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CYTOTOXICITY AND OSTEOGENIC POTENTIALS OF MESENCHYMAL STEM CELLS VIA A NOVEL FORMULATION OF MINERAL TRIOXIDE AGGREGATE: AN IN VITRO STUDY

Ahmed M. Elgendi[1] | Ehab E. Hassanien[2,3] | Mohamed M. Nagy[2,3] | Iman A. Fathy[4] | Ashraf M. Abu-Seida[5]* | Elhassan E. Hassanein[6]

Objectives: Root perforations can lead to periodontitis and alveolar bone loss. This study compared cytotoxicity and osteogenic potential of zirconia-containing mineral trioxide aggregate (MTA) Matreva (MAT group) to MTA Angelus (ANG group) as root perforation repair materials.

Methods: Bone marrow mesenchymal stem cells (BM-MSCs) were extracted from two albino rats and cultivated. The cytotoxicity of the studied materials was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay at three time points: 3, 7, and 14 days. The evaluated materials were employed to guide the osteogenic differentiation of BM-MSCs into osteoblasts. The osteogenic impact was evaluated by assessing calcium deposition with Alizarin red staining (ARS) and expression of receptor activator of nuclear factor-kappa B (NF-κB) ligand (RANKL) protein in BM-MSCs seeded on the investigated materials for 14 days. Genes analyzed included tumor necrosis factor (TNF-α), transforming growth factor beta (TGF-β), and interleukin 1 beta (IL-1β). The data was statistically examined.

Results: Cytotoxicity and osteogenic potential did not differ significantly between the ANG and MAT groups ($p > 0.05$). The MAT group had the greatest average% calcific area percentage (90%), followed by the ANG group (68%) and negative controls (NC, 48%). BM-MSCs in the MAT group had the greatest amount of RANKL protein expression, followed by the ANG group. The ANG group had considerably higher levels of TNF-α and IL-1β compared to NC. TGF-β levels were considerably higher in the MAT group compared to the NC.

Conclusions: Zirconia-based MTA exhibits comparable cytotoxicity and osteogenic capability to standard MTA Angelus.

Keywords: Mesenchymal stem cells, Cytotoxicity, Mineral trioxide aggregate, Osteogenesis, Root perforation.

Corresponding author:
Ashraf M. Abu-Seida, e-mails: ashrafseida@cu.edu.eg, ashrafseida@gu.edu.eg

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The authors declare no conflicts of interest.

1. Faculty of Dentistry, Ain-Shams University, Cairo, Egypt.
2. Department of Endodontics, Faculty of Dentistry, Galala University, New Galala City, Suez, Egypt.
3. Department of Endodontics, Faculty of Dentistry, Ain Shams University, Cairo, Egypt.
4. Department of Oral Biology, Faculty of Dentistry, Ain-Shams University, Cairo, Egypt.
5. Department of Surgery, Anesthesiology & Radiology, Faculty of Veterinary Medicine, Cairo University, Giza, Egypt. PO: 12211.
6. HHU Heinrich Heine Universität Düsseldorf, Germany.

CYTOTOXICITÉ ET POTENTIELS OSTÉOGENIQUES DES CELLULES SOUCHES MÉSENCHYMATEUSES VIA UNE NOUVELLE FORMULATION D'AGRÉGAT DE TRIOXYDE MINÉRAL: ÉTUDE IN VITRO

Objectifs: Les perforations radiculaires peuvent entraîner une parodontite et une perte osseuse alvéolaire. Cette étude a comparé la cytotoxicité et le potentiel ostéogène de l'agrégat de trioxyde minéral (MTA) contenant de la zircone Matreva (groupe MAT) à ceux du MTA Angelus (groupe ANG) comme matériaux de réparation des perforations radiculaires.

Méthodes: Des cellules souches mésenchymateuses de moelle osseuse (CSM-MO) ont été extraites de deux rats albinos et cultivées. La cytotoxicité des matériaux étudiés a été déterminée par dosage au bromure de 3-(4,5-diméthylthiazol-2-yl)-2,5-diphényltétrazolium (MTT) à trois moments précis : 3, 7 et 14 jours. Les matériaux évalués ont servi à guider la différenciation ostéogénique des CSM-MO en ostéoblastes. L'impact ostéogénique a été évalué en mesurant le dépôt de calcium par coloration au rouge d'alizarine (ARS) et l'expression de la protéine ligand de l'activateur du récepteur du facteur nucléaire kappa B (NF-κB) (RANKL) dans les CSM-MO ensemencées sur les matériaux étudiés pendant 14 jours. Les gènes analysés comprenaient le facteur de nécrose tumorale (TNF-α), le facteur de croissance transformant bêta (TGF-β) et l'interleukine 1 bêta (IL-1β). Les données ont été analysées statistiquement.

Résultats: La cytotoxicité et le potentiel ostéogénique ne différaient pas significativement entre les groupes ANG et MAT ($p > 0,05$). Le groupe MAT présentait le pourcentage moyen de surface calcifiée le plus élevé (90 %), suivi du groupe ANG (68 %) et des témoins négatifs (NC, 48 %). Les CSM-MO du groupe MAT présentaient la plus forte expression de la protéine RANKL, suivies du groupe ANG. Le groupe ANG présentait des taux de TNF-α et d'IL-1β considérablement plus élevés que le groupe NC. Les taux de TGF-β étaient considérablement plus élevés dans le groupe MAT que dans le groupe NC.

