Title

Attachment and proliferation of dental pulp stem cells on dentine treated with different regenerative endodontic protocols

Authors

M. A. Alghilan[1, 2], L. J. Windsor[1], J. Palasuk[1], G. H. Yassen[1]

[1]Department of Biomedical and Applied Sciences, Indiana University School of Dentistry, Indianapolis, USA.

[2]Department of Restorative Dental Science, King Saud bin Abdulaziz University for Health Sciences, Riyadh, Kingdom of Saudi Arabia.

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Corresponding author:

Ghaeth H. Yassen
Department of Biomedical and Applied Sciences
Indiana University School of Dentistry
1121 W. Michigan St.
Indianapolis, IN, 46202, USA
Tel: +1-317-437-2240; fax: +1-317-278-7462
E-mail address: gyassen@iupui.edu

Abstract

Introduction To investigate the attachment and proliferation of dental pulp stem cells (DPSC) on dentine treated with various endodontic regeneration protocols.

Methodology Standardized dentine samples were irrigated with sodium hypochlorite (1.5% NaOCl) and ethylenediaminetetraacetic acid (17% EDTA), and randomized into four treatment groups and two control groups. The treatment groups were treated with a clinically used concentration of triple antibiotic paste (TAP), double antibiotic paste (DAP), calcium hydroxide ($Ca(OH)_2$), or diluted TAP in a methylcellulose system (DTAP) for 1 week. Each sample in the treatment groups was then irrigated with EDTA. The two control groups were treated with EDTA or received no treatment. DPSC were seeded on each dentine specimen (10,000 cells). Lactate dehydrogenase activity assays were then performed to evaluate the attached DPSC after 1 day of incubation. Water-soluble tetrazolium assays were used to determine DPSC proliferation after 3 additional days of incubation. Friedman's test followed by Least Significant Difference were used for statistical analyses ($\alpha = 0.05$).

Results TAP and DTAP regeneration protocols, as well as EDTA-treated dentine, caused significant increases in DPSC attachment to the dentine. TAP, DAP and $Ca(OH)_2$ regeneration protocols caused significant reductions in DPSC proliferation on dentine. However, DTAP regeneration protocol did not have any significant negative effects on DPSC proliferation.

Conclusions The clinically used endodontic regeneration protocols that include the use of TAP, DAP or $Ca(OH)_2$ medicament negatively affected DPSC proliferation on dentine. However, the use of DTAP medicament during regenerative endodontic treatment may not adversely affect the proliferation of DPSC.

 Introduction

The utilization of biological factors is the distinctive characteristic of clinical endodontic regeneration procedures in comparison to regular root canal therapy (Kim *et al.* 2013). These biological elements include the formation of blood-based scaffold, the promotion of endogenous growth factors in the local environment, and the delivery of stem cells into the root canal system (Diogenes *et al.* 2013). However, the survival, attachment, proliferation and differentiation of the delivered stem cells are essential to develop new pulp/dentine-like structures (Huang & Garcia-Godoy 2014) and optimize the clinical outcomes during endodontic regeneration (Diogenes *et al.* 2013).

Recent studies have been aimed at reducing the concentrations of various disinfectants used during regeneration procedures (Althumairy *et al.* 2014, Martin *et al.* 2014) as an attempt to create a stem cell-friendly environment that eliminates the infection without undermining the biological properties of the stem cells and the availability of dentine-endogenous proteins (Diogenes *et al.* 2014). Indeed, clinically used concentrations of triple antibiotic paste (TAP), double antibiotic pastes (DAP), sodium hypochlorite (NaOCl) and chlorhexidine were found to have toxic effects on stem cells from apical papillae (SCAP) (Trevino *et al.* 2011, Ruparel *et al.* 2012, Althumairy *et al.* 2014, Martin *et al.* 2014) and dental pulp stem cells (DPSC) (Kim *et al.* 2015). On the other hand, low concentrations of TAP (1 mg/mL), DAP (1 mg/mL), and NaOCl (1.5%), as well as the clinically used concentration of calcium hydroxide ($Ca(OH)_2$), were found to be non-toxic to SCAP and DPSC (Althumairy *et al.* 2014, Martin *et al.* 2014, Kim *et al.* 2015). Multiple studies have suggested that ethylenediaminetetraacetic acid (EDTA) is the most stem cell-friendly irrigation solution and recommended to use it as a final irrigation step during regenerative endodontics to promote stem cell attachment, proliferation and differentiation (Galler *et al.* 2011, Pang *et al.* 2014, Galler *et al.* 2016), as well as to neutralize the cytotoxic

