INTRODUCTION

Biocompatibility refers to the quality of not having toxic or injurious effects on biological systems. A new material in endodontic practice, mineral trioxide aggregate (MTA) (which is grey in color), was developed in the 1990s. The main ingredients of MTA are tricalcium and dicalcium silicates, tri-calcium aluminate, tri-calcium oxide and silicate oxide. Bismuth oxide has been added as a radiopacifier. A number of in vivo and in vitro studies have been done on this material, with MTA having good biocompatibility results. Recently, a novel endodontic cement has been introduced to dentistry: calcium enriched mixture (CEM) cement. CEM comprises water-soluble calcium and phosphate (calcium hydroxide, calcium oxide, calcium phosphate, calcium sulfate, calcium silicate, and calcium carbonate), and forms hydroxyapatite after setting. Its physical properties conform to ISO 6876:2001. The clinical uses of CEM are the same as MTA; they have similar working time, pH, and dimensional stability. Asgary et al. found that CEM had significantly more antibacterial properties than MTA and CEM can stimulate hard tissue healing and can set in humid environments. It has appropriate setting time and good handling characteristics and it has an excellent seal when used as a root-end filling biomaterial. Nevertheless, further biocompatibility assessment should be done to evaluate the risk of using this cement.

Genotoxicity assays are of special importance in evaluating biocompatibility, because they have achieved general acceptance as serious and useful indicators of carcinogenicity. Many different systems for detecting genotoxicity have been developed. Animal systems may more accurately reflect human metabolism; however, they are expensive and time-consuming (requiring up to 3 years to complete); they are, therefore, not used as a first screen for genotoxicity or carcinogenicity. Thanks to the work of previous investigators, the single-cell, gel (comet) assay has been accepted as a rapid, simple, and reliable method of evaluating the genotoxicity of materials. The basic principle of this assay is the migration of DNA fragments in an agarose matrix under electrophoresis. When viewed under a microscope, cells have the appearance of a comet, with a head (the nuclear region) and a tail containing DNA fragments or strands migrating toward the anode. The studies of Ribeiro and coworkers have demonstrated that the single-cell gel (comet) assay is one of the suitable tools to investigate the genotoxicity of compounds used in dental practice.

Previous studies have demonstrated that MTA, whether gray or white, cannot induce geno- or cytotoxicity in mouse lymphoma or Chinese hamster ovary (CHO) cells, even after exposure to human peripheral lymphocytes ex vivo.

Taking into consideration that fibroblasts are in direct contact with MTA or CEM over extended periods of time, it would be interesting to know whether, and to what extent, these two materials can exert geno- and/or cytotoxic effects in fibroblasts. Therefore, the aim of the present study was to evaluate the cytotoxic and genotoxic effects of calcium enriched mixture (CEM) compared with MTA using MTT and single-cell gel (comet) assays with serial ascending concentrations (0 to 1,000 µg/mL) of tested materials. Cytotoxicity data indicated that there is no significant difference between CEM and MTA at all concentrations except for the full concentration (1,000 µg/mL); CEM had lower cytotoxicity. Genotoxic effects were more evident with CEM at concentrations of 15.6 and 250 µg/mL; however, was less than that of MTA at concentrations of 500 and 1,000 µg/mL. The cytotoxicity and genotoxicity effects of the two experimental groups generally increased with consistency. Under the conditions of this study, CEM is biocompatible in terms of cyto- and genotoxicity. It appears to be an alternative to MTA as an endodontic biomaterial offering several advantages.

Keywords: Biocompatible, Calcium enriched mixture, Cytotoxicity, Genotoxicity, Mineral trioxide aggregate

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genotoxic effects of MTA and CEM in L929 mouse fibroblasts by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT) and single-cell gel (comet) assays in vitro.