Conclusions : Le MTA à base de zircone présente une cytotoxicité et un potentiel ostéogénique comparables à ceux du MTA Angelus standard.

Mots-clés: Cellules souches mésenchymateuses, cytotoxicité, agrégat de trioxyde minéral, ostéogenèse, perforation radiculaire.

Introduction

Perforation is an inappropriate connection that occurs between the root canal system and neighboring periodontal tissues, resulting in a pathological condition. Tooth perforation can result from a number of pathological conditions, such as caries and internal or external resorption [1, 2]. Furthermore, it can occur at any point of endodontic therapy or after placement [1]. Nonetheless, furcation region perforation is more common during access preparation techniques attempting to locate a root canal opening [3]. Unintentional perforations account for up to 29% of all root canal iatrogenic cases [4].

Furcal perforation can lead to periodontal inflammation and alveolar bone loss [5]. Depending on the severity, inflammation might result in epithelial growth and granulation tissue. Periodontal pockets and polypoid lesions may develop in the coronal root. If not treated adequately, furcal perforation can anticipate tooth loss [6].

The site and size of the perforation, the time delay before treatment, the presence of microorganism contamination, the osteogenic differentiation potential, the sealing ability of the restorative material, and the tooth's periodontal condition all influence the long-term prognosis of this complication [7]. A material with outstanding physiochemical, mechanical, and biocompatibility qualities is well recommended [8, 9].

Nonsurgical perforation therapy has a success rate greater than 70% and may be preferable. In combination with the patient's clinical characteristics, the material used for the restoration may impact the physiological response of a perforated tooth and nearby tissues [10].

A number of materials, including amalgam, Cavit, intermediate restorative material (IRM), Super-ethoxy benzoic acid (EBA), glass ionomer cements, and resins, have been used for this purpose. Mineral trioxide aggregate (MTA) and other calcium-silicate cements have been successfully used for perforation repair because they create a regenerative environment and stimulate new bone formation through osteoinductive action [3, 6, 7].

Most clinicians recommend MTA for pulp capping, root perforation repair, retrograde filling restoration, apexification, revascularization, resorption repair, and apical surgery because of its sealing ability, marginal adaptation, and biocompatibility as determined by cytotoxicity and genotoxicity tests of cell cultures or *in vivo* studies [2, 3, 6, 7]. The disadvantages of MTA include a long initial setting time (3-4 hours), difficulty handling (depending on the water/powder ratio), high cost, restricted radiopacity, discoloration when in contact with sodium hypochlorite, and wash-out in humid settings [11]. Therefore, MTA may be modified to improve its qualities by adding different concentrations and combinations of methylcellulose and calcium chloride, which improves handling and lengthy setting time [12]. Furthermore, nano modification of MTA greatly decreased the initial and final setup times [13].

A new material, MTA Matreva, has recently been introduced to the market. According to the manufacturer, it has a roughly identical composition to MTA with different amounts of titanium and zirconium oxide instead of bisthmus oxide as a radiopacifer. This modification enhances material manipulation and therapeutic outcomes [14, 15]. Furthermore, it improves biocompatibility [16] while having no effect on antibiofilm action [17]. In addition, adding zirconium oxide to MTA resulted in rapid repair of damaged periodontal tissue [18].

In endodontics, the biocompatibility and regeneration potential of new materials are dependent on their cytotoxicity and osteogenicity being accurately assessed. The oral and dental tissues harbors many types of mesenchymal stem cells residing in various tissues, including the dental pulp stem cells (DPSCs), the periodontal ligament stem cells (PDLSCs), and even the lining of the gingiva. Each type has unique properties. DPSCs, found within the dental pulp, have the ability to differentiate into, nerve, and dentin-forming cells. PDLSCs from the periodontal tissues play a crucial role in tooth stability and can regenerate into cementum, bone and fibers [19, 20]. Isolated stem cells from the apical papilla or periodontal ligament act as sensitive biosensors, demonstrating the endodontic materials' rapid cytotoxic effects on cell survival and proliferation [21]. Beyond cytotoxicity, by driving these stem cells toward an osteogenic lineage and studying their response to endodontic materials, researchers get significant insights into their osteogenic potential, indicating their capacity to induce bone repair [22].

There is a lack of studies assessing the properties of MTA Matreva. Therefore, this research evaluated the cytotoxicity and osteogenic potential of the zirconia-containing MTA-Matreva to the standard MTA Angelus as a root perforation repair material. The null hypothesis of this experimental investigation was that Matreva MTA and MTA Angelus had same cytotoxicity and osteogenic potential.

Materials and Methods

Ethical approval

This study was approved by the institutional research ethical committee (Approval No: FDASU-RecID051907 in 17/7/2019). Moreover, all methods were carried out in accordance with ARRIVE guidelines.

Sample size calculation and classification

Based upon the previous study of Abu Zeid *et al*., the effect size (d) was 1.118 with success of MTA = 69.2%. Using alpha (α) level of (5%) and Beta (β) level of (20%) i.e. power = 80%; the minimum estimated sample size was 28 for tested materials and 14 samples for negative control. Sample size calculation was performed using G*Power Version 3.1.9.2 [23].

