrations compared to those at 50% and this was obviously shown in Fig. 2.

Whereas, there was no significant difference in DNA damage between both tested concentrations of group I at both exposure periods.

4.2.2. Comparison between exposure periods

The effect of tested periods was statistically significant on both DNA damage parameters for all tested sealers types and concentrations. It demonstrated significantly higher values of genotoxicity at 5 days exposure versus 24 h (Fig. 3) except for MTA Plus medium extracts.

5. Discussion

DNA damage in cells could have an important implication on health because they are cumulative and may in turn affect cell functions leading to cell death or slow onset disease overtime [27]. To avoid unwanted side effects following the use of root canal sealers, only materials exerting no or minimum deleterious effects on living cells should be used [1].

Comet assay is a standard, non-invasive, and a powerful technique that directly measures DNA damage in cells.

<table>
<thead>
<tr>
<th>Time</th>
<th>Concentration</th>
<th>Comet parameters</th>
<th>Group I MTA plus Mean ± SD</th>
<th>Group II MTA Fillapex Mean ± SD</th>
<th>Group III RealSeal SE Mean ± SD</th>
<th>Kruskal–Wallis $P$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 h</td>
<td>100%</td>
<td>Tail length</td>
<td>33.8 ± 6.9$^a$</td>
<td>37.3 ± 1.1$^a$</td>
<td>52.4 ± 3.8$^b$</td>
<td>0.034*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tail intensity</td>
<td>27.3 ± 2.7$^a$</td>
<td>49.1 ± 1.3$^b$</td>
<td>59.9 ± 1$^c$</td>
<td>0.049*</td>
</tr>
<tr>
<td>50%</td>
<td>Tail length</td>
<td>11 ± 0.8</td>
<td>12.9 ± 3.1</td>
<td>14.4 ± 0.3</td>
<td>12.8 ± 0.3</td>
<td>0.603</td>
</tr>
<tr>
<td></td>
<td>Tail intensity</td>
<td>14.1 ± 0.1</td>
<td>15.3 ± 1.9</td>
<td>12.8 ± 0.3</td>
<td>12.8 ± 0.3</td>
<td>0.412</td>
</tr>
<tr>
<td>5 days</td>
<td>100%</td>
<td>Tail length</td>
<td>42.3 ± 3.8$^a$</td>
<td>56.7 ± 2.9$^b$</td>
<td>139.9 ± 4.3$^c$</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td></td>
<td>Tail intensity</td>
<td>29 ± 1.6$^a$</td>
<td>72.1 ± 0.7$^b$</td>
<td>84.5 ± 2.3$^b$</td>
<td>84.5 ± 2.3$^b$</td>
<td>0.005*</td>
</tr>
<tr>
<td>50%</td>
<td>Tail length</td>
<td>28 ± 2.9$^a$</td>
<td>31.2 ± 0.9$^a$</td>
<td>55 ± 0.3$^b$</td>
<td>55 ± 0.3$^b$</td>
<td>0.002*</td>
</tr>
<tr>
<td></td>
<td>Tail intensity</td>
<td>21 ± 1</td>
<td>34.3 ± 2.8</td>
<td>35 ± 1.9</td>
<td>35 ± 1.9</td>
<td>0.603</td>
</tr>
</tbody>
</table>

*Significant at $P \leq 0.05$. Mann–Whitney $U$ test: groups with different superscripts in the same row are significant.

Fig. 1. Comet assay micrographs showing cell DNA migration pattern produced by Comet assay after treatment with 100% medium extracts for 24 h (a): samples treated with MTA Plus with less DNA damage, (b): MTA Fillapex, (c): RealSeal SE with more tail length and tail intensity.
damage in almost all kinds of individual cell types. This assay is based on the principle that, DNA damage reduces the size of DNA fragments which is detected by applying an electrophoretic field to lysed cells where damaged cellular DNA fragments are separated from intact DNA, yielding a classic “comet tail” shape under the microscope. The extent of DNA damage is usually estimated by comet tail measurements where image analysis software is available for measuring various parameters [28].

