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Evaluation of Cytotoxicity and Antibacterial Effect of Different Types of Mineral Trioxide Aggregate

Farklı Mineral Trioksit Agregatların Sitotoksisite ve Antibakteriyel Etkilerinin Değerlendirilmesi

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Abstract

Objective: Mineral trioxide aggregate (MTA) has been used as a filling material in endodontic procedures over decades. The newer formulations of MTA were launched in the dental market and their cytotoxic, proliferative and antimicrobial effects need to be revealed. This study compared the possible cytotoxic and proliferative effects on fibroblasts and the antimicrobial activity against *Streptococcus mutans*, *Lactobacillus acidophilus*, and *Enterococcus faecalis* of four different MTAs in the dental market.

Materials and Methods: Cytotoxicity assay was performed on 3T3 fibroblast cell lines were determined using yellow tetrazolium 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, while the antimicrobial activity was tested with the broth microdilution method.

Results: RetroMTA, AngelusMTA, and NeoMTA demonstrated a proliferative effect on 3T3 cells, suggesting induction in tissue repair. Moreover, NeoMTA showed the highest antimicrobial activity against all strains tested.

Conclusion: According to our study, NeoMTA and RetroMTA may be recommended for clinical applications in comparison with the conventional AngelusMTA.

Keywords: Mineral trioxide aggregate, MTA, cytotoxicity, proliferative effect, antimicrobial activity

Öz

Amaç: Mineral trioksit agregat (MTA), endodontik prosedürlerde dolgu malzemesi olarak yıllardır kullanılmaktadır. MTA'nın sitotoksik, proliferatif ve antimikrobiyal etkilerinin yeni formülasyonlarda test edilmesi ihtiyacı oluşturmaktadır. Bu çalışma, fibroblastlar üzerindeki olası sitotoksik ve proliferatif etkileri ile dört farklı MTA'nın *Streptococcus mutans*, *Lactobacillus acidophilus* ve *Enterococcus faecalis* üzerindeki antimikrobiyal aktivitesini karşılaştırmayı amaçlamaktadır.

Gereç ve Yöntemler: 3T3 fibroblast hücre hatları üzerinde sitotoksisite analizi sarı tetrazolyum 3-(4,5-dimetiltiyazol-2-il)-2,5-difeniltetrazolium bromid kullanılarak, antimikrobiyal aktivite ise sıvı mikrodilüsyon yöntemi ile test edildi.

Bulgular: RetroMTA, AngelusMTA ve NeoMTA, 3T3 hücreleri üzerinde proliferatif etki gösterdiği gözlemlenmiştir. Antibakteriyel etki açısından incelendiği zaman NeoMTA en yüksek etkiyi göstermiştir.

Sonuç: Çalışmamıza göre NeoMTA ve RetroMTA'nın klinik uygulamalarda klasik AngelusMTA'ya alternatif olarak önerilebileceği düşünülmektedir.

Anahtar Kelimeler: Mineral trioksit agregat, MTA, sitotoksisite, proliferatif etki, antimikrobiyal aktivite

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Introduction

Majority of endodontic failures occur in a consequence of leakage of irritants into the periapical tissues. An ideal filling material should seal the root canal system and it should not have cytotoxic or genotoxic effects, also it should be compatible with host tissues and dimensionally stable (1). Therefore, an important aim of endodontic therapy is the elimination of microorganisms from the root canal. The amount of microorganisms inside the infected root canal is reduced by intracanal medication, instrumentation and irrigation (2,3). Thus, an endodontic sealer with antimicrobial effects may be beneficial in order to eliminate complications after endodontic processes.

Mineral trioxide aggregate (MTA) was developed during the 1990s. Although initially recommended as a root-end filling material, in the following years it has been used for pulp capping, pulpotomy, apexogenesis, apical barrier formation in teeth with open apexes, repair of root perforations, and as a root canal filling material (1,2).