The investigated materials were methodically crafted into disc-shaped structures (N=28 discs), with each disc acting as a representative sample. The samples were divided into two groups (14 samples each) based on the substance used: group A included MTA Angelus (ANG), and group B contained MTA Matreva. Group C (14 samples) represented the negative control (NC), which contained BM-MSCs without testing material.

Preparation of the tested materials

MTA Matreva contained Tricalcium silicate, Dicalcium silicate, Tricalcium aluminate, Calcium sulfate, and Zirconium dioxide (Matreva BioMTA, Matreva dental labs co., Cairo, Egypt) while MTA Angelus contained Calcium silicates, Tricalcium aluminate, Calcium oxide, Bismuth oxide, Potassium oxide, Silicon oxide, Iron oxide, and Sulfuric acid (MTA Angelus, Angelus, Londrina, Brazil). Under sterile conditions, both materials were mixed in a 3:1 powder-to-liquid ratio and pressed onto 96-well plates that were 4 mm in radius and 2 mm in height to create tiny discs of each cement (Disc dimensions were 4 mm diameter, 2 mm thickness). The materials were incubated with damp gauze until hardened, then dried in a laminar hood for 24 hours before cell seeding. This was done a day before the experiment.

Isolation and culture of bone marrow mesenchymal stem cells

Stem cells were isolated from the bone marrow of two 12-week-old albino rats' tibiae and femurs weighing 200 mg each. BMMSCs showed positive CD29 and CD90 levels of 98.5% and 98.6%, respectively. Only 0.7% of hematopoietic cells expressed CD45, demonstrating that the isolated BM-MSCs were pure mesenchymal stem cells. The two albino rat samples were collected from Faculty of Medicine's animal housing facilities, Ain Shams University, Egypt.

Each rat's femurs and tibiae were immediately dissected, the connected muscles were removed, and the bones were transferred to the laboratory within a 30-minute period after being immersed in an ice-cold solution containing minimum essential medium (MEM) supplemented with 100 U/mL penicillin and 100 μg/ml streptomycin in a 15-ml Falcon tube. All treatments were performed at Faculty of Dentistry's Central Laboratory for Stem Cells and Biomaterials Applied Research, Ain Shams University. When the bones arrived at the laboratory, the bone epiphyses were removed, pulverized using a homogenizer, and incubated in 1 mL of ice cold solution comprising MEM (Gibco, Thermo Scientific, Bio City Leipzig, Germany) in sterile 15 mL Falcon tubes with 0.2% collagenase type I and 0.2% dispase (Sigma Aldrich, Wisconsin, USA) plus 100 U/mL penicillin and 100 μg/mL streptomycin (Gibco, Thermo Scientific) for 30 minutes at 25°C.

To eliminate bone debris, gently flip the tube several times and filter the mixture through a 100 μm cell filter. The cell suspension was centrifuged for 10 minutes at 400 ×g at 25°C in a room, followed by three rinses with cold phosphate buffer saline (PBS, Gibco, Thermo Scientific). The supernatant was then collected and disposed away. The cells were then suspended in DMEM with 10% fetal bovine serum (FBS, Gibco, Thermo Scientific) and antibiotics (penicillin/streptomycin) and incubated at 37°C for 24 hours with 5% CO_2 and 100% humidity [24].

Cell cultures were inspected every 24 hrs to examine cell morphology and rule out infection (Figure 1A-C).

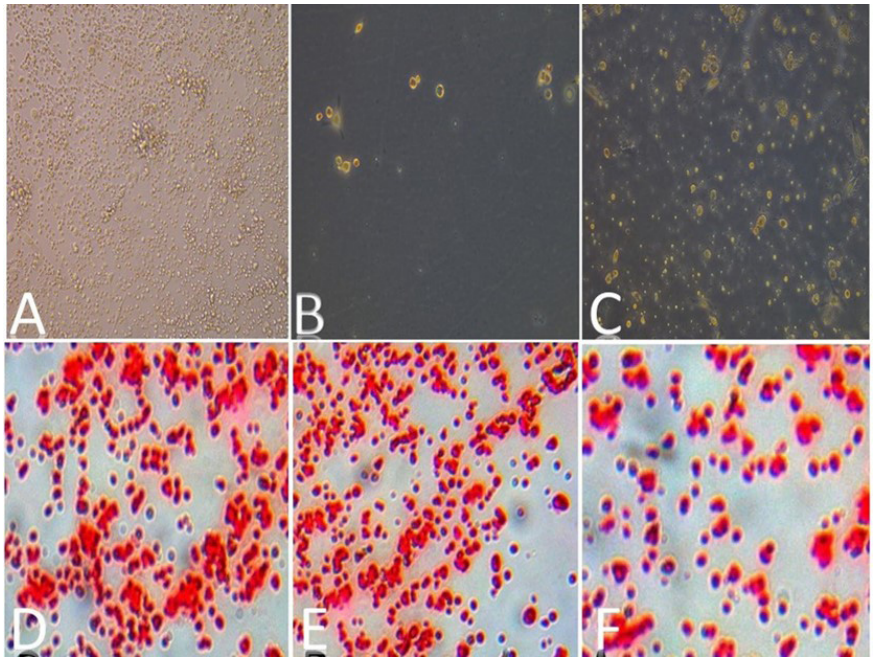

Figure 1. Representative photomicrographs of phase contrast microscopy showing A primary culture at day 0, B fibroblast like cells after 3 days of culture, and C confluent cell culture with variable morphologies. D-F Representative photomicrographs showing the osteogenic differentiation of the BM-MSCs in MTA Angelus group D, MTA Matreva group E and negative control group F. (Alizarin red stain, X400).

