

Cytotoxicity of Triple Antibiotic Paste and Calcium Hydroxide against Cultured Human Dental Pulp Fibroblasts

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Abstract

Objective: In necrotic immature teeth, intra canal medicaments such as triple antibiotic paste (TAP) and calcium hydroxide (CH) are used for root canal disinfection and regeneration treatment. However, the effect of these medicaments on dental pulp fibroblasts has yet to be known. This study aimed to assess the cytotoxicity of CH and TAP against cultured human dental pulp fibroblasts (HDPFs) obtained from third molars.

Methods: In this *in vitro* study, fibroblasts were obtained from the dental pulp of two third molars. Fibroblasts were exposed to 0.1, 1 and 10 mg/mL concentrations of TAP and CH. Six samples were prepared of each medicament and fibroblast viability was evaluated after 72 hours. Data were analyzed using one-way and two-way ANOVA ($p < 0.001$). The percentage of cell viability was calculated and the cytotoxicity of the medicament was categorized as severe (30%), moderate (30-60%), mild (60-90%) and non-toxic (>90%).

Results: In TAP samples, only the 10 mg/mL concentration had a significant difference with the control group in terms of the percentage of cell viability and showed moderate cytotoxicity. In CH samples, the 1 and 10 mg/mL concentrations showed significant differences with the control group and were severely cytotoxic.

Conclusion: Reduction in cell viability of fibroblasts by increase in concentration was significantly greater in CH compared to TAP group. Thus, in regeneration treatments, these medicaments must be used in concentrations with adequate therapeutic and insignificant adverse effects on fibroblasts.

Key words: Calcium hydroxide, Cytotoxicity, Dental pulp, Fibroblast, Triple antibiotic paste.

Please cite this article as:

Hosseini Matin M, Zare Jahromi M, Fesharaki M, Ostad Sharif M. Cytotoxicity of Triple Antibiotic Paste and Calcium Hydroxide against Cultured Human Dental Pulp Fibroblasts. *J Dent Sch* 2015; 33(3): 196-204.

Received: 21.11.2013

Final Revision: 14.06.2014

Accepted: 17.06.2014

Introduction:

Preservation of dental pulp health is a goal in endodontic treatment. Clinical studies have paid a great deal of attention to pulp-dentin complex regeneration. Regeneration treatments aim to provide conditions for biological regeneration of dental tissues and their supporting structures (1). After birth, stem cells can be used for regeneration of root and periodontal ligament (PDL) supporting crowns. Regeneration can be

done using the residual living pulp tissue even in presence of apical periodontitis and sinus tract in necrotic teeth, more commonly seen in open-apex teeth (2).

Repetitive application of CH is a traditional treatment for necrotic, open-apex teeth. At present, mineral trioxide aggregate (MTA) is also used for treatment of these teeth. However, both these medicaments have disadvantages as well (3).

In the recent years, many cases of

revascularization of necrotic pulp have been reported (4). Researchers and clinicians have used different medications for pulp space disinfection including TAP (1:1:1 mixture of ciprofloxacin, metronidazole and minocycline), other antibiotics, CH alone or in combination with antibiotics and formocresol (4).

The efficacy of TAP (mixture of ciprofloxacin, metronidazole and minocycline) for elimination of bacteria in infected dentin and root canals was first described by Hoshino *et al.*, in 1996. TAP can provide a suitable environment for revascularization and cell proliferation by elimination of bacteria from the root canal system of teeth with necrotic pulp or immature apices (5).

One possible reason for failure of CH treatment may be elimination of stem cells in the first steps of treatment (1). However, studies on the cytotoxicity of TAP are more limited than those on CH. Considering the increasing use of TAP for regeneration treatments, this study aimed to assess and compare the cytotoxicity of CH and TAP against HDPPFs.

Methods:

This *in vitro*, experimental interventional study evaluated the cytotoxicity of CH and TAP against HDPPFs obtained from human third molars. The study was undertaken in the Endodontics Department of Islamic Azad University, School of Dentistry of Khorasgan and the central laboratory of Isfahan University of Medical Sciences in the summer of 2012.

