

DILUTED ANTIBIOTICS FOR TREATING TRAUMATIZED IMMATURE TEETH

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## DEDICATION

All praise belongs to God, Who helped me to accomplish this dissertation. May the Lord help me to remember, to praise, and to worship Him. This dissertation is dedicated to my parents, a great source of motivation, inspiration and support, and to my brother Khaldoon, my sisters Arwa and Heba. To all my great friends; Sarah, Amnah, Nadia, Hadeel, Maryam, Eman, Nozha, Fatima Afnan, Laila, and Rawan who were always by my side, May God bless you all.

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## DILUTED ANTIBIOTICS FOR TREATING TRAUMATIZED IMMATURE TEETH

Endodontic regeneration (ERP) has been successfully used in the treatment of traumatized immature teeth. The procedure has three essential steps: disinfecting the root canal (i.e. triple antibiotic paste (TAP) or double antibiotic paste (DAP)), provoking bleeding inside the canal to form a scaffold upon which pulp stem cells will be deposited and continue root growth, and creating a good coronal seal. Previous research has reported that antibiotic pastes (TAP and DAP) are cytotoxic to stem cells in the concentrations commonly used in endodontic regeneration (1000 mg/mL). To decrease the adverse effects on stem cells and increase the rate of success of the regeneration, defining appropriate antibiotic concentrations for ERP is critical. In this project, five *in-vitro* experiments were conducted to determine the breakpoint dilutions of both TAP and DAP medicaments, and to prepare a suitable novel pastes containing diluted TAP or DAP medicaments for ERP.

In the first experiment, we compared the antibacterial effect of TAP, and DAP against early biofilm formation of *Enterococcus faecalis* (*E. faecalis*) and *Porphyromonas gingivalis* bacteria. In the second study, we investigated the antibacterial effect of various dilutions of TAP and DAP antibiotic medicaments against established *E. faecalis* biofilm. In the third experiment, we investigated longitudinally the residual antibacterial activity of human radicular dentin treated with 1000, 1 or 0.5 mg/ml of TAP and DAP. In the fourth study, we investigated the cytotoxic effect of various dilutions of TAP and DAP antibiotic medicaments on the survival of human dental pulp stem cells (DPSC). And in the fifth experiment, we investigated the antibacterial and cytotoxic effect of novel intracanal medicaments consisting of methylcellulose (MC) and/or propylene glycol (PG) mixed with 1mg/ml of TAP or DAP.

1 mg/ml of DAP or TAP medicaments had a significant antibacterial effect against early bacterial biofilm formation, and established bacterial biofilm. Furthermore, 1 mg/ml had a residual antibacterial activity comparable to 1000 mg/ml. The novel intracanal medicaments had comparable antibacterial effect to currently used medicaments (1000 mg/ml). Additionally, the novel intracanal medicaments significantly enhanced DPSC metabolic activity, compared to currently used medicaments in endodontic regeneration procedures.

Jeffrey A. Platt, D.D.S., M.S., Chair

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## INTRODUCTION

### **Necrotic immature teeth**

Immature teeth have been defined as growing teeth with incomplete root development. Root development has been reported to be completed after three years of tooth eruption inside the oral cavity (Schour et al. 1940). Pulp necrosis or death of the pulp of immature teeth will cause root growth cessation which is associated with open apexes and thin dentin walls (Gomes-Filho et al. 2012) which can pose potential problems and complications during and after endodontic treatment (Miller et al. 2012). Appropriate apical seal of necrotic immature teeth using conventional endodontic treatment methods is difficult (Nosrat et al. 2011). In cases of achieving an apical seal, the short immature treated teeth tend to have more frequency of fracture (Cvek 1992).

The developing dentition is at risk of experiencing pulp necrosis due to trauma, caries or developmental anomalies. The incidence of dental trauma is greater between the ages of 7 and 15 years, its prevalence in children is almost 20%-30% (Andersson 2013) and almost 50% of trauma cases are associated with pulp necrosis (Diogenes et al. 2013). This indicates that 1 of 8 children suffers from pulp necrosis of one or more immature teeth. The second most common cause of pulp necrosis is developmental anomalies which have been reported to have a prevalence of 5 to 6.4% (Uslu et al. 2009). Dental caries are less commonly associated with pulp necrosis of immature teeth. This might be related to significant variations in the levels of caries experience between populations due to socio-economic inequality and differences in economic development (Iwaya et al. 2001; Do 2012).

### **Treatment of necrotic immature teeth**

Apicoectomy or surgical intervention has been described as the oldest treatment approach of immature necrotic teeth (Friend 1967; Fregnani et al. 2008). This approach involves surgical exposure of the root end followed by retro-filling of the root canal with a restorative material (i.e, zinc oxide cement) (Fregnani et al. 2008). Limited success has been associated with the surgical

treatment approach because the treated teeth remain thin, short and fragile and tend to have unfavorable crown root ratios that may lead to tooth fracture (Rafter 2005).

Establishing an apical barrier has been used as an alternative treatment of necrotic immature permanent teeth. This approach has been done by using calcium hydroxide  $\text{Ca(OH)}_2$  to induce an apical hard tissue barrier (apexification) (Walia et al. 2000) or by an artificial apical barrier technique using mineral trioxide aggregate (MTA) (Nosrat et al. 2011). The placement of non-setting  $\text{Ca(OH)}_2$  stimulates the formation of mineralized fibrous tissue that can form an apical barrier at the root end of immature teeth (Kontakiotis et al. 1995). However, the use of non-setting  $\text{Ca(OH)}_2$  needs patient compliance due to the long treatment time. The increased risk of coronal leakage, reinfection of the root canals, and the tendencies of root fracture are other disadvantages of  $\text{Ca(OH)}_2$  apexification (Hatibovic-Kofman et al. 2008; Yassen et al. 2013). MTA artificial apical barrier technique has been used as an alternative to  $\text{Ca(OH)}_2$  apexification to decrease the time of the treatment (Bakland et al. 2012). Difficult handling properties, high cost, extrusion of the material toward the periapical area and the tendency for root fracture are disadvantages of using the MTA artificial apical barrier technique (Parirokh et al. 2010).

Unfortunately, neither of these treatment strategies increases the root thickness and length nor promotes the continuation of normal root development (Nosrat et al. 2011; Scarparo et al. 2011; Gomes-Filho et al. 2012). Endodontic regeneration, an alternative clinical approach to apexification, has received great attention in recent years because it may allow a continuation of root development (Thibodeau et al. 2007; Nosrat et al. 2011; Lenzi et al. 2012) which might reduce the risk of fracture and premature tooth loss associated with apexification treatment. Endodontic regeneration has been defined as biologically based procedures designed to replace damaged dentin and root structures, in addition to the cells of the pulp–dentin complex (Murray et al. 2007). This treatment protocol involves initial irrigation of the infected root canal with a root canal irrigant, and the placement of a canal dressing with antibacterial properties for 1 to 4 weeks. After canal disinfection, the canal is irrigated again to remove the canal dressing remnant, and

bleeding is induced inside the root canal to create a matrix for the ingrowth of new vital pulp tissue in the root canal space (Murray et al. 2007; Lovelace et al. 2011). Stem cells in the remaining vital pulp or apical papilla have been hypothesized to mediate tissue regeneration (Lovelace et al. 2011; Nosrat et al. 2011). Many clinical reports have shown favorable results, including continued dentin pulp complex development (Gomes-Filho et al. 2012; Miller et al. 2012). A recent randomized clinical trial has compared the regenerative potential of necrotic permanent immature teeth after treatment with either mineral trioxide aggregate (MTA) apical plug, or two different regenerative endodontic protocols (blood clot scaffold or blood clot and an injectable scaffold impregnated with basic fibroblast growth factor). The study showed that both protocols for regenerative endodontic results in progressive increase in root width, and length in addition to progressive decrease in apical diameter (Mohamed M. Nagy et al. 2014) as compared to MTA apical plug treatment. Another retrospective clinical study compared root length, thickness and overall survival rate of immature teeth after treatment with Ca(OH)<sub>2</sub> apexification, MTA apical plug, or endodontic regeneration. It has been concluded that endodontic regeneration significantly increases root length and thickness in comparison with Ca(OH)<sub>2</sub> apexification or MTA apical plug (Njeeruphan et al. 2012).

### **Drawbacks of endodontic regeneration**

Although many case studies have shown good clinical outcomes associated with endodontic regeneration (resolution of clinical signs and symptoms, absence of periapical infections on radiographs, continued root development and canal wall thickness of immature teeth) (Shin et al. 2009; Cehreli et al. 2011; Martin et al. 2013; Yang et al. 2013), there are still some drawbacks and unfavorable clinical and biological outcomes associated with endodontic regeneration (Nosrat et al. 2012).

Tooth discoloration represents the most common patient complaint after endodontic regeneration treatment. The discoloration is usually caused by antibiotic intracanal medicaments (Kim et al. 2010; Petrino et al. 2010; Lenherr et al. 2012). Tetracycline antibiotics including

minocycline, a common constituent of triple antibiotic paste (TAP), have been reported to attach to dentin followed by subsequent slow release from the dentin (Mohammadi 2008). According to Bowles and Bokmeyer (Bowles et al. 1997), minocycline binds significantly to collagen. The retained minocycline on root dentin explains its role in tooth discoloration. Upon exposure to air, minocycline tends to oxidize forming black quinone pigments (Good et al. 2003).

Clinical outcomes of endodontic regeneration procedures are not yet predictable. Lack of root development either by length or thickness has been reported in many clinical reports (Petrino et al. 2010; Chen et al. 2012; Lenzi et al. 2012; Nosrat et al. 2012). In a recent study (Chen et al. 2012), five responses of necrotic immature permanent teeth to regeneration procedures have been reported which includes: increased thickness and length of the root, blunt closed apex with no significant continuation of root development, open apex with continued root development, canal calcification (obliteration), or formation of a hard tissue barrier. Furthermore, despite root development and wall thickness increase in some clinical cases, tooth fracture can still happen (Martin et al. 2013).

Histological examination of regenerative tissues revealed that a true histological dentin-pulp formation is absent, and the mineralized tissues formed inside the canal space appeared to be cementum (Martin et al. 2013), bone like tissues (Martin et al. 2013) or periodontal tissue (Tawfik et al. 2013).

### **Medicaments used in endodontic regeneration**

$\text{Ca(OH)}_2$  has been one of the most commonly used intracanal medicaments in endodontic treatment. The antibacterial effect of  $\text{Ca(OH)}_2$  is believed to be related to its high alkalinity (pH=11.8) (Milosevic 1991). Some reports used  $\text{Ca(OH)}_2$  as a canal dressing in endodontic regeneration procedures and showed successful root development (Chueh et al. 2006; Cotti et al. 2008; Chueh et al. 2009; Nosrat et al. 2011; Chen et al. 2012). Unfortunately, calcification of the root canal happens occasionally (Chueh et al. 2006; Chueh et al. 2009).

When treating radicular dentin with  $\text{Ca(OH)}_2$ , significantly higher phosphate/amide I ratios were observed (Yassen et al. 2013) which suggests that  $\text{Ca(OH)}_2$  has a denaturation effect on the dentin organic matrix. The denaturation of the various growth factors and dentin embedded collagen fibers might have an effect on the attachment and differentiation of pulp stem cells. A recent animal study suggested that EDTA conditioning of dentin surfaces may enhance the attachment and differentiation of dental pulp stem cells during endodontic regeneration (Galler et al. 2011). Therefore,  $\text{Ca(OH)}_2$  treatment of radicular dentin may inhibit stem cell attachment and hence interfere with the regeneration process.

Triple antibiotic paste (TAP), a mixture of metronidazole, ciprofloxacin, and minocycline antibiotics, is the most widely used intracanal medicament for endodontic regeneration procedures (Lovelace et al. 2011; Garcia-Godoy et al. 2012; Miller et al. 2012). TAP use has been reported in 51% of published cases of endodontic regeneration (Diogenes et al. 2013). TAP was developed by Hoshino et.al (Hoshino et al. 1996; Sato et al. 1996) and reported to be effective against the pathogens commonly found inside the root canal system (Bose et al. 2009; Vijayaraghavan et al. 2012). TAP produces a significantly greater increase in root wall thickness than  $\text{Ca(OH)}_2$  (Bose et al. 2009; Nosrat et al. 2011). Double-antibiotic paste (DAP) is another antibiotic mixture (metronidazole and ciprofloxacin) that has been used successfully in endodontic regeneration (Iwaya et al. 2001). Its use has been recommended to overcome the discoloration caused by minocycline in TAP by excluding it (Trope 2010). Both metronidazole (nitro-imidazole) and ciprofloxacin (fluoro-quinolones) prevent DNA synthesis in bacteria, while the minocycline prevents protein synthesis through binding to the 30S ribosomal subunit (Ings et al. 1974; Bosso 2005). Antibacterial action occurs through binding of the antibiotic or its metabolite (i.e., metronidazole) to essential enzymes in the bacterial cells and preventing their growth (Ings et al. 1974).

Previous clinical reports of endodontic regeneration have used both TAP and DAP in a concentration of approximately 1000 mg/ml (Diogenes et al. 2013). Due to the critical role of



stem cells in endodontic regeneration, their viability in the presence of antibiotic pastes is essential for success. Recently, studies have suggested that intracanal medicaments (TAP, DAP) are directly toxic to dental papilla stem cells, human periodontal ligament fibroblasts, human dental pulp cells, and apical papilla cells (Ruparel et al. 2012; Yadlapati et al. 2013; Labban et al. 2014; Phumpatrakom et al. 2014) and indirectly toxic to dental papilla stem cells (Althumairy et al. 2014) in the concentrations currently used in endodontic regeneration. Most of the studies proposed that 0.1 -2 mg/ml of TAP or DAP did not have any cytotoxic effect on dental pulp cells (Ruparel et al. 2012; Labban et al. 2014; Phumpatrakom et al. 2014).

Additionally, recent studies suggested that high concentrations of both TAP and DAP (1000 mg/ml) have adverse effects on the chemical and mechanical structure of radicular dentin (Yassen et al. 2013; Yassen et al. 2013). Compared to Ca(OH)<sub>2</sub>, both TAP and DAP produced a significant reduction in phosphate/amide I ratios (Yassen et al. 2013) which suggests a demineralization effect of both medicaments on radicular dentin. Although demineralization can be beneficial for attachment and differentiation of pulp stem cells through the exposure of collagen and growth factors, long term exposure to TAP and DAP can increase the susceptibility of roots to fracture (Yassen et al. 2013).

### **Requirements of intracanal medicaments for endodontic regeneration procedures**

Endodontic regeneration techniques are based on the presence of osteo/odonto progenitor stem cells in the apical papilla that are resistant to the infection and necrosis caused by proximity to periodontal blood supply (Huang et al. 2008). The ideal goal is to prepare an appropriately clean environment inside the root canal space that promotes repopulation of these stem cells, regeneration of pulp tissue, and continuation of root development (Nosrat et al. 2012).

Periapical periodontitis is considered a biofilm induced disease (Chavez De Paz 2007) due to the resistance of bacterial biofilm to treatment (Stewart et al. 2001). Biofilm is an aggregate of microbial cells that are attached to a surface or other microbial cells and surrounded with a matrix of proteins and polysaccharides protecting bacteria from antibiotics or the host

immune system (Stewart et al. 2001; Huang et al. 2011). A recent study using electron microscope imaging demonstrated that bacteria can penetrate the root canal, move in a coronal-apical and internal-external direction through the main root canal, and establish its biofilm within and in between dentinal tubules (Kwang et al. 2014). The mechanism of biofilm formation inside the root canal has been described to mimic the mechanism of biofilm formation of dental plaque (Ramachandran Nair 1987) which involves: initial bacterial attachment to conditioned surfaces, early biofilm formation, established biofilm formation or maturation, and dispersion of biofilm cells (Huang et al. 2011; Mohammadi et al. 2013). The inability of mechanically debriding the root canal of necrotic immature teeth, to eradicate bacteria, necessitates the use of effective antibiotic medicaments that can chemically interfere with bacterial infection. Therefore, the success of antibiotic medicaments is measured by their ability to prevent early biofilm formation and eradicate established bacterial biofilm. The lack of canal filling during the regeneration process may facilitate the repopulation of residual bacteria inside the canal space (Fouad et al. 2014). Therefore, maintaining an aseptic canal space after the disinfection procedures for a longer period of time (i.e., residual antibacterial effect) can be beneficial for the success of endodontic regeneration (Fouad 2011).

