AL-AZHAR
Dental Journal
For Girls

The Official Publication
of The Faculty of Dental
Medicine For Girls,
Al-Azhar University
Cairo, Egypt.

Print ISSN 2537-0308 • Online ISSN 2537-0316

ADJ-for Girls, Vol. 6, No. 4, October (2019) — PP. 409:416

Cytotoxicity Evaluation of Three Biocompatible and Bioactive Retrofilling Materials Using Periodontal Ligament Stem Cells: An In-Vitro Study

Safwat S Elwasseef [1*], Wael H Kamel [2], Mahmoud H Mohamed [3]

Codex : 50/1910

azhardentj@azhar.edu.eg

http://adjg.journals.ekb.eg

DOI: 10.21608/adjg.2019.6359.1108

ABSTRACT

Purpose: this study aimed to evaluate the biocompatibility of Mineral Trioxide Aggregate (*MTA*) versus Biodentine and *Retro MTA* extracts at three observation periods (24h, 48h, and 72h) using MTT-Assay. **Materials and Methods**: 72 wells in three culture insert plates were used in this study. MTA, Biodentine and Retro MTA cements were evaluated in a fresh state. Passage four of human PDL cells were included in this study. After cells preparation and culturing, an extract of the three tested materials was prepared and cultured with the complete media in addition to 5×10^4 cells were implanted into each well 24h earlier and incubated in a stem cell incubator. MTT Assay was used to evaluate in vitro cytotoxicity, calculated the viable cells by hundred percent using ELISA system and the inverted light microscope used to reassure their confluence. After three different observation periods (24h, 48h, and 72h), evaluation of cell viability was done after exposure to the material extracts. **Results:** MTA and Biodentine cements showed no statistically significant differences at all the observation periods. However, Retro MTA cement resulted in a statistically significant difference at all the observation periods but none of them below the cut level. Meanwhile, all the materials extracts resulted in a statistically significant increase in cytotoxicity at 24h and then decrease at 48h and finally a little increase in cytotoxicity at 72h. **Conclusions**: All the tested materials showed low cytotoxicity and are comparable to the old standard MTA.

KEYWORDS

Retrograde filling, Bioactive material, Cytotoxicity.

INTRODUCTION

Apicectomy is considered as a must in presence of a periradicular lesion or when failure resulted from non-surgical retreatment[(1)]. The desired success of apicectomy mainly assisted by the regeneration of a functional periodontal attachment apparatus, including cementum

- Paper extracted from PHD thesis titled: "Evaluation of The Cytotoxicity, Push out Bond Strength and Healing Potential of Three Bioactive Retrograde Filling Materials".

1. * Assistant Lecturer of Endodontics, Faculty of Dental Medicine, Al-Azhar University (Assuit Branch), Egypt. Email: drsafwat86@gmail.com
2. Professor and Head of Endodontic Department, Faculty of Dental Medicine for girls Al Azhar University.
3. Professor and Head of Endodontic Department, Faculty of Dental Medicine Al-Azhar University (Assuit Branch).

overlying the resected root-end surface, alveolar bone, and PDL[2]. To achieve this target, it was suggested to place a retrofilling material that not only allows for the deposition of a normal cementum but also prevents entrance of any stimulus to form a well seal in the apical piece[3]. Materials used as retrofilling must possess some criteria such as dimensional stability, untoxic, unaffected by moisture, easy to manipulate, biocompatible, radio-opaque and has antibacterial activity as well as having good bond strength. No existence-filling material fulfilled the ideal requirements[4].

MTA represents the standard benchmark to which other repair materials are compared. It has many favorable properties that makes it the material of choice such as its biocompatibility, antimicrobial activity and enhance regeneration of the original tissue when placed in close to any vital tissue. Although MTA has high biocompatibility and sealing ability in comparison to the other usable biomaterials. It has some defects for instance extended setting and manipulation time, sophisticated handling properties. Although the numerous applicators in the market to aid in its clinical application, endodontists are still finding MTA difficult to use in certain surgical situations such as small-sized root-end preparation[5].