effects of NaOCl (Trevino *et al.* 2011) and intracanal medicaments (Kim *et al.* 2015). Limited information is available regarding the effects of the entire endodontic regeneration protocol, including the use of intracanal medicament followed by extensive EDTA irrigation, on the attachment and proliferation of DPSC on dentine. Therefore, the aim of this study was to determine the effects of various endodontic regeneration protocols on DPSC attachment and proliferation on dentine surfaces. The null hypothesis was that all endodontic regeneration protocols have significant negative effects on the attachment and proliferation of DPSC on dentine.

Materials and Methods

Dentine specimen preparation

After obtaining institutional review board approval, 60 intact human molars were selected. Radicular dentine specimens (4×4×2 mm) were obtained from intact human molars using a low-speed diamond saw (Isomet, Buehler, Lake Bluff, IL, USA) under constant irrigation (n=60). The non-pulpal surfaces of the dentine were ground flat using 1200-grit abrasive paper (MDFuga, Struers, Cleveland, OH, USA) in an automated grinding/polishing machine (Rotoforce-4, Struers). The pulpal surfaces were sequentially grounded with abrasive papers (500-4000 grit) and finally polished using a polishing cloth with diamond suspension (1 μm; Struers). The specimens were then sonicated in neutral detergent solution and rinsed with deionized water for 3 minutes, sterilized using a high-pressure steam autoclave (Bolortuya *et al.* 2011) and maintained in 100% humidity at 4°C until used.

Treatment of dentine specimens

The pulpal surface of each dentine sample was irrigated with 1 mL of sterile water, 1.5% NaOCl (Value Bleach, Kroger, Cincinnati, OH, USA), 17% EDTA (Henry Schein, Melville, NY, USA) and sterile water. Then, the dentine specimens were individually placed into wells of a sterile 96-well plate (Celltreat, Shirley, MA, USA) with the pulpal surface facing upward and randomized into 4 treatment groups and 2 control groups (n=10 per group). Dentine specimens in the 3 treatment groups were treated with 200 µL of $Ca(OH)_2$ (UltraCal, Ultradent, South Jordan, UT, USA), clinically used concentration of TAP that was prepared by mixing 1000 mg of equivalent portions of metronidazole, ciprofloxacin and minocycline powders (Champs Pharmacy, San Antonio, TX, USA) with 1 mL of sterile water (Althumairy *et al.* 2014, Yassen *et al.* 2015a), clinically used concentration of DAP that was prepared by mixing 1000 mg of equivalent portions of metronidazole and ciprofloxacin powders (Champs Pharmacy) with 1 mL of sterile water (Althumairy *et al.* 2014) or diluted concentration of TAP (1 mg/mL, DTAP) in a methylcellulose system. The DTAP was prepared as described in previous studies (Prather *et al.* 2014, Algarni *et al.* 2015, Yassen *et al.* 2015a, Yassen *et al.* 2015b). In summary, 25 mg of TAP powders was dispersed into 25 mL of sterile water. Then, 2 gm of methylcellulose powder (Methocel 60 HG, Sigma-Aldrich, St Louis, MO, USA) was incorporated into the antibiotic solution under magnetic stirring to create a homogenous 1 mg/mL of DTAP with a pasty consistency that can be injected into a root canal using commercial delivery tips (NaviTip, Ultradent, USA) (Algarni *et al.* 2015).

The remaining dentine specimens of the two control groups were treated with 200 µL of sterile water. All dentine specimens were then incubated for 7 days at 37°C and 100% relative humidity. After the incubation period, all groups were rinsed with 2 mL of sterile water to remove the medicaments. Then, the pulpal surface of each dentine specimen in the three

treatment groups and one of the control groups was irrigated with 20 mL of 17% EDTA for 10 min. To ensure complete removal of medicaments from dentine surface, the medicament layer was gently peeled off from the surface of each dentine specimen before EDTA irrigation. The complete absence of medicaments after EDTA irrigation was further confirmed in a pilot study using scanning electron microscopy. Additionally, the complete removal of the medicaments used in the current study from flat dentine samples was assessed and reported in a recent study (Yassen *et al.* 2015a). After that, all dentine specimens received final irrigation with 500 µL of phosphate-buffered saline (PBS) and were transferred into individual wells of a sterile ultra-low attachment 96-well plate (Costar, Corning, NY, USA) with the treated pulpal surface facing upwards.

