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Assessment of Gingival Fibroblast Attachment to Root Surfaces Restored with Three Different Dental Materials: An in Vitro Study

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Abstract

Background and aims. The aim of this study was to compare the biocompatibility of calcium-enriched mixture cement (CEM), composite resin and nano-particled mineral trioxide aggregate (NP-MTA) using human gingival fibroblasts.

Materials and methods. A comparative in vitro cell culture study was carried out using 60 single-rooted teeth which were assigned to the following four groups: 1) untreated healthy group (control); 2) restored with composite resin; 3) CEM cement; 4) NP-MTA. The MTT assay was used to measure the viability of fibroblasts attached to each specimen and scanning electron microscopy (SEM) was used for describing cell morphology.

Results. After 24 hours of incubation, the survival rates for composite resin and NP-MTA were 74.1% and 76.9%, respectively, which were significantly lower than the value in the control group, while both were equally biocompatible. No statistically significant difference was found between the control group and CEM cement samples (94.3%). After 3 days of incubation, some increases in the viability of fibroblasts were detected in the composite resin and NP-MTA groups, with their survival rates being 89% and 93%, respectively. Conversely, in the CEM cement group, the survival rate decreased to 80.7%, which was significantly lower than that in the control group (P = 0.0001).

Conclusion. The results of in vitro tests indicated that on days 1, 3 and 5 after incubation, composite resin, CEM cement and NP-MTA exhibited acceptable biocompatibility, provided they were allowed to set for 24 hours before exposure to the cells.

Key words: **Gingival fibroblast attachment, Mineral trioxide aggregate, Calcium-enriched cement.**

Introduction

Damage to the periodontal attachment may result from periodontal disease and/or iatrogenic factors such as tooth bleaching; orthodontic movements, endodontic flare-ups and acute trauma.[1] These damages may lead to defects like cervical resorption and root perforation. They may appear just below the junctional epithelium or on the root surface, which then may result in associated bony defects.[1,2] There is an ongoing debate on the most suitable material for repair of root defects depending on the extent and depth of resorption.[3-7] The material used is critical as it should be biocompatible, with good sealing ability.[8] Various materials such as MTA, compomer, amalgam, composite resin, resin-modified glass-ionomer, reinforced zinc oxide-eugenol cement and glass-ionomer cement are frequently used to treat these defects.[7,9,10]

While MTA has proven to have ideal biocompatibility with many other favorable properties,[11-20] the difficulties in its handling and long setting time have led to numerous attempts in finding a proper alternative material.[4-21] CEM is a novel endodontic cement with promising properties.[22] Many studies have compared CEM and MTA and found that CEM has favorable properties, such as good biocompatibility, flow and sealing abilities similar to MTA. Also, CEM has good handling properties and shorter setting time compared to MTA, and does not lead to discoloration of the teeth.[23-28] Although CEM has been compared to MTA in many different aspects, most such studies have analyzed this material only in comparison to those of similar compositions (different brands of MTA and Portland cement). Studies comparing the qualities of CEM with those of other materials such as composite resins are lacking. Composite resins as esthetic dental materials are in demand. But, due to the release of unbound monomers and co-monomers, biocompatibility has remained a major concern.[29-35] However, some studies have shown that composite resin cytotoxicity is only high for a short period and diminishes over time.[36] In addition, many different additives have been used to overcome undesirable properties of MTA.[37] One attempt was the use of different nano-particle elements in the composition of MTA.[38,39] Nevertheless, NP-MTA is a relatively new material with insufficient evidence and research to support its use in the clinical setting.

Reformation of a normal attachment apparatus, including junctional epithelium and connective tissue attachment, is mainly carried out by fibroblast cells of gingival connective tissue. Most of the available studies used PDL fibroblasts for testing cell attachment properties of dental materials. However, the behavior and reactions of gingival and PDL fibroblasts are not similar from various aspects. The aim of this study was to compare the biocompatibility properties of CEM, composite resin and NP-MTA using human gingival fibroblasts.

Materials and Methods

This study was designed as a comparative cell culture study in vitro. Extracted teeth were collected after obtaining written informed consent from patients, and then 60 single-rooted teeth based on the inclusion criteria (healthy single-rooted teeth with no history of periodontal disease or any sort of defects) were selected for this study. They were assigned to the following four groups: 1) untreated healthy group (control); 2) restored with composite resin; 3) restored with CEM cement; 4) resorted with NP-MTA.