A root canal in an infected immature tooth has a large, blunder buss apical foramen, which may cause the disinfecting medicament to pass through the open apex (Chang et al. 2013) and induce a cytotoxic effect to the stem cells present in the periapical area. Therefore, the unique clinical situation of necrotic immature teeth mandates achieving a balance between the antibacterial effect and the cytotoxic effect of any suggested intracanal medicament.

The use of diluted antibiotic medicaments of both TAP and DAP has been suggested (Diogenes et al. 2013) to improve the survival rate of human stem cells in the apical papillae. However, diluting antibiotic pastes beyond the breakpoint dilution (Athanasiadis et al. 2010) might decrease the antibacterial effectiveness required for canal disinfection. Therefore, the overall aim of this project was to determine the breakpoint dilution of both TAP and DAP

medicaments and to investigate the suitability of using novel medicament pastes carrying the determined dilution of TAP or DAP for endodontic regeneration procedures by measuring their antibacterial effect and the cytotoxic effect *in-vitro*.

### **Specific Aims**

Specific Aim 1: To determine the minimum inhibitory concentrations (MIC), minimum biofilm inhibitory concentrations (MBIC), and minimum bacteriocidal concentrations (MBC) of TAP and DAP medicaments, and establish their antibacterial effect against early bacterial (*E. faecalis*, and *P. gingivalis*) biofilm formation over time.

Our null hypothesis stated that TAP and DAP antibiotic medicaments have no antibacterial effect against early biofilm formation.

Specific Aim 2: To investigate the antibacterial effect of various dilutions of TAP and DAP antibiotic medicaments (10, 1, 0.5, 0.25, and 0.125 mg/ml) in eradicating established *E. faecalis* bacterial biofilm.

Our null hypothesis stated that tested dilutions of TAP or DAP antibiotic medicaments (10-0.125 mg/ml) have no antibacterial effect against established bacterial biofilm formation.

Specific Aim 3: To investigate longitudinally the residual antibacterial activity of human radicular dentin treated with 1000, 1 or 0.5 mg/ml of TAP and DAP.

Our null hypothesis stated that radicular dentin treated with 1000, 1 or 0.5 mg/ml of either TAP or DAP have no residual antibacterial activity at all tested time points.

Specific Aim 4: To investigate the direct cytotoxic effect of various dilutions of TAP and DAP antibiotic medicaments (10, 1, 0.5, 0.25, and 0.125 mg/ml) on the survival of human dental pulp stem cells (DPSCs) using WST-1 and LDH cytotoxicity assays, and the indirect cytotoxic effect of various dilutions of TAP and DAP antibiotic medicaments (1000, 1, and 0.5 mg/ml) on the survival of human dental pulp stem cells (DPSCs). Our null hypotheses stated that tested concentrations of TAP or DAP antibiotic medicaments have no cytotoxic effect on DPSCs, and

that tested concentrations of TAP or DAP antibiotic medicaments (1000, 1, and 0.5 mg/ml) have no indirect cytotoxic effect on DPSCs.

Specific Aim 5: To investigate the antibacterial effect of novel intracanal medicaments consisting of methylcellulose (MC) and/or propylene glycol (PG) mixed with 1mg/ml of TAP or DAP in eradicating *E. faecalis* from radicular dentin and to establish their cytotoxic effect against DPSC metabolic activity.

Our null hypothesis stated that intracanal medicaments consisting of 1 mg/ml of TAP or DAP in MC or MCPG vehicles have no antibacterial effect on infected radicular dentin or cytotoxic effect against DPSCs.

CHAPTER ONE: THE EFFECTIVENESS OF ANTIBIOTIC MEDICAMENTS AGAINST  
EARLY BIOFILM FORMATION OF *ENTEROCOCCUS FAECALIS* AND  
*PORPHYROMONAS GINGIVALIS*

## **MATERIALS AND METHODS**

### **Bacterial Strains and Media**

*E. faecalis* and *P. gingivalis* were used in this study because they are the most commonly isolated bacteria from root canal infections (Molander et al. 1998). Anaerobic blood agar (CDC, BioMerieux, Durham, NC USA) plates were used to initially grow *E. faecalis* (ATCC 29212) and *P. gingivalis* (ATCC 33277) strains. Brain heart infusion (BHI) media supplemented with vitamin K+ hemin 5% v/v (Becton, Dickinson and Company, Franklin Lakes, NJ, USA); was used to grow the bacteria. Bacterial strains were grown at 37°C in an anaerobic environment using gas generating sachets (GaPak EZ, Becton, Dickinson and Company) to produce the required environment.

### **Saturated solutions preparations**

Saturated solutions of Ca(OH)<sub>2</sub> paste (UltraCal XS, Ultradent, South Jordan, UT, USA) were prepared by mixing 16 mg of Ca(OH)<sub>2</sub> with 10 ml of distilled water. For TAP (CHAMPS Medical, San Antonio, TX, USA), 300 mg of USP grade antibiotic powder compounded of equal portions of metronidazole, ciprofloxacin, and minocyclin was dissolved in 3 ml of distilled water. For DAP (CHAMPS Medical), 300 mg of USP grade antibiotic powder compounded of equal portions of metronidazole and ciprofloxacin was dissolved in 3 ml of distilled water. Ca(OH)<sub>2</sub>, TAP and DAP mixtures were stirred for 4 h at room temperature. The mixtures were then centrifuged at 3000 RPM for 15 minutes to clarify the solutions, and the aqueous supernatant layers were filtered using a sterile 25 mm syringe filter (Fisher Scientific, Newark, DE, USA).

### **Determination of minimum biofilm inhibitory concentration and biofilm formation**

The minimum biofilm inhibitory concentration (MBIC) is the lowest concentration of an agent that inhibits the visible biofilm formation of a microorganism (Xu et al. 2010). To determine the MBIC, overnight *E. faecalis* and *P. gingivalis* cultures (10<sup>6</sup> CFU/ml) in BIH were treated with 0, 1:10, 1:20, 1:40, 1:80, 1:160, 1:320, 1:1000, 1:2000, 1:4000, 1:8000, 1:16000, 1:32000, and 1:64000 dilutions of Ca(OH)<sub>2</sub>, TAP and DAP solutions for 24 h in 96-well

microtiter plates. Biofilm was gently washed twice with saline, fixed with 10% formaldehyde (Vasilev et al. 2010), washed twice with saline again, and stained with 0.5% crystal violet for 30 min. After washing the biofilm three times with saline, crystal violet was extracted from the biofilm cells by 200  $\mu$ l of 2-propanol for 1 h. The extract was diluted (1:5) with 2-propanol and read at 490 nm with 2-propanol used as a blank control (Huang et al. 2012). The method of biofilm formation was similar to the method of MBIC, but the TAP, DAP and  $\text{Ca}(\text{OH})_2$  dilutions used in biofilm formation were 0, 1:10, 1:20, 1:40, 1:80, 1:160, and 1:320. Biofilm formation was read at three time intervals; 24, 48, and 72 h in order to ascertain the ability of the medicaments to inhibit biofilm over time. Optical absorbance of the diluted crystal violet stain represents the actual bacterial biofilm volume. A higher absorbance indicates higher biofilm volume.

### **Statistical Analysis**

Each experiment was conducted in triplicate and repeated individually at least three times. The results were presented as the mean and standard error of means. Two way-ANOVA and pair-wise comparison were used for statistical analyses. Significance level was set at 0.05.

### **Results**

The calculated concentration of the saturated solution were 16, 96, and 46 mg/ml for  $\text{Ca}(\text{OH})_2$ , TAP and DAP, respectively.  $\text{Ca}(\text{OH})_2$  was not inhibitory in the MIC and MBC assays against *E. faecalis* or *P. gingivalis* at any dilution used in this study. Visible bacterial growth was observed in all wells treated with  $\text{Ca}(\text{OH})_2$ . However, the MBIC values for  $\text{Ca}(\text{OH})_2$  were 1:10 (1.6 mg/ml) for *E. faecalis* and 1:80 (0.2 mg/ml) for *P. gingivalis*. For TAP, the MIC and MBIC values were 1:32000 (0.003 mg/ml) against *E. faecalis* and 1:16000 (0.006 mg/ml) against *P. gingivalis*. The MBC values for TAP were 1:320 (0.3 mg/ml) against both bacteria. The MIC and MBIC values for DAP were 1:32000 (0.001 mg/ml) against *E. faecalis* and *P. gingivalis*. The MBC values for DAP were 1:320 (0.14 mg/ml) against both bacteria.

Biofilm formation was significantly decreased ( $p < 0.00001$ ) with TAP and DAP at all dilutions (Figures 1-6). Furthermore, TAP and DAP effects on biofilm formation were not

significantly different.  $\text{Ca(OH)}_2$  significantly decreased *E. faecalis* biofilm formation in a concentration dependent gradient (Figure 1-3), but it inhibited *P. gingivalis* biofilm at only higher dilutions (Figure 4-6).



CHAPTER TWO: THE EFFECT OF DILUTED ANTIBIOTIC MEDICAMENTS AGAINST  
*ETEROCOCCUS faecalis* ESTABLISHED BACTERIAL BIOFILM

## **MATERIALS AND METHODS**

### **Bacterial Strain and Media**

*E.faecalis* (ATCC 29212) strain was initially grown on anaerobic blood agar plates (CDC, BioMerieux, Durham, NC USA). Then, bacteria cultures were grown in brain heart infusion broth supplemented with 5 g/l yeast extract (BHI-YE) at 37°C in an anaerobic environment using gas generating sachets (GaPak EZ, Becton, Dickinson and Company, Franklin Lakes, NJ, USA).

### **Antibiotic medicament preparation**

For TAP, 100 mg of USP grade antibiotic powder compounded of equal portions of metronidazole, ciprofloxacin, and minocycline (CHAMPS Medical, San Antonio, TX, USA) was dissolved in 1 ml of sterile water (33.3 mg of each antibiotic/ml). For DAP (CHAMPS Medical), 100 mg of USP grade antibiotic powder compounded of equal portions of metronidazole and ciprofloxacin was dissolved in 1 ml of distilled water (50 mg of each antibiotic/ml). The stock solution of each antibiotic mixture was used to prepare the five experimental concentrations (10 mg/ml, 1 mg/ml, 0.5 mg/ml, and 0.25 mg/ml and 0.125 mg/ml) by mixing the appropriate amount of the antibiotic solution with BHI-YE culture media for *E.faecalis* biofilm assay. The ranges of dilutions used in this study were selected according to most recent available literature regarding both antibacterial and cytotoxic effect of these medicaments (Sato et al. 1996; Ruparel et al. 2012; Sabrah et al. 2013; Labban et al. 2014). The different dilutions were filter sterilized using a sterile 20 µm filter (Fisher Scientific, Newark, DE, USA). A control group of BHI-YE culture media with no antibiotics was also used.

### **Antibiotic effects on established bacterial biofilm**

To determine the effectiveness of the different dilutions of TAP and DAP antibiotic medicaments on the established bacterial biofilm, 100 µl of an overnight *E.faecalis* culture ( $10^6$  CFU/ml) in BHI-YE and 900 µl fresh BHI-YE were incubated for 3 days in one well of 12-well plates (Becton, Dickinson and Company). Triplicate wells for each antibiotic dilution and no

antibiotic control wells were prepared. After the incubation time, bacterial cultures were removed from each well, and the established biofilm was washed with saline solution (5ml over 60 minutes) and treated with antibiotic dilutions for 3 days. At the end of the incubation time, antibiotic dilutions were removed from each well, and the biofilm cells were gently washed with saline. The biofilm cells were collected by cell scrapers (Fisher scientific) and spirally plated on tryptic soy agar plates (TSA). The plates were incubated for 48 h in a 5% CO<sub>2</sub> incubator at 37°C. Then, the number of colony forming units per volume (CFUs/ml) of each treated well was determined using an automated colony counter (Synbiosis, Inc., Frederick, MD, USA) and compared to the values from the control culture. Logarithmic reduction units were calculated as the following equation:

$$\text{Logarithmic reduction units} = \text{CFU/ml of the control} - \text{CFU/ml of treatment group.}$$

### **Statistical Analysis**

Each experimental treatment was conducted in triplicate and repeated individually three times. Data were analyzed using a linear mixed model to test the effects of antibiotic and dilution on bacteria established biofilm. The untreated control group in the experiment was used as reference groups and it was removed from the model. The significance level was set at 0.05.

### **Results**

A significant decrease in the log of CFUs/ml of *E. faecalis* bacteria has been noticed with all used dilutions for both TAP and DAP compared with the untreated control. For TAP, a gradual and significant reduction in antibacterial activity was observed between different dilutions (Figure 7). However, 0.125 and 0.25 mg/ml concentrations of TAP were not significantly different from each other. For DAP, a gradual and significant reduction in antibacterial activity was observed between different dilutions (Figure 7). However, 0.125, 0.25, and 0.5 mg/ml concentrations of DAP were not significantly different from each other.

The calculated logarithmic reduction units in log CFU/ml for DAP and TAP (Figure 8) showed that only 10 and 1 mg/ml produced 5 units reduction in CFU/ml of *E. faecalis* established biofilm.

CHAPTER THREE: EVALUATION OF RESIDUAL ANTIBACTERIAL EFFECT OF  
HUMAN RADICULAR DENTIN TREATED WITH TRIPLE AND DOUBLE ANTIBIOTIC  
PASTES AT VARIOUS CONCENTRATIONS

## **MATERIALS AND METHODS**

### **Bacterial Strains and Media**

Anaerobic blood agar plates (CDC, BioMerieux, Durham, NC) were used to initially grow and maintain *E. faecalis* bacteria (ATCC 29212). Brain heart infusion broth supplemented with 5 g/l yeast extract (BHI-YE) was used to grow the bacterium at 37°C in an anaerobic environment using gas generating sachets (GasPak EZ, Becton, Dickinson and Company, Franklin Lakes, NJ) to produce the required environment.

### **Antibiotics Pastes Preparation**

For TAP, 1000 mg, 1 mg, or 0.5 mg of USP grade antibiotic powder compounded of equal portions of metronidazole, ciprofloxacin, and minocycline (Champs Medical, San Antonio, TX) were mixed with 1 mL of distilled water at room temperature to form three different concentrations of the antibiotic paste. For DAP, 1000 mg, 1 mg, or 0.5 mg of USP grade antibiotic powder compounded of equal portions of metronidazole and ciprofloxacin (Champs Medical) were mixed with 1 mL of distilled water to form the same concentrations of the antibiotic paste.

### **Human Dentin Specimens Preparation and Treatment**

Intact human permanent premolars were collected and stored in 0.1% thymol solution at 4°C after obtaining Indiana University Institutional Review Board approval (IRB, 1303010841). The crowns were cut-off using a water-cooled diamond saw and each root was divided longitudinally into two halves. Each root half was used to prepare dentin slabs with the dimensions of 4x4x1 (16 mm<sup>3</sup>). The pulpal side of dentin specimen was sequentially polished with 500, 1200, and 2400 grit SiC abrasive papers using a Struers Rotopol 31/Rotoforce 4 polishing unit (Struers, Cleveland, OH). Specimens were sonicated for nine minutes and washed for another nine minutes under running water. All specimens were kept in water throughout the procedure to avoid dehydration.

A total of 315 specimens were randomly assigned into six treatment groups and a no-treatment control group (n = 45 per group). The specimens were sterilized in ethylene oxide and each specimen was placed inside one well of a sterile 96 well plate with the pulp surface facing outward. For treatment groups, specimens were treated with 200 µl of TAP or DAP at various concentrations (1000, 1 or 0.5 mg/mL) for two weeks at 37°C and 100% humidity. Thus, the pulpal surface of each specimen was covered with an approximately 0.55 mm layer of antibiotic. For the no-treatment group, the specimens were kept in 200 µl of saline for two weeks under the same conditions.