METHODS AND MATERIALS

Cell line and reagents

The L929 mouse fibroblast cell line was obtained from the Pasteur Institute (Tehran, Iran, NCBI code 161). ProRoot MTA and CEM were provided by Dentsply Tulsa Dental (Tulsa, OK) and BioniqueDent (Tehran, Iran), respectively. High-glucose Dulbecco’s Modified Eagles Medium (DMEM, 4.5 g/L) and fetal bovine serum (FBS) were purchased from Gibco (Carlsbad, CA). The MTT and other cell culture materials were purchased from Sigma (St. Louis, MO). Low melting point (LMP) agarose and normal melting point (NMP) agarose were obtained from Fermentas (Glen Burnie, MD). Other chemicals, mainly ethylene diaminetetraacetic acid disodium salt (Na₂EDTA), Tris (hydroxymethyl) aminomethane (Trizma base), t-octylphenoxy poly-ethoxyethanol (Triton X-100), dimethyl sulfoxide (DMSO), sodium laurylsarcosinate (sarkosyl), and ethidium bromide were purchased from Merck (Darmstadt, Germany).

Cell culture

The L929 cells were cultured in DMEM supplemented with 2 mM Glutamine, 10% FBS, and 100 units/mL of penicillin/streptomycin. Cultures were supplied with fresh medium every other day and incubated in a 37°C humidified incubator with 5% CO₂ and 95% air. Confluent cells were detached with a mixture of 0.25% trypsin or trypsin/EDTA. Aliquots of separated cells were then subcultured. Cell cultures between the fourth and sixth passages were used for all experimental procedures.

Cell proliferation (MTT) assay

The MTT assay is a colorimetric assay for measuring the activity of enzymes that reduce MTT to formazan dyes, giving a purple color. A major application allows assessment of cell viability (cell counting) and cell proliferation (cell culture assays). It can also be used to determine the cytotoxicity of potential medicinal agents and toxic materials, since those agents stimulate or inhibit cell viability and growth.

The L929 cells were seeded in 96-well tissue culture plates with 5×10⁴ cells in 100 µL media per well and, after 24 h, the cells were treated with MTA or CEM for 24 h. White ProRoot MTA (Dentsply, Tulsa Dental, Tulsa, OK, USA) was prepared according to the manufacturer’s direction: as a mixture of powder (20 mg) and normal Ringer (0.2 mL) in a slurry form. A recently introduced biomaterial called CEM cement (BioniqueDent, Tehran, Iran) was also prepared as a creamy mixture with phosphate solution (i.e. CEM vehicle; 20 mg/0.2 mL normal Ringer). Then, 0.05 mL of the mixtures was separately added to the recording chamber containing extracellular solution.

The materials tested were prepared in serial dilutions ranging from 1,000 to 0 µg/mL (1,000, 500, 250, 125, 62.5, 31.25, 15.6, 7.8, 0). These doses were chosen based on the IC₅₀ (concentration giving 50% inhibition) of each material, calculated from earlier experiments. Cell culture without treating and were treated with methyl methansulfonate used as negative and positive control, respectively. MTT solution in phosphate-buffered saline (PBS, 5 mg/mL) was added to a final concentration of 0.05%. After 2 h, the formazan precipitate was dissolved in DMSO containing 10% glycine buffer (pH=10.5). The microplates were then gently shaken in the dark for 30 min, and the absorbance at 570 and 620 nm (background) was measured using a StatFAX303 plate reader.

All experiments were carried out in triplicate; the percentage of viable cells was calculated as the mean±SEM, with controls set at 100%. Morphological deformation of the cells was also examined.