Preparation of Root Specimens

After removing the crown and the apices of the teeth, the middle third of each root was sectioned longitudinally in the buccolingual plane, and rectangular root plates were obtained, measuring approximately 5×5×7 mm. The defects were then artificially created on 45 root slices (except in controls). Before filling, all the slices were sterilized in 70% ethanol containing 4% antibiotic (penicillin-streptomycin) for 30 minutes. Then they were rinsed three times with phosphate-buffered saline (PBS) solution. Afterwards, the defects were restored with composite resin, NP-MTA and CEM under aseptic conditions. The materials were allowed to set for 24 hours before exposure to the cells.

Preparation of Test Materials

The materials tested were CEM (Bionique Dent, Tehran, Iran), composite resin (Z350, 3M ESPE, USA), and NP-MTA (experimental product that has not yet been released into the market). For each material, 15 dental blocks with cavities were prepared, except for the control group which had smooth surfaces with no cavities prepared. CEM and NP-MTA were prepared according to the manufacturers' instructions under aseptic conditions and placed in the cavities created in the dental blocks. The surfaces were smoothed-out using a sterile plastic instrument. For the composite resin group the blocks were first acid-etched (Dentsply, York, PA, USA) and rinsed with water. After removing the excess water, dentin bonding agent (Scotchbond, 3M, MN, USA) was applied and light-cured; then, composite resin was placed in the cavi-

ties and light-cured according to the manufacturer's instructions. The materials were allowed to set for 24 hours.

Preparation of Samples

Before cell seeding on filled dental slices, all of them were placed in a 24-well culture plate (SPL, Korea) and UV-irradiated for 20 minutes on each side. Human gingival fibroblast cells (HGF) (NCBI: CIS2) were purchased from the cell bank (Pasteur Institute, Tehran, Iran). The cells were kept in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, USA) and 1% penicillin-streptomycin (Gibco, USA) and incubated at 37°C, 95% humidity and 5% CO_2. In the logarithmic phase of growth, the cells were extracted by Trypsin-EDTA enzyme (Gibco, USA) and transferred to each well of a 24-well plate on a dental slice (50,000 cells in 1 mL of DMEM per well).

Quantitative Cell Viability and Proliferation Assay

Methyl-tetrazolium bromide (MTT) assay was performed to evaluate the survival and proliferation of gingival fibroblasts on days 1, 3 and 5 after the cell culture. After incubation (1, 3 and 5 days), root slices at each time were placed in a new 24-well culture plate. The fresh culture medium with 10% MTT dye was added to each well. The plates were incubated at 37°C for 3 hours. Afterwards, the medium of each well was extracted and replaced by dimethyl sulfoxide (DMSO) solvent, solubilizing the formazan crystals. Next, 100 µL of each purple solution (from each well of the 24-well plate) was transferred into each well of a 96-well Elisa reader plate in triplicate. The absorbance of solution dye was measured by an Elisa reader (Anthous 2020, Austria) at 570 nm. The cell viability percentage of each experimental group was expressed as a ratio relative to that of the control group (100% viability).

Scanning Electron Microscopy

SEM analysis was performed on fibroblasts attached to the root surfaces. The samples were rinsed with PBS and fixed on the root surfaces using 2.5% glutaraldehyde (Merck, Germany) for 2 hours. Then, the slices were placed in 1% osmium solution (TAB, England) for 1 hour. Gradient concentrations (30%, 50%, 70%, 90%, and 100%) of ethanol were used to dehydrate the specimens. The specimens were dried and then sputter-coated with gold.

Statistical Analysis

The MTT assay was used to measure the viability of the fibroblasts attached to each specimen. Data were expressed as mean ± SD. The results were analyzed using one-way ANOVA, followed by post hoc Tukey-Kramer test. P-values < 0.05 (indicated with a star on graphs) were considered significant.

Results

The results after 24 hours of incubation indicated cellular attachment and primary toxicity of the materials, while the results of 3-day and 5-day incubation periods were indicative of cellular proliferation and viability as well as the materials' cytotoxicity. The materials with normalized viability rates below 70% were considered cytotoxic. Nevertheless, the results of our study indicated that after 24 hours of setting time, all the tested materials were biocompatible (Table 1).

