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Comparison of Cytotoxicity between Mineral Trioxide Aggregate Mixed with Chlorhexidine and Common Endodontic Regeneration Medicaments on Periodontal Ligament Stem Cells: an in Vitro Study

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A B S T R A C T

Objectives: The combination of mineral trioxide aggregate (MTA) and 2% chlorhexidine (CHX) has been recently introduced as an intracanal medicament. The aim of this study was to evaluate the potential cytotoxic effects of MTA mixed with 2% chlorhexidine gel on human periodontal ligament stem cells (PDLSCs) and compare it with other common endodontic regeneration medicaments.

Materials and Methods: Minimum inhibitory concentration and minimum bactericidal concentration of six experimental groups against *Enterococcus faecalis* was determined. The study groups consisted of RetoMTA mixed with 2% chlorhexidine gel (MTA+CHX), calcium hydroxide (CH), CH mixed with CHX gel, two concentrations of double antibiotic paste, and 2% CHX. The direct cytotoxic effect of minimum bactericidal concentration was evaluated by MTT on PDLSCs on days 1, 3, and 7. One-way ANOVA and post hoc tests were used for data analysis ($P<0.05$).

Results: The viability of cells treated with MTA+CHX decreased significantly over time ($P<0.05$) making this group the most cytotoxic intracanal medicament on the 3rd and 7th days of treatment. On day one, the highest viability percentage was detected in the CH+CHX group followed by the CHX group. On day 3, CH+CHX and CHX groups displayed the highest viability percentage. On day 7, the highest viability was observed in the CHX group, which showed no significant difference with the control group ($P=0.12$).

Conclusion: Regarding the antimicrobial potency of intracanal medicaments at minimum bactericidal concentration levels, CHX gel appears to be the least cytotoxic drug, while MTA+CHX shows the highest reduction in viability percentage.

Keywords: Mineral Trioxide Aggregate; Chlorhexidine; Calcium Hydroxide; Anti-Bacterial Agents; Regenerative Endodontics; Stem Cells; Cytotoxicity Tests, Immunologic

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INTRODUCTION

The chief etiology of periapical diseases is invasion of pathogens through root canals into periradicular tissues due to the progression of pulpal inflammation or bacterial infection [1]. Therefore, all endodontic treatment strategies aim to remove these bacteria and their by-products from root canal spaces [2]. Regenerative endodontics combines the use of tissue engineering, stem cells, biomimetic scaffolds and bioactive growth factors in the canal system to rejuvenate the pulp tissue

affected by infection, trauma or developmental anomalies [3]. It is important that viable cells are not destroyed during root canal treatment by the cytotoxicity of irrigants or intracanal medicaments to allow tissue regeneration. However, disinfection of the root canal space with irrigation solutions and medicaments is one of the major steps during endodontic treatment as well as regenerative treatments [4]. During this process, different medications, including a mixture of different antibiotics (triple or double) and calcium hydroxide (CH), are used within the root canals [5].

CH has been vastly utilized as an intracanal medication and has been recommended in regenerative treatments due to its favorable antibacterial properties [6,7]. These properties have been attributed to its high pH (about 12.5), which prevents the growth and survival of most of bacteria because they cannot tolerate highly alkaline environments [8]. CH is used as a paste [9], and incomplete removal from the root canal network is one of its drawbacks [10]. On the other hand, studies have shown that there are concerns about its ability to serve as a strong and persistent antibacterial agent in some regeneration treatment cases [11].