To control seeding density, the total number of cells was determined by trypan blue stain as follows: Adding 25 $\mu l$of the harvested cells and 25 μl of trypan blue solution in buffer to the hemocytometer. The chamber was then placed in the Invitrogen Countess Automated Cell Counter [25]. Nonviable cells were stained; however, the viable cells did not take up the stain. The cells were counted, and the dead cells were measured to estimate the number of viable cells as follow:

% of viable cell = [1.00 – (number of blue cells ÷ Number of total cells)] × 100. The number of viable cells per ml of culture estimated by the formula:

Number of viable cells × 10^4 × 1.1 = cells/mL culture

The following day, 1x10^4 BMMSCs were seeded in 200 μL of mesenchymal stem cell (MSC) osteogenic differentiation medium (OM, Gibco, Thermo Scientific), which included optimized MSC osteogenic differentiation basal medium, mesenchymal stem cell-qualified fetal bovine serum, penicillin, streptomycin, glutamine, ascorbate, β-glycerophosphate, and dexamethasone. The culture plates were incubated at 37°C in a 5% CO_2 environment for the times specified. BM-MSCs grown on OM without MTA material served as negative controls.

Assessment of cell viability and cytotoxicity

The MTT was used to assess cell viability after 3, 7, and 14 days. Cell proliferation was evaluated with the Vybrant MTT Cell Proliferation Assay Kit (Cat no: M6494, Thermo Fisher, Bio City Leipzig, Germany). A 96 well tissue culture plate was inoculated with 1 X 10^5 cells / ml (100μl/well) where cells were allowed to set on discs of tested reagents for different time intervals according to study design and incubated at 37°C to develop a complete monolayer sheet [26]. After the incubation time, 100 μL of media was replaced with fresh media. Next, 20 μl of MTT solution (1 mg/mL) was applied to each well. At each time point, a cell proliferation test was performed. The experimental design was intended to analyze the proliferative capability of BMMSCs on the studied MTA materials at three time points: 3, 7, and 14 days. The plates were incubated for four hours at 37°C with 5% CO_2. After removing the MTT solution, 100 μL of sodium dodecyl sulfate with hydrochloric acid (SDS-HCl) was applied to each well. The optical density (OD) of 570 nm was measured to detect cell viability.

Assessment of osteogenic potential

Assessment of osteogenic potential through evaluation of calcium deposition by Alizarin red staining (ARS)

The tested materials were used to direct the osteogenic differentiation of BM-MSCs into osteoblasts. The cells were grown for fourteen days. Following the incubation period, calcium deposition in differentiated osteoblasts was examined by ARS, which evaluated calcium buildup in mature osteoblasts.

Cells were planted onto MTA Matreva and MTA Angelus discs in 96-well plates with a density of 1 X 10^5 cells / ml (100 μl/ well) and cultured for 14 days at 37°C in a 5% CO_2 incubator in osteogenic media [25]. After 14 days, the growth media were removed and the cells were washed three times with PBS. After 15 minutes of fixation with 4% formaldehyde at room temperature, the cells were washed three times with deionized water (diH_2O). A single 40 mM mL of ARS was applied and shaken at room temperature for 30 minutes. After five washes with diH_2O, the cells were examined under an LX400 fluorescence microscope (Labomed, Los Angeles, California, United States).

The assessments were repeated three times to ensure accuracy. For each group, the H-score method was utilized to identify individual cells and their subcellular compartments, followed by the relative expression of ARS [area fraction] (Loba Chemie Pvt. Ltd., Mumbai, Maharashtra 400005, India) to rate the staining intensity as high (3+), medium (2+), low (1+), or no staining (0). The H-score was calculated by dividing the sum of area fraction intensity by the total number of detected cells [27].

Assessment of osteogenic potential by RANKL protein expression

The expression of receptor activator of nuclear factor-kappa B (NF-κB) ligand (RANKL) protein in BM-MSCs seeded on the two MTA materials for 14 days was evaluated. The tested materials were used to direct the osteogenic differentiation of BM-MSCs into osteoblasts. The cells were grown for fourteen days. After incubation, cells were collected using 0.25% trypsin, washed three times with PBS, and centrifuged at 1000×g for 10 minutes. The resultant cell pellet was then resuspended in culture medium, with the cell count set to1x10^6 cells/ml. RANKL protein expression was evaluated in collected cell homogenates using an enzyme-linked immunosorbent assay (ELISA) kit (Fine test, Wuhan, China). The BM-MSCs in the two separate groups were seeded for 14 days, as per the research design. The BM-MSCs were quickly immunostained with a rabbit anti-RANKL antibody (Invitrogen; Thermo Fisher Scientific, Hilden; Germany) and examined under a microscope. The H-score median value was calculated [28].

Assessment of osteogenic potential through gene expression

The tested MTA materials were used to direct differentiation of BM-MSCs into osteoblasts. The cells were grown for fourteen days. Following the incubation time, total mRNA was extracted using RNeasy Mini Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions.

