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ORIGINAL ARTICLE

Human Stem Cells of Apical Papilla Viability Following the Removal of Triple Antibiotic Paste in a 3D Root Canal Culture Model

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ABSTRACT

Objective: This study investigated the residual effects of various concentrations of triple antibiotic paste (TAP) on the viability of human stem cells of the apical papilla (hSCAPs) in a 3D root canal culture model.

Methods: Sixty-four single-rooted segments were prepared and allocated into five groups (n=12): four concentrations of TAP (1 mg/ml, 2.5 mg/ml, 5 mg/ml, 10 mg/ml) and a control group with vehicle alone. TAP was prepared by mixing USP-graded antibiotic powder with vehicle (macrogol and propylene glycol). The canals were filled with the prepared medication for 28 days. After removing TAP, fibrin gels containing hSCAPs were loaded into the canal and incubated for 7 days. Cell morphology was observed using confocal laser scanning microscopy (CLSM), whilst cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

Results: The vehicle alone and the 1 mg/ml TAP groups showed viable and proliferative cell morphology. However, higher concentrations of TAP displayed non-proliferative cells as observed by CLSM. For the cell viability test, 1 mg/ml TAP did not demonstrate a different percentage of cell viability from the control group. However, 2.5 mg/ml, 5 mg/ml and 10 mg/ml TAP exhibited significantly lower percentages of cell viability compared with the control ($p<0.001$).

Conclusion: Cells can survive at low TAP concentrations of ≤1 mg/ml. However, harmful effects become evident at TAP concentrations of ≥2.5 mg/ml.

Keywords: 3D culture, cell viability, confocal laser scanning microscopy, human stem cells of apical papilla, regenerative endodontics, triple antibiotic paste

HIGHLIGHTS

- This was the first study to investigate TAP cytotoxicity at concentrations of 1-10 mg/ml in a 3D root canal model.
- TAP concentrations ≥ 2.5 mg/ml generated a cytotoxic effect on hSCAPs.
- The control (vehicle alone) and low concentration TAP (1 mg/ml) were not toxic to hSCAPs.
- The CLSM analysis confirmed that the control and low concentration TAP (1 mg/ml) cells were viable and able to form a spindle shape in the fibrin scaffold.

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INTRODUCTION

Triple antibiotic paste (TAP) is the medicament of choice for Regenerative Endodontic Procedures (REPs) because of its effective antimicrobial activity (1). Previous studies demonstrated that high concentration TAP (≥10 mg/ml) completely eradicated bacterial biofilm in root dentine (2–4). However, the use of TAP showed a detrimental effect on stem cells and might impair the regeneration phase of REPs that are crucial for the treatment outcome (5–8). Therefore, the optimum concentration of TAP used in REPs has been discussed to balance the bactericidal efficacy and the adverse effects on stem

cells. The cytotoxic effect of various concentrations of TAP on stem cells of the apical papilla (SCAPs) has been investigated. A study using a direct contact method suggested that a non-toxic concentration of TAP should be 0.1 mg/ml, whereas 1 mg/ml and 10 mg/ml TAP showed a dramatic reduction in SCAP survival (5, 8). Recently, the residual effect of TAP in a 3D culture model demonstrated that 1 mg/ml TAP did not affect SCAPs, whereas 1,000 mg/ml resulted in no viable cells. However, this study did not investigate the cytotoxic effect of TAP between 1–1,000 mg/ml (6).

To our knowledge, 10 mg/ml TAP is effective to eradicate bacterial biofilm, however, it might be harmful to stem cells. In contrast, 1 mg/ml TAP, which showed less cytotoxic effect on stem cells, was insufficient to eradicate the bacterial biofilm. Moreover, most previous studies employed a direct contact method and a 2D culture model, which may not be directly applicable to clinical situations. Therefore, the purpose of this study was to evaluate the residual effect of freshly-mixed 1 mg/ml, 2.5mg/ml, 5 mg/ml and 10 mg/ml TAP on human stem cells of the apical papilla (hSCAPs) survival in a 3D root canal culture model.