At the end of the treatment period, the specimens were irrigated for one minute with 5 mL of sterile saline and were blotted dry with sterile gauze. The 45 dentin specimens from each group were further randomized into five subgroups (n=9) and the five subgroups were tested for residual antibacterial activity either immediately or after 3, 7, 14 or 30 days. Dentin specimens were immersed in 200 µl phosphate buffered saline (PBS) at 37°C until the allocated time of antibacterial testing.

### ***E. faecalis* Growth on Treated Root Specimens**

After PBS immersion, the specimens from each subgroup were transferred into a new well of a sterile 96 well plate. Then, 190 µl of fresh BHI-YE growth media and 10 µl of an overnight *E. faecalis* culture ( $10^6$  colony forming units (CFU/mL)) were added to each well and incubated anaerobically for 72 h at 37°C. After that, the culture media was removed and each dentin specimen was gently washed twice with sterile saline to remove unattached bacteria. Each dentin specimen was then transferred to a new plastic test tube containing 200 µl of sterile saline. The tubes were sonicated for 20 seconds and vortexed for 30 seconds to detach biofilm cells. The detached biofilm cells were diluted and spirally plated on blood agar plates (CDC, BioMerieux). The plates were incubated for 48 h in 5% CO<sub>2</sub> at 37°C and the number of CFUs/mL was determined using an automated colony counter (Synbiosis, Inc., Frederick, MD).

### **Antibiotic release from conditioned dentin specimens**

In order to assess the antibiotic effect of the released antibiotics from treated dentin specimens, the PBS solutions were collected after different incubation times (t=3 d, t=7 d, t=14 d and t=30 d). 10 µl of an overnight *E. faecalis* culture ( $10^6$  CFU/ml) was added to 190 µl BHI-YE mixed with PBS from different groups in a ratio of 10:1 and incubated in an anaerobic environment for 48 h in 96-well microtiter plates. Biofilm was gently washed twice with saline, fixed with 10% formaldehyde, washed twice with saline again, and stained with 0.5% crystal violet for 30 min. After washing the biofilm three times with saline, crystal violet was extracted from the biofilm cells by 200 µl of 2-propanol for 1 h. The extract was diluted (1:5) with 2-propanol and read at 490 nm with 2-propanol used as a blank control Using a spectrophotometer (SpectraMax 190; Molecular Devices, Sunnyvale, CA). Optical absorbance of the diluted crystal violet stain represents the actual bacterial biofilm mass. A higher absorbance indicates higher biofilm mass (Sabrah et al. 2013).

### **Statistical Analysis**

Data were analyzed using a linear mixed model ANOVA and multiple pairwise comparisons to test the effect of treatment type and immersion time in PBS after treatment on biofilm growth. The significance level was set at 0.05.

### **Results**

The mixed linear model indicated significant effects of treatment type, and post treatment time in PBS and their interaction on the residual antibacterial properties of radicular dentin ( $P < 0.0001$ ). All dentin specimens treated with antibiotics showed gradual reduction in residual antibacterial effect over time ( $P < 0.0001$ - 0.0137) (Figure 9). Table 1 shows that dentin treated with 1000 mg/mL of TAP demonstrated significant residual antibacterial effect compared to the untreated control group in up to 14 days of post treatment ( $P = 0.0001$ -0.002). Dentin treated with 0.5 or 1 mg/mL of TAP demonstrated significant residual antibacterial effect compared to the untreated control group up to seven days of post treatment. Dentin specimens treated with 1000



mg/mL of DAP demonstrated significant residual antibacterial effect compared with untreated control group up to 30 days ( $P = 0.0044$ ). Dentin specimens treated with 0.5 or 1 mg/mL of DAP exhibited significant residual antibacterial effect compared to the untreated control group up to 14 days ( $P = 0.0001-0.007$ ).

Dentin treated with 1 mg/mL of TAP or DAP demonstrated no significant difference in residual antibacterial effect compared to dentin treated with 0.5 and 1000 mg/mL of TAP or DAP, respectively at all time points. Dentin treated with 0.5 mg/mL of TAP had significantly lower residual antibacterial effect compared to dentin treated with 1000 mg/mL of TAP after 14 days ( $P=0.005$ ). Dentin treated with 0.5 mg/mL of DAP had significantly less residual antibacterial effect compared to dentin treated with 1000 mg/mL of DAP after 3 and 7 ( $P<0.0001$  and  $P=0.007$ ).

The antibiotics released in PBS solutions caused a gradual and significant biofilm inhibition of *E. faecalis* bacteria up to 14 d and 30 d for 1000 and 0.5-1 mg/ml of TAP or DAP respectively. However, the activities of released antibiotics from dentin treated with TAP or DAP lower concentrations (0.5-1 mg/ml) start falling at 30 d. (Table 2).

CHAPTER FOUR: THE DIRECT AND INDIRECT CYTOTOXIC EFFECTS OF DILUTED  
ANTIBIOTIC MEDICAMENTS ON DENTAL PULP STEM CELLS

## **MATERIALS AND METHODS:**

### **Antibiotic medicament preparation**

For direct cytotoxic effect, 100 mg of TAP USP grade antibiotic powder compounded of equal portions of metronidazole, ciprofloxacin, and minocycline (CHAMPS Medical, San Antonio, TX, USA) was dissolved in 1 ml of sterile water (33.3 mg of each antibiotic/ml). For DAP (CHAMPS Medical), 100 mg of USP grade antibiotic powder compounded of equal portions of metronidazole and ciprofloxacin was dissolved in 1 ml of sterile water (50 mg of each antibiotic/ml). The stock solution of each antibiotic mixture was used to prepare the five experimental concentrations (10 mg/ml, 1 mg/ml, 0.5 mg/ml, and 0.25 mg/ml and 0.125 mg/ml) by mixing the appropriate amount of the antibiotic solution with BHI-YE culture media for *E.faecalis* biofilm assay. The ranges of dilutions used in this study were selected according to most recent available literature regarding both antibacterial and cytotoxic effect of these medicaments (Sato et al. 1996; Ruparel et al. 2012; Sabrah et al. 2013; Labban et al. 2014). The different dilutions were filter sterilized using a sterile 20 µm filter (Fisher Scientific, Newark, DE, USA). A control group of BHI-YE culture media with no antibiotics was also used.

For the indirect cytotoxic effect, the released antibiotics in PBS solutions after treatment of dentin specimens were utilized and their associated cytotoxic effects on DPSC were determined after 3, 7, 14, and 30 days (Chapter 3).

### **Human dental pulp stem cells (DPSC)**

DPSCs (General BioTechnology LLC, Indianapolis, IN, USA) obtained in liquid nitrogen were thawed and cultured in DMEM media supplemented with 10% fetal bovine serum (Atlanta Biologicals Inc. Flowery Branch, GA, USA), 5% penicillin-streptomycin (Life Technologies Corporation, Grand Island, NY, USA) and 2% Amphotericin B (Life Technologies Corporation). Cells at passages between 3 and 5 were utilized in this experiment. Sub-confluent cells were detached from the culture plate with 0.05% trypsin and ethylene di-amine tetra acetic acid (EDTA) (Life Technologies Corporation). The evaluation of cytotoxic effects of the various TAP

and DAP dilutions were determined using lactate dehydrogenase activity assay (LDH) and cell metabolic activity colorimetric assay utilizing water soluble tetrazolium salts (WST-1).

### **Direct cytotoxic effect**

#### **LDH assays**

Sub-confluent DPSCs were seeded into a 96-well plate (10,000 cells/well) and incubated for 24 hours in DMEM supplemented with 10% fetal bovine serum. After 24 hours, the old media was removed, and the cells were incubated with serum free DMEM in the presence of different concentrations of TAP or DAP mixed with DMEM culture media. Additionally, DPSCs incubated without any medicament in serum free DMEM were used as a negative control. After 3 days, the media were collected and utilized for the LDH assays. The levels of LDH released by the cells into the media due to membrane damage were determined using the Pierce™ LDH cytotoxicity assay kit (Thermo Scientific, Pierce Biotechnology, Rockford, IL, USA). Briefly, 50 µl of DPSCs culture media were transferred into another 96-well plate. Then, 50 µl of LDH assay mix was prepared according to the manufacturer's protocol, added and mixed in each well, followed by incubation for 30 minutes at room temperature. A spectrophotometer (SpectraMax 190; Molecular Devices, Sunnyvale, CA, USA) was used to quantitatively measure the colorimetric change at a wavelength of 490 nm. Positive control wells (Maximum LDH release) were also used by incubating DPSCs with the lysis solution provided by the manufacturer for 45 minutes (100 µl of serum-free DMEM and 10 µl lysis solution). The following equation was used to calculate the percentage of cytotoxicity due to LDH release from the treated cells relative to the untreated control:

$$\text{Cytotoxicity (\%)} = (\text{experimental value} - \text{negative control}) / (\text{positive control} - \text{negative control}) \times 100.$$

The positive control cytotoxicity percentage was considered to be 100%, while the percentage cytotoxicity of negative control was 0%.

**WST-1 assays:**

After collecting the media for LDH assays and washing the cells with phosphate buffer solution, the DPSCs were incubated with 10  $\mu$ l WST-1 and 90  $\mu$ l serum-free DMEM for 4 h in a humidified atmosphere at 37°C and 5% CO<sub>2</sub>. After that, a spectrophotometer was used to measure the optical absorbance of each well at 450 nm. Negative controls consisting of 10  $\mu$ l WST-1 incubated with 90  $\mu$ l serum-free DMEM without the presence of cells were used as a blank. The following equation was used to calculate the percentage of the absorbance values of the antibiotic-treated cells at each concentration in comparison to the absorbance values of the untreated cells.

The percentage of cell metabolic activity (%) = (absorbance value of the treated cells / absorbance value of untreated cells)  $\times$  100. The percentage cell metabolic activity of the untreated cells was considered 100%.

**Indirect cytotoxic effect**

Sub-confluent DPSCs were seeded into a 96-well plate (10,000 cells/well) and incubated for 24 hours in DMEM supplemented with 10% fetal bovine serum. After 24 hours, the old media was removed, and the cells were incubated in serum free DMEM mixed with PBS solutions collected in previous chapter (Chapter 3) in a ratio of 10:1 for 72 h. The metabolic activity of DPSC was measured using WST-1. DPSC were incubated with 10  $\mu$ l WST-1 and 90  $\mu$ l serum-free DMEM for 150 minutes in a humidified atmosphere at 37°C and 5% CO<sub>2</sub>. Spectrophotometer was used to measure the optical absorbance of each well at 450 nm. Negative controls consisted of 10  $\mu$ l WST-1 incubated with 90  $\mu$ l serum-free DMEM without the presence of cells was used as a blank. The following equation was used to calculate the percentage of the absorbance value of the treated cells at each concentration in comparison to the absorbance value of the untreated cell (positive control).

The percentage of cell metabolic activity (%) = (absorbance value of the treated cells / absorbance value of untreated cell)  $\times$  100.

The percentage cell metabolic activity of the untreated cell was considered 100%.

### **Statistical Analysis**

For the direct cytotoxic effect, each experimental treatment was conducted in triplicate and repeated individually three times. Data were analyzed using a linear mixed model to test the effect of groups on DPSC metabolic activity (WST-1) and cell membrane integrity (LDH). For the indirect cytotoxic effect, data were analyzed using linear mixed model and multiple pairwise comparison to test the effect of group (DAP/TAP; 0.5, 1, and 1000 mg/ml) and time (3 d, 7 d, and 14 d) on DPSCs metabolic activity. The untreated control groups in both experiments were used as reference groups and they were removed from the model. The significance level was set at 0.05.

### **Results**

For the LDH cytotoxic assays, the three lowest tested dilutions of DAP (0.125, 0.25 and 0.5) did not have a significant cytotoxic effect on DPSC compared to the untreated control (Figure 10). For TAP, the two lowest tested dilutions (0.125 and 0.25) did not have a significant cytotoxic effect on DPSC compared to the untreated control. For WST-1 assays, all dilutions of TAP and DAP were significantly cytotoxic to DPSC except 0.125 mg/ml (Figure 11).

For the indirect cytotoxic effect, PBS solutions collected from dentin specimens treated with either TAP or DAP various concentrations (0.5, 1, and 1000 mg/ml) had no significant reduction in DPSC metabolic activity at all tested time points ( $P > 0.05$ ) (Table 3).

CHAPTER FIVE: THE CYTOTOXIC EFFECT AND ANTIBACTERIAL EFFECT OF NOVEL  
DILUTED ANTIBIOTIC PASTES

## **MATERIALS AND METHODS**

### **Human dental pulp stem cells (DPSC)**

DPSCs (General BioTechnology LLC) obtained in liquid nitrogen were thawed and cultured in DMEM media supplemented with 10% fetal bovine serum (Atlanta Biologicals Inc.), 5% penicillin-streptomycin (Life Technologies Corporation) and 2% Amphotericin B (Life Technologies Corporation). Cells at passages between 6 and 8 were utilized in this experiment. Sub-confluent cells were detached from the culture plate with 0.05% trypsin and EDTA (Life Technologies Corporation) and seeded in a 96 well plate (10,000 cells/well).

### **Bacterial Strain and Media**

Anaerobic blood agar plates (CDC, BioMerieux, Durham, NC) were used to initially grow and maintain *E. faecalis* bacteria (ATCC 29212). Brain heart infusion broth supplemented with 5 g/l yeast extract (BHI-YE) was used to grow the bacterium at 37°C in an anaerobic environment using gas generating sachets (GasPak EZ, Becton, Dickinson and Company, Franklin Lakes, NJ) to produce the required environment.

### **Determining the cytotoxic effect of Methylcellulose (MC) and Propylene glycol (PG) on DPSC metabolic activity using WST-1 assays**

Two concentrations of MC were prepared by mixing 0.5% (4 mg/ml) or 2% (20 mg/ml) by weight (% wt) of MC (Acros Organics, Thermo Fisher Scientific, New Jersey) in sterile water using a magnetic stirrer. For PG (Acros Organics), five concentrations were prepared by mixing 80%, 60%, 40%, 20% or 10% of PG in sterile water (by volume v/v). The DPSC were incubated with 100 µl of the different vehicles mixed with serum-free DMEM culture media at a concentration of 1:10 for 72 hours. At the end of the incubation time, culture media were removed, and cells were washed with PBS solution. Then DPSC were incubated with 10 µl of WST-1 reagent and 90 µl serum-free DMEM culture media for 4 h in a humidified atmosphere at 37°C and 5% CO<sub>2</sub>. After that, a spectrophotometer was used to measure the optical absorbance of



each well at 450 nm and the percentage of cell metabolic activity (%) was calculated as previously described.

### **Antibiotic Paste Preparation**

For TAP, 1000 mg of USP grade antibiotic powder compounded of equal portions of metronidazole, ciprofloxacin, and minocyclin (Champs Medical, San Antonio, TX) were mixed with 1 ml of distilled water at room temperature. For DAP, 1000 mg of USP grade antibiotic powder compounded of equal portions of metronidazole and ciprofloxacin (Champs Medical) were mixed with 1 ml of distilled water. For Ca(OH)<sub>2</sub> paste, premixed UltraCal intracanal medicament was used according to manufacturer instructions (UltraCal XS, Ultradent Products Inc. South Jordan, UT).

Depending on the results of the cytotoxicity of both MC and PG, four new medicament pastes were prepared. The first paste consisted of 2% (w/v) MC and 1 mg of DAP (Champs Medical) mixed in 1 ml of distilled water at room temperature (D1+MC). The second paste consisted of 2% (w/v) MC and 1 mg of TAP (Champs Medical) mixed in 1 ml of distilled water at room temperature (T1+MC). The third paste consisted of 2% (w/v) MC, and 1 mg of DAP mixed with 1 ml of 10 % PG in distilled water (v/v) at room temperature (D1+MCPG). The fourth paste consisted of 2% (w/v) MC, and 1 mg of TAP mixed with 1 ml of 10 % PG in distilled water (v/v) at room temperature (T1+MCPG). All medicaments were mixed using a magnetic stirrer (300 rpm) for 24 h to ensure proper mixing of the different pastes. Sterile containers and stir bars were used and all mixing procedures were done in laminar flow cabinet.