Mineral trioxide aggregate (MTA) has been successfully applied to repair perforations on the lateral and furcal areas of the root, as pulp-cap medication, and as a filling material for root-ends. Some studies have demonstrated that application of MTA to infected partially incomplete tooth roots, can induce hard tissue formation and apical seal. MTA is a biocompatible material, induces osteoblastic activity, and has a favorable sealing ability [12]. Despite all these positive characteristics, MTA has some disadvantages, including prolonged setting time, a discoloration aptitude, difficulties in handling, and steep pricing [13]. Moreover, some studies have demonstrated that the antibacterial features of MTA are limited [14,15]. Bidar et al. [16] indicated that chlorhexidine (CHX) mixed with MTA, and CEM cement confers antimicrobial effects against *Enterococcus faecalis* (*E. faecalis*), while MTA and CEM cement alone do not have such activity. Mahmoud et al. [17] showed that the addition of 2% CHX to calcium silicate-based cements prevents its setting reaction in an 84-day period. Calcium ion release and flowability was superior to CH paste and it was successfully removed from the root canals.

Since MTA combined with 2% CHX has potential as an intracanal medicament, the present study aimed to evaluate its cytotoxicity against periodontal ligament stem cells (PDLSCs) compared to commonly used medicaments applied in regenerative endodontic treatment.

MATERIALS AND METHODS

The study protocol was approved by the Research Ethics Committee of Tehran University of Medical Sciences (approval no: IR.TUMS.REC.1399.158). All experiments were performed in triplicate.

Minimum inhibitory concentration and minimum bactericidal concentration assessment

E. faecalis (ATCC 29212) was used to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the test materials.

MIC, a chemical's lowest concentration preventing visible bacterium growth [18], was determined using the Broth Microdilution method [19]. Following incubation of different concentrations of each of the materials with *E. faecalis* at 37°C for 24 hours, the wells were examined for bacterial growth and the minimum concentration of substances that inhibited bacterial growth was recorded as MIC. In cases where bacterial growth was found in all concentrations of a substance, higher concentrations of the primary stock were used. MBC represents the lowest concentration of any antibacterial substance needed to eliminate a particular bacterium [19,20]. Cultures (100µl) from the wells with test-substance concentrations equal to and higher than MIC were transferred to plates containing Müller Hinton agar media (Merck-Germany) and stored for 24 hours at 37°C. At the end of the incubation period, the minimum concentration at which the bacteria did not survive was reported as MBC [21].

Primary Cell Culture

PDL stem cells were purchased from the National Cell Bank of Iran (Lig-01 code 11329, Pasteur Institute of Iran, Tehran) and cultured in 96-well plates (SPL, SPL Life Science Co., Korea) containing Dulbecco's modified eagle's medium (DMEM) (Gibco; Carlsbad, California, United States) supplement with 10% fetal bovine serum at 10,000 cells/well for 24 hours. Following attachment, the cells were treated with serum-free DMEM and minimum bactericidal concentrations of the test groups [22].

Medicament Preparation

The study groups (N=8, each) consisted of:

• *MTA+CHX*: RetroMTA (bioMTA, Seoul, South Korea) was mixed with 0.1% CHX gel (Morvabon, Tehran, Iran) at a 1:1 ratio by dissolving 25mg of Retro MTA and 25mg of 2% CHX gel in 5ml PBS (Sigma-Aldrich, USA) to obtain a concentration of 10mg/ml.

• *CH*:10mg CH powder (Golchadent, Tehran, Iran) was dissolved in 1ml PBS (Sigma-Aldrich, USA) to reach a concentration of 10mg/ml.

• *CH+CHX*: CH (Golchadent, Tehran, Iran) was mixed with 0.1% CHX gel (Morvabon, Tehran, Iran) at a 1:1 ratio. Equal weights of both drugs, i.e., 5mg of calcium hydroxide (Golchadent, Tehran, Iran) and 5mg of 2% chlorhexidine gel, were mixed together and then dissolved in 5ml PBS (Sigma-Aldrich, USA) to obtain a concentration of 10mg/ml.

• *CHX*: 10 mg CHX 2% gel (Morvabon, Tehran, Iran) was dissolved in 1ml of PBS (Sigma-Aldrich, USA) to obtain a concentration of 10 mg/ml of this drug

• *Double antibiotic paste (DAP) 500/500*: this was a combination of 500mg ciprofloxacin (Tehran Darou, Tehran, Iran) and metronidazole 500mg (Pars Darou, Tehran, Iran). For preparation, first the coating of both tablets was removed with a sterile surgical blade. The tablets were then crushed as much as possible and combined in equal weights (5mg) and finally dissolved in 1ml PBS (Sigma-Aldrich, USA) to obtain an initial stock at a concentration of 10mg/ml.