Samples were obtained from two impacted maxillary and mandibular third molars of a 19 year-old female. The teeth were sound and immature and were surgically extracted by a maxillofacial surgeon with minimal trauma. The teeth surfaces were immediately disinfected using gauze soaked with 70% ethanol immediately after extraction and then rinsed with sterile distilled water (Gibco, Karlsruhe,

Germany). The teeth were maintained by a forceps and a section was made at the cemento-enamel junction. A fracture was induced along the section to extract dental pulp (6). Dental pulp was removed using a sterile endodontic file and transferred to the lab in phosphate buffered saline (PBS) transfer medium (Gibco, Karlsruhe, Germany) (7). In the lab, dental pulp was rinsed with PBS and minced into 1mm pieces. Next, 3mg/mL of type I collagenase (Sigma, Berlin, Germany) was added and the mixture was stored in a bain marie bath at 37°C for 60 minutes. Then, the cells were centrifuged for 10 minutes using EBA20 centrifuge (HettichZentrifugen, Tuttlingen, Germany) at 1800 rpm. In the next step, cells were rinsed with sterile PBS twice and filtered using 0.2 micron filter followed by centrifugation for another 10 minutes. The obtained fibroblasts were cultured in a culture medium containing Dulbecco's Modified Eagle's Medium and Ham's F-12 nutrient mixture enriched with 10% fetal bovine serum, 1% L-glutamine, 1% penicillin G, streptomycin and 1% Fungizone and stored in an incubator at 37 °C with 1% CO₂ (6). The culture medium was refreshed every three days until the cells reached 80% confluence (8).

Third passage cells were detached from the bottom of the dish using 5% trypsin (Gibco, Karlsruhe, Germany). After counting, 10,000 cells were transferred to a 24-well plate (Greiner Jet Bio, Munich, Germany) containing the culture medium and stored in an incubator at 37°C with 5% CO₂ for 24 hours (9).

To prepare TAP, 250mg ciprofloxacin tablet (Aria Darou, Tehran, Iran), 250mg metronidazole tablet (Pars Darou, Tehran, Iran) and 100mg minocycline capsule (TeoPharma, Pavia, Italy) were used. Similar amounts of the three antibiotics (1:1:1) were placed on a mixing pad and mixed using a spatula. Next, 10, 1 and 0.1 mg/mL concentrations of the mixture were prepared. Also, 10, 1 and 0.1 mg/mL

concentrations of CH (Merck, Germany) were prepared (10).

In the next step, the prepared concentrations of TAP and CH were added to wells and cells were placed in an incubator for 24 hours (7).

Cells were washed with PBS twice to eliminate the dead cells. Next, 40µL of 0.05% MTT solution (Sigma, Berlin, Germany) and 400 µL of Dulbecco's Modified Eagle's Medium and Ham's F-12 nutrient mixture were added to each well. After 4 hours of incubation at 37°C and 5% CO₂, the overlaying solution was extracted. To dissolve the deposited formazan crystals, 100µL of dimethyl sulfoxide (Sigma, Germany) was added to each well and centrifuged for 10 minutes. The obtained solution was transferred to a 96-well plate and the optical density at 540 nm wavelength was read using ELISA Reader (BioRad, Germany) (9). For reproducibility of results, at least 6 samples were prepared of each medicament and after 72 hours, the viability and proliferation of cells were evaluated.

To compare the results of ELISA in the intervention and control groups, one-way ANOVA and Dunnett's T3 test were used. To assess the effect of type of material and concentration, two-way ANOVA was applied. Considering the significant interaction effect of variables ($p < 0.001$), one-way ANOVA and Tamhane's test were used to compare different concentrations of each material. To compare the same concentrations of the two materials, independent sample t-test was used.

The cell viability (survival fraction) for the two medicaments was calculated as percentage using the formula below:

$$SF = \frac{OD_{\text{test}} - OD_{\text{in cell free wells}}}{OD_{\text{in cell free wells}}} \times 100 \quad (11)$$

And the cytotoxicity of the two medicaments was categorized accordingly as severe (<30%), moderate (30-60%), mild (60-90%) and non-toxic (>90%) (12).

Results:

In this study, fibroblasts were isolated from the pulp of two impacted maxillary and mandibular third molars (Figure 1) and in the next step, TAP and CH were added to the culture medium. The amount of residual cells after exposure with medicaments was evaluated using an ELISA Reader.