### **Cytotoxic effect of different pastes on DPSC metabolic activity**

The DPSCs were incubated with 100 µl of the different pastes mixed with serum-free DMEM culture media at a concentration of 1:10 for 7 d. At the end of the incubation time, cultures media were removed, and cells were washed with PBS solution. DPSCs were incubated with 10 µl of WST-1 and 90 µl serum-free DMEM for 4 h in a humidified atmosphere at 37°C and 5% CO<sub>2</sub>. After that, a spectrophotometer was used to measure the optical absorbance of each

well at 450 nm and the percentage of cell metabolic activity (%) was calculated as previously described.

### **Antibacterial effect of different pastes on infected radicular dentin**

The study protocol has been approved by the Institutional Review Board (IRB) at Indiana University (IRB number 1303010841). Intact human permanent premolars were stored in 0.1% thymol solution at 4°C after extraction. The crowns were removed using water-cooled diamond saw rotating at 300 rpm and each root was divided longitudinally into two halves. Each root half was used to prepare dentin specimens with the dimensions of 4x4x1 (16 mm<sup>3</sup>). Pulpal side of each dentin specimens were sequentially polished using 500 and 1200 grit SiC abrasive papers using a Struers Rotopol 31/Rotoforce 4 polishing unit (Struers, Cleveland, OH). Specimens were sonicated for nine minutes and washed for another nine minutes under running water. All specimens were kept in water during all procedures to avoid dehydration.

A total of 70 specimens were randomly assigned to nine treatment groups and one saline control group (n = 7 per group). The specimens were sterilized in ethylene oxide and each specimen was placed inside one well of a sterile 96 well plate with the pulp surface facing outward. Specimens were inoculated with 190 µl of fresh BHI-YE growth media and 10 µl of overnight *E. faecalis* culture (10<sup>6</sup> CFUs/ml) incubated anaerobically for 7 days at 37°C. A fresh BHI-YE media was added every 2-3 days.

Infected dentin specimens were then transferred into new wells of sterile 96 well plate and treated with 50 µl of different paste medicaments for 7 days at 37°C and 100% humidity. Thus, the pulpal surface of each specimen was covered with 50 µl layer of antibiotic pastes using NaviTip application tip (Ultradent), and the cementum surface was in contact with 50 µl layer of BHI-YE growth media that was replaced every 2-3 days.

At the end of the treatment period, the specimens were washed for one minute with 5 ml of sterile saline to remove the medicament. Each dentin specimen was transferred to a new plastic test tube containing 200 µl of sterile saline. The tubes were sonicated for 20 seconds and vortexed

for 30 seconds to detach biofilm cells. The detached biofilm cells were diluted and spirally plated on blood agar plates (CDC, BioMerieux). The plates were incubated for 48 h in a 5% CO<sub>2</sub> at 37°C and the number of colony forming units per volume (CFU/ml) was determined using an automated colony counter (Synbiosis, Inc., Frederick, MD).

### **Statistical Analysis**

Data for the three different experiments were summarized and one way ANOVA with multiple pairwise comparisons was used for analysis. The significance level was set at 0.05.

### **Results**

For vehicle cytotoxic effect, all concentrations of PG exhibited a significant reduction in DPSC metabolic activity ( $P < 0.0001$ ) except for 10% of PG ( $P = 0.6519$ ). However, both 0.5% and 2% of MC demonstrated a significant increase in DPSC metabolic activity ( $P = 0.007-0.009$ ) (Figure 12).

For pastes cytotoxic effect, the metabolic activity of DPSC was significantly reduced after exposure to the three commonly used intracanal medicaments (TAP, DAP, and Ultracal) as compared to saline control group after 7d ( $P < 0.0001$ ). However, all newly formed medicament pastes (T1+MCPG, D1+MCPG, T1+MC, and D1+MC) had a significant double fold increase in DPSC metabolic activity as compared to saline control group ( $P < 0.0001$ ) (Figure 13).

For antibacterial effectiveness of different pastes, dentin specimens treated with antibiotic pastes (TAP, DAP, T1+MC, D1+MC, T1+MCPG, D1+MCPG) exhibited significant reduction in the log CFU/ml of *E. faecalis* as compared to saline, MC, MCPG and Ca(OH)<sub>2</sub> ( $P < 0.0001$ ). The reduction in the log CFU/ml with DAP treatment was 5.96 followed by D1+MCPG (5.77), D1+MC (5.4), T1+MCPG (5.17), TAP (4.45), and T1+MC (3.91) compared to saline treatment group. Dentin specimens treated with Ca(OH)<sub>2</sub> had a significant reduction in the log CFU/ml of *E. faecalis* (1.63) compared with saline and MC ( $P = 0.013-0.014$ ). However, dentin specimens treated with Ca(OH)<sub>2</sub> had no significant reduction in log CFU/ml of *E. faecalis* compared with MCPG ( $P = 0.122$ ) (Figure 14).

## DISCUSSION

Biofilm is a slimy layer of polysaccharide, protein and microbial cells forming a matrix that provides bacteria with some protection from antibiotics or host immune response (Stewart et al. 2001). In order to compare the effectiveness of antibacterial agents, biofilm inhibition is among the most important criteria to examine. Therefore, the purpose of chapter one was to compare the antibacterial effect of TAP, DAP and  $\text{Ca(OH)}_2$  against early biofilm formation of two bacterial species; *E. faecalis* and *P. gingivalis*, and to determine their MIC, MBC, and MBIC concentrations.

No MIC and MBC for  $\text{Ca(OH)}_2$  were obtained in this study which suggests a poor antimicrobial activity of the medicament. This agrees with previous studies that found  $\text{Ca(OH)}_2$  to be ineffective against *E. faecalis* (Distel et al. 2002; Gomes et al. 2002; Gomes et al. 2003; De Souza et al. 2005; Upadya et al. 2011; De Lucena et al. 2012) and *P. gingivalis* (Gomes et al. 2002; De Souza et al. 2005). Low numbers of *E. faecalis* bacteria still survived even after exposure to 100%  $\text{Ca(OH)}_2$  for 24 h (Upadya et al. 2011). Mixing  $\text{Ca(OH)}_2$  paste with glycerin results in significantly better antibacterial effects (Gomes et al. 2002). However, Ultracal XS aqueous  $\text{Ca(OH)}_2$  paste was used in this study, which is one of the most common clinically used materials.

Hoshino et al. (1996) reported that TAP was effective at a concentration of 0.075 mg/ml, while Sato et al. (Sato et al. 1996) found that 0.150 mg/ml of TAP was required to sterilize *E. coli* infected root dentin *in situ*. Our results demonstrate that TAP was effectively bactericidal at a concentration of 0.300 mg/ml against both *E. faecalis* and *P. gingivalis*. The different methodology and bacterial species used explains the differences noticed between our reported values and previous studies (Hoshino et al. 1996; Sato et al. 1996). In this study, DAP was effectively bactericidal at a concentration of 0.140 mg/ml against both bacteria. Interestingly, DAP and TAP demonstrated significant reduction in early biofilm formation of *E. faecalis* and *P. gingivalis* at all tested dilutions over different time periods (24, 48, and 72h). Both DAP and TAP were effective against early biofilm formation at dilutions up to 1 in 32000 (0.001-0.003 mg/ml).

Furthermore, there was no significant difference between TAP and DAP antimicrobial activity against either bacteria. Therefore, the null hypothesis stating that TAP and DAP antibiotic medicaments have no antibacterial effect against early biofilm formation was rejected.

Ca(OH)<sub>2</sub> significantly inhibited the biofilm formation of both bacteria compared to the control, but biofilm inhibition was significantly lower than both TAP and DAP. The relative low sensitivity of *E. faecalis* and *P. gingivalis* biofilms to Ca(OH)<sub>2</sub> may be explained by the high resistance of some endodontic bacterial biofilms to an alkaline challenge, which was reported in previous studies (Chavez De Paz 2007; Chavez De Paz et al. 2007; Brandle et al. 2008). A recent study suggested that the amount of hydroxyl ions released from Ca(OH)<sub>2</sub> is not high enough to promote antimicrobial activity against intraoral-infected dentin biofilm (Ronald Ordinola-Zapata 2013).

Collectively, both TAP and DAP were effective against early bacterial biofilm at high dilutions, which indicates that low concentrations of antibiotics might be sufficient to obtain the required antibacterial effect. However, these relatively low concentrations of medicaments may not be effective against established bacterial biofilm. The use of minimum or no instrumentation during endodontic regeneration requires an effective chemical challenge to eradicate the endodontic pathogens. Therefore, the aim of chapter two was to investigate the antibacterial effect of various concentrations of TAP and DAP against *E. faecalis* established biofilm. *E. faecalis* was used as *in-vitro* model because it is a commonly isolated microorganism from persistent endodontic infections (Stuart et al. 2006), it poses various survival and virulence factors that enables it to resist nutritional deprivation (Kayaoglu et al. 2004) and hence resist treatment. Additionally, *E. faecalis* is described as a high biofilm producer (Al-Ahmad et al. 2009; Al-Ahmad et al. 2014).

Both TAP and DAP at a concentration of 10 mg/ml were able to eradicate all the established biofilm of *E. faecalis* after 3 days. All other concentrations of TAP and DAP significantly reduced the established *E. faecalis* biofilm but did not completely eradicate it. The

antibiotic concentration required to completely eradicate the biofilm in this study (10 mg/ml) was higher than the concentrations reported in previous studies (Hoshino et al. 1996; Sato et al. 1996), which ranged from 0.075 to 1.5 mg/ml of TAP. However, both studies did not test the medicament against established bacterial biofilm (Hoshino et al. 1996; Sato et al. 1996) which indicates higher resistance of bacterial biofilm to treatment.

A concentration of 1 mg/ml of both TAP and DAP was also effective in eradicating more than 80% of *E. faecalis* established biofilm. A material can be considered effective in eradicating the bacterial biofilm when it can produce a reduction of more than 5 logarithmic units in the log CFU/ml (British standard 2006; Arias-Moliz et al. 2009; Athanassiadis et al. 2010). The lowest three concentrations of both TAP and DAP (0.125-0.5 mg/ml) significantly reduced *E. faecalis* established biofilm compared to the untreated control. Those concentrations did not produce a reduction of 5 logarithmic units in the log CFU/ml which indicates that the established bacterial biofilm of *E. faecalis* is resistant to concentrations lower than 1 mg/ml of TAP and DAP. However, the antibacterial effect measured in this study was accomplished after only 3 days of antibiotic application. The use of these concentrations in clinical situations might be more efficient since the application time of intracanal medicaments usually range between 1 to 4 weeks (Diogenes et al. 2013). The null hypothesis stating that tested dilutions of TAP or DAP antibiotic medicaments (10-0.125 mg/ml) have no antibacterial effect against established bacterial biofilm formation was rejected.

Residual antibiotic activity represents the capability of the antibiotic medicament to bind to the tooth structure and the subsequent release in an active form (Baker et al. 1983).

Tetracycline antibiotics, including minocycline and doxycycline, are broad spectrum antibiotics that have been widely used in endodontics. Different tetracycline containing irrigants (i.e., doxycycline in MTAD and Tetraclean) have a residual antibacterial effect after treating the radicular dentin for at least 28 days (Mohammadi 2008; Mohammadi et al. 2010; Mohammadi et al. 2012; Mohammadi et al. 2012). Tetracycline antibiotics have been reported to attach to dentin

(Mohammadi 2008) and minocycline binds significantly to collagen (Bowles et al. 1997). Therefore, the presence of minocycline in TAP is expected to provide a retention property to root dentin. More than 85% of TAP has been reported to significantly bind to radicular dentin to a depth of approximately 350  $\mu\text{m}$  from the root canal surface (Berkhoff et al. 2014) which indicates that antibiotic medicaments might have a residual effect on radicular dentin. Therefore, the aim of chapter three was to investigate and compare the residual antibacterial effect of 1000, 1 and 0.5 mg/ml concentrations of TAP or DAP on human radicular dentin over different time points. In order to simulate the clinical situation we used 1000 mg/ml concentration because it is the concentration commonly used in clinical treatment. 1 and 0.5 mg/ml were compared to 1000 mg/ml in order to examine the ability of diluted medicament to provide residual antibacterial properties to radicular dentin.

In this study, dentin specimens treated with lower concentrations of TAP (1 and 0.5 mg/ml) had a significant residual antibacterial effect for up to 7 days. However, 1000 mg/ml of TAP have a significant residual antibacterial activity for up to 14 days. Previous reports proposed the presence of residual antibacterial activity of radicular dentin treated with different tetracycline containing medicaments and irrigants (Stabholz et al. 1993; Stabholz et al. 1993; Stabholz et al. 1998; Mohammadi 2008). Additionally, longer residual antibacterial activity has been associated with higher concentrations of irrigants (Mohammadi 2008; Mohammadi et al. 2012; Mohammadi et al. 2012). For DAP, dentin specimens treated with 1000 mg/ml had significantly reduced biofilm growth even after 30 days of the removal of the antibiotic medicaments, which indicates a longer residual antibacterial activity of DAP compared to TAP. The presence of residual antibacterial activity with DAP treatment indicates that antibiotic components other than minocycline (ciprofloxacin and metronidazole) may also bind to the dentin surface. Additionally, the longer residual activity of DAP compared to TAP may also indicate that ciprofloxacin and metronidazole can bind or adsorb more efficiently to dentin compared to minocycline. It is well documented that ciprofloxacin is less soluble compared to tetracycline (Varanda et al. 2006; Cac



et al. 2008; Prakash et al. 2014). Therefore, DAP may be harder to remove from radicular dentin compared to TAP. A previous study reported that TAP was easier to remove from the root canal compared to DAP based on images from scanning electron microscopy (Yassen et al. 2013).

Intriguingly, dentin treated with 1 mg/ml of either TAP or DAP demonstrated no significant difference in biofilm growth compared to 1000 mg/ml of both TAP and DAP at all measured time points. This indicates that a relatively low concentration of DAP or TAP (1 mg/ml) has comparable ability to reduce bacterial biofilm growth on radicular dentin compared to the currently used concentration of these medicaments (1000 mg/ml). The null hypothesis stated that radicular dentin treated with 1000, 1 or 0.5 mg/ml of either TAP or DAP have no residual antibacterial activity at all tested time points was rejected. Collectively, 1 mg/ml can be considered the best dilution of TAP and DAP medicaments that can provide a significant antibacterial properties.

Due to the critical role of stem cells in endodontic regeneration, its viability in the presence and after the removal of antibiotic medicaments is essential for the success of endodontic regeneration. Therefore, it is important to determine the direct and indirect cytotoxic levels of various dilutions of TAP and DAP on the survival of human dental pulp stem cells in order to achieve a balance between the antibacterial effect and the cytotoxic effect of any suggested intracanal medicament. The aim of chapter four was to investigate the direct cytotoxic effect of various dilutions (0.125-10 mg/ml) of both TAP and DAP on the survival of DPSC, and to investigate the indirect cytotoxic effect on the viability of DPSC.

For LDH cytotoxicity, the three lowest tested concentrations of DAP (0.125, 0.25 and 0.5 mg/ml) and the two lowest concentrations of TAP (0.125 and 0.25 mg/ml) were not toxic to DPSC cell membrane integrity. A recent study reported that 0.3-2.5 mg/ml of TAP and 0.5-1 mg/ml of DAP were not toxic to dental pulp cells using LDH assays (Labban et al. 2014). The difference in detected cytotoxic concentrations using LDH assays between our study and the previous study could be explained by the different type of cells used (pulp stem cells versus pulp

fibroblasts). For WST-1 assays, only 0.125 mg/ml of both antibiotic mixtures did not affect the metabolic activity of DPSCs. This is in agreement with previously reported concentrations of both DAP and TAP (0.1 mg/ml ) that did not have any toxic effects against stem cells of dental papilla (SCAP) (Ruparel et al. 2012). In another study, the safest concentration of TAP and DAP on dental pulp cells was 2 and 0.3 mg/ml, respectively (Labban et al. 2014). The null hypothesis stated that tested concentration of TAP or DAP antibiotic medicaments (10-0.125 mg/ml) have no cytotoxic effect on DPSCs was rejected.