• ***DAP 250/500***: was composed of 250mg metronidazole (Pars Darou, Tehran, Iran) and 500mg ciprofloxacin (Tehran Darou, Tehran, Iran). Preparation was similar to the pervious group.

MTT assay

Fifty mg of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-2H-tetrazoliu bromide) powder (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in 1 ml PBS solution (Sigma-Aldrich) until a final concentration of 5 mg/ml was reached. Next, the test and control groups were incubated with 50µl of the 5mg/ml MTT solution for 3 hours at 37°C and 5% CO2 followed by addition of 250µl dimethyl-sulfoxide (DMSO) solution to dissolve formazan crystals. The optical density was read by an Elisa Reader (Anthons2010, Biochrom, UK) at 570nm and recorded [22]. Viability percentage was calculated on days 1, 3, and 7, using the following formula:

Cell viability (%) = absorbance value of treated cells/absorbance value of untreated cells × 100.

Comparisons were performed by one-way analysis of variance (ANOVA) and post hoc tests (Tukey and Games-Howell). Statistical power was set at $P<0.05$.

RESULTS

Based on the results of the MTT assay (Fig. 1), the highest viability percentage on day 1 was detected in the CH+CHX group (98.62%± 2.64%) followed by the CHX group (93.37%±8.39%). The controls presented no significant difference with the CH+CHX ($P=0.75$) and CHX ($P=0.38$) groups.

On day 3, viability percentage was highest in the CH+CHX (75.61%±15%) and CHX (69.68%±8.94%) groups. However, the controls demonstrated significantly higher viability percentage compared to the other groups ($P<0.05$). On day 7, the highest viability percentage was observed in the CHX group (93.94±5.35) and there was no significant difference between the CHX group and controls ($P=0.12$). In the MTA+CHX group viability percentage significantly decreased with time and its highest and lowest amounts were observed on days 1 and 7, respectively. This decrease was also observed in the CH+CHX group from day 1 to day 3 to day 7 ($P<0.05$). Viability percentage in the CH group was significantly higher on the first day