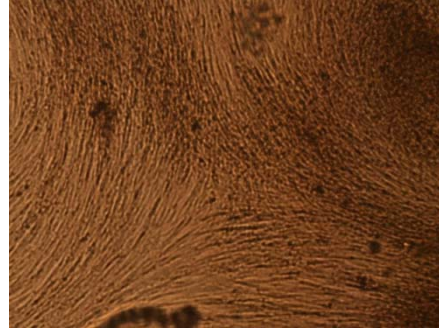
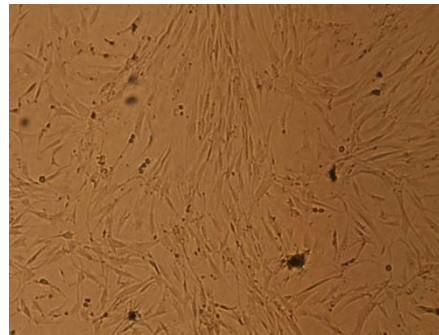


Figure 1- Morphology of fibroblast cells isolated from the dental pulp in culture medium before exposure (third passage cells at 4X magnification)

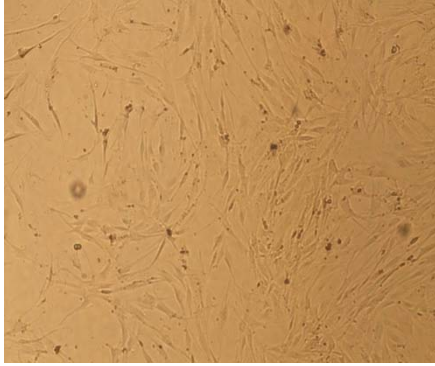
Figure 2 shows samples of fibroblast cells in the culture medium after exposure to TAP.



A (01)

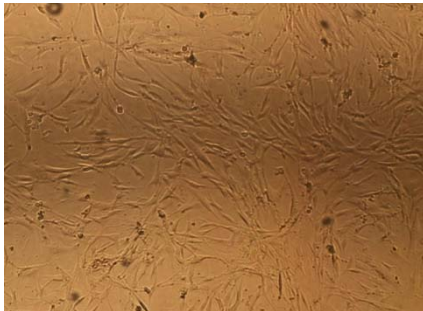


B (1)

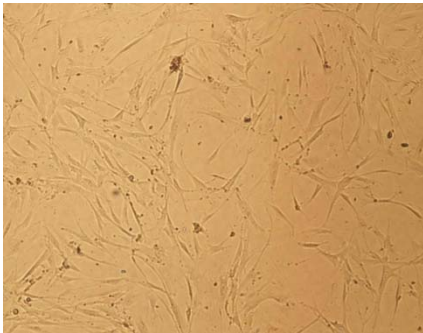


C (10)

Figure 2- Samples of fibroblast cells in the culture medium after exposure to different concentrations of TAP (0.1, 1 and 10)



A (0.1)



B (1)



C (10)

Figure 3- Samples of fibroblast cells in the culture medium after exposure to different concentrations of CH (0.1, 1 and 10)

Assessment of cytotoxicity of different concentrations of TAP and CH against fibroblasts showed that this cytotoxicity was concentration-dependent and the higher the concentration of medicaments the lower the cell survival and cell viability. Cytotoxicity of 0.1 mg/mL CH (2.43 (0.12)) was mild compared to that of the control group (2.78 (0.071)). CH at 1 mg/mL concentration (0.37 (0.04)) was severely cytotoxic compared to the control group (2.78 (0.07)). CH at 10 mg/mL concentration (0.06 (0.01)) was also severely toxic compared to the control group (2.78 (0.07)) ($p < 0.001$).

These findings revealed no significant difference in cytotoxicity of 0.1 mg/mL CH and that of control group and thus, 0.1 mg/mL concentration of CH was found to be non-toxic. The difference in this regard between the control group and 1 and 10 mg/mL concentrations of CH was significant indicating the severe cytotoxicity of CH at 1 and 10 mg/mL concentrations (Diagram 1).

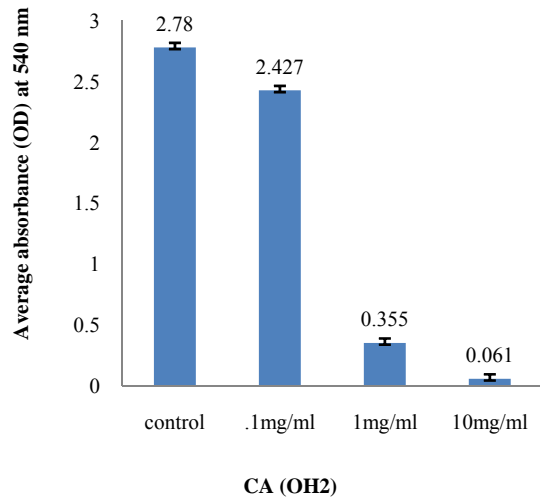


Diagram 1- Cell viability following 72 hours of exposure to different concentrations of CH in comparison to the control group. Data are shown as mean (SD). $p < 0.001$ was considered significant ($n=6$).