Single-cell gel electrophoresis (SCGE, comet) assay

The alkaline SCGE assay was conducted based on the method described previously by Hosseinzadeh et al. Briefly, the L929 cells (3×10⁵) were preincubated with different concentrations of MTA or CEM for 24 h. The materials tested were prepared in serial dilutions ranging from 0 to 1,000 µg/mL (1,000, 500, 250, 125, 62.5, 31.25, 15.6, 7.8, 0). After the medium was removed, the cells were washed 3 times with cold PBS, harvested, and centrifuged at 3,000 rpm for 5 min at 4°C. The pellets were then resuspended in PBS at a cell density of 1×10⁵. For the comet assay, 100 µL NMP agarose was quickly layered on conventional slides, covered with cover slips, then the slides were placed on ice to allow the agarose to gel. Ten microliters of the nuclear suspension, prepared as above, was mixed with 100 µL LMP agarose, and the mixture was quickly layered over the NMP agarose layer after removing the cover slips. Finally, another layer of LMP agarose was added on top. The slides were immersed immediately in a chilled lysing solution (pH=10) made up of 2.5 M NaCl, 100 mM Na₂EDTA, 10 mM Trizma, 1% sarkosyl, 10% DMSO, and 1% Triton X-100, and kept at 0°C in the dark overnight. Then the slides were placed on a horizontal gel electrophoresis platform and covered with a prechilled alkaline solution made up of 300 mM NaOH and 1 mM Na₂EDTA (pH>13). They were left in the solution in the dark at 0°C for 40 min, and then electrophoresed at 0°C in the dark for 30 min at 25 V and approximately 300 mA. The slides were gently rinsed 3 times with 400 mM Trizma solution (adjusted to pH 7.5 with HCl) to neutralize the excess alkali, stained with 50 µL of 20 µg/mL ethidium bromide, and covered with a cover slip.

For comet analysis, 100 nuclei were randomly selected from 2 replicate slides (50 nuclei on 1 slide), examined, and photographed through a fluorescence microscope (Nikon, Kyoto, Japan) at 400× magnification, equipped with an excitation filter of 520–550 nm and a barrier filter of 580 nm. Undamaged cells resemble an intact nucleus without a tail, and damaged cells have
the appearance of a comet. The percentage of DNA in the comet’s tail (percentage of tailed DNA), which is an estimation of DNA damage, was analyzed using computerized image analysis software (CASP software). The experiments were done in triplicate.

**Statistical analysis**
The Shapiro-Wilk test was used to assess the normality of sample distribution. The results indicated that the samples were distributed normally. Levene’s test was also used to assess the equality of variance in different samples. The resulting p value (0.415) indicated that there was no difference between the variance in population. The results are presented as mean±standard error. The values were compared by 2-way ANOVA. The level of statistical significance was set at 5%.

**RESULTS**
The cytotoxicity of CEM compared with MTA was measured in mouse fibroblasts using the MTT assay prior to determining chemically induced genotoxicity. The results from the dose-response relationship for each material at concentrations ranging from 0 to 1,000 µg/mL are shown in Fig. 1. No statistically significant difference was found between CEM and MTA at any concentration (0 to 500 µg/mL; p=0.346) except for the highest concentration (1,000 µg/mL; p=0.019).

The single-cell gel (comet) assay was used to measure DNA damage in mouse fibroblasts produced by CEM compared with MTA in vitro. DNA damage was represented as percentage of tailed DNA in 300 comets per sample. Data are expressed as mean±standard deviation in Table 1. The results from the dose-response relationship for each material at concentrations ranging from 0 to 1,000 µg/mL are shown in Figs. 2 and 3. Genotoxic effects were more evident in the CEM group at low and medium concentrations (15.6 and 250 µg/
mL) compared with MTA ($p<0.001$); however, it was less evident at high concentrations (500 and 1,000 µg/mL) ($p<0.001$).

Images of comet assay results of non-damaged and damaged DNAs in CEM and MTA group are presented in Figs. 4 and 5, respectively.

**DISCUSSION**

Most materials used in dentistry are in contact with oral tissues over extended periods of time. Biochemical assays that evaluate the effects of dental materials on genetic tissues are relevant for minimizing risks to patients and clinicians. Antibacterial activity, cytotoxicity, clinical applications, and subcutaneous
tissue reactions of CEM have been evaluated in several studies\textsuperscript{22,23}. To our knowledge, however, this is the first study in which the genotoxic effects of CEM have been demonstrated \textit{in vitro} by a single-cell gel electrophoresis (comet) assay. Therefore, and taking into account the lack of data currently available, the assessment of the potential genotoxicity of CEM is justified. In addition, it has been reported in previous studies that MTA has no cytotoxic\textsuperscript{7,15,20,31,32} and no genotoxic\textsuperscript{15,20,33,34} effects \textit{in vitro}. For these reasons, the aim of this study was to evaluate the biocompatibility of CEM compared with MTA.