Biodentine with silicate based Technology was available in the market since 2009. It is considered as a bioceramic material, which could overcome the MTA drawbacks. Biodentine has excellent bioactivity with high mechanical properties and biocompatibility. In addition to Biodentine has an excellent handling properties and reasonable setting time making it easier than MTA in use[6]. Anew generations of MTA-based products, including Retro MTA. It has been reported to have a cheaper price than the Pro Root MTA, faster-setting time than MTA, with a preliminary setting time of about 3.5 minutes[7-9].

Biocompatibility is considered as an important criterion of retrofilling materials. Biocompatibility tests measure the ability of a material to maintain high percent of cell viability[8-10]. Limited studies exist about the biocompatibility of MTA, Biodentine and Retro MTA. This study was performed to evaluate the biocompatibility of MTA, Biodentine and Retro MTA on human PDL fibroblasts using MTT assay test.

MATERIALS AND METHODS

Fibroblasts of humankind PDL were pick out from impacted third molars in healthy patients presented to the clinics of the Department of Oral Surgery AL-Azhar University who underwent surgical extractions. Extracted periodontal ligament pieces were cultured in $25cm^2$ flask with 10ml DMEM-F12 supplemented to 10% Foetal Bovine Serum and 1% streptomycin, penicillin, and fungizone, then the cultured pieces were incubated at 99F, 5% CO_2 atmosphere and 97% humidity inside a biological incubator.

Every three days, the changes in the Culture Media was detected using an inverted light microscope. When the cell formation units reached 90% confluence, the cells were passed into 75-cm^2 flask and this was passage one. The fourth Cell passage were used in this study. During passaging, fibroblasts were transported from flask to another flask with trypsenization process using Trypsin EDTA solution aiming to de-adhering cells from the flask [11, 12].

The three materials MTA, Biodentine, and Retro MTA were mixed according to the directives given by the manufacturer into soft thickness under completely aseptic conditions. Once the materials were prepared, they were mixed with the supplemented medium DMEM-F12 to a concentration of 2.5 mg/mL and this weighted with a sensitive balance and agitated overnight. The medium obtained was spun using the vortex device at 13,500 rpm for 6 min. The resulted supernatant was allowed to precipitate again and the medium was spun, again, and the supernatant obtained at the

second spun was filtered with 0.20 um membrane and stored inside the incubator at 4°C for its later use. The resulted extract contained the products released from the material which allow evaluation of cytotoxicity and cell adhesion with great predictability (cell viability assay) this is called the extract fresh form of the materials according to study in 2017[9].

Classification of the sample

The PDL cells treated with different materials were divided into four groups, group 1: 18 well in three Culture plates (6 each) treated with MTA extract, Group 2: 18 well in three Culture plates (6 each) treated with Biodentine extract, Group 3:18 well in three Culture plates (6 each) treated with RetroMTA extract, Group 4: (Negative control group) consisting of 18 well in three culture plates (6 each) without biomaterials. They were evaluated by 24, 48, and at 72hours.

Once the fibroblast cultures were found at 80-90% confluence, they were separated from the flask with trypsin-EDTA. Then 5×10^4 cells were overlaid in 100 μL of culture medium onto insert culture plates and incubated for 5h period at 99F, 6% CO_2, and 97% humidity [12]. Initially, the complete media was added without material extract, to allow adaptation of the cells to the plate for 24h. After 24 hours, the media was removed and replaced with 100μL of culture medium with the type of cement evaluated.

Fibroblasts cell line fourth passage were seeded into 24 well of three 96-insert culture plates at a concentration of 5×10^4 cells/well. Cells was seeded in complete media included DMEM supplemented with 1% pen/ strep/ fungizone antibiotic, 10% FBS, for 24h at 99 F, 5% CO_2 and 97% humidity.

After 24h from incubation, the culture medium was replaced with 100μL of material extractions were replaced in wells related to the three test groups in addition to the negative control group. Complete media was used as the control group, instead of material extraction. The plates were incubated at 5% CO_2, 98% humidity, and 37°C, for 24, 48, and for 72h then were evaluated by MTT Assay.

