Genotoxicity of Mineral Trioxide Aggregate and Three Endodontic Cements on Human Gingival Fibroblast Cells

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Abstract: New endodontic cements have been formulated for a possible substitution of Mineral Trioxide Aggregate (MTA), although their properties such as genotoxicity must be evaluated *in vitro* before applying in human beings in detailed. The aim of this study was to compare the genotoxicity of Calcium Aluminate α-Aluminate Cement (CAAC), Calcium Aluminate α-Aluminate plus Cement (CAAC plus) and a mixture of wollastonite and CAAC cement (WOLCA) and Mineral Trioxide Aggregate (MTA) using human gingival fibroblast cells and single cell gel (COMET) assay. In this experimental *in vitro* study, MTA, CAAC, CAAC plus and WOLCA cement prepared in final concentrations of 1, 10, 100 and 1000 μg mL^{-1} then these materials were added to the human gingival fibroblast cells for studying their effect on DNA. Then, COMET assay was done for examining the cement genotoxicity. An automatic analysis system (COMET Assay) was used in order to calculate DNA damage. Tail moment (result of tail DNA/total DNA by the center of gravity) was considered to determine DNA damage. The potential DNA damage associated with exposure to different materials were calculated and statistically analyzed by Kruskal-Wallis and Mann-Whitney tests. DNA damage values for MTA were in 0.94-0.97 range; of CAAC in 0.96-1.02; of CAAC-Plus in 1.18-1.23 and of WOLCA was in 0.99-1.01 range. MTA had the least DNA damage values compared to others, although CAAC cement showed DNA damage similar to MTA. CAAC Plus and WOLCA had significantly higher genotoxicity than MTA, however the values obtained for CAAC cement was comparable to MTA suggesting its possible use *in vivo*.

Key words: Genotoxicity, mineral trioxide aggregate, calcium aluminate α-aluminate cement, calcium aluminate α-aluminate plus cement, wollastonite cement, Iran

INTRODUCTION

Mineral Trioxide Aggregate (MTA) was developed as a root perforation repair material by Torabinejad *et al.* (1993) at the University of Loma Linda for the 1st time. MTA is composed primarily of calcium, silicon, bismuth and oxygen. Several studies have demonstrated the excellent biological properties of MTA (Torabinejad *et al.*, 1995a, 1997; Koh *et al.*, 1997, 1998) and its good marginal adaptation (Torabinejad *et al.*, 1995b) and sealing ability (Torabinejad *et al.*, 1993). MTA was introduced to the US market under the brand name ProRoot MTA (Dentsply/Tulsa Dental Specialties, Tulsa, OK).

MTA is used extensively in endodontics for pulp capping, pulpotomy, repair of root perforations, root end filling, root canal filling and apical barrier in teeth with necrotic pulps and open apices (AAE, 2004; Yavari *et al.*, 2009).

As the materials used in root canal treatments, especially root end filling are in direct contact with soft and hard periodontal tissues; a root filling material is needed to be highly biocompatible and nontoxic (Camilleri and Ford, 2006).

Although with specific advantages mentioned for MTA, some shortcomings are listed for this root-end filling material including possible tooth discoloration, presence of some toxic elements in its composition, higher costs, long setting time, difficult handling and more difficulties in its removal after setting (Kratchman, 2004; Camilleri *et al.*, 2005) all these encouraging researchers to develop new filling materials for a possible substitution with MTA.

In this regard, new cements have been formulated at the Torabinejad Dental Research Center at Isfahan University of Medical Sciences (IUMS), Isfahan, Iran. These cements are Calcium Aluminate α-luminate Cement (CAAC), Calcium Aluminate α-Aluminate Plus Cement

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(CAAC Plus) and a mixture of 1-1 Wollastonite and CAAC cement (WOLCA). CAAC contains calcium aluminates (60-70% CA, 10-15% CA2 and 0-5% C12A7) and alpha aluminate (α-Al$_2$O$_3$, 5-15%). CAAC plus is a mixture of CAAC and 5% by weight sodium-hexametaphosphate (Na-HMP) to improve physical properties of CAAC.

In addition, wollastonite is a natural calcium silicate (CaSiO$_3$) with a theoretical composition of 48.3% CaO and 51.7% SiO$_2$ (Maxim and McConnell, 2005).

Physical properties like hardness, pH, setting time, plasticity and flow rate of these new materials have been studied but more studies are required to approve their use in clinical studies.

Furthermore, genotoxicity can be studied through *in vitro* and *in vivo* assessments developed to detect compounds that induce genetic damage such as DNA damage, gene mutation, chromosomal breakage, altered DNA repair capacityand cellular transformation. Genotoxicity methods are highly accepted as a main and useful indicator of carcinogenicity (Auletta and Ashby, 1988). Therefore, genotoxicity assessments are required for complete risk analysis of MTA and other endodontic cements.

The present study compared the genotoxicity effect of MTA and three new endodontic cements of sorel, light and alumina in human gingival fibroblasts cells.

