

In vitro cytotoxicity of four calcium silicate-based endodontic cements on human monocytes, a colorimetric MTT assay

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Objectives: This study was performed to evaluate the cytotoxicity of four calcium silicate-based endodontic cements at different storage times after mixing. **Materials and Methods:** Capillary tubes were filled with Biodentine (Septodont), Calcium Enriched Mixture (CEM cement, BioniqueDent), Tech Biosealer Endo (Tech Biosealer) and ProRoot MTA (Dentsply Tulsa Dental). Empty tubes and tubes containing Dycal were used as negative and positive control groups respectively. Filled capillary tubes were kept in 0.2 mL microtubes and incubated at 37°C. Each material was divided into 3 groups for testing at intervals of 24 hr, 7 day and 28 day after mixing. Human monocytes were isolated from peripheral blood mononuclear cells and cocultured with 24 hr, 7 day and 28 day samples of different materials for 24 and 48 hr. Cell viability was evaluated using an MTT assay. **Results:** In all groups, the viability of monocytes significantly improved with increasing storage time regardless of the incubation time ($p < 0.001$). After 24 hr of incubation, there was no significant difference between the materials regarding monocyte viability. However, at 48 hr of incubation, ProRoot MTA and Biodentine were less cytotoxic than CEM cement and Biosealer ($p < 0.01$). **Conclusions:** Biodentine and ProRoot MTA had similar biocompatibility. Mixing ProRoot MTA with PBS in place of distilled water had no effect on its biocompatibility. Biosealer and CEM cement after 48 hr of incubation were significantly more cytotoxic to on monocyte cells compared to ProRoot MTA and Biodentine. (*Restor Dent Endod* 2014;39(3):149-154)

Key words: Biodentine; Biosealer; Calcium enriched mixture cement; Cytotoxicity; Mineral trioxide aggregate

Introduction

Mineral trioxide aggregate (MTA) is suggested for use in many challenging endodontic procedures such as apexogenesis, apexification, perforation repair and apical surgery in the expectation it will promote healing of pulpal and periradicular tissues.^{1,2} Indeed, several studies have demonstrated the biocompatibility and good biologic properties of MTA.³⁻⁶

Recently, new materials have been introduced as alternatives to MTA. Biodentine (Septodont, Saint Maur-des Fossés, France) is a relatively new calcium silicate cement. The main component of the powder is tricalcium silicate, with addition of CaCO₃ and ZrO₂. The liquid is composed of water and CaCl₂ that reduces the setting time.⁷ Biodentine has been reported to provide good biocompatibility, bioactivity, quick

setting and high compressive strength.^{8,9}

Calcium Enriched Mixture (CEM cement, BioniqueDent, Tehran, Iran) consists of several calcium compounds, i.e. calcium oxide, calcium phosphate, calcium carbonate, calcium silicate, calcium sulfate and calcium chloride.¹⁰ It has been reported to have good handling characteristics, and an ability to form hydroxyapatite in contact with tissue fluid.¹¹ Mozayeni *et al.* and Ghoddosi *et al.* demonstrated that MTA and CEM had similar favorable biologic responses when cocultured with L929 fibroblasts.^{12,13} In another study using electronic scanning microscope, human gingival fibroblast cells displayed a favorable biologic response when in contact with MTA and CEM.¹⁴ MTA and CEM were also well tolerated following implantation in subcutaneous tissues in albino rats.¹⁵ In addition, Rahimi *et al.* implanted MTA and CEM in rat femoral bone and concluded that biocompatibility of both biomaterials are comparable.¹⁶

Tech Biosealer (Tech Biosealer Endo, Isasan SRL, Revello Porro, Italy) is another MTA-like cement. Its powder is a mixture of tricalcium silicate, beta dicalcium silicate (β -Ca₂SiO₄), anhydrous calcium sulfate, calcium carbonate and bismuth oxide. Its liquid is composed of Dulbecco's phosphate buffered saline (PBS).¹⁷ According to the manufacturer's instructions, Tech Biosealer Endo can be used for vital pulp therapy, perforation repair and root-end filling (www.isasan.com). Recently, Hakki *et al.* concluded that Tech Biosealer Endo significantly decreased the viability of cementoblasts.¹⁸

Considering the clinical applications of these materials, it is essential for them to be non-toxic. Since monocytes/macrophages play a key role in the healing process by participating in the innate and acquired immune systems, the increase of their phagocytic activity may accelerate wound healing.¹⁹⁻²¹ Therefore in the current study these cells were used to evaluate the cytotoxicity of the experimented materials.