In \textit{vitro} studies are easy and inexpensive to perform, they provide a significant amount of information, they can be conducted under controlled conditions, and they may clarify mechanisms of cellular toxicity\textsuperscript{15}. The results obtained from \textit{in vitro} assays might be indicative of the effects observed \textit{in vivo}. Cell culture studies are commonly used in the evaluation of cytotoxicity and potential genotoxicity.

In this study, the MTT assay was used to determine the biocompatibility of these materials. It is a useful method for evaluating the cytotoxicity of a material, owing to its simplicity, speed, and precision\textsuperscript{35}. The assay identifies viable cells after coming in contact with extracts of the test materials. Also in this study, increasing concentrations of the test materials (MTA and CEM) were prepared, and L929 mouse fibroblasts were used to simulate the root-end environment. The dose-response relationships of the 2 compounds on cell viability were assessed by MTT at concentrations ranging from 0–1,000 µg/mL. Osorio and his coworkers, using a radiochromium method and MTT assay, found that MTA was nontoxic to cultures of L929 fibrosarcoma cells\textsuperscript{32}. Cytotoxicity data obtained in our study demonstrated that there was no significant difference between CEM and MTA at all concentrations from 0 to 1,000 µg/mL except for the final concentration (1,000 µg/mL).

The single-cell gel (comet) assay is a sensitive method for detecting DNA damage induced by genotoxic compounds in cells. The alkaline version, used in this study, can detect a variety of DNA lesions and incomplete repair sites\textsuperscript{22,23}. Since the introduction of the alkaline comet assay in 1998\textsuperscript{20}, a number of advances have greatly increased the flexibility and utility of the technique for detecting various forms of DNA damage. According to Kumaravel percentage of tailed DNA is a virtual measure calculated by a computerized image analysis system considering the tail intensity (percentage of DNA in the comet tail)\textsuperscript{30}.

In other studies, treatment time with the test substances varied from 1 h\textsuperscript{22,23,34} to 3 h\textsuperscript{15,20,33,34}, but we exposed cells for a longer time (24 h), as described by Hosseinizadeh et al\textsuperscript{22,23}. This may explain the different results for MTA in our study.

The genotoxic effects of MTA and other root-end filling materials were assessed in previous studies\textsuperscript{15,20,21,33,34}. Although their findings indicated that MTA cannot induce genotoxicity in mouse lymphoma or Chinese hamster ovary cells or even following exposure to human peripheral lymphocytes, but some studies suggest that MTA is not an inert material\textsuperscript{37}. Balto\textsuperscript{38} evaluated the morphology of human fibroblast in contact with MTA and observed that only a small number of viable cells remained adhered to the fresh material and that some cells exhibited morphological alterations. Ovir et al\textsuperscript{39} have demonstrated that white MTA was more biocompatible than grey MTA. They attributed this result to the different components present in the formulation cements.

Duarte et al\textsuperscript{40} reported that MTA displayed very low amount of arsenic release thus demonstrating no contraindication for the use of MTA in clinical practice in terms of this chemical element. In the present study damage of cells by MTA was greater than that of CEM at higher concentrations. Maybe the reason for this feature is the high amount of arsenic in the medium containing MTA.

In spite of the critical role of calcium ions in the tissue repair process and also its combination with water leading to the setting expansion of material such as MTA and CEM, a high release of these ions in the culture medium may result in irreversible damage to the cell culture\textsuperscript{41}.

It should be emphasized that the results obtained from this preliminary genotoxicity test had limitations therefore further studies employing CEM are required to demonstrate its genotoxicity in different methods of evaluation such as LDH, analysis of qRT-PCR, micronucleus, necrosis and apoptosis assay by FACS, conflocal laser microscope.

**CONCLUSION**

Under the limitation of this \textit{in vitro} study, we conclude that the favorable results for CEM compared with MTA indicate that CEM appears to be an alternative to MTA as an endodontic biomaterial, with several advantages.

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