MATERIALS AND METHODS

In this *in vitro* experimental study, the human gingival fibroblast cells were obtained from Iran Pasture Institute and collected in the culture specific flasks. The cells were incubated at 37°C for being grown in the culture medium of RPMI with 95% air and 5% CO$_2$. They were cultured 5 days before they will be tested with the selected materials. Cell suspension was counted using a Neubauer chamber. Then, they were exposed to 15% Trypsin in order to be separated.

MTA (Proroot, Dentsply Tulsa Dental, Tulsa, OK), CAAC, CAAC Plus and WOLCA cements were prepared according to the manufacturer's instructions under the aseptic conditions. All materials tested were prepared in final concentrations of 1, 10, 100 and 1000 µg mL^{-1}.

The selected materials were added to the gingival fibroblast cells for studying their effect on DNA for about 1 h at 37°C temperature. All groups were centrifuged for 5 min, washed with freshly culture medium and re-suspended in a fresh media. Then, COMET assay (single-cell gel electrophoresis) was done for examining the cement genotoxicity (Fig. 1).
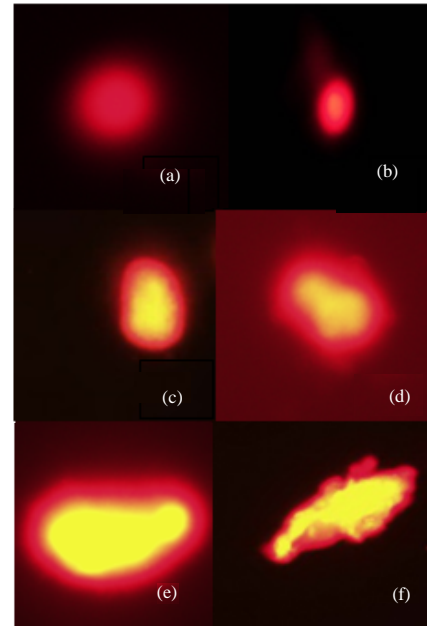

Fig. 1: Photographs from COMET assay showing human fibroblast gingival cells damage from undamaged a, to apoptotic f, cells

The protocol of COMET assay followed the standard recommendations existed in the literature. In brief, a volume of 10 µL of cells in each material was added to 120 µL of 0.5% low-melting point agarose at 37°C layering on a precoated slide with 1.5% regular agaroseand the lamel was placed onto it. After agarose setting in a refrigerator, the coverslip was removed and the slides immersed in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris-HCI buffer, pH 10, 1% sodium sarcosinate with 1% Triton X-100 and 10% DMSO) for 1 h. Prior to electrophoresis procedure, the slides were stored into alkaline buffer solution (pH>13) for 40 min and electrophoresed for 40 min at 25 V (0.86 V cm^{-1}) and 300 MA then. At the completion of electrophoresis, the slides were fixed in an absolute ethanol and stored at room temperature until the analysis will be done blindly in a fluorescence microscope at 400X magnification. An automatic analysis system (COMET Assay II softwar, perceptive.

Instruments, Haverhill, UK) was used in order to calculate DNA damage. Tail moment (result of tail DNA/total DNA by the center of gravity) was considered to determine DNA damage from 50 cells per treatment (Tice *et al.*, 2000; Hartmann *et al.*, 2003; De Oliveira *et al.*, 2007; Yavari *et al.*, 2009; Torabinejad and Parirokh, 2010). To decrease additional DNA damage from ambient ultraviolet radiation, all steps were performed with reduced illumination. All procedures were repeated another 2 times for all cements.

Results of COMET assay subjected to Kruskal-wallis (for overall analysis) and Mann-Whitney U (for paired comparisons in total concentrations) nonparametric tests using SPSS software (SPSS Inc., Chiago, IL). In this analysis, the level of statistical significance was determined to be 5%.

RESULTS AND DISCUSSION

The results of single cell gel (COMET) assay were shown in Table 1. No significant effect was observed regarding the cement studied concentrations at 1, 10, 100 and 1000 $\mu g\ mL^{-1}$ (p = 0.33) on the DNA damage. Then, the effect of different concentrations was ignored in the statistical assessments. However, the effect of different cement types on DNA damage was statistically significant (p<0.0001).

Significantly higher values of DNA damage were noted in the positive control group (2.25±0.67) when compared to the experimental cements suggesting the validity of the COMET assay in genotoxicity experiments. In the paired comparisons, no significant differences were noted between MTA and CAAC materials in terms of DNA damage in total concentrations (Mann-Whitney U test; p = 0.08) however, MTA resulted in significantly lower values of DNA damage when compared to CAAC-Plus (p<0.0001) and WOLCA cements (p<0.04). Furthermore, CAAC cement caused significantly lower values of DNA damage than CAAC-Plus (p<0.0001) and WOLCA was associated with significantly lower values of DNA damage than CAAC-Plus (p<0.0001). Furthermore, no significant differences were observed between CAAC and WOLCA cements regarding their genetoxicity expressed through DNA damage (p = 0.69). In total, MTA showed the least genotoxicity and CAAC-Plus demonstrated the most values of genotoxicity when exposed to human fibroblasts.