Firstly, complementary DNA (cDNA) was generated by reverse transcription with a QuantiTect II RT Kit (Qiagen, Hilden, Germany). Secondly, gene expression analysis involved quantifying the gene expression levels of the transforming growth factor β subunit (TGF-β), tumor necrosis factor α subunit (TNF-α), and interleukin 1β (IL-1β). The mRNA was subjected to gene expression amplification utilizing the QuantiTect primer set, namely, the HsTNF-α, TGF-β, and IL-1β primer sets (cat no: 249900), together with the QuantiTect SYBR Green PCR Kit (Cat no: 204141, Qiagen, Germany). The ACTB_1_SG QuantiTect Primer Assay Kit (catalog number 249900) was used as the reference gene. A 5 plex Rotor-Gene PCR Analyzer (Qiagen, Germany) was used to assess all the samples. The 2ΔΔCt technique was used to analyze gene expression levels, with ACTB serving as an endogenous reference control for normalization [29].

Statistical analysis

The data were presented as mean, standard deviation, median, and range. The Kruskal Wallis test was used to compare between groups, followed by the Mann Whitney U test for pairwise comparisons. The significance level for statistical tests was set at $p < 0.05$. The statistical analysis was performed using SPSS software (IBM Corp. Released 2017. IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY: IBM Corp).

Results

Results of cell viability and cytotoxicity using the MTT assay

According to OD measurement at 570 nm, the MAT group had the highest level of viable cells and proliferation after 3, 7 and14 days followed by the ANG group. However, the NC group had the lowest OD (Figure 2).

The mean and standard deviation values of the percentage of viable cells after 3, 7, and 14 days revealed statistically significant differences ($p = 0.027$) between the three groups (Table 1).

MAT groups had substantially higher percentages of viable cells after 3, 7, and 14 days compared to NC groups ($p < 0.05$). There were no

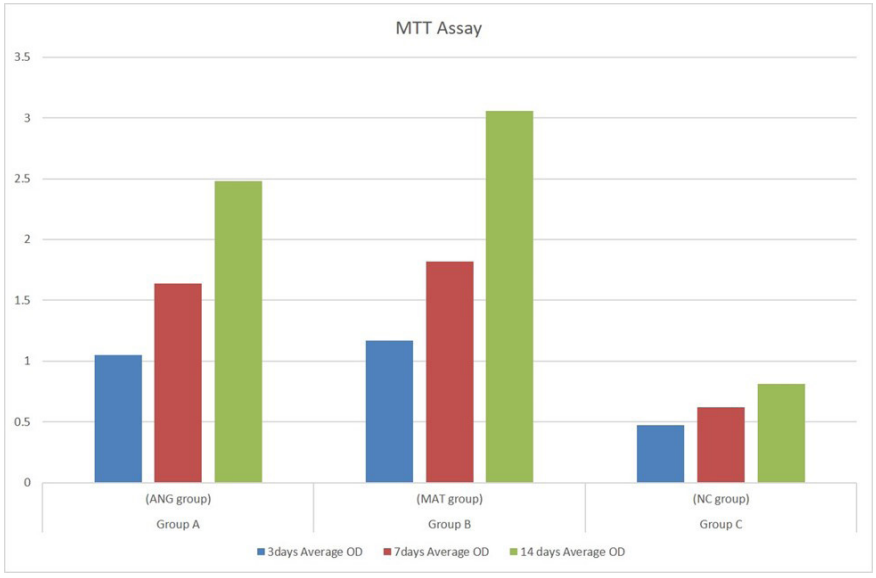

Figure 2. Cell viability results (MTT assay) after 3-, 7- and 14-day's incubation of BM-MSCs cells showing higher level of viable cells and proliferation in MTA Matreva group than MTA Angelus group after 3, 7 and14 days. OD: optical density, NC: Negative control, MAT: MTA Matreva, ANG: MTA Angelus.

Table 1. Descriptive statistics and result of Kruskal Wallis test for comparison of percentage of viable cells between the three groups after 3, 7 and 14 days.

Incubation time	Data	Group A (ANG Group)	Group B (MAT Group)	Group C (NC Group)	*p*-value
3 days	Mean (SD)	224.4^{ab} (10.2)	248.2^{a} (10.1)	100.8^{b} (0.1)	0.027*
	Median (Range)	224.4 (214.2- 234.6)	248.2 (238.1- 258.3)	100.8 (100.66- 100.86)	
7 days	Mean (SD)	265^{ab} (11.8)	293.3^{a} (0)	100.3^{b} (0)	0.027*
	Median (Range)	262 (255- 278)	293.27 (293.26- 293.31)	100.26 (100.24- 100.28)	
14 days	Mean (SD)	335.2^{ab} (14.2)	412.9^{a} (0.9)	100.2^{b} (0.5)	0.027*
	Median (Range)	335.28 (320.98- 349.29)	413.3 (411.9- 413.44)	100.1 (99.7- 100.65)	

Different lower case letters in the same row indicate statistical significance. *Significant at $p < 0.05$. NC: Negative control, MAT: MTA Matreva, ANG: MTA Angelus.

significant differences between the ANG and MAT groups, nor between the ANG and NC groups after 3, 7, and 14 days (p >0.05).