Upon the end of the observing periods 24h, 48h and 72h the predictable MTT Assay was processed in a sterile cabinet. The extracts of the cement were removed from wells of the culture plate, and replaced with the sterile MTT solution (5mg/mL) diluted in cell culture media (1:10 ratio). Then, the plates were incubated inside the biological incubator for 2h at 99F at 97% humidity and 5% CO_2. During this period, the metabolically active cells bio-reduced MTT salt to formazan [12]. After this period MTT solution was removed from the cells and 100ul of dimethyl sulfoxide solution was added to each group to dissolve the insoluble formazan crystal formed inside the cells. The assay was subsequently read on a microplate reader at 580nm, with 620nm as a reference wavelength, using an ELISA plate reader.

The background absorbance was subtracted from the primary values to attain the main optical density of the test well. All tests were compared with the negative control cells untreated with cement. The result was confirmed with the inverted light microscope. All of tests were repeated in triplicate for 24, 48 and for 72h. Cytotoxicity was expressed with the percentage (%) of the viable cells using the following formula:

Statistical Analysis

The statistical data were collected; mean and standard deviation (SD) of cell viability for each group were calculated. Statistical analysis was performed using one-way analysis of variance (ANOVA). Repeated measures ANOVA test was used to compare between cytotoxicity, which in inverse relation with cell viability of the four groups as well as to study the changes by time within each group. Qualitative data were presented as frequencies and percentages. Chi-square test or Fisher's Exact test when applicable were used for the comparisons. The significance level was set at

P ≤ 0.05. Statistical analysis was performed with IBM SPSS Statistics Version 21 for Windows.

RESULTS

In MTA, Biodentine and Retro MTA groups, the highest cell viability was recorded at 48h; meanwhile the lowest cell viability was recorded at 72h. In all groups, a statistically significant change in cytotoxicity was found by time. Comparisons between the three time-periods revealed statistically significant decrement in cell viability after 24h, a statistically significant rising in cell viability after 48h followed by a statistically significant decrement in cell viability after 72h. The mean cell viability after 72h was statistically significant less than after 24h Fig: (1,2)

In the negative Control group, a statistically significant alteration in cell viability by time was observed (P-value <0.001, Effect size = 0.581). Pairwise comparisons between the three time-periods demonstrated that there was a statistically significant decrease in cell viability after 48h as well as from 48 to 72h Fig: 3.

After 24, 48 and 72h, the highest cell viability was recorded with MTA extract meanwhile the lowest cell viability was recorded with Retro MTA extract but the difference between material is not significant. Retro MTA group showed the statistically significant lowest cell viability compared with the negative control group at all the observation periods but none of them below the cut level 70%.

Table 1: *The mean, standard deviation (SD) values and results of repeated measures ANOVA test for comparison between cell viability % at different time periods within each group.*

Group	24 Hours		48 Hours		72 Hours		*P*-value	*Effect size (r)*
	Mean	SD	Mean	SD	Mean	SD		
MTA	89.03 ^{B}	2.04	90.98 ^{A}	2.03	87.39 ^{C}	2.29	<0.001*	0.432
Biodentine	88.13 ^{B}	3.01	90.72 ^{A}	2.29	85.24 ^{C}	3.32	<0.001*	0.636
Retro MTA	82.24 ^{B}	3.03	88.45 ^{A}	3.08	78.67 ^{C}	3.98	<0.001*	0.854
Control	95.00 ^{A}	1.49	92.58 ^{B}	1.55	91.05 ^{C}	1.26	<0.001*	0.581

**: Significant at P ≤ 0.05, Different superscripts in the same row are statistically significantly different*

Table 2: *The mean, standard deviation (SD) values and results of the Kruskal-Wallis test for comparison between cell viability (HU) in the four groups*

Time	MTA (n = 18)		Biodentine (n = 18)		Retro MTA (n = 18)		Control (n = 18)		*P*-value	*Effect size (r)*
	Mean	SD	Mean	SD	Mean	SD	Mean	SD		
24 Hours	89.03 ^{B}	2.04	88.13 ^{B}	3.01	82.24 ^{C}	3.03	95.00 ^{A}	1.49	<0.001*	0.779
48 Hours	90.98 ^{B}	2.03	90.72 ^{B}	2.29	88.45 ^{C}	3.08	92.58 ^{A}	1.55	<0.001*	0.303
72 Hours	87.39 ^{B}	2.29	85.24 ^{C}	3.32	78.67 ^{D}	3.98	91.05 ^{A}	1.26	<0.001*	0.718