Cytotoxicity was detected earlier using WST-1 assays compared to LDH assays. This could be explained by the capability of the WST-1 to detect change in mitochondrial metabolic activities inside the cells that may precede the cell membrane destruction detected by LDH assays. Previous studies that used pulp fibroblasts or various other types of cells have also reported earlier detection of cytotoxicity using WST-1 assays compared to LDH assays (Huhtala et al. 2002; Kikkawa et al. 2005; Labban et al. 2014). Various cytotoxic assays may give different cytotoxic results depending on the measured physiological endpoint. Therefore, any clinical extrapolation of a detected cytotoxic concentration should be based on more than a single cytotoxic assay.

The use of WST-1 and LDH assays to detect the cytotoxic effect of different materials on human cells have been reported in many studies (Labban et al. 2014; Khaled et al. 2013; Allam et al. 2013). LDH is an enzyme present in the cytoplasm of many different cell types. Any damage to the plasma cell membrane will release LDH from cytoplasm into the cell culture media. Extracellular LDH in the media is then quantified by a coupled enzymatic reaction in which LDH catalyzes the conversion of lactate to pyruvate via NAD<sup>+</sup> reduction to NADH. Diaphorase then uses NADH to reduce a tetrazolium salt to a red formazan product that can be measured at 490nm. The level of formazan formation is directly proportional to the amount of LDH released into the medium, which is indicative of cytotoxicity according to the manufacturer. WST-1 assay reagent is used to measure mitochondrial dehydrogenase activities for cells. WST-1 is a water

soluble tetrazolium salt with an electron coupling agent diluted in PBS. The mitochondrial dehydrogenase cleaves the tetrazolium salts into formazan dye which is also detected by measuring the optical absorbance at 450nm. However, both tests give an indication of the cell growth in the presence of the antibiotic medicaments rather than cytotoxicity. For cells with high proliferative ability, such as stem cells, the cytotoxic level to cell membrane can be masked by the increased or decreased numbers of stem cells. A previous study (Ruperal et al. 2012) has reported that the non-cytotoxic concentration of TAP or DAP is 0.1 mg/ml depending on cell counting under the microscope which is similar to the concentration detected by WST-1 assay for metabolic activity. Therefore, 0.1 mg/ml is considered the nontoxic concentration of TAP or DAP against mesenchymal stem cells (Ruperal et al. 2012).

The large, blunder buss apical foramen of immature teeth, and the lack of an apical seat may cause the disinfecting medicament to pass through the open apex (Chang et al. 2013) to the surrounding periapical tissues.  $\text{Ca(OH)}_2$  extrusion into the apical region has been associated with inflammatory reactions (Sonat et al. 1990). Histological analysis demonstrated that the extrusion of intracanal medicament from immature teeth into periapical region is common even if the medicaments were placed shorter than the root apex (Felippe et al. 2005). Similarly, antibiotic medicaments may also be extruded from the open apices of immature teeth toward the periapical region. Although it is difficult to determine the amount of extruded antibiotic medicaments in each clinical case, it is expected that extrusion of small amount of intracanal medicaments (1000 mg/ml) may have a detrimental effect on stem cells compared to the extruded amount from lower concentration (1 mg/ml). The use of high concentrations of antibiotic medicaments (1000 mg/ml) for prolonged time (1-4 weeks) can expose the stem cells for toxic concentrations repeatedly (Diogenes et al. 2014) which decrease the number of viable cells.

After the removal of the antibiotic medicaments from the root canal, the next step will be provoke bleeding in order to recruit the stem cells into the canal space and initiate the regeneration process (Lovelace et al. 2011). Therefore, the viability of stem cells after the

removal of the antibiotic (i.e., indirect effect) is considered more important than the direct effect due to the direct contact of stem cells with previously treated radicular dentin (Althumairy et al. 2014). Our results demonstrated that there were no significant indirect cytotoxic effects on DPSC associated with retained antibiotic medicaments on radicular dentin. A recent study reported that the use of TAP and DAP at a concentration of 1000 mg/ml had an indirect cytotoxic effect on the stem cells of apical papilla (Althumairy et al. 2014). However, 1 mg/ml of both TAP and DAP did not have any cytotoxic effect (Althumairy et al. 2014). In our experiment we examined the indirect cytotoxic effect of retained antibiotic which was released in PBS solutions at different time points. In the other experiment (Althumairy et al. 2014), the indirect cytotoxic effect was measured by culturing the stem cells directly on treated dentin specimens with either 1000 or 1 mg/ml of TAP or DAP which can explain the different results from our study. Therefore, there were not enough evidence to reject the null hypothesis stated that tested concentration of TAP or DAP antibiotic medicaments (1000, 1, and 0.5 mg/ml) have no indirect cytotoxic effect on DPSCs.

Collectively, 1 mg/ml of TAP or DAP can be suggested as an efficient concentration of TAP or DAP medicaments with less potential for direct and indirect cytotoxicity on stem cells. The American Academy of Endodontists (AAE) has recommended the use of antibiotic medicaments in endodontic regeneration in concentrations ranging from 0.01 to 0.1 mg/ml (Diogenes et al. 2013; Endodontists 2013). However, those concentrations might not be effective against established bacterial biofilm. Recently, it is recommended that no more than 1 mg/ml of antibiotic medicaments to be used in endodontic regeneration (Diogenes et al. 2014) which is in agreement with our findings. However, no clinical studies have reported the use of this recommended diluted concentration (1 mg/ml) of antibiotics. Furthermore, a full concentration of TAP (1000 mg/ml) is still used in recently published clinical studies (Becerra et al. 2014; Nagata et al. 2014). One of the challenges associated with the use of diluted antibiotic concentrations in immature infected teeth is that they are in liquid form and do not have a pasty consistency.

Incorporation of the diluted antibiotics within a vehicle may be a good option to efficiently deliver the intended concentration of antibiotics into the root canal. Another approach to deliver these low antibiotic concentrations into the root canal could be through the use of a three-dimensional scaffold system. Recent studies have proposed the use of a bio-resorbable antibiotic-loaded scaffold to disinfect the root canal space in endodontic regeneration (Bottino et al. 2013; Bottino et al. 2013).

The aims of using different vehicles for delivering intracanal medicaments are mainly to maintain therapeutically active concentrations, and increase the residence time of the medicament inside the root canal system. Additionally, the use of different vehicles to form viscous medicaments has been suggested to decrease the toxicity to periapical tissues (Athanasiadis et al. 2007) by retaining the medicament inside the canal space in contact with dentinal tubules (Ferraz et al. 2001; Gomes et al. 2001). Different vehicles have been used to deliver intracanal medicaments in endodontics. Those can be classified as: water soluble vehicles (i.e., water, saline, methylcellulose (MC)), water soluble viscous vehicles (i.e., propylene glycol (PG)), and oily vehicles (i.e. camphorated paramonochlorophenol) (Athanasiadis et al. 2007). The aim of chapter five was to determine the cytotoxic level of two commonly used vehicles in intracanal medicament formulation (MC and PG) and to investigate their possible use as vehicles for diluted antibiotic (1mg/ml of TAP or DAP) delivery.

PG ( $C_3H_8O_2$ ), a synthetic alcohol, has been used widely as an effective vehicle for delivery of antibiotic medicaments with reported moderate to mild tissue reaction (Takushige et al. 2004; Marques et al. 2011; Pinky et al. 2011; Gomes-Filho et al. 2012). PG has been reported to significantly penetrate into dentinal tubules (Cruz et al. 2002) which make it a suitable vehicle for delivering intracanal medicaments. However, the direct cytotoxic effect on dental pulp stem cells needs to be determined before being used in endodontic regeneration procedures. Our results demonstrated that only 10 % of PG in distilled water (v/v) did not cause any reduction in DPSC metabolic activity. All other tested concentrations (80, 60, 40, and 20 v/v %) caused a significant

reduction in DPSC metabolic activity. This is in agreement with previous studies demonstrated that high concentrations of PG have an adverse effect on the metabolic activity and gene expression of cells (Morshed et al. 1998; Aye et al. 2010). Lower concentrations of PG have been used as a cryoprotectant agent (Woods et al. 2009) for different cell types which indicates minimal cytotoxicity at lower concentrations.

MC, a synthetic polymer of cellulose, has been used as a vehicle to deliver intracanal medicaments (Blanscet et al. 2008; Athanassiadis et al. 2010; Athanassiadis et al. 2010). Most commercially available calcium hydroxide intracanal medicaments, such as UltraCal XS and Pulpdent, are methyl cellulose based medicaments. Our data demonstrated that the two tested concentrations of MC (0.5 and 2 % w/v) significantly increased DPSC metabolic activity compared to control group. MC has been widely used as a supplement added to serum free culture medium (Thirumala et al. 2010). Its incorporation in culture medium has been associated with higher cells metabolic activity, numbers, and viability (Bryant 1969) which is in agreement with our findings. MC is also used as a cryoprotectant agent for different cell types (Thirumala et al. 2010) and as a medium for cell culturing and counting of CFU which indicates that MC has a minimal cytotoxicity and can be safely used as a vehicle for endodontic regeneration procedures.

Four novel pastes have been prepared using 2 % MC alone or in combination with 10 % of PG to add a viscosity for diluted TAP or DAP. The cytotoxicity of the novel pastes on DPSC have been compared to the three commonly used pastes in endodontic regeneration (TAP, DAP, and Ca(OH)<sub>2</sub> (UltraCal XS)). The metabolic activity of DPSC after exposure to TAP, DAP, or Ultracal XS was significantly reduced after 7d of treatment which is in agreement with previous studies report the cytotoxicity of those medicaments (Ruparel et al. 2012; Althumairy et al. 2014; Labban et al. 2014). Although Ca(OH)<sub>2</sub> have been reported to be not cytotoxic to the stem cells of dental papillae (Ruparel et al. 2012), a recent report suggested that calcium hydroxide is cytotoxic to the dental pulp cells at a concentration of 5 mg/ml (Labban et al. 2014). The percentage of

Ca(OH)<sub>2</sub> in UltraCal XS pastes is 35 wt% (35g/100ml) which is higher than the cytotoxic level identified in previous study.

Interestingly, all MC containing and MCPG containing pastes and vehicles had significantly increase the metabolic activity of DPSC after 7 days. Therefore, the null hypothesis stated that intracanal medicaments consisting of 1mg/ml of TAP or DAP in MC or MCPG vehicles have no cytotoxic effect against DPSCs was accepted. The increased metabolic activity of DPSC may be explained by the role of PG and MC in accelerating citric acid cycle that took place inside the mitochondria organelles (Zosel et al. 2010; Ghosh 2013). PG is enzymatically oxidized inside the cells to form lactic acid then converted to Pyruvate which is the first molecule in the citric acid cycle (Zosel et al. 2010). MC has also been reported to accelerate the formation of citric acid which is the third molecule in citric acid cycle (Ghosh 2013). The end result of citric acid cycle is the production of energy, reduced coenzymes, and multiple metabolites used by the cells in different metabolic pathways (i.e., NADH, FADH<sub>2</sub>) (Madeira 2012; Pirinen et al. 2012).

For the antibacterial effect, dentin specimens treated with the different diluted antibiotic pastes (D1+MC, T1+MC, D1+MCPG, and T1+MCPG) had significant reduction in bacterial biofilm as compared to MC, and MCPG vehicles. This indicates that the antibacterial action is attributed to the presence of 1 mg/ml of antibiotic medicaments of TAP or DAP and not to the vehicle used. Our results are in agreement with a previous study that reported MC vehicle to be ineffective against bacterial biofilm (Athanasiadis et al. 2010). The antibacterial action of different MC or poly ethylene glycol containing medicaments is mainly attributed to the active ingredients in a medicament (Athanasiadis et al. 2010).

Ca(OH)<sub>2</sub> paste (UltraCal XS) significantly reduced the log CFU/ml compared to the control, but biofilm reduction was significantly lower than TAP, DAP, and diluted antibiotic pastes (D1+MC, T1+MC, D1+MCPG, and T1+MCPG). Previous studies have reported a significant reduction of *E. faecalis* biofilm compared to the control group. However, the action of Ca(OH)<sub>2</sub> was less effective than other intracanal medicaments (i.e. Chlorhexidine, and TAP)

(Delgado et al. 2010; Jhamb et al. 2010; Madhubala et al. 2011; Pacios et al. 2012). Diluted TAP pastes (T 1+MC, and T1+MCPG) had a comparable antibacterial effectiveness to 1000 mg/ml of TAP. Similarly diluted DAP pastes (D 1+MC, and D1+MCPG) had a comparable antibacterial effectiveness to 1000 mg/ml of DAP which indicates that those novel diluted antibiotic pastes are promising intracanal medicaments for endodontic regeneration procedures. However, clinical studies are still needed to confirm the effective use of diluted antibiotic medicaments in endodontic regeneration procedures. The null hypothesis stated that intracanal medicaments consisting of 1mg/ml of TAP or DAP in MC or MCPG vehicles have no antibacterial effect on infected radicular dentin was rejected.

Future studies should focus on examining the possible side effect of adding MC or PG on the stability and the residual antibacterial properties of the antibiotic medicaments. Although the *in-vitro* bacterial model used in this project is rarely involved in primary root canal infection, *E. faecalis* is considered one of the most resistant bacteria to treatment. A recent studies has reported that anaerobic black pigmented bacteria (i.e., *P.gingivalis* and *Fussobacterium nucleatum*) is sensitive to lower concentrations of ciprofloxacin, metronidazole and minocycline compared to *E. faecalis* bacteria (AL-Ahmad et al. 2014). However, the polymicrobial nature of endodontic infections showed be taken into consideration and require further investigation. Furthermore, endodontic infections could be a potential reservoir for antibiotic resistance genes that can be transferred by horizontal gene transfer (AL-Ahmad et al. 2014).

The presence of stem cells in the remaining vital tissues inside the root canal has been described as a potential source for endodontic regeneration (Diogenes et al. 2013). In our study we considered the presence of a dilution factor when the medicament is placed inside the root canal space due to the presence of a rich blood supply in the apical area surrounding the stem cells. However, in the case of the presence of vital tissues inside the root canal, the dilution factor is not relevant and the stem cells are in direct contact with the antibiotic medicaments. Therefore, examining the cytotoxic effect of the new antibiotic pastes without dilution in DMEM growth



media is recommended. A limitation of *in-vitro* studies is that DPSC growth is measured immediately after the removal of the antibiotic medicaments. However, in clinical situations the growth ability of the DPSC may be recovered over time due to the dilution of the extruded antibiotic medicaments by the blood flow in the apical area. Therefore, examination of the cell growth after exposure to antibiotic medicaments showed be delayed and examined after different time intervals.

DPSC is a multipotent stem cells that are able to differentiate into many other cell types including; osteoblasts, adipocytes, odontoblasts, and chondroblasts. Histological examination of regenerative tissues revealed that a true histological dentin-pulp formation is absent, and the mineralized tissues formed inside the canal space appeared to be cementum (Martin et al. 2013), bone like tissues (Martin et al. 2013) or periodontal tissue (Tawfik et al. 2013). Achieving adequate disinfection, while maintaining the microenvironment suitable for stem cell metabolic activity and differentiation, appears to remain a challenge in clinical situation. Therefore, to create an ideal environment for the stem cell differentiation to form an organized pulp like tissue, the effect of antibiotic medicaments on cell differentiation should be further investigated.