 DPSC culture and seeding on treated dentine

Previously characterized DPSC (Cook General BioTechnology, Indianapolis, IN, USA) harvested from third molars were used (Perry *et al.* 2008, Woods *et al.* 2009). DPSC from the 6th and 7th passages were cultured in low-glucose Dulbecco's modified Eagle's medium (1 g/L, DMEM) supplemented with heat inactivated 10% foetal bovine serum (FBS, Atlanta Biologicals Inc., Flowery Branch, GA, USA), L-glutamine (4 mM , Thermo Scientific, HyClone, Waltham, MA, USA), amphotericin B (2.5 g/mL, Fungizone, BioWhittaker, Walkersville, MD, USA), penicillin (100 unit/mL, Thermo Scientific), and gentamicin (50 g/mL, Thermo Scientific), and grown to 80% confluency. Subconfluent DPSC were then trypsinized, centrifuged, resuspended in serum plus DMEM, counted, and seeded on the treated pulpal surface of each dentine sample (10,000 cells per sample). The DPSC seeded on treated dentine were then incubated for 24 hours at 37°C and 5% CO_2. After a 24-hour attachment period, the media containing the unattached cells were used to determine the amount of attached DPSC. Each dentine sample was then

irrigated with 100 µL of serum plus DMEM, 200 µL of serum plus DMEM was added and all samples were incubated for an additional 3 days. After incubation, DPSC proliferation on the treated dentine surfaces was determined.

Assessment of DPSC attachment to dentine

To quantify the amount of unattached and attached DPSC after the 24-hour seeding period, the release of lactate dehydrogenase from unattached DPSC was measured using Lactate Dehydrogenase permeability assays (LDH, Roche Applied Science, Indianapolis, IN, USA) as described in a recent study (Kim *et al.* 2015). In summary, the media containing the unattached cells were individually transferred into a sterile 96-well plate, mixed with 100 µL of LDH reaction solution prepared according to the manufacturer's protocol and incubated for 30 minutes at 23ºC. Maximum LDH release (high control) was obtained by adding 10 µL of the provided lysis solution (Roche Applied Science) to 10,000 cells. The culture media from 3 wells without DPSC were used as low control. A microplate reader (Molecular Devices, Menlo Park, CA, USA) was then used to measure the absorbance at 490 nm. The percentages of unattached DPSC after various dentine treatments were calculated relative to the total amount of cells (DPSC with no dentine) according to the following equation: Unattached DPSC (%) = (experimental absorbance value – low control absorbance value) / (high control absorbance value – low control absorbance value) ×100. The percentage of attached cells was then calculated by subtracting the percentage of unattached cells from the percentage of total cells. The percentage of total cells was deemed to be 100%.

Assessment of DPSC proliferation on dentine

After 3 days of additional incubation, DPSC proliferation on the treated dentine samples was evaluated using water soluble tetrazolium salts assays (WST-1, Roche Applied Science, Penzberg, Germany) as previously described (Kim *et al.* 2015). Briefly, DPSC on dentine specimens were washed with 200 µL of DMEM, incubated in a mixture of 100 µL of DMEM and 10 µL of WST-1 reagent for 2 hours at 37°C and 5% CO_2. Also, 10 µL of WST-1 reagent was added to 10,000 newly counted cells suspended in 100 µL of DMEM (n=3) to obtain maximum WST-1 signal (high control wells). Furthermore, WST-1 reagent was also added to low control wells containing 100 µL of DMEM with no cells. Following incubation, 100 µL of the reaction mixture was transferred from each well into another sterile 96-well plate and absorbance values were read at 450 nm. The percentage of DPSC proliferation on treated dentine specimens in comparison to high control was calculated using the following equation:

Proliferation of attached DPSC (%) = (experimental absorbance value – low control absorbance value) / (high control absorbance value – low control absorbance value) ×100.

Furthermore, one dentine sample from each group was processed for scanning electron microscopy (SEM) rather than WST-1 assays to evaluate the morphology of DPSCS proliferated on dentine. Briefly, dentine specimens were gently washed with PBS to remove unattached DPSC cells and fixed with 2% Glutaraldehyde and 2% Paraformaldehyde in Phosphate Buffer. Specimens were then dehydrated through ascending ethyl alcohol and hexamethyldisilazane (Electron Microscopy Sciences, Fort Washington, PA, USA). Samples were then sputter coated with gold/palladium for 3 minutes and images were taken with a JEOL 6390LV scanning electron microscope (Peabody, MA, USA) in secondary electron imaging mode.