Different types of MTA were released from different manufacturers since the first MTA production. Revealing the possible cytotoxic or proliferative effects of MTA is important as it is in close contact with the gingiva and the fibroblasts which are the predominant cell type in the area (3,4). This study aimed to investigate and compare different MTAs in the dental market according to their possible cytotoxic and proliferative effects on fibroblasts, also to reveal antibacterial activity against *Streptococcus mutans* ATCC 25175, *Lactobacillus acidophilus* ATCC 4356 and *Enterococcus faecalis* ATCC 29212, which are the most frequently recovered microorganisms from refractory periapical periodontitis. The null hypothesis is that there is no difference between different types of MTA according to their possible cytotoxic and proliferative effects, and antibacterial activity.

Materials and Methods

Tested cements

MTA-Angelus (AngelusMTA; Angelus Soluções Odontológicas, Londrina, Brazil), EndocemMTA (CemMTA; Maruchi, Wonju-si, Korea), RetroMTA (RetroMTA; BioMTA, Daejeon, Korea) and NeoMTA (NeoMTA; Avalon Biomed Inc. Bradenton, FL, USA) were tested to reveal their antibacterial activity and cytotoxicity. Since the materials used in this study do not related with any patient, ethical and informed patient approval was not required.

Bacterial strains

Streptococcus mutans American Type Culture Collection (ATCC) 25175, *Lactobacillus acidophilus* ATCC 4356 and *Enterococcus faecalis* ATCC 29212, which are primary dental pathogens were chosen in order to compare MTAs antibacterial activity and these strains purchased from Refik Saydam National Public Health Agency, Turkey.

Antimicrobial Broth Microdilution Test

Antimicrobial activity of the selected MTAs were performed according to the instructions of the Clinical Laboratory Standards Institute. Ten mg/mL of each MTA was dissolved in Brain Heart Infusion Broth, and 180 uL of each MTA dissolved medium was added to the first well of the relevant row of the 96 well plate. Serial dilutions were done for each MTA dissolved medium up to 8 fold. Overnight broth cultures of *S. mutans*, *L. acidophilus* and *E. faecalis* were adjusted to the turbidity of a 0.5 McFarland standard. Twenty uL of each strain were inoculated each well. Broth without MTA materials was served as controls for comparison. Plate incubated overnight and bacteria levels in each well were measured with a spectrophotometer at 600 nm (5).

Cytotoxicity Assay

The cytotoxic effects of RetroMTA, AngelusMTA, NeoMTA and CemMTAs on 3T3 embryonic mouse fibroblast cell lines were determined using yellow tetrazolium 3- (4-5-dimethyl thiazolyl-2) -2,5-diphenyltetrazolium bromide (MTT). The 3T3 cell line was cultured in "Dulbecco's Modified Eagle's Medium/High Glucose" (DMEM/High, Gibco 41966), containing 10% (v/v) fetal bovine serum (heat-inactivated), 1% (v/v) penicillin-streptomycin antibiotic. All incubations were done at 37 °C in a humidified atmosphere of 5% CO_2. MTT Assay was performed according to the recommended protoco (6). Flow-chart for MTT cytotoxicity assay peocedure was shown in Figure 1.

Statistical Analysis

When the tests were performed twice, 10 uL of distilled water was applied to the control group which was considered as 100% viable.

Cell viability % = [Absorbance570 (treated wells)/ Absorbance570 (control wells)] x 100

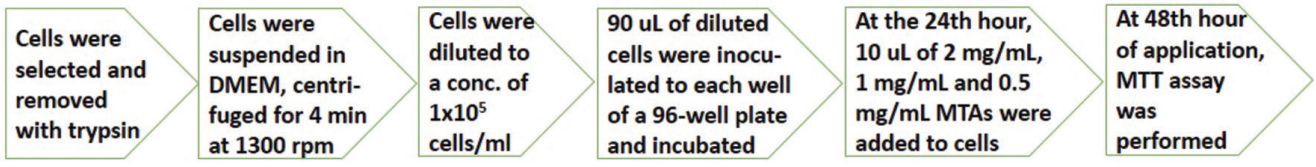

Figure 1. Flow chart for MTT cytotoxicity assay procedure
MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

One-way ANOVA test was utilized to analyze the consistency between the data obtained from MTT tests to determine the effects of MTAs on 3T3 cell viability using IBM SPSS software version 22.0 (IBM Corporation, New York, USA). The significance of the experimental groups according to the control group was analyzed by Dunnett's test.