MATERIALS AND METHODS

Tooth Preparation

This study was conducted in accordance with the Declaration of Helsinki. The study protocol was approved by the Faculty of Dentistry and Faculty of Pharmacy, Mahidol University, Institutional Review Board (MU-DT/PY-IRB 2022/029.2705). Sixty-four single-rooted mandibular premolars were collected. Teeth with root fracture, root caries, root resorption, immature root formation, multiple root canals, canal calcification and previous root canal treatment were excluded. The teeth were stored in 0.1% (w/v) Thymol solution until used. To obtain 3-mm root segments, the teeth were cut at the cementoenamel junction (CEJ) and 3 mm apically from the CEJ with a low-speed precision cutter (Isomet 1000, Buhler, New York, USA). The root canals were standardized to a 1.7 mm diameter by sequentially enlarging the canal with a Peeso reamer No. 1–6 (Dentsply Maillefer, USA) under irrigation with sterile phosphate-buffered saline (PBS; Sigma Aldrich, St. Louis, MO, USA). Smear layer removal was performed as previously described (9). The specimens were sterilized by autoclaving at 121°C 15 psi for 20 min. Four specimens were randomly collected and incubated in cell culture medium at 37°C for 24 hours to confirm the sterile condition by the absence of turbidity.

TAP Preparation

TAP was prepared at 1 mg/ml, 2.5 mg/ml, 5 mg/ml and 10 mg/ml using the antibiotic powder (USP-grade; Sigma Pharmaceutical, North Liberty, Iowa, USA) together with macrogol and propylene glycol (M:P ratio = 1:1) as a vehicle. The composition of a 1 mg/ml TAP preparation is shown in Table 1. The prepared TAP was used immediately after preparation.

Medication Procedure

The specimens were randomly allocated into 5 groups (n=12), i.e., 1 mg/ml, 2.5 mg/ml, 5 mg/ml, 10 mg/ml TAP and vehicle alone. The medication preparation was performed in a biosafety cabinet with a laminar flow (NU-126-300E, NUAIRE, Minnesota, USA). Each specimen was placed in a 48-well plate, 600 µl TAP or vehicle was added into the root canal using micropipettes and incubated at 37°C with 5% CO_2 and 95% humidity for 28 days. After incubation, the medicament was removed by irrigating the canals with 20 ml of 17% EDTA for 5 min and 20 ml of sterile normal saline for 5 min using a sterile syringe and 25-gauge needle before cell seeding.

Cell Seeding in Fibrin Gel

Primary hSCAPs were isolated from extracted human mandibular third molars and cultured as previously described (10). Cells at passages 3–6 were used in this study. The cells were stained with green fluorescent cytopainter dye (ab176735: Abcam, Cambridge, UK) for 30 min before loading into a fibrin gel. Eight mg/ml fibrinogen, 2.5 mM Ca++ and 2 NIH Units/ml thrombin were diluted (Tisseel kit Baxter, Zurich, Switzerland) in Tris-Buffered saline, pH 7.4. To prepare hSCAPs in 1% fibrin gel (11), 6×10^3 hSCAPs were added to a thrombin solution prior to mixing with fibrinogen solution at a 1:1 ratio. Immediately after mixing, 15 µl of the mixture was loaded into the root canal and incubated for 30 min to allow complete gelation of the fibrin scaffold. Subsequently, 800 µl culture medium was added and the specimens were incubated at 37°C for 7 days.

Fluorescent Imaging

To investigate the cell morphology of viable hSCAPs, four specimens from each group were randomly selected and observed using a confocal laser scanning microscope (CLSM; Stellaris 8, Leica Microsystem, Wetzlar, Germany) at 10X and 20X magnification and resolution of 1024×1024 pixels. The images were analyzed with Leica Application Suite X (LAS X) software.