Statistical analysis

Both attachment and proliferation experiments were conducted in triplicates of n = 3 (final n = 9 per group). Data were checked for normality using the Shapiro–Wilk test, and the normality assumption was not satisfied. Therefore, the nonparametric Friedman's test was used to compare DPSC attachment and proliferation after various dentine treatments. Pair-wise comparisons were performed using Least Significant Difference (LSD) and the significance level was set at 5%. The high and low control groups in both experiments were excluded from the statistical model and only used as reference groups to calculate the percentages of attachment and proliferation.

Results

DPSC attachment to dentine

Figure 1 demonstrates that dentine treated with $Ca(OH)_2$ and untreated control dentine had a significantly lower percentage of DPSC attachment in comparison to dentine treated with TAP (P < 0.0001), or DTAP (P < 0.0001) regenerative protocols, as well as EDTA treated dentine (P < 0.01). Dentine treated with $Ca(OH)_2$ had a significantly lower percentage of DPSC attachment in comparison to dentine treated with DAP (P < 0.01). However, no significant differences in cell attachment were found between dentine treated with TAP, DTAP, and EDTA treated dentine. No significant difference in cell attachment was found between dentine treated with DAP and EDTA treated dentine. Furthermore, no significant differences were found between $Ca(OH)_2$ treated dentine and untreated control dentine.

DPSC proliferation on dentine

Figure 2 indicates that dentine treated with TAP or DAP regeneration protocols had significantly lower DPSC proliferation in comparison to that treated with DTAP ($P < 0.0001$) and $Ca(OH)_2$ ($P < 0.01$) regeneration protocols, as well as EDTA treated dentine ($P < 0.0001$) and untreated control dentine ($P < 0.0001$). Additionally, dentine treated with $Ca(OH)_2$ regeneration protocols had significantly lower DPSC proliferation compared to that treated with the DTAP regeneration protocol, EDTA, and untreated control dentine (all $P < 0.0001$). No significant differences in DPSC proliferation were found between dentine treated with DTAP regeneration protocol, EDTA treated dentine and untreated control dentine. Furthermore, no significant difference in DPSC proliferation was found between dentine treated with TAP and DAP regeneration protocols.

Visualization of DPSC on dentine

Representative SEM images demonstrated cellular adhesion on dentine surfaces among all treatment and control groups. Most surface areas of dentine treated with DTAP and $Ca(OH)_2$ regeneration protocols, as well as EDTA treated dentine and untreated control dentine were covered with spindle shaped large and overlapped DPSC with elongated cytoplasmic processes (Figures 3 A-D). On the other hand, dentine surfaces treated with TAP or DAP regeneration protocols showed relatively fewer numbers of DPSC that were sporadic and smaller in size (Figures 3 E-F).

Discussion

The relatively long application time (1-4 weeks) and the pasty consistency of intracanal medicaments in comparison to root canal irrigation solutions render them as potential disinfectants that can modify the attachment, proliferation and differentiation of stem cells within

the root canal environment. Therefore, different regeneration protocols in the current study were established by using various intracanal medicaments. Additionally, extensive EDTA irrigation for 10 minutes was used in the current study as a final irrigation step following intracanal medicaments to determine the ability of EDTA to reverse the potential cytotoxic effects of some intracanal medicaments. Indeed, the current clinical recommendations suggest the use of EDTA irrigation for 10 minutes during regenerative endodontics (Diogenes *et al.* 2014). Furthermore, recent *in vitro* studies have demonstrated the beneficial effects of using EDTA irrigation for 10 minutes on adhesion, migration and differentiation of DPSC (Galler *et al.* 2016). Additionally, 10 minutes irrigation with EDTA was also found to promote the release of various growth factors from human dentine (Galler *et al.* 2015). However, 10 minutes irrigation with EDTA may cause excessive dentine erosion and negatively affect the integrity of the dentine surface (Ozdemir *et al.* 2012). Therefore, shorter EDTA irrigation time is usually advocated during regular root canal treatment and manufacturer's directions must be followed when using EDTA to irrigate root canals.

Ultra-low attachment 96-well plates were used in the current study in order to promote the attachment of DPSC to dentine rather than to the bottom surface of the wells. These well plates are coated with hydrophilic neutrally charged hydrogel layer to maintain the cells in unattached suspended status and have been extensively used in various stem cells studies (Ivanov *et al.* 2014, Tsunoda *et al.* 2015, Yue *et al.* 2015). Thus, the total percentages of both unattached and attached DPSC to dentine were determined in the current study.