The concentrations of the substances were applied to the apse and the cell viability (%) data was placed on the ordinate and the graphics created with GraphPad Prism 8.0 program (Graph-Pad Software, Inc., San Diego, CA, USA).

Results

Antibacterial Broth Test

Antimicrobial susceptibility tests revealed that 10 mg/mL NeoMTA inhibited the growth of *S. mutans*, *L. acidophilus* %100 and *E. faecalis* at a rate of 89% and had the most powerful effect against bacterial growth. Also, it has been observed that NeoMTA had an inhibitory effect on the growth of *S. mutans* and *E. faecalis* at the lowest concentration tested which was 1.25 mg/mL.

In addition, 10 mg/mL AngelusMTA inhibited the growth of *L. acidophlius*, *S. mutans* and *E. faecalis* at the following rates 100%, 99% and 62%, respectively. Also, it had an inhibitory effect on the growth of *S. mutans* and *E. faecalis* until the lowest concentration tested which was 1.25 mg/mL.

Moreover, 10 mg/mL RetroMTA had an inhibitory effect on *S. mutans*, *L. acidophilus* and *E. faecalis* at the rates of 89%, 70% and 75%, respectively. RetroMTA's inhibitory effects were observed at descending rates until 1.25 mg/mL for *S. mutans* and *E. faecalis*, and 2.5 mg/mL for *L. acidophilus*.

Finally, according to antimicrobial susceptibility tests 10 mg/mL CemMTA had an inhibitory effect on *L. acidophilus* and *E. faecalis* at the rates of 94% and 11%. However, it did not show any inhibition on the growth of *S. mutans*.

All the results of antimicrobial susceptibility assays for *S. mutans*, *L. acidophilus* and *E. faecalis* were given in Table 1.

Cytotoxicity Assay

The highest application dose (2 mg/mL) of NeoMTA and RetroMTA's cytotoxic effect could not be measured in the spectrophotometer due to the sediment formed resulting from the interaction between MTA and the medium.

Table 1. Inhibitory effects (%) of different types of MTA against the growth of *S. mutans*, *L. acidophilus* and *E. faecalis*

	MTA	10 mg/mL	5 mg/mL	2.5 mg/mL	1.25 mg/mL
S. mutans ATCC 25175	NeoMTA	99.00%	93.48%	52.31%	28.14%
	RetroMTA	88.87%	38.16%	11.61%	10.65%
	AngelusMTA	98.73%	69.16%	14.15%	5.56%
	CemMTA	0.00%	0.00%	0.00%	0.00%
L. acidophilus ATCC 4356	NeoMTA	100.00%	83.02%	15.84%	0.00%
	RetroMTA	70.42%	30.73%	1.15%	0.00%
	AngelusMTA	100.00%	51.34%	-9.16%	0.00%
	CemMTA	94.27%	84.73%	31.49%	0.00%
E. faecalis ATCC 29212	NeoMTA	89.10%	67.29%	28.41%	22.53%
	RetroMTA	74.89%	26.54%	19.66%	16.64%
	AngelusMTA	62.41%	20.09%	8.90%	4.73%
	CemMTA	11.33%	-68.29%	30.42%	0.00%

MTA: Mineral trioxide aggregate

Table 2. Cell viability % results of four MTAs

	2 mg/mL	1 mg/mL	0.5 mg/mL
RetroMTA	Not measured	74.33±5.21	105.48±6.34
AngelusMTA	Not measured	86.52±3.97	104.89±4.49
NeoMTA	Not measured	73.29±4.78	106.35±8.39
CemMTA	Not measured	Not measured	88.32±3.5

MTA: Mineral trioxide aggregate

Cytotoxicity assays revealed that the application dose (1 mg/mL) of RetroMTA, AngelusMTA and NeoMTAs were determined to have 26%, 14% and 27% cytotoxic effect on 3T3 cells, respectively (**p<0.01, **p<0.01, ***p<0.001, respectively).