**: Significant at P ≤ 0.05, Different superscripts in the same row are statistically significantly different.*

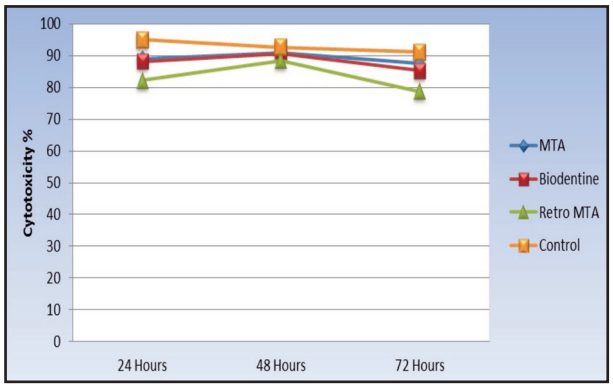

Figure (1) Bar chart representing a comparison between cell viability in the four groups.

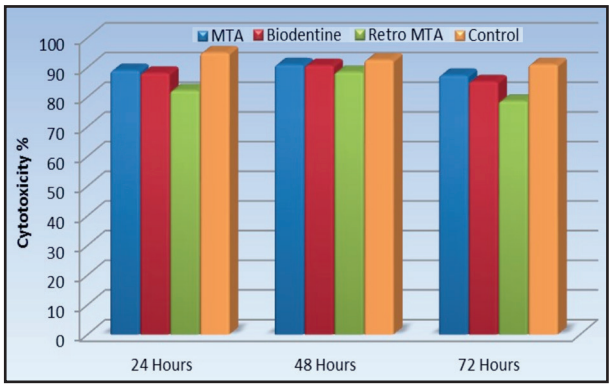

Figure (2) Bar chart representing a comparison between cell viability in the four groups.

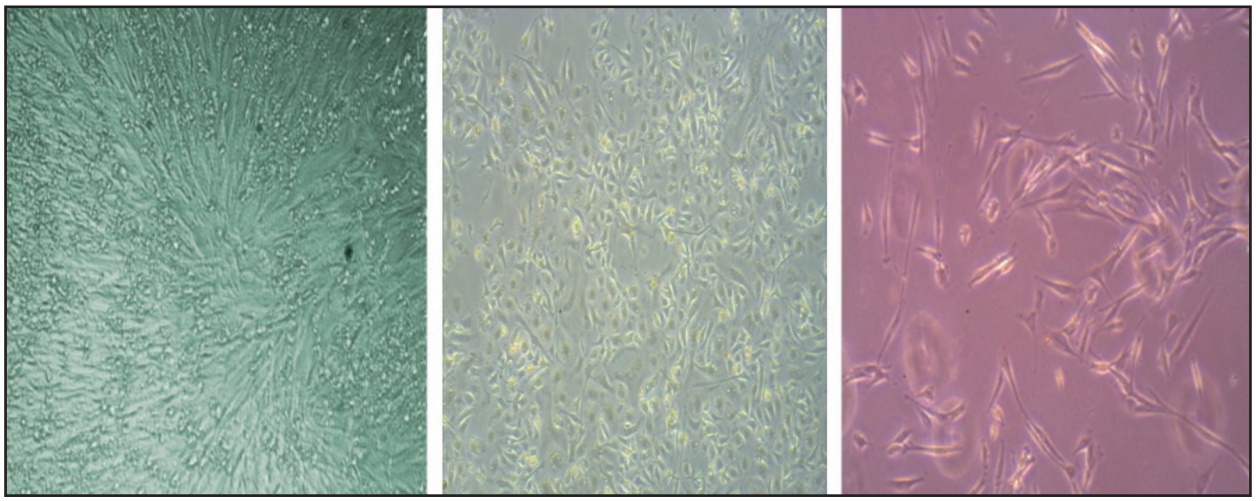

Figure (3) Representative photomicrographs (20-x magnification) of fibroblasts viable cells after incubation wit complete media without any material extracts at 24, 48, and 72h.From left to right respectively. Photomicrographs reveal decrease in cell viability after 48h as well as from 48 to 72h.