This study demonstrated that dentine treated with $Ca(OH)_2$ regeneration protocol had a significantly lower percentage of DPSC attachment in comparison to that treated with antibiotic regeneration protocols and EDTA treated dentine. This indicates that the application of $Ca(OH)_2$

did not improve cell attachment even after extensive EDTA irrigation, which can be justified by the limited ability of $Ca(OH)_2$ to demineralize radicular dentine and expose growth factors encapsulated by hydroxyapatite. Furthermore, the high pH and low molecular weight of $Ca(OH)_2$ may lead to degradation of dentine organic components (Yassen *et al.* 2013) and minimize the reservoir of endogenous dentineal proteins that are usually released after EDTA irrigation.

Another interesting finding of this study is that $Ca(OH)_2$ regeneration protocol caused significantly lower percentage of DPSC proliferation on dentine in comparison to untreated control dentine, EDTA treated dentine and dentine treated with the DTAP regeneration protocol. Recent studies proposed that $Ca(OH)_2$ has no cytotoxic effects on SCAP (Ruparel *et al.* 2012, Althumairy *et al.* 2014). These studies, however, either directly mixed $Ca(OH)_2$ with SCAP in the absence of dentine (Ruparel *et al.* 2012) or seeded SCAP on a synthetic scaffold within the root canal lumen after $Ca(OH)_2$ treatment (Althumairy *et al.* 2014). In the current study, DPSC were directly seeded on dentine treated with $Ca(OH)_2$, which is the expected clinical scenario during regenerative endodontics. One possible hypothesis that can explain the adverse effects of $Ca(OH)_2$ on DPSC proliferation despite its non-cytotoxic nature is that $Ca(OH)_2$ can significantly change the physio-mechanical properties of surface dentine (Kawamoto *et al.* 2008, Yassen *et al.* 2015a), which may cause unfavorable interactions between DPSC and the brittle dentine surface. Indeed, recent studies suggested that the viscoelastic properties of substrates can significantly modify the cellular ability to attach, spread, and differentiate (Trappmann *et al.* 2012, Swift *et al.* 2013, Chaudhuri *et al.* 2015). Furthermore, the $Ca(OH)_2$ regeneration protocol was found to cause significant reductions in dentine wettability in comparison to other regeneration protocols (Yassen *et al.* 2015b). It is also worth noting that a previous clinical study found significantly less increases in dentineal wall thickness among endodontic regeneration cases treated with

Ca(OH)$_2$ in comparison to cases treated with antibiotic medicaments (Bose *et al.* 2009). On the other hand, previous *in vitro* studies have shown that Ca(OH)$_2$ improved cellular attachment to dentine (Kitikuson & Srisuwan 2016) and release TGF-β1(Graham *et al.* 2006, Tomson *et al.* 2007, Galler *et al.* 2015), which suggest positive influences of Ca(OH)$_2$ on dentine regeneration. The different experimental conditions and designs might explain the discrepancies in the available literature regarding the biological effects of applying Ca(OH)$_2$ on dentine. Further studies are warranted to explore the pros and cons of using Ca(OH)$_2$ in endodontic regeneration.

The current study also demonstrated that dentine treatment with TAP and DTAP regeneration protocols caused significant improvements in DPSC attachments, which can be explained by the demineralization effects of TAP (Yassen *et al.* 2013) and its ability to increase surface roughness of dentine (Nerness *et al.* 2015). However, TAP regeneration protocol caused significant and severe reductions in DPSC proliferation, which indicates that the majority of initially attached DPSC were unable to survive and/or divide even after extensive EDTA irrigation. This can be explained by the strong affinity of TAP to chemically bind to dentine (Berkhoff *et al.* 2014) and subsequently be released over time (Sabrah *et al.* 2015) causing deleterious effects on the survival of DPSC. DAP (minocycline-free) regeneration protocol was also found to cause significant reduction in DPSC proliferation in the current study, which indicates that the negative effects of TAP is not solely mediated by the presence of minocycline in TAP. The present findings mirror those of previous studies suggesting cytotoxic effects of dentine treated with clinically used concentrations of TAP and DAP on SCAP and DPSC (Althumairy *et al.* 2014, Kim *et al.* 2015). On the other hand, DTAP did not exert significant negative effects on DPSC proliferation, which generally agrees with a previous study (Althumairy *et al.* 2014). It is worth mentioning that the latter study used a liquid form of DTAP