After 24 hours of incubation, the survival rates for composite resin and NP-MTA were 74.1% and 76.9%, respectively, which were significantly lower than the value for the control group, while both were equally biocompatible. Nevertheless, no statistically significant difference was found between the control and CEM cement groups (94.3%). After 3 days of incubation, increases in the viability of fibroblasts were detected in composite resin and NP-MTA groups, and their survival rates were found to be 89% and 93%, respectively. Conversely, in the CEM cement group, the survival rate decreased to 80.7%, which was significantly lower than that in the control group ($P = 0.0001$). This outcome can be an indicator of a decline in the proliferation rate of the fibroblasts attached to the surfaces treated with CEM cement overtime. At the end of the 3rd day, composite resin showed no statistically significant difference from CEM cement and NP-MTA ($P > 0.05$), whereas the viability rate of NP-MTA was significantly high-

Table 1. Effect of three different dental materials (MTA, composite resin and CEM) on attachment and viability of HGF cells

	%Viability (Mean ± SD)			
	Control	**Composite resin**	**CEM cement**	**NP-MTA**
Day 1	100.0	74.1±7.1	94.3±12.8	76.9 ±8.2
Day 3	100.0	89.0±11.7	80.7±6.7	93.9±10.0
Day 5	100.0	77.3±4.6	78.5±5.0	89.9±7.0

HGF= Human gingival fibroblast, NP-MTA=Nano-particle mineral trioxide aggregate, CEM= Calcium enriched mixture

Table 2. Data obtained from different published articles on fibroblast cell attachment to different filling materials