The percentage of cell viability showed a significant reduction as well (Diagram 2).

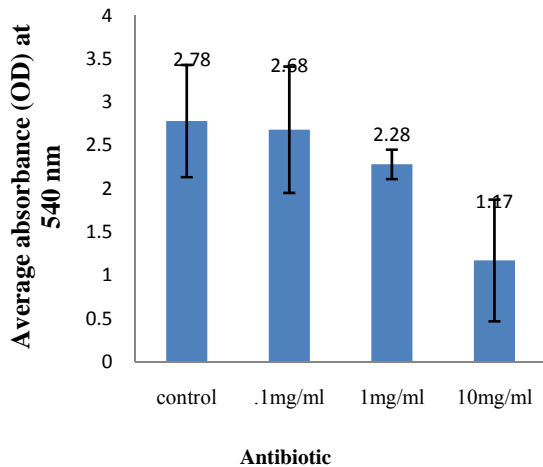


Diagram 2- Cell viability following 72 hours of exposure to different concentrations of TAP in comparison to the control group. Data are shown as mean (SD). $p < 0.001$ was considered significant ($n = 6$).

At 0.1 mg/mL concentration (2.68 (0.64)), TAP showed mild cytotoxicity compared to the control group (2.78 (0.07)). The 1mg/mL concentration of TAP (2.28 (0.18)) also showed mild cytotoxicity compared to the control group (2.78 (0.07)) while the 10 mg/mL concentration of TAP had medium cytotoxicity compared to the control group (2.78 (0.07)) ($p < 0.001$). These findings showed that the control group did not have significant differences with the 0.1 and 1 mg/mL concentrations of TAP in terms of cytotoxicity ($p < 0.001$) but the difference between the cytotoxicity of the control group and the 10 mg/mL concentration of TAP was significant ($p < 0.001$). The percentage of cell viability experienced a greater reduction at 10 mg/mL concentration compared to the other two concentrations (Diagram 3).

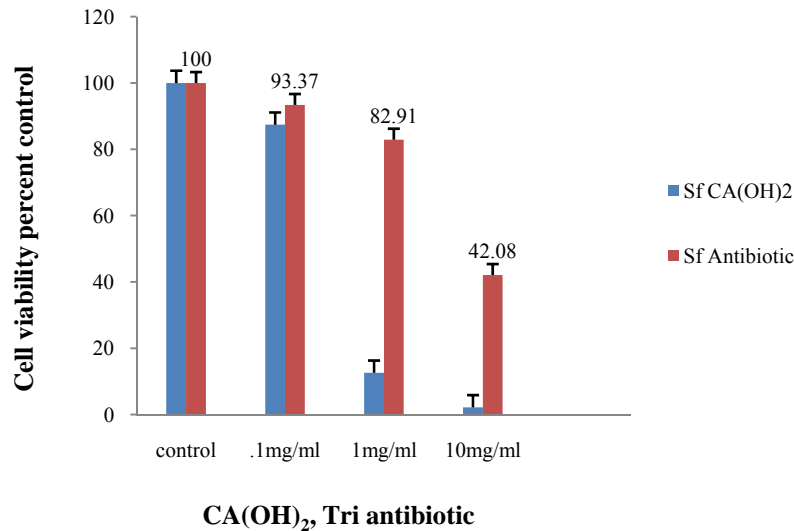


Diagram 3- Percentage of cell viability following exposure to different concentrations of TAP and CH compared to the control group (100% viability). Data are shown as mean (SD) ($n = 6$).

Discussion:

Despite the increase in cases of regeneration treatment, no standard protocol has been published for this treatment so far. This treatment is the first step for a successful infection control by the application of intracanal

medicaments. A study by Trevino *et al.* (2011) showed that the intracanal irrigants had long-lasting effects on cell viability and they stated that the cytotoxicity of intracanal irrigants must be investigated along with their antimicrobial properties (13). Although some studies have evaluated the cytotoxicity of TAP and CH,

number of studies on the cytotoxicity of TAP is scarce indicating the need for further evaluation of this topic.

Bogovic *et al.* in 2011 discussed that CH has high cytotoxicity but it is still known to be biocompatible. Their study was performed on rat fibroblasts (14). Guigand *et al.* in 1999 demonstrated that CH constituents were as toxic as the CH itself (15).

On the other hand, Miranda *et al.* in 2009 reported the cytotoxicity of MTA to be slight or grade I and that of CH to be mild or grade 2. They evaluated cytotoxicity based on cell lysis rather than the percentage of viable cells. Mild or grade 2 cytotoxicity was defined as cell lysis at 5 mm distance and severe cytotoxicity was defined as lysis at 10 mm distance from the specimen (16). Eldeniz *et al.* in 2007 demonstrated that CH- and resin-based sealers were more toxic than other sealers (17).