Results of osteogenic potential

Results of calcium deposition using ARS

After 14 days of osteogenic development, cultivated BM-MSCs were stained with ARS to detect calcified nodules. The NC group showed no colorimetric alterations in response to ARS. Both the ANG and MAT groups had scattered red-stained nodules. The cells seeded in the MAT group had the greatest average% area fraction (90%), followed by the ANG group (68%) and the NC group (48%). The MAT group seemed to have larger and more intense nodules than the ANG and NC groups (Fig. 1D–F). The mean and standard deviation H-scores were calculated and there was a significant difference between the three groups (p = 0.027) as shown in Table (2). Pairwise comparisons revealed that MAT group had significantly greater H scores than those of NC group (p<0.05). There were no significant differences between ANG and MAT groups or between ANG and NC groups (p > 0.05).

Results of RANKL protein expression

The MAT group had the greatest mean level of RANKL protein expression (800.2 ± 74.65), followed by the ANG (593.43 ± 27.28) and NC groups (192.8 ± 4.6). There were significant differences across the three groups (p = 0.027). The MAT group showed significantly higher mean RANKL levels than the NC group (p <0.05). Table (3) shows that there were no significant differences between the ANG and NC groups or the ANG and MAT groups (p > 0.05).

Results of gene expression

Results of the gene expression analysis are shown in [Figure 3]. Table (4) shows significant differences (p = 0.027) between the three groups in terms of TNF-α and interleukin 1-β levels. The ANG group had substantially higher mean TNF-α level than the NC group (p < 0.05). There was no significant difference between the MAT and NC groups, or between the MAT and ANG groups (p >0.05).

Table 2. Descriptive statistics and result of Kruskal Wallis test for comparison of H-Score denoting calcium deposition using ARS between the three tested groups.

Data	Group A (ANG group)	Group B (MAT group)	Group C (NC group)	p - value
Mean (SD)	68^{ab} (1)	90^{a} (2)	42^{b} (1)	0.027*
Median (Range)	68 (67 - 69)	90 (88 - 92)	42 (41 - 43)	

Different lower case letters in the same row indicate statistical significance. *Significant at p <0.05. NC: Negative control, MAT: MTA Matreva, ANG: MTA Angelus.

Table 3. Descriptive statistics and results of Kruskal Wallis test for comparison of RANKL levels between the three tested groups.

Data	Group A (ANG group)	Group B (MAT group)	Group C (NC group)	p - value
Mean (SD)	593.43^{ab} (27.28)	800.2^{a} (74.65)	192.8^{b} (4.6)	0.027*z
Median (Range)	597.9 (564.2 - 618.2)	818.2 (718.2 - 864.2)	190.5 (189.8 - 198.1)	

Different lower case letters in the same row indicate statistical significance. *Significant at p <0.05. NC: Negative control, MAT: MTA Matreva, ANG: MTA Angelus.

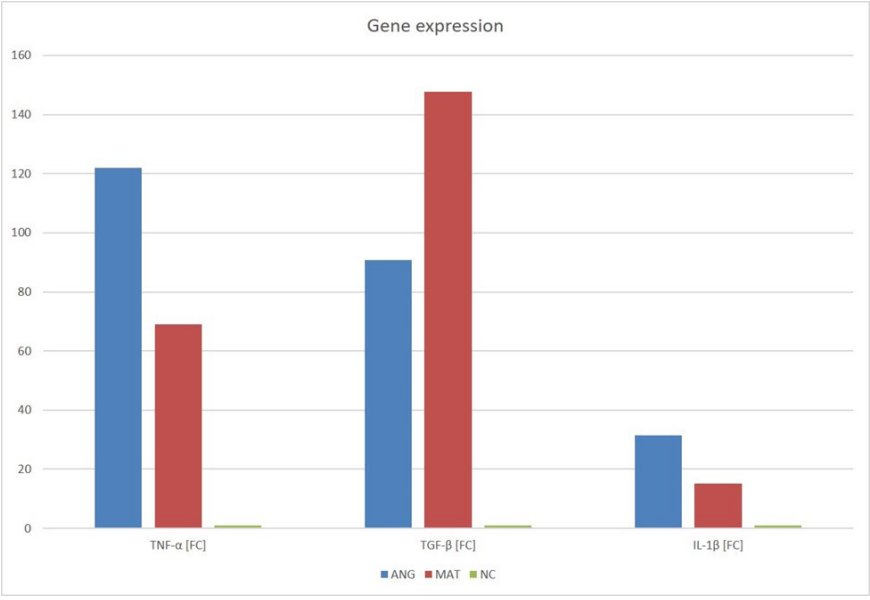

Figure 3. Mean values of the gene expression analysis showing significant differences between the three tested groups in terms of TNF-α and interleukin 1-β levels. FC: Fold change in gene expression compared to the negative control. TNF-α: Tumor necrosis factor alpha, TGF-β: Transforming growth factor beta, IL-1β: Interleukin 1-β. NC: Negative control, MAT: MTA Matreva, ANG: MTA Angelus.