that cannot be used clinically as an intracanal medicament. In the current study, 1 mg/mL of TAP loaded into methylcellulose system that can be delivered into the root canal and used as interappointment medicament was used. The antibacterial properties of 1 mg/mL of various antibiotic mixtures loaded into methylcellulose delivery vehicle have been confirmed in recent studies (Algarni *et al.* 2015, Tagelsir *et al.* 2016). The current study indicates significant improvements in the attachment of DPSC to dentine after EDTA treatment, which generally agrees with recent studies (Pang *et al.* 2014, Galler *et al.* 2016). Nevertheless, EDTA treatment did not improve DPSC proliferation on dentine.

The dentine samples used in the current study were sterilized using autoclave sterilization, a method that has been used in previous studies to sterilize dentine before seeding various types of human cells (Bolortuya *et al.* 2011, Kitikuson & Srisuwan 2016). However, there are some concerns that this approach might negatively affect some of proteins present within dentine. Previous study proposed that autoclave sterilization can negatively affect collagenase present in dentine but did not affect the presence and activity of various dentinal gelatinases (Sulkala *et al.* 2007). It is also worth noting that autoclave sterilization might negatively affect the endogenous growth factors present within radicular dentine, which might render some of these growth factors unavailable after EDTA irrigation.

Conclusions

The null hypothesis stated that all endodontic regeneration protocols have negative effects on the attachment and proliferation of DPSC on dentine was rejected. The findings from the current study suggest that dentine treated with TAP, DAP or $Ca(OH)_2$ regeneration protocols had unfavorable effects on DPSC proliferation. On the contrary, DTAP regeneration protocol did not adversely affect the proliferation of DPSC on dentine.

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Figure 1. The percentages (mean ± SE) of DPSC attachment to radicular dentine treated with various regenerative endodontic protocols. For each group, three independent experiments were performed in triplicate (final n=9). Asterisks denote statistical differences between groups.

Figure 2. The percentages (mean ± SE) of DPSC proliferation on radicular dentine treated with various regenerative endodontic protocols. For each group, three independent experiments were performed in triplicate (final n=9). Asterisks denote statistical differences between groups.