TABLE 1. The composition of TAP in each concentration for 1 ml preparation

	Antibiotic powder (mg)			Vehicle (ml)	
	Metronidazole	Ciprofloxacin	Minocycline	Macrogol	Propylene glycol
1 mg/ml TAP	0.33	0.33	0.33	0.5	0.5
2.5 mg/ml TAP	0.83	0.83	0.83	0.5	0.5
5 mg/ml TAP	1.66	1.66	1.66	0.5	0.5
10 mg/ml TAP	3.33	3.33	3.33	0.5	0.5

TAP: Triple antibiotic paste

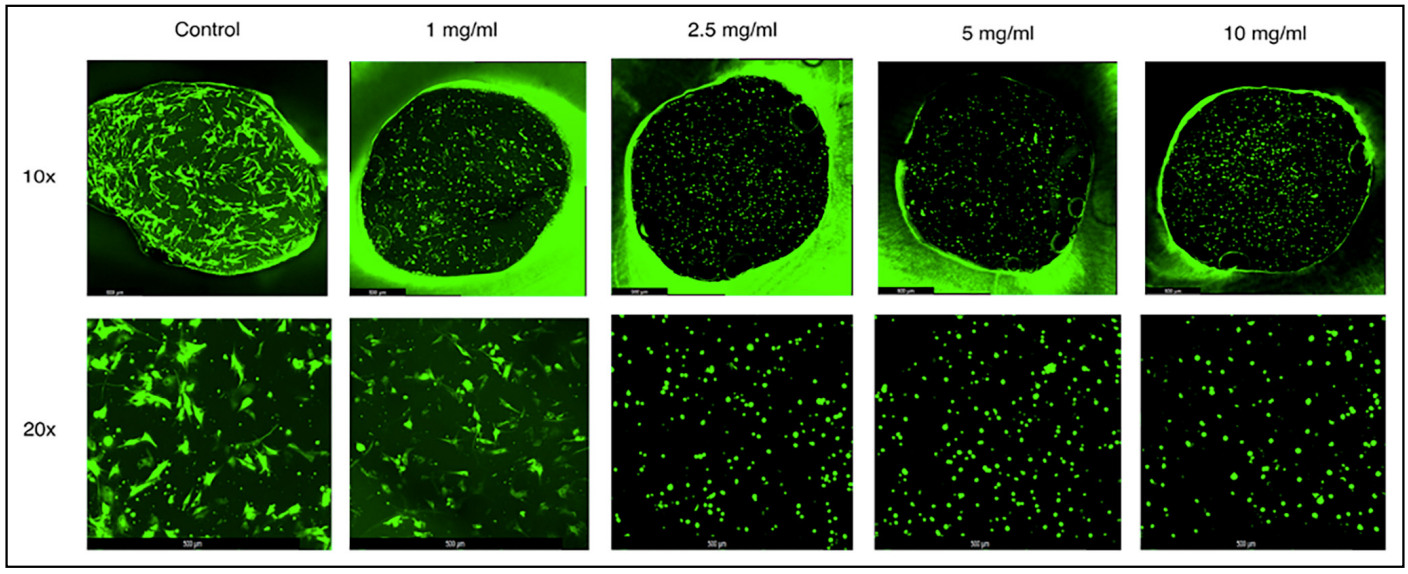

Figure 1. Representative CLSM images of viable hSCAPs (green colour) in each experimental group (n=4/group) under 10x and 20x magnifications

CLSM: Confocal laser scanning microscopy, hSCAPs: Human stem cells of apical papilla.

MTT Assay

After removing the old culture medium, the specimens were washed twice with PBS. Eight hundred microliters of 0.5 mg/ml MTT (Sigma, Berlin, Germany) in DMEM (Gibco, Life Technologies, Grand Island, NY, USA) were added to each well and incubated at 37°C for 4 hours. After discarding the supernatant, the precipitation was dissolved by adding 400 µl dimethylsulfoxide (DMSO) and mixed for 1 hour to ensure complete solubilization. The solution was transferred to a 96-well plate to measure the absorbance at OD570 nm by a spectrophotometer. The percentage of cell viability was calculated according to the following equation.

$$\text{Percentage of cell viability} = \frac{\text{OD570–OD690 value of each experimental group} \times 100}{\text{OD570–OD690 value of control}}$$

Data Analysis

Statistical analysis was performed using SPSS version 23.0.0.0 (SPSS Inc., IL, USA). One-way ANOVA and Games-Howell test were used to analyse the cytotoxic effect of residual TAP. The statistical significance level was set at $p<0.05$. The cell morphology of each experimental group was also described.