The lowest application dose (0.5 mg/mL) of RetroMTA, AngelusMTA and NeoMTA was determined to have 5%, 5% and 6% proliferative effect on 3T3 cells, respectively (***p<0.001, **p<0.01, ***p<0.001, respectively).

Moreover, CemMTA's cytotoxic effect in 2 mg/mL and 1 mg/mL concentrations could not be measured spectrophotometrically, because of the sediment formed in the medium. However, it was observed that 0.5 mg/mL CemMTA had a %12 toxic effect on 3T3 cell line. The cytotoxic effects of all tested MTA's were given in Figure 2. In addition, cell viability % results of four MTAs were given in Table 2.

Discussion

MTA was developed as a root-end filling material and it has been used for a long time. Different types of new MTAs were released from different manufacturers (1,2). However, these new MTAs should be investigating a focus on biocompatibility and antibacterial activity.

According to the findings of the present study, we tested three oral bacteria that were commonly associated with oral diseases. It was reported in previous studies that initial carious lesion is associated with *S. mutans* and cavitated lesion are associated with both *S. mutans* and *Lactobacillus* (1,3). MTA is used as a direct/indirect pulp capping material. the materials used in deep cavities should be able to maintain the vitality of the pulp, prevent the entry of residual bacteria into the root canal system and reduce the pulp inflammation (7,8).

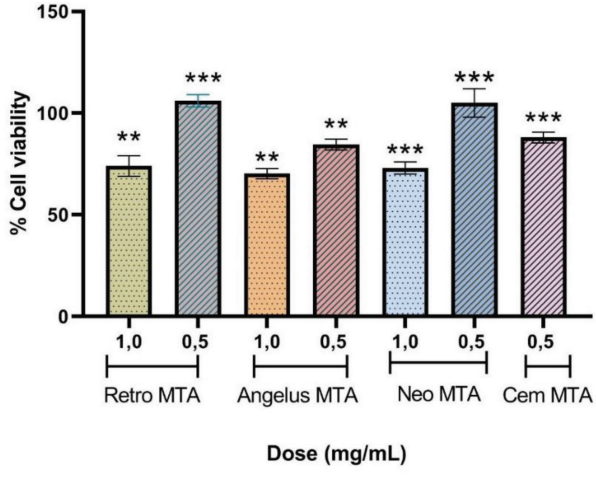

Figure 2. Cytotoxic effects of four kind of MTA's in different concentrations

*MTA: Mineral trioxide aggregate, **p<0.01, ***p<0.001, vertical bars demonstrating standard deviation values*

NeoMTA, AngelusMTA and RetroMTA have similar antibacterial effects to *S. mutans*. The only exception was the non-significant antibacterial activity of CemMTA against the *S. mutans*. These results agree with previous studies. Luczaj-Cepowicz et al. (9) reported that AngelusMTA had a good antibacterial effect against the standard strains of *S. mutans*. Donyavi et al. (10) reported that RetroMTA had antibacterial activities against the *S. mutans*. To the best of our knowledge, there is no study about the antibacterial activity of CemMTA and NeoMTA.

There are no reports in the literature of studies that have examined the antimicrobial properties of the MTAs against *L. acidophilus*. Our study was the first study to collect this data. NeoMTA and AngelusMTA had the greatest antibacterial effect against the growth of *L. acidophilus*. The concentration (CFU/mL) of this species in the presence of this biomaterial was zero (100% reduction). RetroMTA and CemMTA demonstrated acceptable antibacterial activity against the standard strains of *L. acidophilus*. Despite the fact that NeoMTA and AngelusMTA were very successful in inhibiting the growth of *L. acidophilus*, either RetroMTA and CemMTA could be used for pulp capping in deep caries lesion.

We investigated the antibacterial effect of *E. faecalis* because it is the most isolated microorganism from the infected root canals. Antibacterial properties of root-canal sealers gain importance in preventing the regrowth of bacteria in the root canals (11). It was reported that MTA has an antibacterial effect against the *E. faecalis* in previous studies (12,13). Donyavi et al. (10) reported that RetroMTA had antibacterial activities against the *E. faecalis*. Koçak et al. (14) reported that AngelusMTA had acceptable MBCs against *E. faecalis*. In the present study, NeoMTA, RetroMTA and AngelusMTA showed similar antibacterial activity against *E. faecalis*. Our results agree with previous studies.