Author & year	Cell type	Material	Assessment test	Results
Huang et al, 2002 (43)	HGF	-Fuji II LC (FLC) Resin-modified (Glass-ionomer) -Compoglass (CG) (Compomer) -Spectrum TPH (TPH) (Composite Resin)	**Cytotoxicity**: MTT Cell Attachment **Assay:** MTT Cell Proliferation **Assay:** MTT	Cell viability: Control(no treatment)>CG>FLC>TPH Cell number: Control>CG>FLC>TPH Cell attachment: Control>CG>FLC>TPH
Haglund et al, 2003 (9)	L929 mouse fibroblasts	-MTA -IRM -Amalgam -Retroplast	Cell number	Total cell number in the control groups (no treatment) was significantly greater ($P < .01$) than that in the fresh material groups. **Total cell number (×1000) after 3 days of incubation:** Set: Control>MTA)>Amalgam> Retroplast>IRM
Camp et al, 2003 (7)	Human PDLfi-broblasts, HGFs	-Geristore® -ProRoot™ -Tytin® amalgam -SuperEBA™	Cell attachment	Greater attachment of gingival fibroblasts wasnoted to Geristore® than to the other root materials at all time periods ($p > 0.05$) Greater attachment of PDL fibroblasts was noted to Geristore® than to the other materials at all time periods ($p > 0.05$) Integrins did not mediate direct attachment of gingival fibroblasts to the root-end–filling materials.
Lin et al, 2004 (10)	hPDL fibroblasts	-MTA -Fuji II SC GIC -Amalgam -IRM -Super EBA	Cytotoxicity Flow cytometric analysis of DNA	Biocompatibility: MTA>> self-curing Fuji II GIC >amalgam. GIC and amalgam: mild cytotoxicity. IRM, GIC and amalgam: induced apoptosis of PDL cells, as revealed by the presence of sub-G0/G1 DNA content in flow cytometric histogram. Twenty-four-hour exposure to IRM and Super EBA elevated the MDH activities to 156%and117ofthatofcontrol. Eugenol, aphenolic ingredient in SuperEBA and IRM also increased MDH activity of PDL fibroblasts by 45% and 51%, at concentrations of 0.5 and 1 mM. However, at concentrations higher than 0.5 mM, eugenol decreased the number of viable PDL fibroblasts.
Al-Sabek et al 2005 (44)	HGF	-Geristore -KetacFil -IRM	SEM Growth Assay Cytotoxicity Assays :MTS	SEM analysis: HGFs attached and spread well over Geristore with relatively normal morphology. Fibroblasts did not attach and spread well over Ketac-Fil or IRM as cells appeared much fewer with rounded and different morphology than fibroblasts grown on Geris- tore. Cytotoxicity assays: HGFs proliferated in the presence of Geristoreeluates and not in the presence of Ketac-Fil or IRM eluates. Geristore is less cytotoxic to gingival fibroblasts.
Ghoddusi et al,2007 (40)	L929 mouse fibroblasts	-MTA -NEC	Cellular viability: MTT assay	NEC and MTA had similar cytotoxic effects onL929cellculture.
AL-HIYASAT et al,2010 (3)	Balb/C 3T3 mouse fibro-blasts	-Retroplast (Resin composite -Geristore (Resin-modified glass iono-mer) -KetacFil (Plus Glass ionomer cement) -IRM®(Reinforced zinc oxide-eugenol cement) -Super EBA (Reinforced zinc oxide-eugenol cement) -9PROROOT MTA Portland cement derivative)	Fibroblast cell attach-ment: SEM	Best fibroblast attachment on MTA and Geristore surfaces (cells exhibited characteristic elongated fibroblastic morphology, with projections of lamellipodia, filopodia, blebs, and microvilli from their surfaces, reflecting good attachment to the material). Poor fibroblast attachment to surfaces of IRM, Super EBA, KetacFil and Retroplast. Cells did not attach well to tooth structure next to IRM and Super EBA
Pontes Raldi et al, 2010 (1)	HGF	-MTA - Irradiation with Er:YAG laser (42mJ, 10 Hz, 10 s) - Irradiation with high-power diode laser (1 W, 10 s)	Cell adhesion assay	Adhesion: Er:YAG>diode> control (no treatment) > MTA
Wang et al, 2011 (46)	hPDL fibroblast	-SRP -SRP & HBD-3 (100ng/ml) -SRP & HBD-3 (200ng/ml)	PDL fibroblast cell viability PDL fibroblast Cell number attachment	Cell viability: no significant difference between HBD-3-treated PDL cells and non-treated control groups ($p > 0.05$) Cell number attachment: *Day 1*: Healthy group: 3.50±1.50. Diseased group: 0.27±0.12. SRP: 0.27±0.12. SRP & HBD-3 (100ng/ml): 0.27±0.12. SRP & HBD-3 (200ng/ml):3.73±1.81 *Day 3:* Healthy group: 9.33±2.52. Diseased: 0.17±0.29. SRP: 4.77±2.66. SRP & HBD-3 (100ng/ml):7.40±2.42. SRP & HBD-3 (200ng/ml): 8.13±0.95 *Day 7*:Healthy group:117.80±11.70. SRP & HBD-3 (100ng/ml):44.07±12.07. SRP& HBD-3 (200ng/ml): 73.80±7.00. SRP group:22.93±3.26.No cells were found in the untreated diseased group
S. S. Hakki et al 2011 (8)	hPDL fibroblast	-MTA -Compomer material (Dyract) -Super Bond C&B -Amalgam -IRM	MTT Confocal microscopy RNA isolation cDNA synthesis quantitative RT-PCR (Q-PCR) analysis	MTT assay: MTA was associated with a significantly higher cell density when compared with other materials ($P < 0.05$). Amalgam, Dyract, IRM and C&B groups had a lower cell density when compared with the control (non-restored) and MTA group at 96 h ($P < 0.05$). MTA was the most biocompatible material with a significantly higher cell density ($P < 0.05$) when compared with Amalgam, Dyract, IRM and C&B and even the control at 48 hours. Confocal microscopy: MTA: largest viable cell population over the restoration site Gene analysis: Increased Runx2 mRNA expressions were noted in MTA ($P < 0.001$) and IRM ($P < 0.01$) groups when compared with control and other tested materials. No significant difference was observed in amalgam, Dyract and C&B groups when compared with the control. Collagen type I transcripts were increased in IRM ($P < 0.01$), Dyract, C&B and MTA ($P < 0.001$) when compared with the control. No significant change was observed in amalgam group when compared with the control. MTA :
Asgary et al, 2012 (42)	HGF	-MTA -CEM -Glass cover slips (control group)	HGF attachment SEM	HGF cells displayed a favorable biologic response(adhesion , attachment and spread) to MTA and CEM, showing no significant difference No cytotoxic effects were seen on CEM or MTA
Mozayeni et al, 2012 (26)	L929 mouse fibroblasts	-IRM -MTA CEM	Cytotoxicity: MTT	The lowest cytotoxic values recorded were expressed by MTA subgroups followed by CEM cement; IRM subgroups were the most cytotoxic root-end/dental material ($P < 0.001$). CEM cement and MTA are reasonable alternatives to IRM because of lower cytotoxicity. CEM cement also has good biocompatibility as well as lower estimated cost to MTA and seems to be a promising dental material.
Ghasemi et al, 2014 (45)	HGF	-DSHP+WMTA -CEM -WMTA	MTT ELISA	MTT assay Control>WMTA+DSHP>WMTA>CEM cement BMP-2 (pg/ml): WMTA>CEM cement> Control >WMTA +DSHP 3167±274.46