## CONCLUSIONS

1. Our results demonstrated that TAP and DAP were more effective than  $\text{Ca(OH)}_2$  against early biofilm formation of *E. faecalis* and *P. gingivalis* bacteria. DAP can be considered an effective and comparable antibacterial substitute to TAP. Both DAP and TAP were effective in inhibiting early biofilm formation at a concentration of 0.001-0.006 mg/ml respectively.
2. Our data suggests that higher concentration of TAP and DAP ( $\geq 1\text{mg/ml}$ ) are required to eradicate established bacterial biofilm.
3. Significant residual antibacterial activities of dentin treated with 1 and 1000 mg/ml of TAP was detected for up to 7 and 14 days, respectively.
4. Significant residual antibacterial activities of dentin treated with 1 and 1000 mg/ml of DAP was detected for up to 14 and 30 days, respectively.
5. The use of TAP or DAP at a concentration of 1 mg/ml can provide a comparable residual antibacterial activity to the concentration of TAP or DAP currently used in endodontic regeneration procedures (1000 mg/ml).
6. Our data demonstrated that exposure of DPSC to higher than 0.125 mg/ml of TAP or DAP will reduce cell metabolic activity according to WST-1 assays.
7. Our data demonstrated that exposure of DPSC to higher than 0.5 mg/ml of DAP or 0.25 mg/ml of TAP will produce a cytotoxic effect to the cell membrane integrity according to LDH cytotoxicity assays.
8. Our data demonstrated that dentin treated with 0.5, 1 or 1000 mg/ml of TAP or DAP had no indirect cytotoxicity on DPSC.
9. Only 10 % of PG in sterile water (v/v) had no direct cytotoxicity on DPSC according to WST-1 assays.
10. Both 0.5 and 2% MC (w/v) had significantly increased the metabolic activity of DPSC.
11. All diluted antibiotic pastes (D1+MC, T 1+MC, D1+MCPG, and T1+MCPG), MC, and MCPG produced a significant increase in the metabolic activity of DPSC compared to the untreated control and currently used intracanal medicaments (TAP, DAP, and  $\text{Ca(OH)}_2$ ).

12. Dentin specimens treated with the different diluted antibiotic pastes (D1+MC, T 1+MC, D1+MCPG, and T1+MCPG) had a significant reduction in bacterial biofilm CFU/ml compared to saline, MC, MCPG, and Ca(OH)<sub>2</sub> groups.

13. Dentin specimens treated with the different diluted TAP pastes (T 1+MC, and T1+MCPG) had no significant biofilm reduction compared to 1000 mg/ml of TAP.

14. Dentin specimens treated with the different diluted DAP pastes (D1+MC, and D1+MCPG) had no significant biofilm reduction compared to 1000 mg/ml of DAP.

## TABLES AND FIGURES

**Table 1.** The means and standard errors of log CFU/ml of *E. faecalis* bacteria grown on treated dentin specimens with different antibiotic concentrations after 0, 3, 7, 14 or 30 days of incubating specimens in PBS solutions.

Time (days)**					
Group*	0	3	7	14	30
DAP 1000 mg/ml	0.51 (0.34) <sup>aA</sup>	0.82 (0.41) <sup>aA</sup>	1.58 (0.42) <sup>aAB</sup>	3.42 (0.5) <sup>aBC</sup>	4.23 (0.22) <sup>aC</sup>
TAP 1000 mg/ml	0.95 (0.48) <sup>abA</sup>	2.5 (0.56) <sup>abAB</sup>	2.42 (0.56) <sup>abAB</sup>	3.95 (0.22) <sup>abC</sup>	6.11 (0.11) <sup>abcC</sup>
DAP 1 mg/ml	1.78 (0.59) <sup>abA</sup>	2.55 (0.44) <sup>abAB</sup>	3.17 (0.76) <sup>abABC</sup>	4.13 (0.64) <sup>abC</sup>	5.79 (0.43) <sup>abC</sup>
DAP 0.5 mg/ml	1.63 (0.33) <sup>abA</sup>	4.4 (1.07) <sup>bB</sup>	4.04 (0.94) <sup>bB</sup>	4.72 (0.48) <sup>abBC</sup>	6.07 (0.49) <sup>abcC</sup>
TAP 1 mg/ml	2.87 (0.8) <sup>bA</sup>	3.93 (0.89) <sup>bAB</sup>	4.44 (0.72) <sup>bAB</sup>	5.55 (0.09) <sup>abcBC</sup>	7.65 (0.21) <sup>bcC</sup>
TAP 0.5 mg/ml	3.09 (0.79) <sup>bA</sup>	4.47 (1) <sup>bAB</sup>	4.39 (0.83) <sup>bAB</sup>	6.46 (0.47) <sup>bcBC</sup>	7.49 (0.26) <sup>bcC</sup>
Saline	7.14 (0.22) <sup>cA</sup>	7.22 (0.18) <sup>cA</sup>	7.29 (0.19) <sup>cA</sup>	7.16 (0.15) <sup>cA</sup>	7.76 (0.13) <sup>cA</sup>

\* Different lower-case letters indicate a significant difference between various treatments/dilution within a single time point.

\*\* Different upper-case letters indicate a significant difference between the five time points of each treatment/dilution.

**Table 2.** The mean and standard errors of the percent of biofilm inhibition caused by released antibiotics in the PBS solutions at different time points.

<b>Time (days)**</b>				
<b>Group *</b>	<b>3</b>	<b>7</b>	<b>14</b>	<b>30</b>
<b>DAP 1000 mg/ml</b>	44.3 (7.7) <sup>aA</sup>	55.9 (2.1) <sup>aA</sup>	63.2 (1.7) <sup>aA</sup>	75 (5) <sup>aA</sup>
<b>TAP 1000 mg/ml</b>	37.2 (6.8) <sup>abA</sup>	54.2 (3.9) <sup>aA</sup>	36.14 (9) <sup>aA</sup>	69.9 (2.4) <sup>aA</sup>
<b>DAP 1 mg/ml</b>	31.8 (6.4) <sup>abA</sup>	40.1 (7.6) <sup>abA</sup>	49.1 (3.6) <sup>aA</sup>	37.7 (9.3) <sup>abA</sup>
<b>DAP 0.5 mg/ml</b>	25.9 (4.3) <sup>abA</sup>	30.2 (8.4) <sup>abA</sup>	38.6 (8.1) <sup>aA</sup>	12.56 (13.3) <sup>bA</sup>
<b>TAP 1 mg/ml</b>	31.8 (5.4) <sup>abA</sup>	36.4 (2.4) <sup>abA</sup>	49.8 (1.7) <sup>aA</sup>	27.1 (16.9) <sup>bA</sup>
<b>TAP 0.5 mg/ml</b>	20.6 (7.1) <sup>abA</sup>	37.4 (1.1) <sup>abA</sup>	47.0 (1.1) <sup>aA</sup>	-78.16 (13.3) <sup>cB</sup>
<b>Saline</b>	0 (3.7) <sup>abA</sup>	0 (11.2) <sup>bA</sup>	0 (15.5) <sup>bA</sup>	0 (8.1) <sup>bA</sup>

\* Different lower-case letters indicate a significant difference between various treatments/dilution within a single time point.

\*\* Different upper-case letters indicate a significant difference between the five time points of each treatment/dilution.

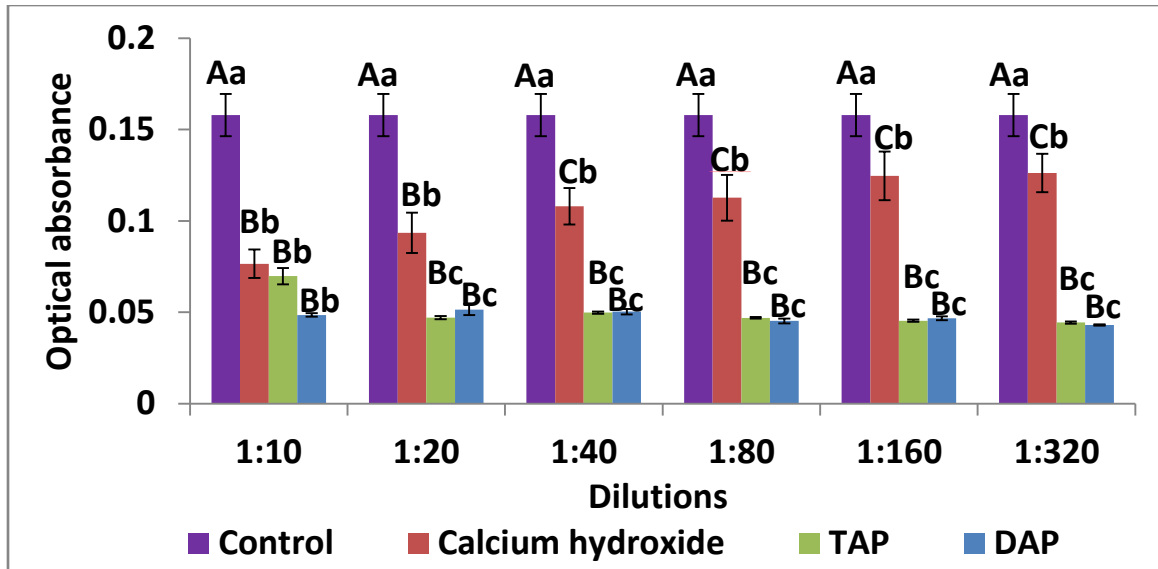
**Table 3.** The mean and standard error of the percent of metabolic activity of DPSC after incubation with PBS solutions at different time points.

<b>Time (days) **</b>				
<b>Group *</b>	<b>3</b>	<b>7</b>	<b>14</b>	<b>30</b>
<b>DAP 1000 mg/ml</b>	<b>126 (8) aA</b>	<b>146 (9) aA</b>	<b>140 (32) Aa</b>	<b>93 (9) aA</b>
<b>TAP 1000 mg/ml</b>	<b>95 (19) aA</b>	<b>134 (10) aA</b>	<b>102 (20) aA</b>	<b>112 (4) aA</b>
<b>DAP 1 mg/ml</b>	<b>86 (6) aA</b>	<b>118 (6) aA</b>	<b>114 (20) aA</b>	<b>93 (2) aA</b>
<b>DAP 0.5 mg/ml</b>	<b>90 (6) aA</b>	<b>100 (8) aA</b>	<b>115 (23) aA</b>	<b>96 (3) aA</b>
<b>TAP 1 mg/ml</b>	<b>122 (7) aA</b>	<b>118 (15) aA</b>	<b>92 (5) aA</b>	<b>94 (4) aA</b>
<b>TAP 0.5 mg/ml</b>	<b>114 (9) aA</b>	<b>117 (13) aA</b>	<b>86 (5) aA</b>	<b>90 (2) aA</b>
<b>Saline</b>	<b>100 (4) aA</b>	<b>100 (8) aA</b>	<b>100 (20) aA</b>	<b>100 (20) aA</b>

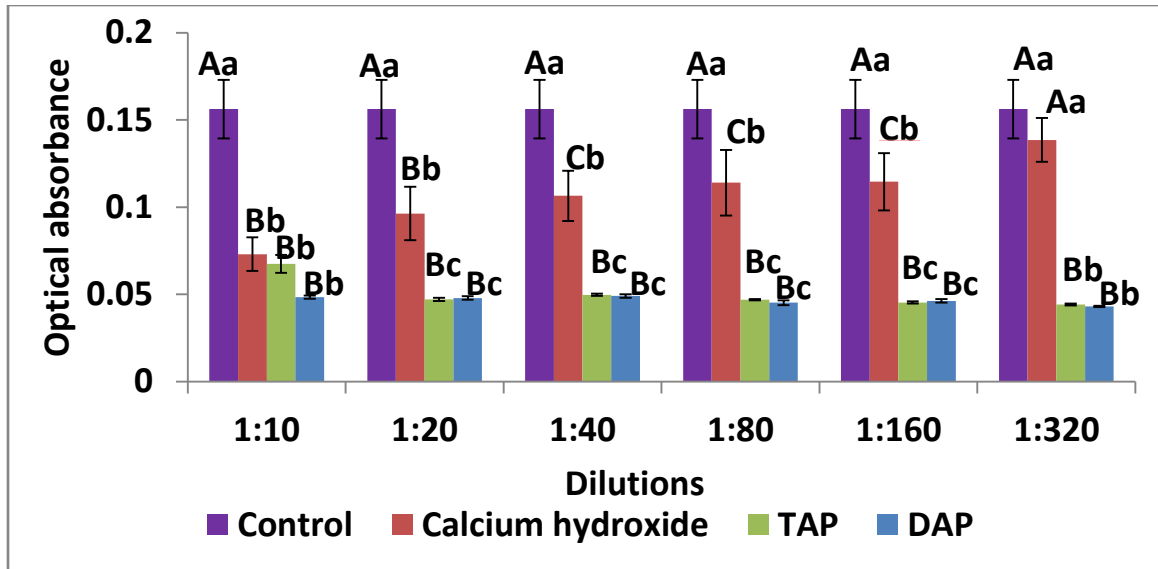
\* Different lower-case letters indicate a significant difference between various treatments/dilution within a single time point.

\*\* Different upper-case letters indicate a significant difference between the five time points of each treatment/dilution.



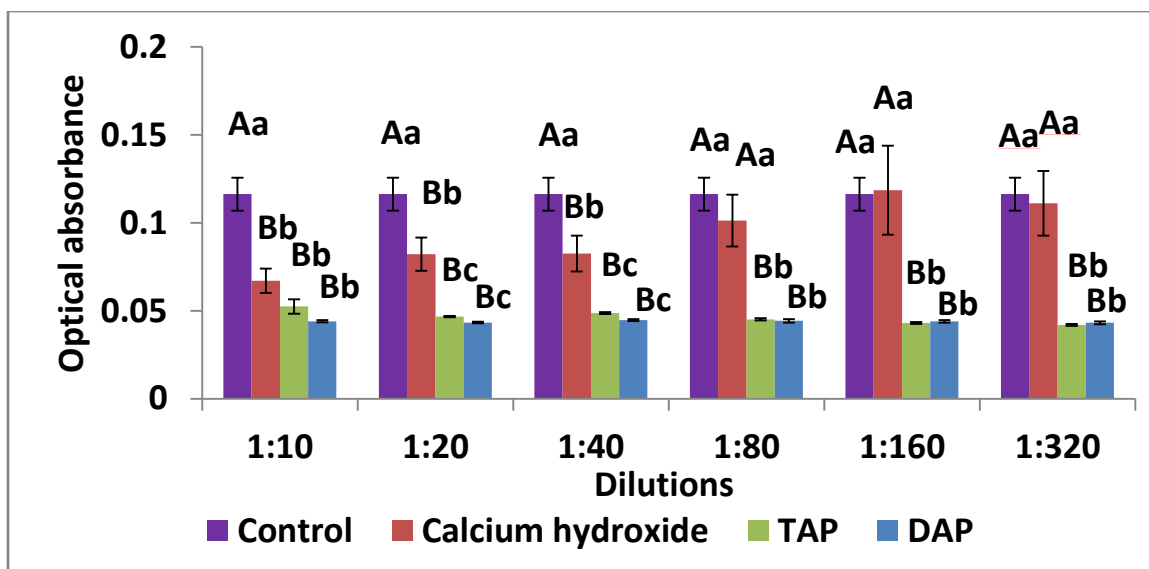


**Figure 1.** The effect of  $\text{Ca}(\text{OH})_2$ , TAP and DAP on *Ef* biofilm formation after 24 hours. Lowercase letters compare various materials and the control at each dilution. Uppercase letters compare all materials and the control at various dilutions. X-axis represents different dilutions of the antibiotic medicaments, and Y-axis represents the optical absorbance measure by spectrophotometer.



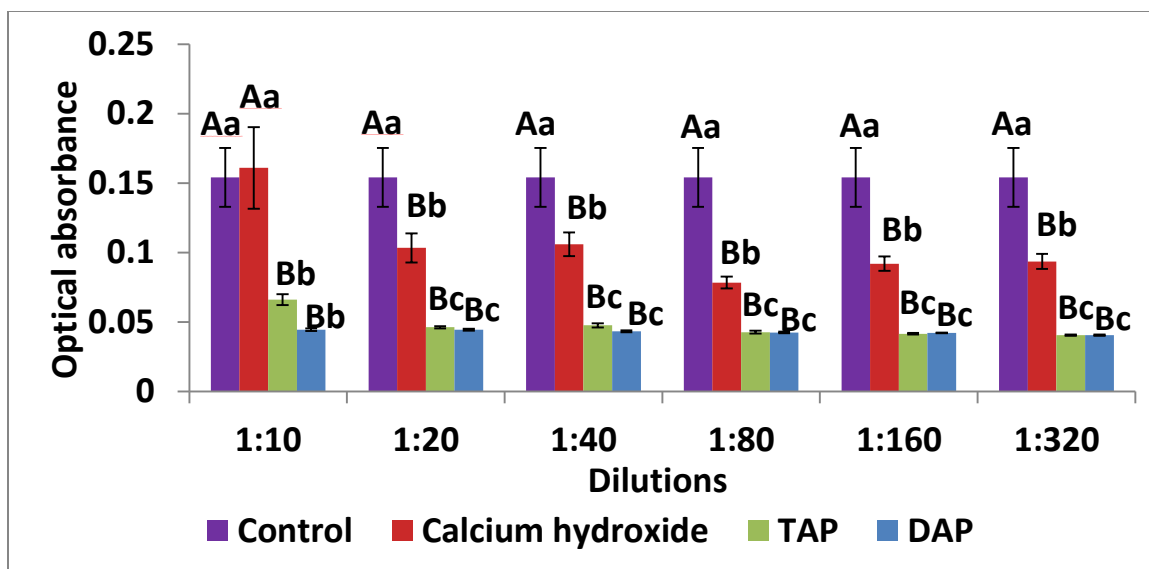
**Figure 2.** The effect of  $\text{Ca}(\text{OH})_2$ , TAP and DAP on *Ef* biofilm formation after 48 hours.