Wang *et al.* in 2007 evaluated the cytotoxicity of 5 intracanal medicaments and concluded that CH with more than 90% cell viability had slight cytotoxicity especially compared to formocresol and phenol. The difference between their study and ours was the use of PDL fibroblasts of mature teeth. In the current study, pulp fibroblasts of immature teeth were used (18).

Khashaba *et al.* in 2009 evaluated the biocompatibility of a CH-based sealer against gingival and L929 murine fibroblasts and stated that CH was still toxic even after 5 weeks and its cytotoxicity did not decrease over time. In their study, gingival fibroblasts were more susceptible to CH compared to L929 murine fibroblasts (19).

In another study on the cytotoxicity of canal irrigants containing CH against L929 murine fibroblasts, the solutions were diluted and 1/2, 1/5, 1/10 and 1/20 concentrations were prepared. At 24 hours, CH at 1/2 concentrations was cytotoxic but other concentrations were biocompatible. The mentioned study was done by Barbosa *et al.* in 2009. They attributed the

cytotoxicity of CH to its high concentration of 1mg/ml, which is in accord with the current study results (20).

Silva *et al.* in 2012 evaluated the cytotoxicity of CH and similar to the current study, a great reduction in cell viability was observed (21).

Hirschman *et al.* in 2012 evaluated the percentage of viable cells for assessment of the cytotoxicity of CH and reported it to be 37%. Their study was conducted on skin fibroblasts (22).

On the other hand, Al-Shaher *et al.* in 2004 reported that CH at 0.4 mg/mL concentration was cytotoxic and less than 25% of cells survived this concentration. Their study was conducted on pulp and PDL fibroblasts (7).

Literature shows that CH is toxic against different types of fibroblasts such as the skin, L929 murine, gingival and pulp fibroblasts. In the current study, the cytotoxicity of CH was concentration-dependent and the higher its concentration, the lower the cell viability. CH at 0.1 mg/mL concentration was slightly toxic but 1 and 10 mg/mL concentrations were severely toxic.

Cytotoxicity of CH may be attributed to its high pH because the high pH of CH and the released hydroxyl ions can cause cell necrosis and apoptosis in adjacent cells. This finding is in accord with the results of Silva (2012)(22).

According to a study by Bose *et al.* in 2009, 89% of all cases of regeneration treatments were successful. TAP and CH (limited to the coronal half of the root) were both successful but TAP caused greater root thickness compared to CH and formocresol (23).

In 2008, Kenneth *et al.* showed that TAP can cause 99% reduction in the mean number of colony forming units and approximately, 75% of root canals would have no culturable microorganisms after the application of this paste. They also stated that application of CH to the root canal prevented revascularization at the coronal area where CH had been applied. In

general, most cases of successful regeneration treatments were in young individuals (8-13 years). Successful cases were also seen in 33 to 37 year-olds as well but the success rate significantly decreased in subjects older than 38 years (24).

Gomes-Filho *et al.* in 2012 evaluated the tissue response of rat to CH and TAP; both medicaments demonstrated moderate inflammation after 7-15 days similar to the control group. This inflammation subsided after 30 days and CH and TAP were reported to be biocompatible. However, their study was conducted on rat fibroblasts (25).

Ruparel *et al.* in 2012 reported greater destructive effect of TAP on stem cells compared to CH. However, they used Trypan blue for assessment of cell cytotoxicity (26).

Most studies consider TAP to be biocompatible for fibroblasts. In the current study, TAP had less cytotoxicity than CH, and this cytotoxicity was concentration-dependent. The higher the concentration, the lower the cell viability. The 0.1 and 1 mg/mL concentrations had mild

cytotoxicity but TAP with 10 mg/mL concentration had moderate cytotoxicity. This means that TAP at 10 mg/mL concentration was not biocompatible and showed higher cytotoxicity.

The lower cytotoxicity of TAP may be attributed to the inhibition of cell collagenase and matrix metalloproteinase. In fact, doxycycline present in its formulation increases collagen formation and angiogenesis in the process of wound healing; this finding was also observed in a study by Gomes-Filho (2012) (25).

Conclusion:

The results of the current study showed that the percentage of cell viability was correlated with the concentration of TAP and was higher than that in presence of CH. Also, it appears that the use of TAP is more practical than CH.

Conflict of Interest: “None Declared”

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