DISCUSSION

The cytotoxicity of retrofilling materials plays avital role especially in endodontic surgery because the material can evoke the immune response, causes partial necrosis of the periapical tissue and delay healing of wounds. After endodontic surgery, ideal healing includes alveolar bone regeneration and PDL regrowth on the amputated root surface[13].

Human PDL cells are considered an additional advantage to reduce alignment concerning species origin and non-tissue specific cell lines[14]. Therefore, we select the humankind PDL cells from extracted molar to simulate the clinical condition, Cell lines with more passage numbers show alterations in cell morphology. Therefore, we preferred using the passage four of cell lines to decrease cell changes due to cell culture manipulation[15].

In this study, the DMEM-F12 was used as a main culture media, which is considered a rich medium that supports the growth of multiple types of cells in both serum and serum-free formulation[16]. Meanwhile, the other usable media was α-MEM (α-Minimum Essential Media). This media must be augmented with serum to be suitable for multiple types of mammalian cells. DMEM-F12 has the amino acids of double concentration and vitamins four times the amount compared to α-MEM[17, 18].

FBS was introduced by biologists in the late 1950 to aid in cellular growth and tissue culturing. Nowadays FBS contains supportive components for maintenance and cell proliferation such as transport proteins, trace elements, hormones, vitamins, spreading and growth factors. FBS has been included as a supplement in culture media for human, animal cells in research. Although bovine sera of alternative origin are available, FBS is most widely used due to its low content of Ig and complement factors in addition to its availability and low cost as recommended by a study in 2018[19.20].

1% streptomycin, penicillin and fungizone were used to support the culture media sterilization during all procedures. A Sterile cabinet was used for all procedures to avoid any interfering microorganism. All cell culture were incubated in a humidified incubator with 99 F, 5% CO_2 to simulate the human body[21]. The Inverted light microscope used in this study as it provides a 2D image with a narrow depth of focus. Daily detection by the inverted light microscope is a very important step to follow interactions between the cells and the materials visually and the confluence of the cell to prevent its crowding and guarantee it's feeding[22]. Material extracts could be a viable solution to assess the cytotoxicity indirectly and to allow fibroblast to proliferate safely in the culture media[9].

Extract of the materials was used after mixing with the vortex and filtering with the micro-filter 0,20 u/ml to leave the extract containing only the cytotoxic products of the materials as described by some studies[9]. Control group was added as a reference point to the entire testing group. We observed the percentage of living PDL cells, which had direct contact with the bioactive materials[23].

The most accepted method to evaluate cell viability is the MTT assay and it is considered as a first-generation tetrazolium derivative. It is a quantitative, reliable, and sensitive colorimetric (calorimetry is the color measurement in a solution) assay that measure activation, viability and cell proliferation[24]. The formazan amount produced from MTT reduction is in direct matching to the viable and active cells percentage. The MTT assay exposed a greater advantage in the detection of viable cells, which are not only dividing but also metabolically active preparing for division[25].

In this study, we evaluated cell viability using an ELISA plate reader. The calorimetric absorbance is inversely proportional to the cytotoxicity in culture. It can be used to evaluate the percent of viable cells in culture suspension[26].

The results of the present study showed statistically significant highest cell viability associated with the control group. Meanwhile there was no statistically significant difference between MTA and Biodentine. Retro MTA group showed statistically significant lowest cell viability compared with the control group but none of them below the cut level 70 %[10]. The viable cells remained in all the evaluated periods showing greater similarity at 24h, a higher percentage of cell proliferation was at 48h and the lowest at 72h. All groups are biocompatible comparable to the negative control group. The microscopic photographs taken during the culturing procedures were consistent with the results of the MTT assay.