Lowercase letters compare various materials and the control at each dilution. Uppercase letters compare all materials and the control at various dilutions. X-axis represents different dilutions of the antibiotic medicaments, and Y-axis represents the optical absorbance measure by spectrophotometer.



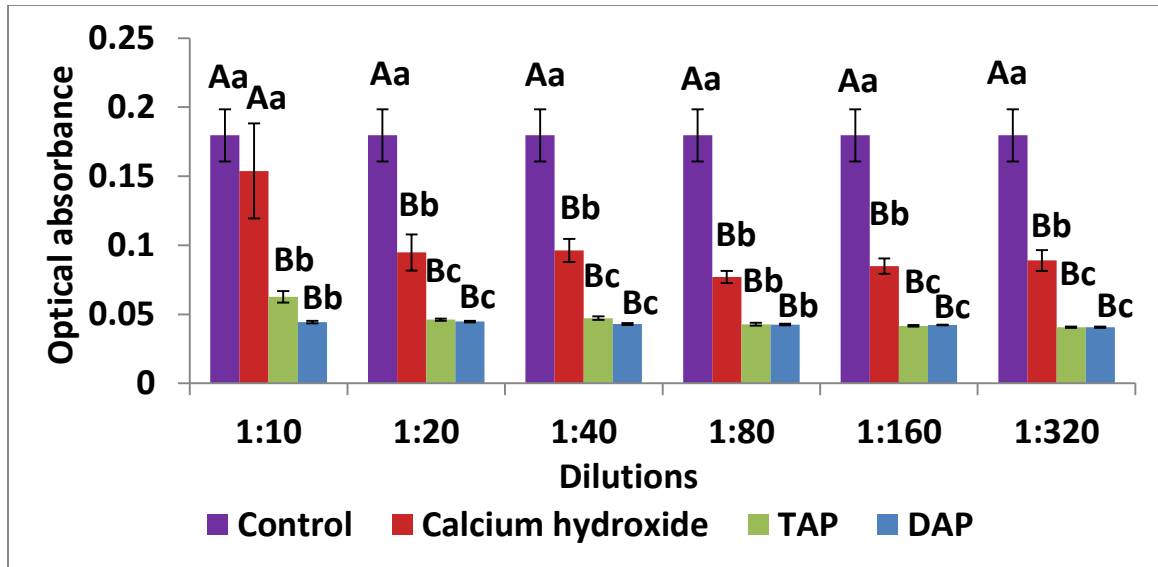
**Figure 3.** The effect of  $\text{Ca}(\text{OH})_2$ , TAP and DAP on *Ef* biofilm formation after 72 hours.

Lowercase letters compare various materials and the control at each dilution. Uppercase letters compare all materials and the control at various dilutions. X-axis represents different dilutions of the antibiotic medicaments, and Y-axis represents the optical absorbance measure by spectrophotometer.



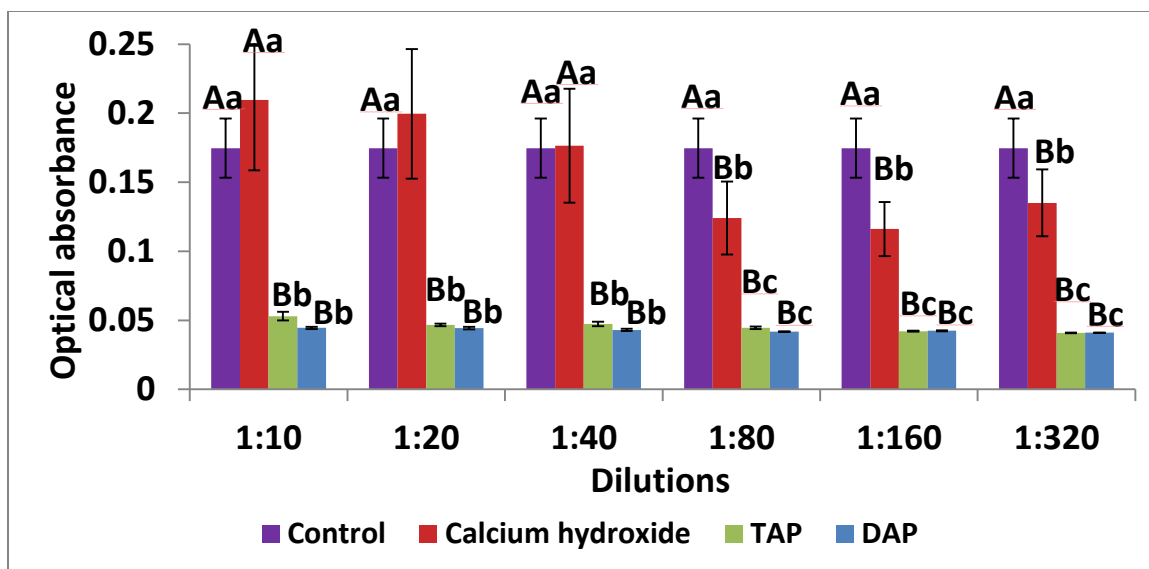
**Figure 4.** The effect of  $\text{Ca}(\text{OH})_2$ , TAP and DAP on *Pg* biofilm formation after 24 hours.

Lowercase letters compare various materials and the control at each dilution. Uppercase letters compare all materials and the control at various dilutions. X-axis represents different dilutions of the antibiotic medicaments, and Y-axis represents the optical absorbance measure by spectrophotometer.



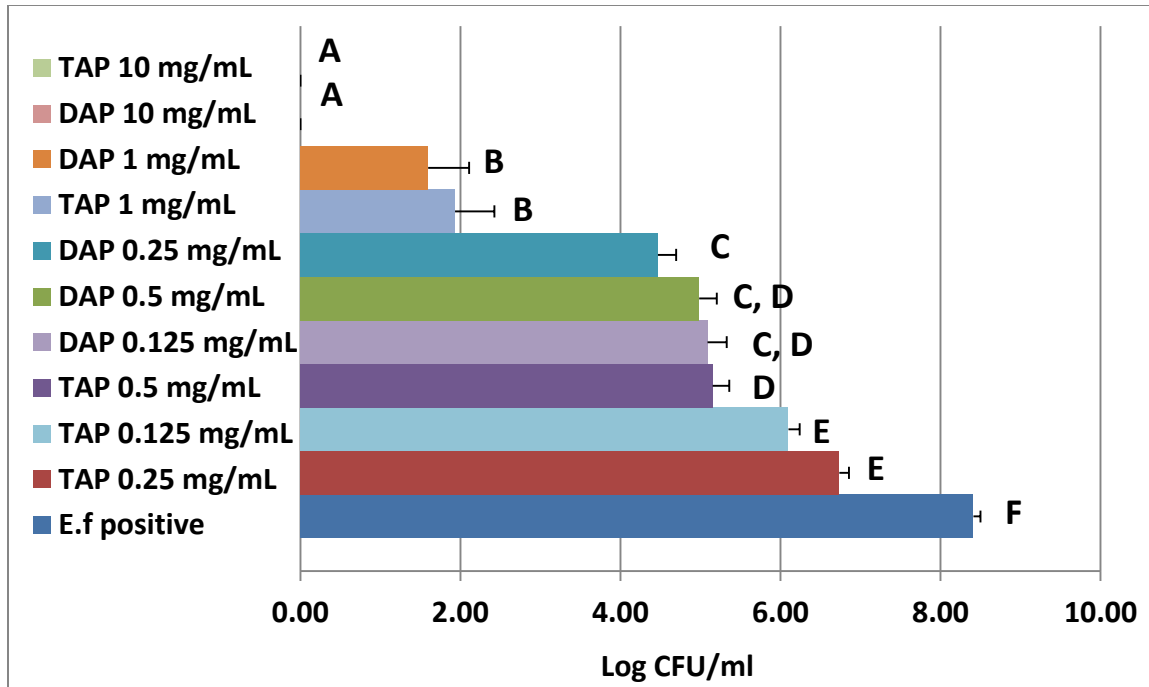
**Figure 5.** The effect of  $\text{Ca}(\text{OH})_2$ , TAP and DAP on *Pg* biofilm formation after 48 hours.

Lowercase letters compare various materials and the control at each dilution. Uppercase letters compare all materials and the control at various dilutions. X-axis represents different dilutions of the antibiotic medicaments, and Y-axis represents the optical absorbance measure by spectrophotometer.



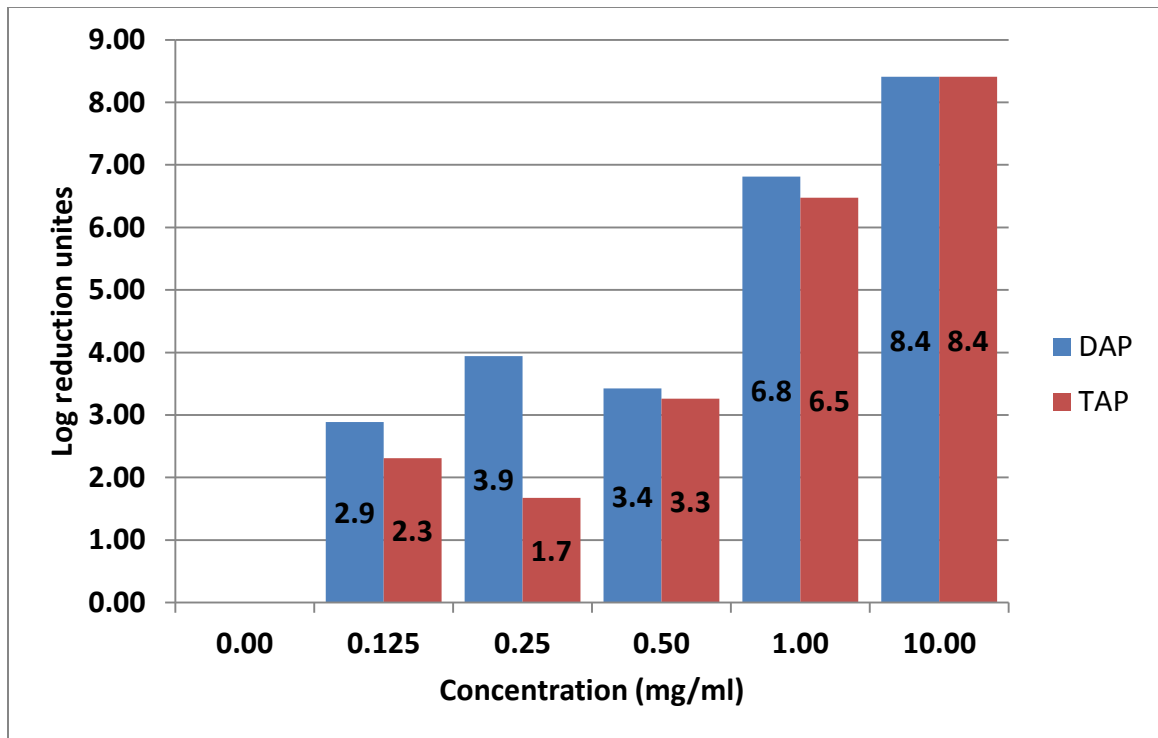
**Figure 6.** The effect of  $\text{Ca}(\text{OH})_2$ , TAP and DAP on *Pg* biofilm formation after 72 hours.

Lowercase letters compare various materials and the control at each dilution. Uppercase letters compare all materials and the control at various dilutions. X-axis represents different dilutions of the antibiotic medicaments, and Y-axis represents the optical absorbance measure by spectrophotometer.



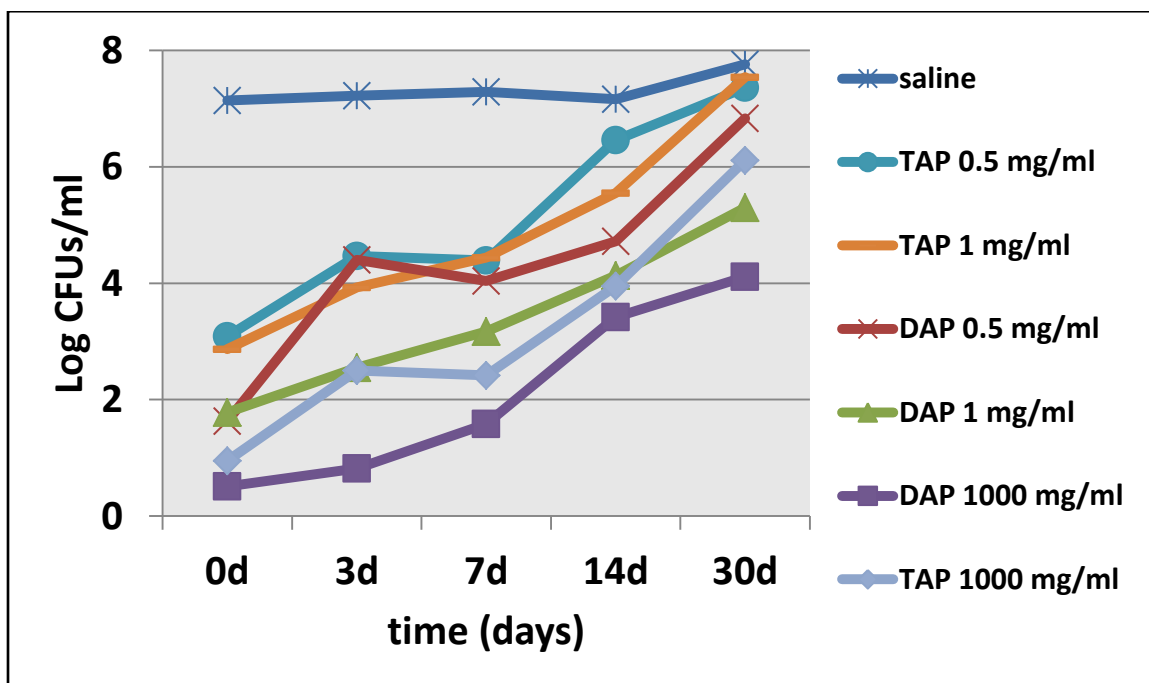
**Figure 7.** The mean log CFU/ml of *E. faecalis* after incubation with different TAP and DAP antibiotic medicament dilutions for 72 h.

The different groups are color coded in ascending order representing the increase in the mean log CFU/ml. Different letters represent statistically significant differences.



**Figure 8.** The Log reduction units in log CFU/ml of bacterial biofilm, a material can be considered effective in eradicating the bacterial biofilm when it can produce a reduction of more than 5 logarithmic units in the log CFU/ml.