HGF= Human Gingival Fibroblast, hPDL= human periodontal ligament, MTT=Methyl-tetrazolium bromide assay, ELISA=enzyme-linked immunosorbent assay, MTA=mineral trioxide aggregate, IRM=interm-ediate restorative material, SEM=Scanning Electron Microscopy, CEM= calcium enriched matrix, WMTA=ProRoot MTA, DSHP=disodium hydrogen phosphate, BMP-2= bone morphogenic protein-2, NEC= New Endodontic Cement, SRP =scaling and root planning, HBD-3= human beta-defensin-3, MDH= mitochondrial dehydrogenase

er than that of the CEM cement group ($P = 0.027$).

Reduction in the number of fibroblasts attached to CEM cement continued for up to 5 days of incubation. There was also a reduction in the survival rate of the cells attached to NP-MTA and composite resin. NP-MTA and composite resin resulted in lower viability compared to the control group; the survival rates in the afore-mentioned 2 groups were 89.9% and 77.3%, respectively. Nevertheless, the survival rate in the NP-MTA group was significantly higher than that in the CEM cement (P = 0.0005) and composite resin (P = 0.0002, Figure 1) groups.

Discussion

Root defects, namely perforations and resorption, are now being treated using various kinds of materials such as MTA, compomer, amalgam, composite resin, resin-modified glass-ionomer, reinforced zinc oxide-eugenol cement and Plus glass-ionomer cement.[7,9,10] Numerous studies have compared the effect of these materials on gingival fibroblast cell adhesion (Table 2). Huang et al[43] evaluated the effect of resin-modified glass-ionomer cement, compomer, and composite resin on human gingival fibroblasts by performing an in vitro MTT assay. The results in treated groups indicated the best cell viability and attachment in the compomer group. In the in vitro study carried out by Haglund et al,[9] cell growth, cell morphology and cytokine [interleukin (IL) 1β and IL-6] production in murine fibroblasts in contact with MTA, amalgam, IRM, and Retroplast were evaluated. The total cell numbers cultured with fresh IRM were significantly less than that cultured with other fresh materials and there was no statistically significant difference in cell count between the fresh MTA group and the fresh amalgam group. In addition, IL-1β or IL-6 production was not detected in any of the root-end filling material groups. Camp et al[7] assessed the attachment of human gingival fibroblasts and PDL fibroblasts to different root-end filling materials (Geristore, ProRoot, Tytin amalgam and SuperEBA) and also evaluated whether integrins were responsible for any attachment. They reported greater cell attachment to Geristore and concluded that integrins did not mediate direct attachment of gingival fibroblasts to the root-end filling materials. Similar results were obtained in a study carried out by Al-Sabek et al[44] and Al-Hiyasat et al.[3] In another research, Raldi et al[1] evaluated the fibroblast attachment and the morphological changes of simulated cervical root resorption after irradiation with high-power lasers and the use of MTA. They concluded that irradiation with Er:YAG and diode lasers caused morphological changes in the dentinal surfaces of simulated resorption areas that favored cell adhesion and that MTA showed lower biocompatibility than the irradiated groups but allowed cell adhesion. Mozayeni et al[26] assessed the cytotoxic effects of IRM, MTA and CEM on L929 mouse fibroblasts using the MTT assay. The assay was based on the reduction of the soluble yellow MTT tetrazolium salt (Sigma, Germany) to a purple insoluble formazan crystals produced by mitochondrial succinic dehydrogenase enzyme. The sensitivity of this method is above 95%. They showed that the lowest cytotoxic values recorded were expressed by MTA subgroups followed by CEM cement; while IRM subgroups were the most cytotoxic root-end/dental material. Ghasemi et al[45] evaluated the effect of CEM and ProRoot MTA (WMTA) + disodium hydrogen phosphate (DSHP) on the induction of bone morphogenic protein-2 (BMP-2) by human gingival fibroblasts (HGF) in comparison to WMTA. The results indicated greater amounts of BMP-2 using WMTA followed by WMTA+DSHP and CEM.