The aim of this study is to evaluate the cytotoxicity of Biodentine, CEM cement, Tech Biosealer Endo and two different mixtures of MTA on human monocytes after 24 and 48 hours of incubation. Various additives and storage medium have been suggested for use with MTA to improve its physical properties and several studies have concluded that the interaction of MTA with PBS resulted in the formation of apatite crystals.²²⁻²⁷ Thus, although the manufacturer's instructions suggest that distilled water (DW) should be mixed with MTA, an additional experimental group was created in which MTA was mixed with PBS to compare its difference in cytotoxic effect. The null hypothesis of this study was that the new materials evaluated are similar to MTA in terms of cytotoxicity and with increasing the storage time their cytotoxicity will decrease.

Materials and Methods

Preparation of samples

Materials included tooth colored ProRoot MTA (Dentsply Tulsa Dental, Tulsa, OK, USA), Biodentine (Septodont), CEM cement (BioniqueDent) and Tech Biosealer Endo (Isasan). The materials were mixed on a sterile glass slab and introduced into capillary tubes having a 1-mm diameter and a 4-mm length. The materials at both ends of the tubes were flattened using a spatula and a moist cotton pellet was placed over each end with minimal pressure.²⁸ All samples were placed into 0.2 mL microtubes (Eppendorf-Elkay, Shrewsbury, MA, USA) and a moist cotton pellet was then placed above but not in contact with the samples.²⁹ All samples were placed in an incubator (37°C, 95% humidity and 5% CO₂). The samples of each material were divided into 3 groups for testing at three storage times (24 hours, 7 days and 28 days after mixing). All groups were then exposed to isolated human monocytes for 24 and 48 hours.

The experimental groups were prepared in sterile condition as follows:

ProRoot MTA + DW: Mixing of MTA was standardized by placing 1 g of tooth colored ProRoot MTA powder and 0.33 mL of DW in a plastic mixing capsule containing a plastic pestle. The capsules were placed in an amalgamator (Promix, Dentsply Caulk, York, PA, USA) and the material and liquid were mixed mechanically for 30 seconds.³⁰

ProRoot MTA + PBS: Preparation of these three groups was the same as groups 'ProRoot MTA + DW' but PBS (Merck, Darmstadt, Germany, pH = 7.4) instead of DW was used for mixing.

Biodentine: According to the manufacturer's instruction, 5 drops of Biodentine liquid were added to the capsule containing the Biodentine powder (1 g). The plastic capsules were placed in an amalgamator and mixed mechanically for 30 seconds.

Tech Biosealer: According to the manufacturer's instruction, 2 drops of Tech Biosealer Endo liquid was added to Biosealer powder (1 g) and mixed with a spatula on a glass slab for 1 minute.

CEM cement: CEM cement samples were prepared by mixing 1 g of powder with 0.33 mL of the liquid supplied using a spatula on a glass slab for 1 minute.

Before the cytotoxicity assay, all samples were sterilized by gamma radiation using a 25 kilo Grays dose (ISO 11137-2012) and then exposed to blood monocytes.

Isolation of human monocytes

Fresh buffy coats (Tehran Blood Transfusion Center, Tehran, Iran) from consented healthy donors were used for isolation of human monocytes. The buffy coats were diluted

with PBS and peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation over a Ficoll-Paque gradient (Lymphodex 100 mL, inno-Train Diagnostic GmbH, Kronberg, Germany). CD14⁺ monocytes were positively selected from PBMCs using MACS CD14 microbeads (Miltenyi Biotec, Teterow, Germany). The purity of separated monocytes was assessed by flow cytometry (> %95 of recovered cells were CD14⁺ monocytes).

Cytotoxicity assay

2 × 10⁵ monocytes were seeded into the wells of four 96-well microplates containing 200 μL of culture medium (RPMI 1640, GIBCO, Carlsbad, CA, USA). The prepared samples of test and control groups were then individually added to wells (n = 6) 30 minutes after seeding. Plates were incubated at 37°C in 5% CO₂ for 24 or 48 hours. Four hours before the end of the incubation time, 20 μL 3-(4, 5-dimethylthiazol-2-yl) 2,5diphenyltetrazolium bromide solution (MTT, Sigma-Aldrich, St. Louis, USA, 5 mg/ml) was added to each well. After 4 hours of incubation, plates were centrifuged and culture medium was removed. Precipitated formazan crystals were dissolved by adding 200 μL solvent (Dimethyl sulfoxide) to each well. The microplates were shaken at room temperature for 10 minutes and prepared for reading by a microplate reader at 570 nm. The percentage of metabolic activity was calculated using the formula: (Test optical density / control optical density)*100. Monocytes that were cultured in the empty capillaries were considered as negative controls. As a

positive control, cells were cultured in medium containing capillaries filled with Dycal (Dentsply DeTrey, Konstanz, Germany).⁵ Statistical analysis of the data was performed by using two-way analysis of variance and Tukey multiple comparison post test, with significance of p < 0.05.