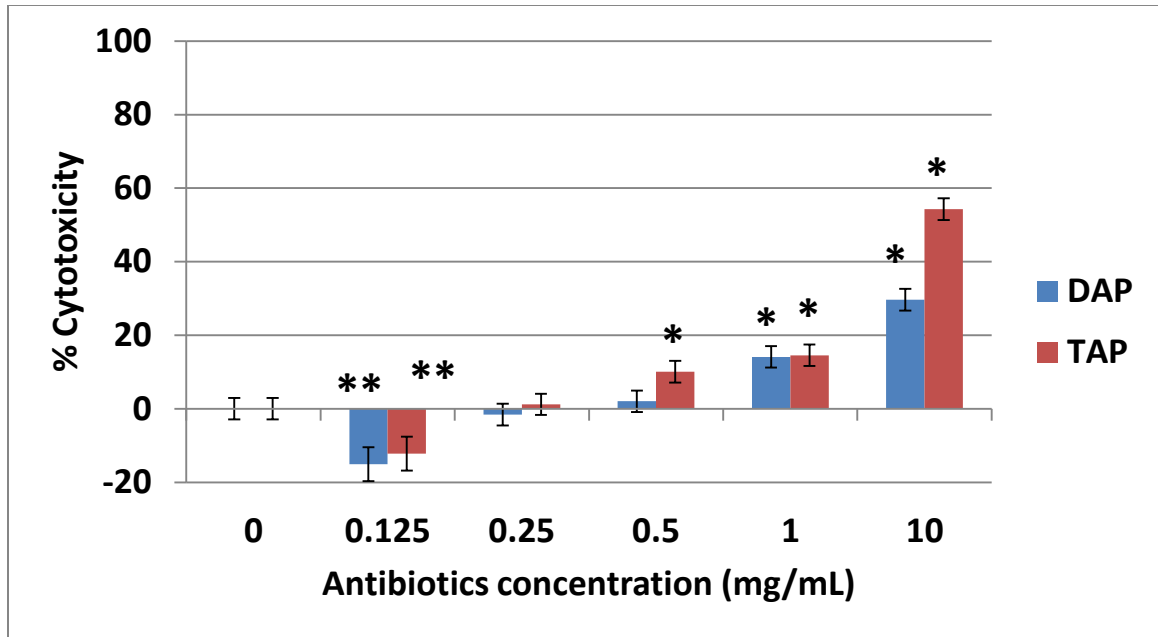
**Figure 9.** The residual antibacterial properties of different antibiotic medicaments and concentration represented as the mean of the log CFU/ml over time.

The mean function of the log CFU/ml is represented by.

$$\text{Log CFU/ml} = 2.857 - 0.731 \times I(\text{DAP 1mg/ml}) - 2.112 \times I(\text{DAP 1000 mg/ml}) + 0.878 \times I(\text{TAP 0.5mg/ml}) + 0.428 \times I(\text{TAP 1mg/ml}) - 1.358 \times I(\text{TAP 1000 mg/ml}) + 4.255 \times I(\text{Saline}) + 0.129 \times \text{time} + [-0.006 \times I(\text{DAP 1mg/ml}) - 0.006 \times I(\text{DAP 1000 mg/ml}) + 0.003 \times I(\text{TAP 0.5mg/ml}) + 0.016 \times I(\text{TAP 1mg/ml}) + 0.024 \times I(\text{TAP 1000 mg/ml}) - 0.110 \times I(\text{Saline})] \times \text{time}.$$

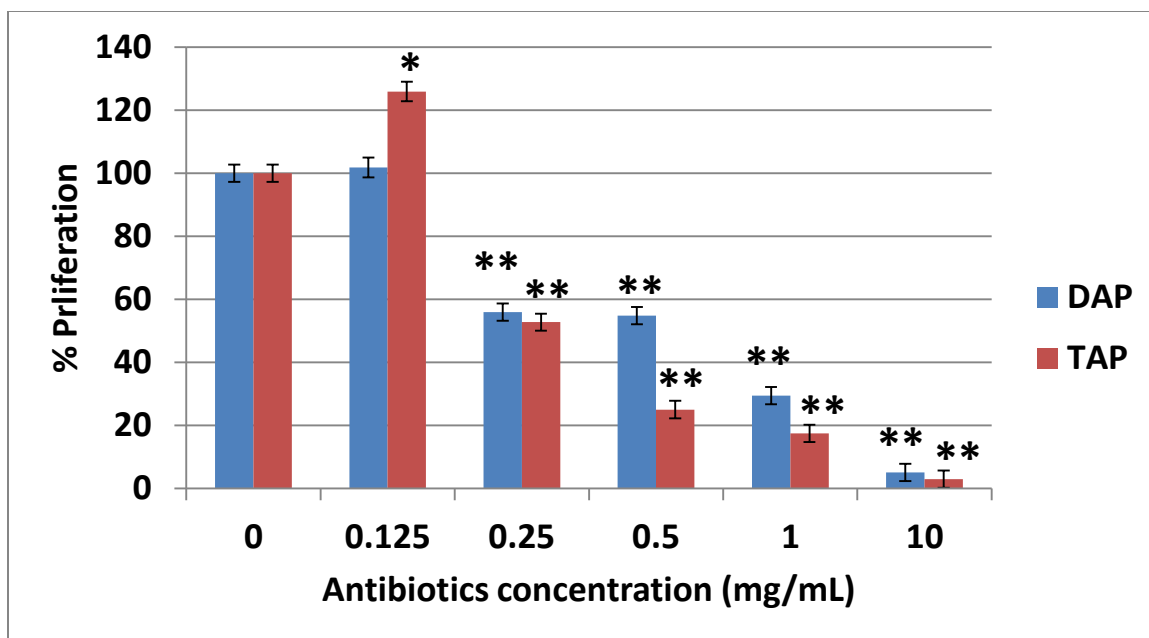
Where  $I(\cdot)$  is an indicator function, which is either 1 or 0. Therefore the mean functions of the log CFU/ml for all groups are:

- Group DAP 1000 mg/ml:  $\text{Log CFU/ml} = 0.745 + 0.123 \times \text{time}$
- Group DAP 1 mg/ml:  $\text{Log CFU/ml} = 2.851 + 0.066 \times \text{time}$
- Group DAP 0.5 mg/ml:  $\text{Log CFU/ml} = 2.857 + 0.129 \times \text{time}$
- Group TAP 1000 mg/ml:  $\text{Log CFU/ml} = 1.499 + 0.153 \times \text{time}$
- Group TAP 1 mg/ml:  $\text{Log CFU/ml} = 3.285 + 0.145 \times \text{time}$
- Group TAP 0.5 mg/ml:  $\text{Log CFU/ml} = 3.735 + 0.132 \times \text{time}$
- Group Saline:  $\text{Log CFU/ml} = 7.112 - 0.019 \times \text{time}$



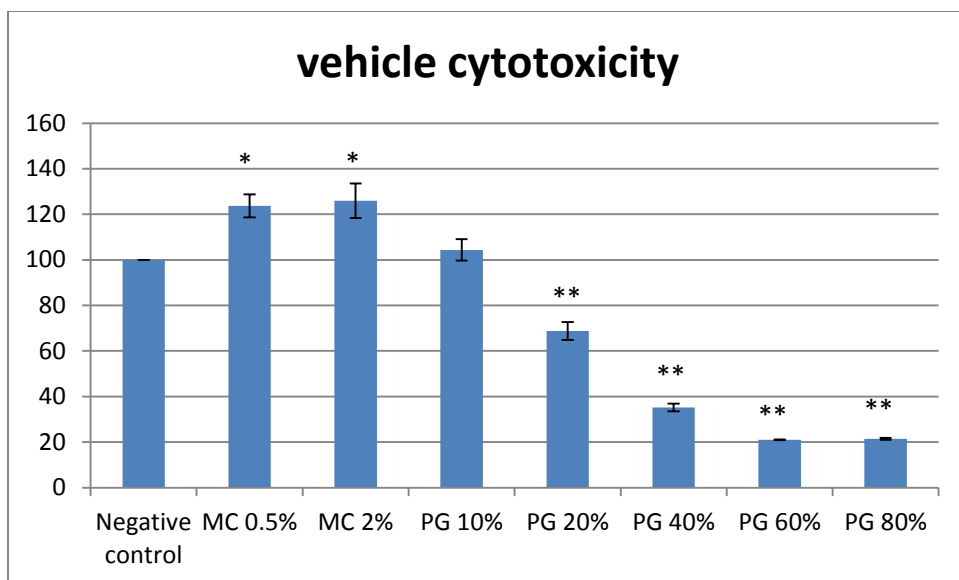
**Figure 10.** The percent cytotoxicity to DPSC in the presence of different TAP and DAP antibiotic dilutions for 72 h using LDH assay compared to the negative control set at 0% and the positive control set at 100% cytotoxicity.

\* Significantly higher than the control. \*\* Significantly lower than the control.



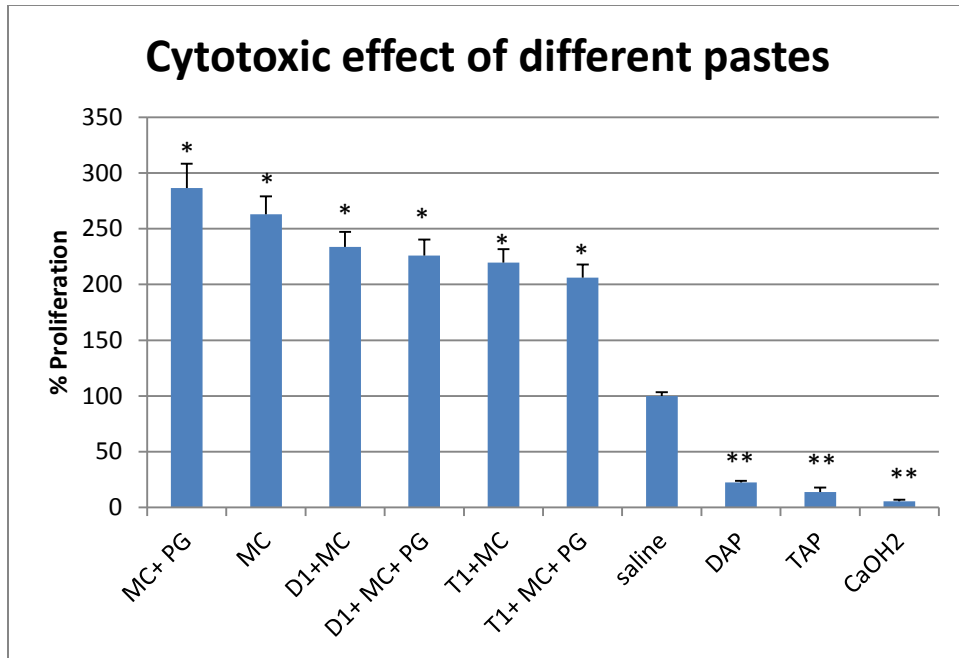
**Figure 11.** The percent metabolic activity of DPSCs in the presence of different TAP and DAP antibiotic dilutions for 72 h using WST-1 assay compared to a negative control set at 100% and a positive control set at 0% metabolic activity.

\* Significantly higher than the control. \*\* Significantly lower than the control.



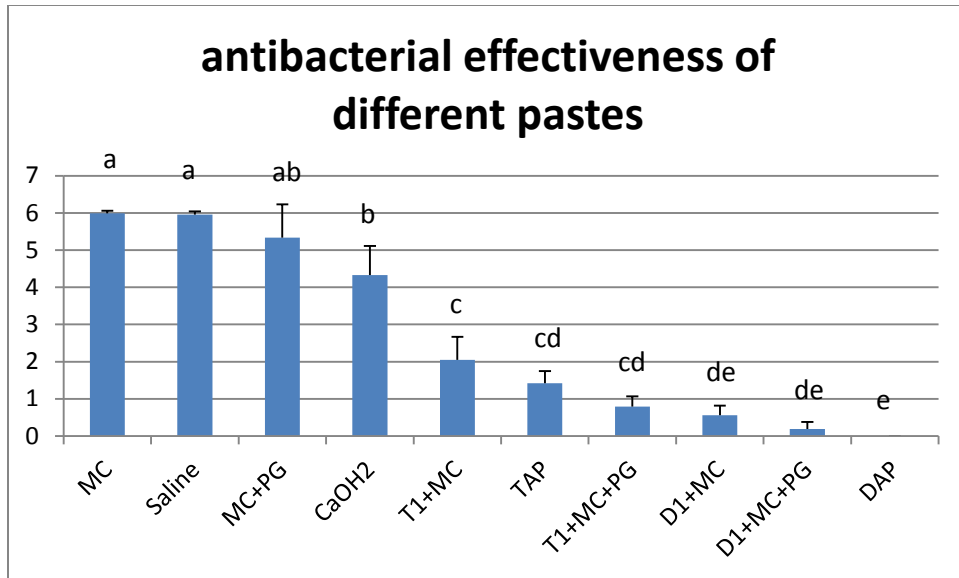
**Figure 12.** The percent metabolic activity of DPSCs in the presence of different MC and PG concentrations for 72 h using WST-1 assay compared to a negative control set at 100% and a positive control set at 0% metabolic activity.

\* Significantly higher than the control. \*\* Significantly lower than the control.



**Figure 13.** The percent metabolic activity of DPSCs in the presence of different intracanal medicaments for 7 d using WST-1 assay compared to a negative control set at 100% and a positive control set at 0% metabolic activity.

\* Significantly higher than the control. \*\* Significantly lower than the control.



**Figure 14.** The mean log CFU/ml of *E. faecalis* infected radicular dentin after incubation with different antibiotic medicament for 7d.

Different letters indicate statistically significant differences between different antibiotic medicaments.

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## CURRICULUM VITAE

Ala'a Hussein Aref Sabrah

### EDUCATION

- 2011-2014 Doctor of Philosophy (Ph.D.), Indiana University, Indianapolis, Indiana, USA
- 2009-2011 M.S.D. Operative Dentistry, Indiana University, Indianapolis, Indiana, USA
- 2002-2007 B.D.S. Dental Surgery, The University of Jordan, Faculty of Dentistry, Amman, Jordan.

### APPOINTMENTS

#### Academic

- 2013-2014 Bench instructor, Indiana University, Indianapolis, Indiana, USA.
- 2007-2009 Teaching and research assistant, The University of Jordan, Faculty of Dentistry, Conservative Dentistry department. Amman, Jordan.

### HONORS AND AWARDS

- 2014 Mynard Hine award for excellence in dental research, Indiana University, IUPUI, Indianapolis, Indiana, USA.
- 2013 Shofu dental research award, Indiana University, IUPUI, Indianapolis, Indiana, USA.
- 2008 The University of Jordan future faculty scholarship award for completing master and PhD.
- 2006 The University of Jordan annual excellence award, Amman, Jordan.

## PROFESSIONAL MEMBERSHIPS AND SERVICE

- 2011-present Indiana Association of Dental Research
- 2009-2011 American Academy of Operative Dentistry
- 2007-present Jordan Dental Association

## JOURNAL REVIEWER

- 2013-present Journal of Oral Microbiology
- 2013-present Journal of Applied Oral Science

## TEACHING

- 2013 Critical Thinking (PBL Tutor, 6h/w), IUPUI.
- 2007-2009 Teaching assistant, Preclinical labs, The University of Jordan, Amman, Jordan.
- 2007-2009 Advanced Clinical Operative Dentistry (clinical assistant and supervision).

## PEER-REVIEWED PUBLICATIONS

- Oweis Y, Hattar S, Abu Eid R, Sabrah A. Dentistry a second time? Eur J Dent Educ. 2012 Feb;16(1):e10-8
- Sabrah A.HA, Cook B, Luangruangrong P, Hara AT, Bottino M. Full-Contour Y-TZP Surface Roughness Effect on Wear of Hydroxyapatite. Dental Materials (2013), 29:666-673.
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- Sabrah A.HA, Cook B, Luangruangrong P, Hara AT, Bottino M. Full-Contour Y-TZP Surface Roughness Effect on Wear of Hydroxyapatite. Oral presentation, 41<sup>st</sup> AADR annual meeting in Tampa, FL, March 21, 2012.
- Sabrah A. HA, Yassen GH, Gregory RL. Antibacterial Activity of Intracanal Medicaments Used in Endodontic Regeneration. Abstract, 42<sup>nd</sup> AADR annual meeting in Seattle, WA, March 22, 2013.
- Sabrah A. HA, Kelly A, Lippert F, and Hara AT. Comparison between radiotracer and surface profile methods for the determination of dentifrice abrasivity. IUPUI research day 2013.
- Sabrah A. HA, Kelly A, Eckert G, Lippert F, and Hara AT. 3D profilometry analysis of in-vitro NCCLS development by toothbrushing. The 61<sup>st</sup> congress of the European Organization for caries research, Greifswald, Germany.