The single cell gel (COMET) assay is a standard, valid and sensitive method for the diagnosis of DNA damage caused by genotoxic materials in individual cells. The method has distinct advantages in term of its applicability in almost all kinds of cell types including human gingival fibroblast cells which was used in the present study. The use of human cells to investigate the mechanism of DNA damage was done as it has been well documented in the literature (Ribeiro *et al.*, 2006a).

Table 1: Mean±standard deviation of DNA damage (tail moment) in human gingival fibroblast cells exposed to MTA, CAAC, CAAC-Plus and WOLCA cements

Concentration ($\mu g\ mL^{-1}$)	MTA	CAAC	CAAC-Plus	WOLCA
1000	0.97±0.24	1.02±0.24	1.23±0.34	0.99±0.22
100	0.96±0.25	1.02±0.24	1.23±0.35	0.99±0.22
10	0.97±0.25	0.96±0.22	1.19±0.34	1.01±0.24
1	0.94±0.28	0.98±0.24	1.18±0.34	0.99±0.25

In addition as no investigation compared the newly developed cements of CAAC, CAAC-Plus and WOLCA with MTA in terms of their genotoxicity; the conduction of the present study was needed. The results of the present study indicated in the experimental conditions, MTA had the least DNA damage values than other 3 cements, although CAAC cement showed DNA damage similar to MTA. However, significantly higher DNA damage values were noted in CAAC-Plus and WOLCA cements than MTA. Therefore, possibly similar results can be obtained from genotoxicity tests of MTA and CAAC. Despite this finding, more studies are required to obtain more detailed judgment on the genotoxic potential of the studied materials.

DNA damage (tail moment) values of MTA were found to be in 0.94-0.97 range; of CAAC in 0.96-1.02 range; of CAAC-Plus in 1.18-1.23 range and of WOLCA was in 0.99-1.01 range being very close in the studied concentrations of 1, 10, 100 and 1000 $\mu g\ mL^{-1}$. The values of DNA damage reported in the present study was similar to the findings of Ribeiro *et al.* (2005) which were in the range of 0.59-0.85 in the concentrations of 1, 10, 100 and 1000 $\mu g\ mL^{-1}$ for MTA, although their results were lower for concentration of 1 $\mu g\ mL^{-1}$ than ours.

Results of 3 cell culture investigations showed that MTA (gray and white) exhibit no cytotoxicity and genotoxicity on various cell lines (Ribeiro *et al.*, 2005b, 2006b; Da Silva *et al.*, 2006). Other studies reported similar findings and showed that Portland cement as well as MTA have no cytotoxicity and genotoxicity in the concentrations of 1-1000 $\mu g\ mL^{-1}$ for 1 h exposure at 37°C (Braz *et al.*, 2006; Ribeiro *et al.*, 2006c). The findings were proved in the present study on MTA.

CAAC showed similar genotoxicity to MTA in this study. It is a biocompatible compound approved by some *in vitro* investigations assessing the biocompatibility of similar calcium aluminate cements (Kimura *et al.*, 1991; Franz *et al.*, 2006). Furthermore, WOLCA cement is a mixture of CAAC and Wollastonite. Wollastonite is also a naturally occurring calcium silicate and its composition is similar to MTA, although WOLCA genotoxicity was significantly higher than MTA. Previously, the biocompatibility of MTA and other calcium silicate cements has been proved (De Morais *et al.*, 2006; Lalis *et al.*, 2009; Fernandes *et al.*, 2010; McNamara *et al.*, 210).

CAAC Plus contains 5% by weight Na-HMP as a dispersant to get some advantages. Due to the increased DNA damage in this cement; it seems that adding this agent to CAAC increased its genotoxicity.

Hesaraki *et al.* (2009) evaluated the effects of adding Na-HMP on the basic characteristics of calcium

phosphate cement and concluded that although Na-HMP made the cement more stable and improved its injectability, it weakened other basic properties of the cement like compressive strength and increased its setting time.

Previously, the ability of Na-HMP to change the electrical charge of the materials has been shown (Hesaraki *et al.*, 2009) which change the proteins and cells absorbed to the material's surface (Thevenot *et al.*, 2008). This issue can also explain the difference between this cement and CAAC regarding their genotoxicity.

In vitro investigations are simple and inexpensive to perform however, they can provide significant information regarding the possible application of different materials in clinical conditions. In addition, experimental studies can be conducted under controlled conditions deleting the effects of confounding factors then the results obtained from *in vitro* assays can be indicative of the effects shown *in vivo*. The information obtained in the present study about different studied cements can be used as a guide for their possible use in the clinical environment.

CONCLUSION

The results of the present study indicated significantly higher genotoxicity for CAAC-Plus and WOLCA cement than MTA, however the values obtained for CAAC cement was comparable to MTA suggesting its possible use *in vivo*.

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