Results

Cell viability of human monocytes after 24 and 48 hours incubation with control and test groups at three storage times (24 hours, 7 days and 28 days after mixing of the materials) are shown in figure 1. There was no significant difference in monocytes viability amongst the test groups after 24 hours of incubation at three storage time (24 hours, 7 days and 28 days after mixing, Figure 1a). However, at 48 hours of incubation, monocytes cocultured in the presence of ProRoot MTA (with DW and/or PBS) and Biodentine had a significantly greater percentage of viability than Biosealer and CEM cement groups (p < 0.001) (Figure 1b). No significant difference was seen between Biodentine and ProRoot MTA when mixed with DW or PBS. In addition, there was no significant difference between Biosealer and CEM cement specimens in this incubation period. There was no significant difference in cytotoxic effect of ProRoot MTA when mixed with PBS instead of DW regardless of the incubation time.

In all tested groups, monocyte viability improved significantly with increasing the storage time regardless of the incubation time. Therefore the cell viability in samples stored for 28 days was significantly greater than samples

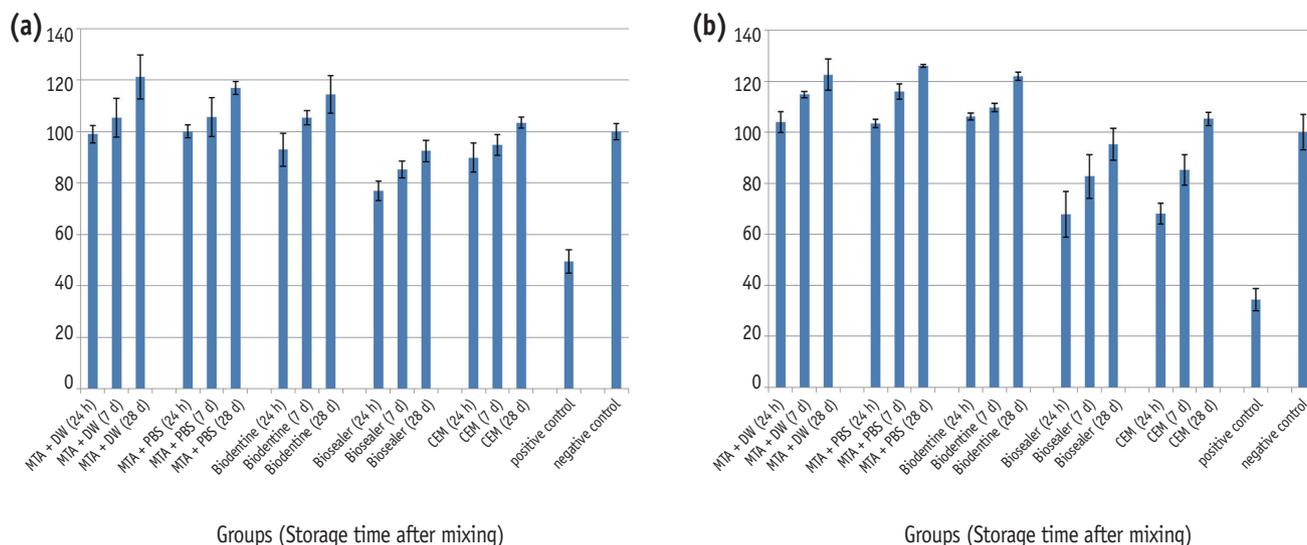


Figure 1. The metabolic activity of human monocytes in different experimental groups and storage times. (a) After 24 hours incubation time; (b) After 48 hours incubation time. MTA, Mineral trioxide aggregate; CEM, Calcium enriched mixture; DW, Distilled water; PBS, Phosphate buffered saline.

stored for 7 days or 24 hours ($p < 0.05$). Furthermore, monocyte viability in 7-day samples was significantly greater than samples stored for 24 hours ($p < 0.05$) and the 24-hour samples showed the lowest monocyte viability ($p < 0.05$). There was no significant difference amongst five tested groups at each storage time (24 hours, 7 days and 28 days after mixing) regarding cytotoxicity when comparing 24 and 48 hours of incubation. In the positive control wells, monocyte viability of the 48-hour incubation samples was lower than the 24-hour incubation samples ($p < 0.05$). Moreover, after 24 and 48 hours of incubation, there was significant difference in monocyte viability between each material and the positive control.