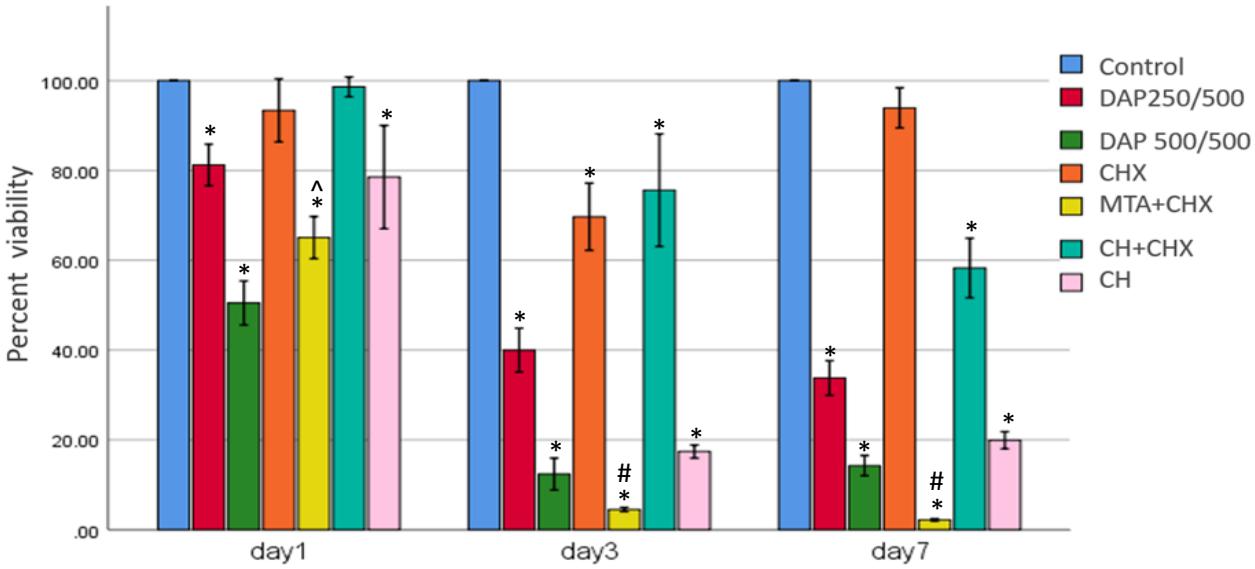

Fig. 1. Viability percentage of periodontal ligament stem cells in experimental and control groups at minimum bactericidal concentration by MTT on days 1, 3 and 7 (error bar: 95% confidence interval). *P<0.05 in comparison to controls; #P<0.05 in comparison to all groups; ^P<0.05 in comparison to all groups, except CH; all comparison results among different time points have been specified in the text. DAP: double antibiotic paste (mg), CHX: chlorhexidine, MTA: mineral trioxide aggregate, CH: calcium hydroxide

compared to the other days and showed gradual reduction until the 7th day. The viability percentage was lowest on the 3rd day, but the difference was not significant with day 7. Viability percentage decreased in the DAP500/500 group with the highest amount found on day one, followed by day 7; however, no statistically significant difference was noticed between days 3 and 7. Survival of PDLSCs in the DAP250/500 group sig-nificantly decreased in a time-dependent manner.

For CHX group, cell viability percentage on the 1st and 7th days were significantly higher than the 3rd day (P<0.001). In general, the CHX group showed the best results with a relatively high survival rate at all time points, while the lowest survival rate (except for day 1) was found in the MTA+CHX group, which demonstrated significant difference with the other groups.

DISCUSSION

Residual bacteria inflict a critical negative effect on the outcome of regenerative endodontic treatments [23]. Since revascularization ceases in the presence of infection, total elimination of bacteria from the root canal plays an important part in a successful revascularization process [24]. Most studies in the field of endodontic regeneration have been limited to case reports or case series and the best intracanal medicament with the lowest cytotoxicity and highest antimicrobial property is yet to be determined. An in vitro study by Mahmoud et al. [17] showed the possibility of using MTA as an intracanal medication by mixing it with 2% CHX to slow down its setting time. The calcium ion release and flowability of this mixture was shown to exceed that of CH. Moreover, its removal from the root canal wall was successfully achieved. This basic study highlighted the potential application of MTA+2% CHX as a root canal medication due to its superior physical properties; however, the need for further investigation especially on its possible cytotoxic effects was highlighted. Therefore, we aimed to assess its potential cytotoxicity on PDLSCs. The reason for selecting these cells was their role in the regeneration of PDL, cementum, and bone in periapical lesions [25]. Additionally, their effects may be similar to apical papilla stem cells [26].

Several methods are used for the evaluation of cytotoxicity, including flow cytometry, MTT/ XTT, WST-1, WST-8 assay, and assessment of lactate dehydrogenase activity. In the present study, MTT assay was utilized to determine the cytotoxic impacts of different experimental groups. This method is widely applied as a standard technique for evaluation of newly developed dental materials and is based on the capability of viable cells to turn water-soluble tetrazolium salts to insoluble formazan crystals through the activity of mitochondrial dehydrogenase enzymes. It helps determine the effect of the experimental biomaterial on the proliferation/metabolic rate of cells. Although this method is straightforward, it has some limitations. For example, cell damage is underestimated and only the apoptotic phase of cell death is considered which makes the generalization of results to clinical conditions rather complicated [27].