The favorable biocompatibility of CEM among other properties has been widely investigated and CEM can be considered as an alternative to MTA.NP-MTA is a relatively new material and only a few studies have tested its physical and biological properties. Composite resins have also been investigated by many studies, but their biocompatibility is often

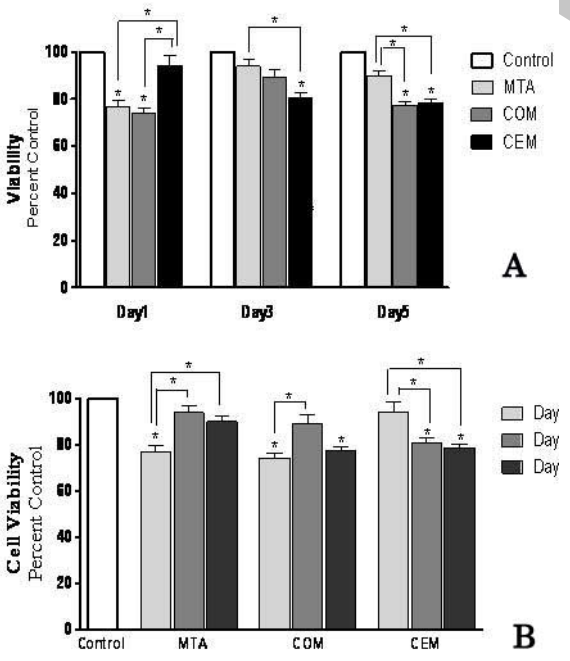

Figure 1. Percentages of cell viability of attached HGF cells on treated teeth (24 hours after setting/treating) in comparison with controls (untreated tooth). A: comparison of materials, B: comparison of time effect.

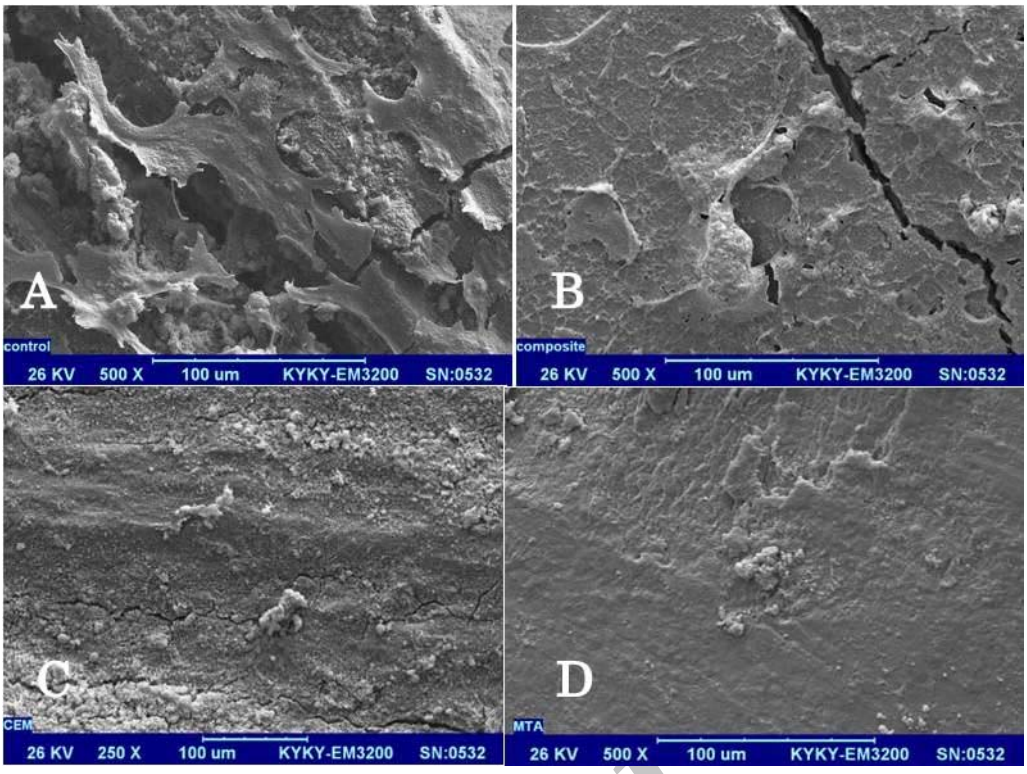

Figure 2. SEM micrographs of HGFs on different materials; A: control tooth surface; B: composite resin-treated surface; C: CEM-treated surface; D: MTA-treated surface (×500).

compared to glass-ionomers or other similar materials. Therefore, there are few studies on the comparison of these two different types of resins and mineral oxides (MTA or CEM cement).