Discussion

ProRoot MTA and Biodentine at 48 hours of incubation had significantly less cytotoxic effect compared to Biosealer and CEM cement. This difference may be due to the specific chemical compositions of these cements and requires more research. For instance CEM cement has more sulphate and calcium oxide in its composition and Biosealer has higher levels of calcium carbonate in comparison to MTA.^{31,17}

Several methods have been used to evaluate cell viability including Trypan blue solution, MTT assay and MTS assay. Trypan blue solution can only distinguish between viable or dead cells.³² MTT is a colorimetric assay based on the ability of mitochondrial dehydrogenase enzymes in living cells to convert the yellow water-soluble tetrazolium salt into dark blue formazan crystals.^{33,34} The amount of formazan produced is directly proportional to the viable cell number.^{34,35} Methylthiazol sulfophenyl assay (MTS) is composed of 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium. Its advantage is that the formazan crystals are soluble in tissue culture media and therefore the solving procedure is omitted.² In this study, viability of cells was determined based on the MTT assay because of its simplicity, precision and accessibility.³³

Although MTA has various promising properties, its handling characteristics and setting time is less than ideal.^{28,36} It has been suggested that different additives or storage media for MTA can improve its properties.^{37,38} Gandolfi *et al.* concluded that soaking MTA samples in PBS improved its setting time and expansion.²⁵ Other studies have demonstrated that the physical properties of MTA improved in contact with PBS; therefore, it might be beneficial to use PBS as a mixing agent with MTA.^{24,27,37} Indeed, the results of the present study revealed that mixing MTA with PBS instead of DW did not alter its biocompatibility.

Biodentine is a relatively new calcium silicate-based material with high compressive strength and short setting time, which is suggested as an appropriate substitute for

dentin.³⁹ Han *et al.* concluded that Biodentine formed a significantly thicker Ca- and Si-rich layer compared to ProRoot MTA.⁴⁰ Zhou *et al.* reported that Biodentine was similar to MTA in terms of gingival fibroblast reaction.⁴¹ In a recent study, it was found that Biodentine and ProRoot MTA had acceptable biologic effects.⁴² In accordance with previous studies, the result of this study revealed no significant difference between the ProRoot MTA specimens and Biodentine regarding cytotoxicity on monocyte cells.^{41,42}

Another material evaluated in this study was CEM cement, which has the same clinical applications as MTA.⁴³ Mozayeni *et al.* investigated the effect of CEM cement, IRM and MTA on fibroblasts viability by MTT assay.¹² They found that set MTA and set CEM cement had similar effects on cell viability, which were enhanced with increasing time after mixing. In the present study, monocyte viability in CEM samples improved significantly with increasing storage time. This finding is consistent with the results of the study by Mozayeni *et al.*, although in their study fresh MTA displayed significantly greater cell viability compared to fresh CEM and in the present study monocyte viability at 48 hours of incubation significantly decreased in CEM samples compared to MTA samples.¹² In the present study, cell cytotoxicity associated with CEM and Biosealer specimens was significantly higher than MTA and Biodentine, but it was significantly lower than Dycal specimens at all storage times. Dycal was chosen as positive control since it has been shown to be toxic to cells.⁵

Tech Biosealer is an MTA-like material available in four types including Tech Biosealer Endo, Tech Biosealer Root End, Tech Biosealer Apex and Tech Biosealer Capping (www.isasan.com). Gandolfi *et al.* reported high releases of calcium and hydroxyl ions in ProRoot MTA and Tech Biosealer Root End.¹⁷ In addition, Tech Biosealer Capping released greater amounts of calcium compared to ProRoot MTA, Pulpdent and Dycal.⁴⁴ Hakki *et al.* evaluated the response of cementoblasts to Tech Biosealer and reported a significant decrease in cell viability.¹⁸ Their results were in accordance with the present findings that Tech Biosealer Endo had the lowest cell viability compared to other calcium silicate cements.

In all tested groups of the present study monocyte viability significantly improved with increasing storage time regardless of the incubation time, and this may be due to the decrease in leached cytotoxic substances from the materials with increasing storage time, thereby decreasing their cytotoxic effects on cells. Overall the results of the current study supported the second half of our hypothesis but regarding the first half, it can be concluded that not all the tested materials showed similar cytotoxic effects. However amongst them, only Biodentine demonstrated similar cytotoxicity to MTA, and CEM Cement and Tech Biosealer were more cytotoxic.

Conclusions

ProRoot MTA and Biodentine at 48-hour incubation was significantly less cytotoxic on monocyte cells compared to Biosealer and CEM cement. Biosealer and CEM cement showed similar biocompatibility as ProRoot MTA and Biodentine. Mixing ProRoot MTA with PBS instead of DW had no effect on biocompatibility.

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