The main components of the antibacterial effect of MTA are tricalcium silicate and dicalcium silicate. When these components are mixed with water, alkaline calcium silicate gel forms. The calcium hydroxide in the silicate matrix releases hydroxide ions. As a result, a highly alkaline environment is formed and bacterial growth is prevented (15-17). In addition to these two main components, materials with different properties have been added to the MTAs used today. Differences in the antibacterial activities of the four MTA types used in this study are probably the result of differences in structure and composition.

The cytotoxicity of end-root filling materials is a major concern for dentists. Antimicrobial components in the root-canal sealers do not have selective toxicity, they may show toxic effects on host cells. The toxic effects of these materials can cause degeneration of periapical tissue and delay wound healing (1-3,18,19). In the present study, the biocompatibility of RetroMTA, AngelusMTA, NeoMTA and CemMTA, was evaluated by using a MTT assay, comparing their cytotoxicity with well-studied AngelusMTA.

MTT test was utilized in order to evaluate the metabolic effects of AngelusMTA, CemMTA, RetroMTA and NeoMTA on 3T3 cells. In living cells, due to the presence of the mitochondrial dehydrogenase enzyme, the tetrazolium ring of the MTT (3- (4,5-dimethylthiazole-2,5-diphenyltetrazolium)) molecule is cleaved, resulting in formation of water-insoluble formazan crystals. Then, formazan crystals are dissolved by DMSO and their absorbances are measured with a spectrophotometer at 570 nm. MTT may activate apoptosis-related factors such as intracellular caspase-8, caspase-3, or intracellular leaks that may occur according to the formation of MTT formazan crystals. Thus, attention should be taken in order not to lose control of cell viability during the MTT test, which is one of the most widely used methods to analyze cell viability and proliferation. However, there may be deviations in the MTT test as a result of the interaction of metabolic rate and mitochondria number with various factors, which is the main disadvantage of the MTT method (20,21).

Eukaryotic cells isolated from animal tissues and having limited ability to reproduce under standard conditions are prevented from aging by providing continuous reproduction ability in cell culture (22). These cells have been used for many years in many biological and biochemical researches, such as drug/chemical agent-dose trials.

The cell line to be used to determine the toxicity of chemical agents on the cell should be related to the natural use of the chemical (23). ISO 10993-5 cytotoxicity tests, the study of the toxicity of dental materials on cells, the use of the cell type used in this study is recommended. Therefore, in our study, the cytotoxicity of MTA materials was analyzed using a 3T3 cell line.

Kouchak Dezfouli et al. (24) compared the cytotoxicity of RetroMTA with ProRootMTA and reported that both of them showed similar biocompatibility. In our study, AngelusMTA showed a better percentage of cell viability. RetroMTA and NeoMTA showed similar cell viability. RetroMTA, AngelusMTA and NeoMTA have a proliferative effect on 3T3 cells, suggesting induction in tissue repair. As a result of our findings, it can be recommended to use MTA as a pulp capping material.

Conclusion

In conclusion, the NeoMTA showed the best antibacterial activity against all strains we tested *in vitro* and it was also found to be the most biocompatible material according to our results. Therefore, based on our findings on the antibacterial effect of tested MTA materials against the main bacteria associated with dental diseases, NeoMTA and RetroMTA may be recommended for dental clinical applications when compared with conventional AngelusMTA in the dental market.

Ethics

Ethics Committee Approval: Since the materials used in this study do not related with any patient, ethical approval was not required.

Informed Consent: Since the materials used in this study do not related with any patient, informed patient approval was not required.

Peer-review: Externally and internally peerreviewed.

Authorship Contributions:

Concept: E.E., Design: Ö.Ü., M.D., Data Collection or Processing: E.E, Analysis or Interpretation: M.D., S.K., Literature Search: E.E., Writing: Ö.Ü., E.E., M.D., S.K.

Conflict of Interest: No conflict of interest was declared by the authors.

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