Table 4. Descriptive statistics and result of Kruskal Wallis test for comparison of TNF-α, TGF-β and IL-1β between the three tested groups

Genes	Data	Group A (ANG group)	Group B (MAT group)	Group C (NC group)	*p*- value
TNF-α	Mean (SD)	121.8[a] (13.1)	69[ab] (13.5)	1[b] (0.8)	0.027*
	Median (Range)	118.6 (110.66 - 136.23)	63.11 (59.3 - 84.44)	1.3 (0.1 - 1.6)	
TGF-β	Mean (SD)	90.9[ab] (10)	147.7[a] (29.1)	1[b] (0.4)	0.027*
	Median (Range)	88.64 (82.13 - 101.82)	151.16 (116.97 - 174.85)	0.8 (0.7 - 1.5)	
IL-1β	Mean (SD)	31.4[a] (4.8)	15.3[ab] (4.6)	1[b] (0.4)	0.027*
	Median (Range)	34.05 (25.81 - 34.29)	15.56 (10.55 - 19.69)	0.95 (0.65 - 1.4)	

Different lower case letters in the same row indicate statistical significance. *Significant at $p < 0.05$. NC: Negative control, MAT: MTA Matreva, ANG: MTA Angelus, TNF-α: Tumor necrosis factor alpha, TGF-β: Transforming growth factor beta, IL-1β: Interleukin 1-β.

The three groups differed significantly in terms of transforming growth factor beta (TGF-β) level ($p = 0.027$). The MAT group had significantly higher mean TGF-β levels than the NC group ($p < 0.05$). There was no significant difference between the ANG and NC groups, or between the ANG and MAT groups ($p > 0.05$).

Discussion

The American Association of Endodontists recommends that considerable biological and clinical studies be conducted before using a novel material [30]. Endodontic materials must be examined *in vitro* before being utilized in clinical settings to determine their biological qualities and reduce the possibility of negative effects. Furthermore, materials that come into regular contact with periapical or pulp tissue must have low toxicity and biocompatible [31]. Zirconia integrating MTA is a novel improved MTA reparative material composition using titanium; hence, the current study evaluated MTA Matreva with MTA Angelus in terms of cytotoxicity and osteogenic potential. MTA Matreva revealed equivalent cytotoxicity and osteogenic potential to MTA Angelus, hence the study's hypothesis was accepted. With similar biocompatibility and bioactivity, MTA Matreva can be a good substitute of MTA Angelus. Other benefits of MTA Matreva include the incorporation of Zirconium oxide as a radiopacifier, which removes issues related to bismuth oxide, such as tooth discoloration and possible cytotoxicity, and enhances mechanical qualities.

Endodontic perforation is the second most common reason of treatment failure. It accounts for 9.6% of endodontic failures and necessitates timely treatment for a positive outcome [32].

In our study, we examined MTA Angelus, the gold standard for perforation repair, and MTA Matreva, a zirconia-containing MTA. The hydrophilic and biocompatible endodontic cement MTA promotes healing and osteogenesis [33, 34].

The aggregate is made up of a finely ground combination of trioxides (tricalcium oxide, silicon oxide, and bismuth oxide) and other hydrophilic particles (tricalcium silicate and tricalcium aluminate), which define its chemical and physical properties [33]. It solidifies when exposed to moisture. Hydrating the powder resulted in a pH 12.5 colloidal gel that developed within 3 to 4 hours. MTA is favorable because to its biocompatibility, bioactivity, hydrophilicity, radiopacity, sealing ability, and low solubility [34]. MTA stimulates fibroblast, osteoblast, cementoblast, bone marrow stromal, and pulp cell growth [35].

Regarding MTA Matreva, a novel recently introduced material; the manufacturer indicated that this product comprises titanium for easy handling and zirconium to avoid bismuth oxide discoloration. Matreva was established to provide cheap access to bioactive and osteogenic materials [14, 15].

In our study, as in others, the cytotoxicity evaluation periods were 3, 7, and 14 days since the healing potential increases with time as the cytotoxic impact decreases and the number of viable cells increases [36].

Cytotoxicity was tested using several assays and techniques, including the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, which transforms MTT into formazan crystals by live cells to quantify mitochondrial activity, cell viability, and proliferation [37]. The MTT test is reliable because it does not exaggerate or underestimate cell viability when assessing the

cytotoxicity of root canal filling materials [38]. Because the formazan crystals may be thoroughly washed and dissolved, MTT is frequently used for adherent cells. In contrast, MTS requires PMS, which can be hazardous to some cell types if improperly optimized; hence, MTT may be more dependable for sensitive primary cells or long-term experiments.

Because MSCs have the ability to multiply naturally, two donors may be adequate in cell culture research. Because of their high rate of proliferation, BM-MSCs can grow significantly *in vitro* from a small initial population. Each donor can produce a significant number of cells thanks to this multiplication, which provides enough material for experimental analysis. Comparable sample sizes have been used in similar research in the area to successfully reach important results. Our results confirm the validity of these well-established works.

In our investigation, the direct contact strategy was employed instead of the elution approach because root healing materials come into touch with cells and tissues in clinical applications, and the direct contact assay is the most sensitive method for detecting mild cytotoxic substance, as previously described [39]. As a result, the cytotoxicity level was accurately determined.

At all test times, the negative control group had the fewest live cells. The absence of repair materials might explain these findings. Due to its biocompatibility, MTA Matreva had the highest proportion of viable cells at all time periods.