According to the previous reports [12,13], only a few studies on putative genotoxicity of MTA-based root canal sealers and methacrylate resin-based sealers have been conducted and the results are conflicting. Thus, the genotoxicity of these materials at different concentrations was assessed in this study. The current results indicated that RealSeal SE root canal sealer was the one that has induced more genotoxic effect on BHK-21 cells at both tested concentrations and periods of exposure. This might be explained by the inherently high resin content of the sealer; UDMA, EBPADMA, PEGDMA, BIS-GMA, which accounts for more than 60% [29]. Additionally, methacrylate-based sealer usually sets in about 30 min inside the root canals in the anaerobic environments. In the present study, the sealer was not fully

![Fig. 2](image_url)  
**Fig. 2.** Photographs from comet assay showing cells exposed for 5 days to medium extracts of 50% RealSeal SE sealer (a) and 100% of the same sealer (b) with more tail length and more DNA damage.
isolated from air, and oxygen may inhibit free radical polymerization of resins that might not be completely polymerized [22]. This was supported by Tyagi et al. [30] who related the toxicity of methacrylate resin-based sealers to the presence of unpolymerized hydrophilic monomers (such as 2-hydroxyethyl methacrylate (HEMA)) that can easily diffuse into the cell and elicit significant toxicity. A conflicting result by Brzovic et al. [1] who demonstrated that Epiphany sealer was not capable of inducing DNA damage might be related to the different assay evaluating genotoxicity where they used chromosomal aberration analysis.

However, after 24 h exposure the genotoxicity of 50% RealSeal SE sealer extracts was not statistically different from other two tested sealers for both comet assay parameters. It was suggested that the concentration of residual monomers leached at this short exposure time and 50% concentration of the polymerized material under the experimental conditions was too low to exhibit genotoxic activity. This finding concurs with other studies which examined genotoxicity of either Epiphany or RealSeal (which is basically Epiphany) sealers with different cytogenetic assays including the number of micronuclei formation [31] and cytotoxicity action [29].

According to the present data, it was found that MTA Fillapex root canal sealer produced more DNA damage and genotoxicity compared to MTA Plus sealer. The genotoxicity of MTA Fillapex was most likely related to the composition of this material where it contains only 30% MTA and resin components such as salicylate which has a potential concern in cellular genotoxicity [12−14]. Furthermore, according to Bramante et al. [32], MTA Fillapex contains high levels of arsenic, heavy metal, element as a contaminant. The release of this arsenic element reacts with protein thiols of the cells and may induce genotoxicity. This finding is consistent with the results of other researches which confirm the genotoxic effect of MTA Fillapex sealer [12−14].

It has been difficult to discuss and compare the present results obtained with MTA Plus sealer because of the lack of previous studies. The current findings showed that MTA Plus has the least genotoxic effect compared to tested sealers. The good compatibility of this sealer might be a sequence of its main components including tricalcium silicate, dicalcium silicate, tricalcium aluminate, calcium oxide, bismuth oxide, and calcium sulfate which are the main components of dentin tissue. Additionally, this type of sealer doesn't include Iron (III) Oxide (Fe₂O₃) even in low concentration compared to other classic MTA that might evoke DNA damage [12].

The present data demonstrated that genotoxicity of both RealSeal and MTA Fillapex significantly increased with time. Additionally it was found that their genotoxicity is dose-dependent where there was significantly more DNA damage associated with 100% concentration compared to that of 50% for both sealers. It could be suggested that the concentration of toxic materials that are leached from the set material of RealSeal and MTA Fillapex (residual monomer and/or arsenic material) at 10% concentration and 5 days exposure was large enough to induce more genotoxic effect. The significant role of sealer concentration and/or exposure time confirmed the findings of some authors [1,13,31,33]. In contrary, Marques et al. [34] revealed that the toxic effect of MTA Fillapex decreased with time. This difference may be attributed to different methodology since they didn't use the Comet assay in evaluating the toxic effect of MTA Fillapex on rat subcutaneous tissue.
On the other hand, in the present study, the genotoxicity of MTA Plus was not found to be dose dependent. This was in consistent with Ribeiro et al. [35] who noted that there was no difference in DNA damage at different concentrations of MTA as depicted by the single cell gel assay, and Aminozarbian et al. [36] who found that MTA genotoxicity did not vary by increasing its concentration.

6. Conclusion

Under the conditions of this study, all tested sealers showed variable degrees of genotoxicity. The DNA damage observed by RealSeal SE and MTA Fillapex sealers was dose and time-dependant. Given the diversity of root canal sealers on the market, additional investigations, especially for MTA Plus, using other parameters of genotoxicity as chromosomal aberration and sensitization tests are recommended to establish an overview on the real potential of these sealers. In vitro results might not be directly extrapolated to in vivo conditions; therefore, it is essential to have long term in vivo studies and clinical investigations to assess the biocompatibility of tested sealers.

Acknowledgment

The authors appreciate the technical assistance of both Dr. Aly Fahmy EL-Sayed, GM of Applied Research Sector, Holding Company for Biological Products, Vaccines, and Drugs (VACSERA), and Dr. Mohamed Rabie, GM of Global Institute Research Services Solution.

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