The results of our in vitro study showed that on day 1, day 3 and day 5 after incubation, all of the tested materials (composite resin, CEM cement and NP-MTA) would exhibit favorable biocompatibility, if allowed to set for 24 hours before exposure to the cells (Figure 2). Furthermore, CEM cement showed

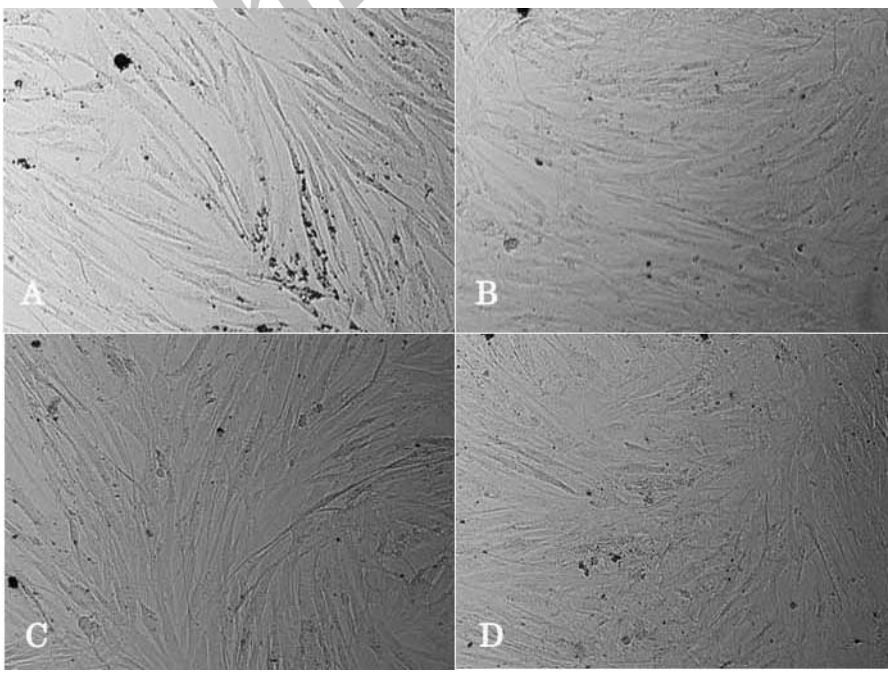

Figure 3. Light microscope images of HGFs on the surface of culture plate in contact with 24h treated (MTA, COM and CEM) and untreated (control) tooth; A: control; B: MTA; C: composite resin; D: CEM.

the best primary viability, while after 5 days of incubation the highest survival rate belonged to the NP-MTA group. This can be interpreted as a time-dependent increase in toxicity of CEM cement and a time-dependent decrease in toxicity of NP-MTA.

The CEM cement group results of our study are consistent with those of others,[26,40-42] all of which have demonstrated the biocompatibility of CEM cement. Still, none of them compared CEM (or other similar materials such as MTA) to a completely different material like composites resin. Although there are many concerns about the cytotoxicity of composite resins due to the release of their monomers, the results of the current study indicated that after 24 hours of setting, composite resins are biocompatible (Figure 3). These results are consistent with those of Gociu et al,[47] Modareszadeh et al[48] and Lee et al.[40] Contradictory results were reported by Huang et al[43] and Gallorini et al.[50] This can be due to the difference in the usage of different types of composite resins and different methods of application; the main reason is probably the lack of suitable polymerization. In the current study, the composite resin was first light-cured and then allowed to continue polymerization for 24 hours before exposure to cells; therefore, higher viability of cells was achieved. Thus, according to the results of the present study, composite resins are biocompatible after proper polymerization (Figure 4).

Conclusion

Under the limitations of this in vitro study, all the tested materials showed favorable biocompatibility after 24 hours of setting, but further studies are needed to confirm the physical and biological properties of NP-MTA. CEM cement showed the best primary viability, while after 5 days of incubation the highest survival rate belonged to the NP-MTA group.

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