In our study, the MTA Matreva and MTA Angelus groups detected more viable cells after 14 days compared to 3 and 7 days. The low number of viable cells in the early stages may be attributed to the predominance of inflammatory infiltration caused by first calcium ion release from calcium silicate-based materials. Furthermore, the increase in pH during the setup of MTA root healing materials, together with the heat created by the reaction, promotes the recruitment and synthesis of inflammatory cells, as well as the generation of proinflammatory cytokines. An earlier research reported similar findings [40].

According to the MTT assays of our examined materials, zirconia containing MTA Matreva exhibited higher cell viability than bismuth oxide containing MTA Angelus, although the difference was not significant. This conclusion is consistent with the findings of Li *et al.*, who discovered that cell viability was much greater in cultures exposed to the nano-ZrO_2-blended MTA sample than in those exposed to the unblended sample [16]. These findings may be explained by previous research findings, such as those reported by Kim *et al.* who demonstrated that bismuth oxide, the radiopacifier in MTA, is toxic to human tooth pulp and periodontal ligament cells *in vitro* [41]. This conclusion was consistent with our research, as MTA Angelus includes bismuth oxide. Moreover, Neto *et al.* discovered that calcium silicate cements with ZrO_2 had better physicochemical properties and more favorable biological responses than bismuth oxide [42].

MTA-Matreva incorporates bioinert material (Titanium) which has no effect on cell viability and toxicity ensuring biocompatibility of the material as stated by the manufacturer. Adding zirconium oxide to calcium silicate enhances compressive strength, antimicrobial activity, and cell proliferation [43]. MTA Angelus includes bismuth oxide as a radioopacifier. It has been established that bismuth oxide has a presumed fatal impact on human osteoblast-like cells when mixed with dicalcium silicate cement compared to when not associated with a radiopacifier [44]. Furthermore, Portland cement containing bismuth oxide enhances dental pulp cell cytotoxicity (parent MTA) [45].

MTT assays demonstrated that mitochondrial activity is dependent on cytoplasmic calcium. Calcium silicate cements' high calcium concentrations may promote osteoblast cell development and proliferation by activating calcium signaling pathways [46]. In this investigation, calcium nodules seemed to be larger and more intense in the MTA Matreva group than in the MTA Angelus and negative control groups. This is seen as an essential consequence because calcium hydroxide, formed by the hydration of calcium silicate cements, dissociates into calcium and hydroxyl ions, which help in stem cell osteogenesis. A pH over 8 can also drive osteoblasts to secrete alkaline phosphatase, which is characteristic of active calcified tissue [47]. Calcium silicate material excretes calcium and silicate ions into the environment, promoting cell differentiation and osteogenic gene expression.

Calcium ions and moderate alkalinity lead to the synthesis of hydroxyl apatite and the release of mineralization-related alkaline phosphatase and bone morphogenetic protein 2 [27]. The H-score determined the amount of calcium deposits in a photograph by taking into consideration both intensity and proportion. It featured a dynamic range of 0 to 300, making it ideal for measurements.

RANKL, or receptor activator of nuclear factor-kappa B ligand, is a membrane-associated cytokine. Its expression is mostly induced in immature cells. RANKL expression has been associated with osteoblastic cell differentiation [48]. RANKL protein expression may be assessed using a variety of techniques, including the ELISA test, which we used in this study.

Our study found that MTA Matreva had higher expression of osteogenic potential genes (RANKL and TGFβ) and lower expression of genes (TNF-αand IL-1β) compared to MTA Angelus. These suggest that MTA Matreva has greater osteogenic potential than MTA Angelus, but not significantly more.

Previous studies found that TGF-β increased osteoblast development, decreased osteoclast production, and delayed bone degradation [49]. These results are consistent with the ARS and show that the BMMSCs' expression and gene expression imply enhanced mineralization and proliferation. Wang *et al.* found that IL-1β enhances osteogenic gene expression and differentiation in BMMSCs without triggering early mortality during bone healing [50]. This might be because the sterile inflammatory response is active at all stages but loses its osteogenic induction effect at high dosages. TNF-α significantly decreases the osteogenic differentiation capacity of MSCs at different dosages

Ex vivo investigations are important for isolating specific pathways in a controlled setting, which can be a crucial step before advancing to *in vivo* models. The study's primary limitation is that it was done *ex vivo*, which may not accurately reproduce clinical settings. We chose this strategy in order to lower variables prior to conducting additional animal research. Although the study offered controlled conditions for evaluating material properties, they could not fully replicate the intricacies of human beings. To address the constraints of the *ex vivo* methodology and short observation duration, future *in vivo* or clinical studies should be conducted to examine systemic effects over extended timeframes. Moreover, this study only compared MTA Matreva to MTA Angelus, therefore additional MTA formulations were not included to offer a more thorough view of MTA Matreva's relative performance and biocompatibility compared to other materials. Lastly, cytotoxicity and osteogenic potential were observed during three, seven, and fourteen days, respectively. Future studies with longer observation periods may tell more about the long-term reactivity and stability of the materials.

Conclusions

Addition of zirconia to MTA improves its biocompatibility and osteogenic potential, however the increase is not considerable. As a result, zirconia containing MTA exhibits similar cytotoxicity and osteogenic potential to regular MTA Angelus, which is considered a reliable material for root perforation repair.

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