The biocompatibility of MTA, Biodentine and Retro MTA influenced by calcium ion release during their setting time and hydroxyapatite formation via calcium binding to phosphorus. These changes seem to initiate alterations in the enzymatic activity of affected cells rather than permeability change[9] and the consanguinity in their chemical composition of these root-end filling materials can be the most important reason for the allocated behavior and the formation of hydroxyapatite in the presence of Ca^{+2} ion during the setting reaction[10].

The three tested exhibited non-significant decrement of cell viabilities at 24h and a higher percentage of cell proliferation were at 48h. This may be expounded in sudden increase in pH and heat of the cement surface that result from mixing

reaction. The alkaline pH and the exothermic reaction can damage the cells directly by apoptosis and (or) necrosis and indirectly damage the cells by denaturing of culture medium proteins making it aimless [27, 28].

Significant decrement in cell viability after 72h in MTA, and Retro MTA may be attributed to calcium hydroxide production due to the hydration reaction in the materials; similarly, when Biodentine rehydrate produce a by-product calcium hydroxide[29]. The gradual release of hydroxyl ions may increase the cytotoxicity in vitro. However, under in vivo conditions, high alkalinity due to hydroxyl ions may be compensated by the body tissue fluid and may not cause significant effects on cell viability[30].

CONCLUSIONS

Within the restrictions of this study, Biodentine and Retro MTA showed good biocompatibility compared to the gold standard MTA.

REFERENCES

1. Seedat C., der-Vyver J., de-Wet A. Micro-endodontic surgery Part 2: root-end filling materials -A literature review. SADJ. 2018; 73:336-42.
2. Setzer FC. Endodontic microsurgery-A technique for the 21st entury .J Orofac Sci. 2018; 10:1-2.
3. Bhagat K., Goel M., Bhagat N. Root-End Filling Materials and Recent Advances: A Review. EC Dental Science.2017; 12:46-57.
4. Solanki N., Venkappa K., and Shah N. Biocompatibility and sealing ability of mineral trioxide aggregate and biodentine as root-end filling material: A systematic review. J Conserv Dent. 2018; 21: 10–15.
5. Loetzen S., Curtius S., Michael H. Root perforation repair concepts and materials: A review. Endodontic Practice Today;2018:12,87-100.
6. Department RD. Biodentine Active Biosilicate Technology Scientific File. Septodont. France, 2009.
7. Souza C., Yadlapati M., Dorn O., Silva R., Letra A. Analysis of radiopacity, pH and cytotoxicity of a new bioceramic material. J Appl Oral Sci.2015; 23:383-9.
8. Pornamazeh T., Yadegari Z., Ghasemi A., Sheykh-Al-Eslamian M., Shojaeian S. In Vitro Cytotoxicity and Setting Time Assessment of CalciumEnriched Mixture Cement, RetroMineral Trioxide Aggregate and Mineral Trioxide Aggregate. Iran Endod J. 2017; 12:488-92.
9. Dezfouli N., Asnaashari E., and Khalilak Z. Comparison of the Biocompatibility of Pro Root MTA, Retro MTA and MTA plus Using an MTT Assay Study. EC Dental Science. 2017; 11: 83-7.
10. Akbulut M., Arpaci P., Eldeniz A. Effects of four novel root-end filling materials on the viability of periodontal ligament fibroblasts. Restor Dent Endod.2018; 43:1-12.
11. Prateeptongkum E., Klingelhoffer C., Muller S., Ettl T., Morsczeck C. Characterization of progenitor cells and stem cells from the periodontal ligament tissue derived from a single person. J Periodontal Res. 2016; 51: 265-72.
12. Rai Y., Pathak R., Kumari N., Sah K., Pandey S., Kalra N., Soni R., Dwarakanath S., Bhatt N. Mitochondrial biogenesis and metabolic hyperactivation limits the application of MTT assay in the estimation of radiation-induced growth inhibition. Sci Rep. 2018: 24:15-31
13. Sultana N., Singh M., Nawal RR., Chaudhry S., Yadav S., Mohanty S., Talwar S. Evaluation of Biocompatibility and Osteogenic Potential of Tricalcium Silicate-based Cements Using Human Bone Marrow-derived Mesenchymal Stem Cells. J Endod. 2018; 44: 446-51.
14. Karimjee K., Koka S., Rallis M., Gound G. Cellular toxicity of mineral trioxide aggregate mixed with an alternative delivery vehicle. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2006; 102:115-20.
15. Geurtsen W., Lehmann F., Spahl W., Leyhausen G. Cytotoxicity of 35 dental resin composite monomers/ additives in permanent 3T3 and three human primary fibroblast cultures. J Biomed Mater Res. 1998; 41: 474- 80.
16. Batouli S., Miura M., Brahim J., Tsutsui T., Fisher L., Gronthos S., Robey PG., Shi S. Comparison of stem-cell-mediated osteogenesis and dentinogenesis. J Dent Res. 2003; 82:976-81.
17. Gronthos S., Mankani M., Brahim J., Robey P., Shi S. Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. Proc Natl Acad Sci USA. 2000; 97:13625-30.
18. Abuarqoub D., Awidi A., Abuharfeil N. Comparison of osteo/odontogenic differentiation of human adult dental pulp stem cells and stem cells from apical papilla in the presence of platelet lysate. Arch Oral Biol. 2015; 60:1545-53.