RESULTS

Effect of Residual TAP on hSCAP Morphology As Determined by CLSM

The effects of 1 mg/ml, 2.5 mg/ml, 5 mg/ml and 10 mg/ml TAP concentrations on cell morphology and viability were evaluated (Fig. 1). In the control group, the cells appeared densely populated with spindle-shaped cells and that formed cell-to-cell interactions. Likewise, 1 mg/ml TAP demonstrated a similar cell density and morphology to the control group. In contrast, a reduction in cell density and structural integrity was observed with increasing TAP concentrations. At higher concentrations, the cells were small and round and isolated from each other, indicating decreased viability or increased cell death.

Effect of Residual TAP on hSCAP Viability As Determined by MTT Assay

The mean percentage of cell viability, with the control group (vehicle alone) set at 100%, is presented in Figure 2. TAP at a concentration of 1 mg/ml resulted in 82.24±22.28% cell viability, which was not significantly different from the control group. In contrast, higher TAP concentrations exhibited a percent cell viability of 15.90±12.22, 18.96±6.30, and 14.25±6.58 for 2.5, 5, and 10 mg/ml, respectively. A notable decrease in cell viability occurred as the TAP concentration increased from 1–2.5 mg/ml. Statistical analysis demonstrated that the percentage of viable cells in the 2.5 mg/ml, 5 mg/ml, and 10 mg/ml groups was significantly lower compared with the 1 mg/ml group and the vehicle alone ($p<0.001$).

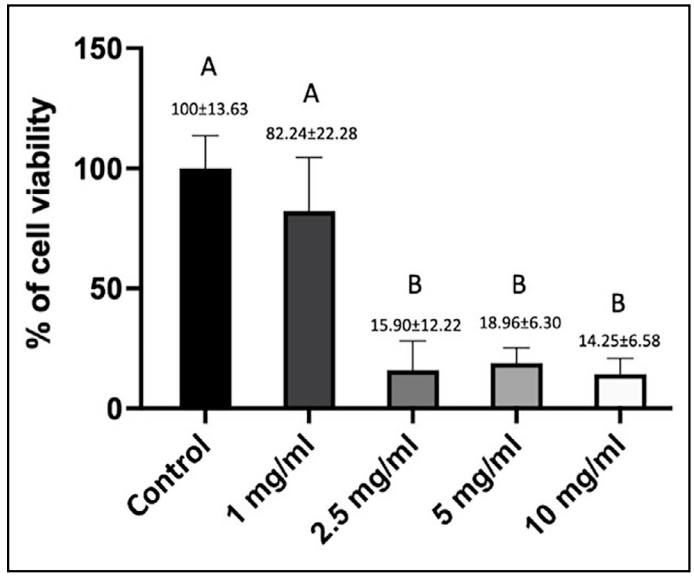

Figure 2. The percentages of cell viability in each experimental group (n=12/group) are represented as means and standard deviations. One-way ANOVA and Games-Howell test were used for statistical analysis.

The different uppercase letters indicate a statistically significant difference at $p<0.05$.

DISCUSSION

High concentrations of TAP are effective in eradicating bacterial biofilm, however, its cytotoxicity may adversely impact the viability of stem cells that migrate from the apical region into the root canal space during pulp tissue regeneration (12). An appropriate concentration of TAP has been discussed in order to balance the bactericidal efficacy and the adverse effects on stem cells. To evaluate the cytotoxicity of TAP on SCAPs, various concentrations of TAP were investigated in this study. The *in vitro* results demonstrated that the antibiotics exhibited cytotoxic effects on stem cells at high concentrations, resulting in significantly reduced cell viability and proliferation. The concentration at 1 mg/ml or lower was non-toxic, similar to the control group loading only vehicle into the canal. It is essential to create a microbe-free environment using the lowest effective concentration of medicament to support pulp-dentine regeneration.