The original goal of this study was to investigate the potential cytotoxicity of retro-MTA mixed with CHX as an intracanal medicament and our results showed that its cytotoxicity increased significantly over time. This may be due to the fact that MTA has strong alkalizing activity [17] which can have an adverse effect on cell viability [28]. According to the result of the current investigation, viability percentage in the MTA+CHX group after 7 days was less than 30%, indicating that the mixture was highly cytotoxic. This is consistent with the results of Hernandez et al. [29], who showed that ProRoot MTA+0.12% CHX increased apoptosis of fibroblasts and macrophages in mice. Maintenance of a high pH over time was suggested to be responsible for this finding. In contrast to our results, Summer et al. [30], reported biocompatibility of MTA+CHX after 60 days implantation into rats.

We prepared a double antibiotic paste using two different doses of metronidazole (500 or 250mg) mixed with 500mg ciprofloxacin, and the results showed that the viability of cells treated with these two pastes were significantly different at different time points. Higher cytotoxicity was observed in the combination containing metronidazole 500mg. It is important to note that the MBC of the two pastes against *E. faecalis* was equal despite the use of different doses of metronidazole. This indicates that while having similar antimicrobial effects, DAP 250/500 produced significantly less cytotoxic effects on PDLSCs. It is noteworthy that we used antibiotic tablets available in the Iranian pharmaceutical market for the preparation of DAP. This has the advantage of similarity to clinical settings; however, clinical doses are much higher and demonstrate stronger cytotoxicity.

When comparing DAP250/500 with CH, our day 3 findings were similar to that of Ruparel et al. [31], but DAP500/500 showed higher cytotoxicity. Similar results were observed at concentrations below 1 mg/ml in a study by Saberi et al. [32] who found no significant difference between their CH and CH+CHX groups in low concentrations, while both groups showed a significant difference with DAP. However, this significant different was not observed at 1mg/ml concentration between the CH and DAP250/500 groups.

There are case reports on the use of CHX as an irrigation solution or CHX+CH as an intracanal medication [33] in successful regenerative endodontic treatments. However, it is not recommended by the American Association of Endodontics (AAE) [6] or European Society of Endodontology (ESE) [7] Despite its broad-spectrum antimicrobial properties and long-lasting effects [34], the biggest drawback of CHX is its lack of tissue-dissolving properties [35]. The combination of CHX with CH increases the antimicrobial properties of the latter, which has been shown to have no antimicrobial properties against *E. faecalis* [36]. However, it has the potential to be used as both a final irrigation and an intracanal medicament in regenerative endodontic treatment.

Finally, among the tested medicaments, CHX had the best results in terms of cytotoxicity on PDLSCs, which was in line with other studies showing dose- [37,38] and time-dependent [39] cytotoxic effects of CHX. However, the concentration used in the present study (MBC) exhibited very low cytotoxicity. Similar to our observations, Giannelli et al. [40] suggested

that very high clinical concentrations of CHX are cytotoxic, but its effective concentration is much lower (about 200-fold). Widbiller et al. [40] used CHX as an irrigant in endodontic regenerative treatment and showed that it had no adverse effect on the viability of stem cells of apical papilla at concentrations below 0.001%, but was cytotoxic at higher concentrations.

CH group had significantly higher cytotoxicity compared to the CH+CHX group. It seems that after mixing, CHX increased the antibacterial properties of CH and reduced the MIC and MBC values, leading to the reduction of CH concentration. The decrease in cytotoxicity in the CH+CHX group can be explained by the reduction in the concentration of CH Based on the MTT results of the current investigation; the viability of cells treated with MTA+2% CHX was lower than the other experimental groups. Further studies using clinical concentrations to evaluate the cytotoxicity and antimicrobial properties of these drugs is recommended.

CONCLUSION

According to the results obtained in the present study, reduction of viability percentage was highest in cells treated with MTA+CHX, followed by DAP500/500, and CH. Two-percent CHX or its mixture with CH showed the lowest viability percentage. These findings should be considered along with other properties of this newly introduced medicament. More comprehensive studies are needed to evaluate and optimize the safety and efficacy of MTA+CHX as a new medicament before using it in clinical practice.

CONFLICT OF INTEREST STATEMENT

None declared

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