19. Puck t., Tjio j. The somatic chromosomes of man. proc Natl Acad Sci USA. 1958: 44:1229-37.

20. Valk J., Bieback K., Buta C., Cochrane B., Dirks W., Fu J., Hickman J., Hohensee C., Kolar R., Liebsch M., Pistollato F., Schulz M., Thieme D., Weber T., Wiest J., Winkler S., Gstraunthaler G. Fetal Bovine Serum (FBS): Past - Present - Future. ALTEX. 2018; 35: 99-118.

21. Park S., Mostoslavsky G. Generation of Human Induced Pluripotent Stem Cells Using a Defined, Feeder-Free Reprogramming System. Curr Protoc Stem Cell Biol. 2018; 45:48-52.

22. Strnad P., Gunther S., Reichmann J., Krzic U., Balazs B., de Medeiros G., Norlin N., Hiiragi T., Hufnagel L., Ellenberg J. Inverted light-sheet microscope for imaging mouse preimplantation development. Nat Methods. 2016; 13:139-42.

23. Perez A., Spears R., Gutmann J., Opperman L. Osteoblasts and MG-63 osteosarcoma cells behave differently when in contact with ProRoot MTA and White MTA. Int Endod J. 2003; 36:564-70.

24. Henriksson E., Kjellen E., Wahlberg P., Wennerberg J., Kjellstrom J. Differences in estimates of cisplatin-induced cell kill in vitro between colorimetric and cell count/colony assays. In Vitro Cell Dev Biol Anim. 2006; 42:320-3.

25. Ishiyama M., Tominaga H., Shiga M., Sasamoto K., Ohkura Y., Ueno K. A combined assay of cell viability and in vitro cytotoxicity with a highly water-soluble tetrazolium salt, neutral red, and crystal violet. Biol Pharm Bull. 1996; 19:1518-20.

26. Avila E., Pugsley M. An overview of colorimetric assay methods used to assess survival or proliferation of mammalian cells. Proc West Pharmacol Soc. 2011; 54:10-4.

27. De-Deus G., Ximenes R., Gurgl E., Plotkowski M., Filho T. Cytotoxicity of MTA and portland cement on human ECV 304 endothelial cells. Int Endod J.2005; 38: 604-9.

28. Chang S., Lee S., Ann H., Kum K., Kim E. Effects of calcium silicate endodontic cements on biocompatibility and mineralization-inducing potentials in human dental pulp cells. J Endod. 2014; 40:1194-200.

29. Camilleri J., Sorrentino F., Damidot A. Investigation of the hydration and bioactivity of radiopacified tricalcium silicate cement, Biodentine and MTA Angelus. Dent Mater.2013; 29:580–93.

30. Mozayeni M., Milani A., Marvasti L. and Asgary. Cytotoxicity of calcium-enriched mixture cement compared with mineral trioxide aggregate and intermediate restorative material. Aust Endod J. 2012; 38:70-5.