The current study supports the idea that high concentrations of TAP, whilst bactericidal, can harm stem cell viability. Our MTT results demonstrated that TAP at 1 mg/ml maintained acceptable cell viability, consistent with a previous study using a 3D culture model, which found no significant impact on SCAP survival at this concentration (6). However, 1 mg/ml TAP was insufficient to eradicate the intracanal bacteria, which requires at least 10 mg/ml, a concentration detrimental to SCAP viability (2–4). Similarly, direct contact studies have shown that TAP concentrations ≥10 mg/ml significantly reduce stem cell survival, whilst lower concentrations (0.01–0.5 mg/ml) exhibit minimal cytotoxic effects (3, 5, 8).

The CLSM results revealed that cells in the 1 mg/ml TAP and control groups maintained a viable, spindle-shaped morphology, with a denser cell population in the control group. This confirms that mesenchymal stem cells can reside in fibrin gel, where they transform into spindle-shaped cells under non-toxic conditions (13). In contrast, cells in fibrin gel with TAP ≥2.5 mg/ml remained round, indicating reduced metabolic activity and a dormant state (14). Combined with the low cell viability from the MTT assay, this suggests potential apoptosis.

This study aimed to replicate the clinical environment for stem cell-based regeneration by addressing the limitations of previous approaches for evaluating the cytotoxicity of TAP and optimizing the use of fibrin gel as a biocompatible scaffold. Most studies on TAP cytotoxicity use direct contact methods (3, 5, 7, 8), which differ from clinical scenarios where TAP must be washed out to reduce toxicity before stem cells can be added. Moreover, the irrigation protocol was also performed according to the AAE guideline with some modification by additionally irrigating with 20 ml of normal saline (15). In the clinical setting, stem cells reside within a blood clot scaffold, forming a 3-dimensional (16) . To replicate this, fibrin gel was chosen for its biocompatibility, nontoxic degradation and ability to form a porous, cross-linked network that supports cell adhesion, nutrient exchange, dental stem cell growth and facilitates pulp-like tissue formation (17).

The formulation of TAP, including the choice of antibiotics and vehicle, plays a critical role in determining its cytotoxic effects on SCAP viability, with differences in the preparation method potentially influencing outcomes. Although USP-grade antibiotic powder was used to ensure accurate concentrations, previous studies have shown higher cytotoxicity in TAP made from USP-grade antibiotics compared with tablet or capsule forms (18). In the present study, MP was used as the vehicle for TAP due to its penetration properties and mild antimicrobial activity of propylene glycol (19, 20) . Additionally, TAP with MP demonstrated lower acidity and reduced cytotoxicity compared with those prepared with distilled water (18). Our results also revealed that MP alone had no cytotoxic effect on stem cells, as seen from the control group.

In our study, as well as in a previous study (6), TAP concentration was limited to 1 mg/ml, which is below the effective range for REP, and reducing it further could compromise disinfection. Clinical studies found that full-strength TAP is associated with higher root development rates (15, 21), emphasizing the importance of disinfection in REPs. Although regeneration can still occur with high TAP concentrations, the survival rate may be affected by either infection or the toxicity of the antibiotic medicament. The current study supported the idea that high-concentration TAP produced high bactericidal efficacy and might be harmful to the viability of stem cells. However, reducing the antibiotic concentration may compromise disinfection efficacy. Therefore, the removal of TAP from the root canal should be a key consideration. This approach allows TAP to be used at an effective bactericidal concentration, followed by its removal prior to stem cell migration. Future research should focus on exploring more efficient TAP removal methods, incorporating adjunctive irrigation techniques, as conventional needle irrigation may be insufficient due to the deep penetration of TAP into dentinal tubules (22, 23).

CONCLUSION

TAP at 1 mg/ml had no significant effect on hSCAP viability, whereas 2.5 mg/ml, 5 mg/ml and 10 mg/ml TAP showed cytotoxicity to hSCAPs, as determined using a 3D root canal culture model.

Disclosures

Ethics Committee Approval: The study was approved by the Faculty of Dentistry and Faculty of Pharmacy, Mahidol University, Institutional Review Board Ethics Committee (no: MU-DT/PY-IRB 2022/029.2705, date: 27/05/2022).

Informed Consent: Informed consent was obtained from all participants.

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