Figure 3. SEM imaging of dental pulp stem cells (DPSC) cultured on human dentine pretreated with various endodontic regeneration protocols as well as untreated control dentine. DPSC adhered on untreated control dentine (A). DPSC adhered on dentine treated with EDTA (B). DPSC adhered on dentine treated with Ca(OH)$_2$ regeneration protocol (C). DPSC adhered on dentine treated with DTAP regeneration protocol (D). DPSC adhered on dentine treated with TAP regeneration protocol (E). DPSC adhered on dentine treated with DAP regeneration protocol (F).
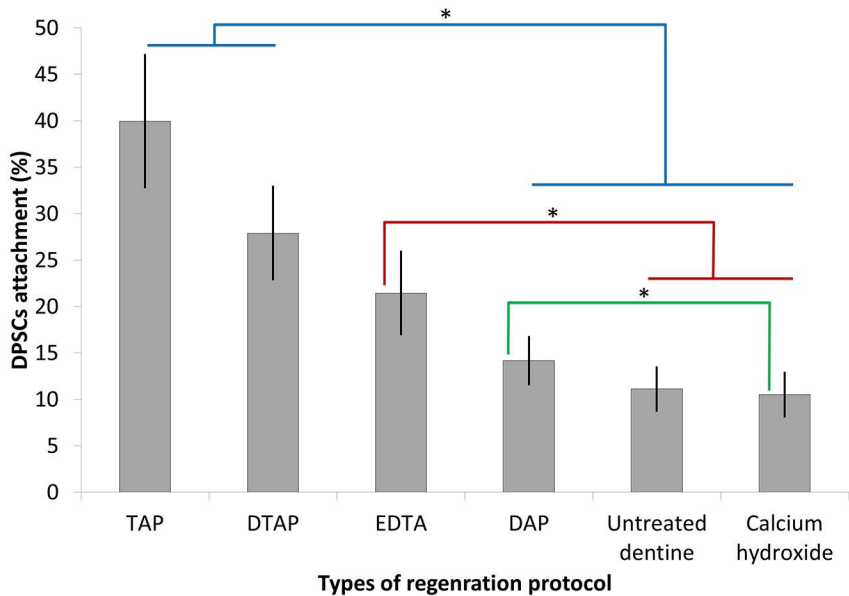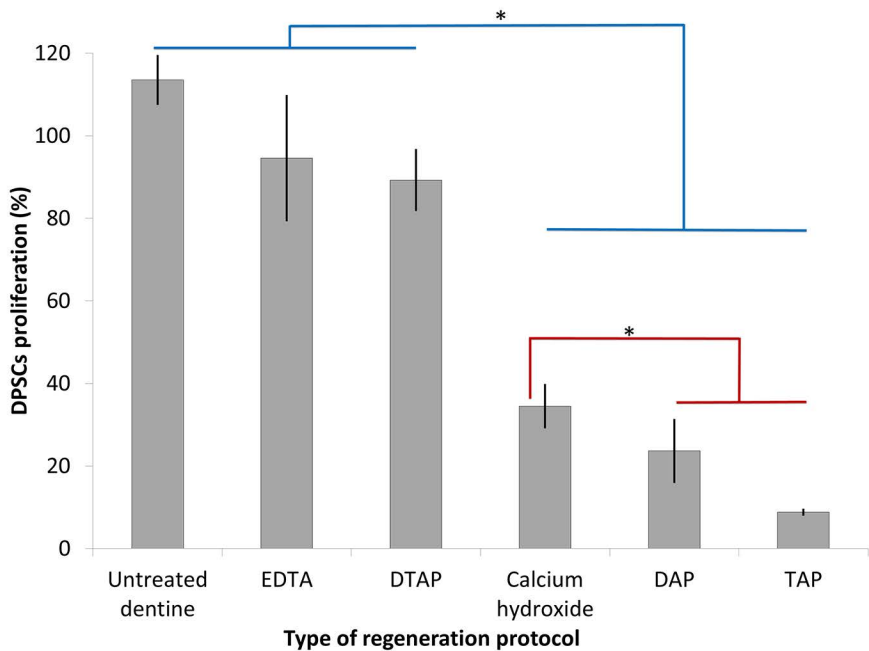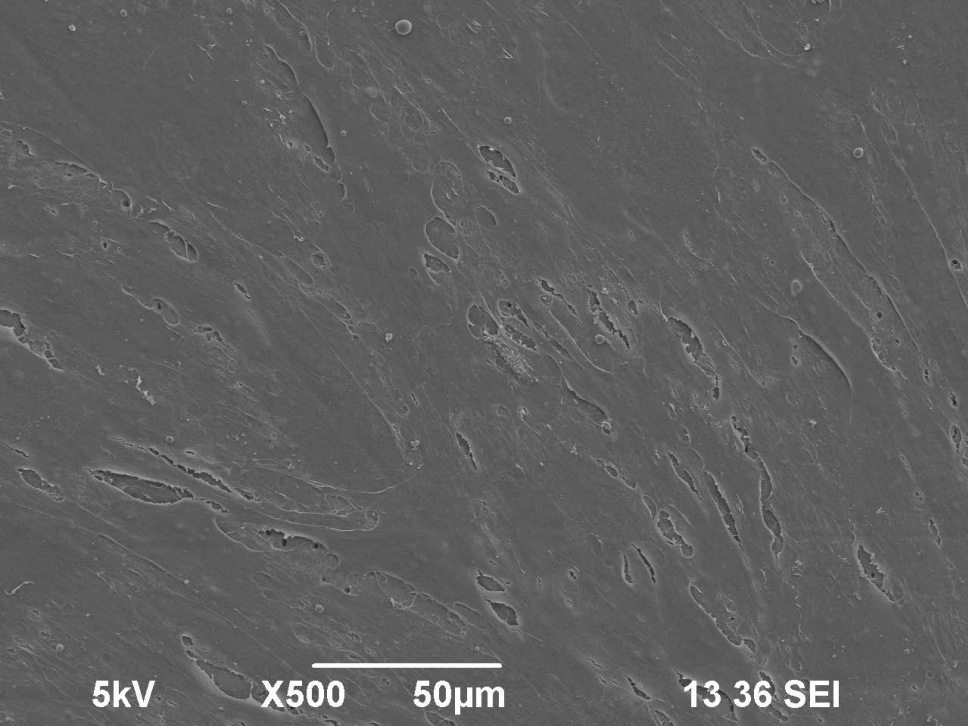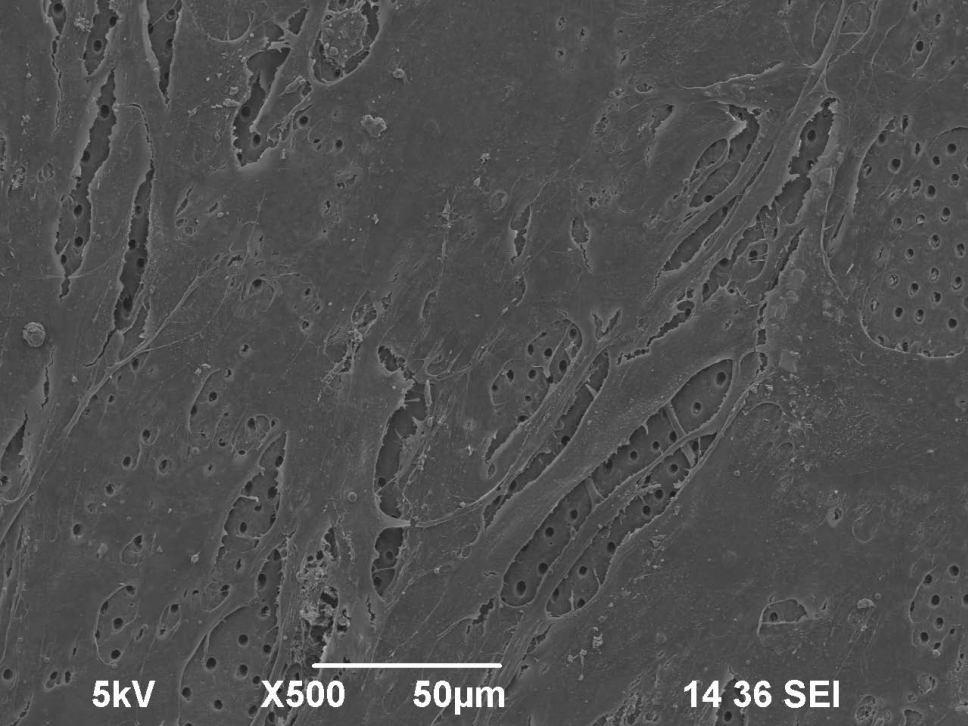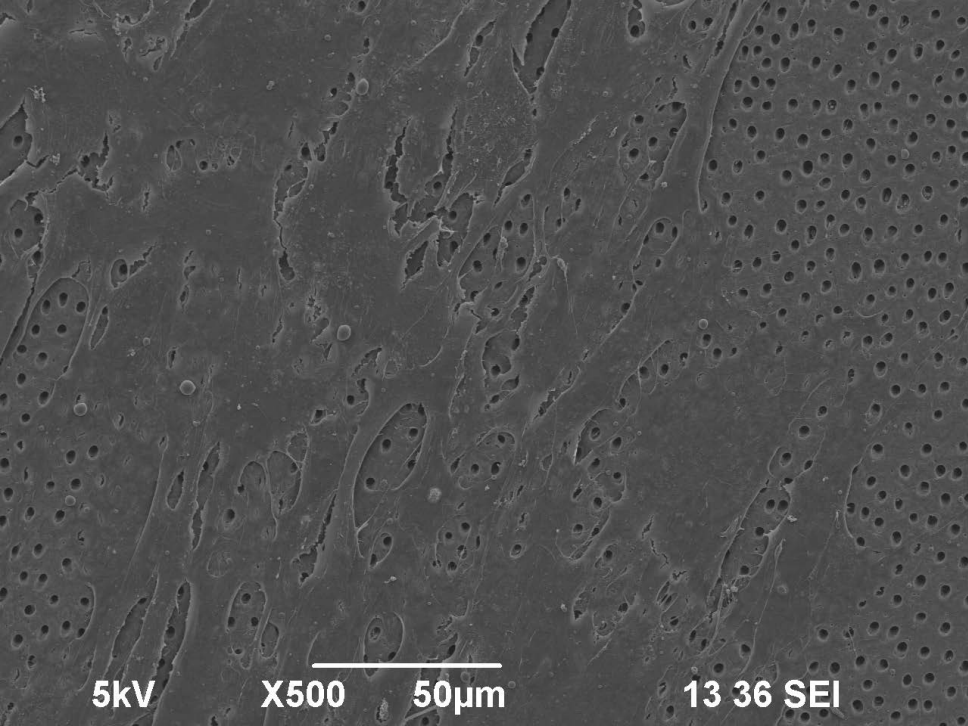
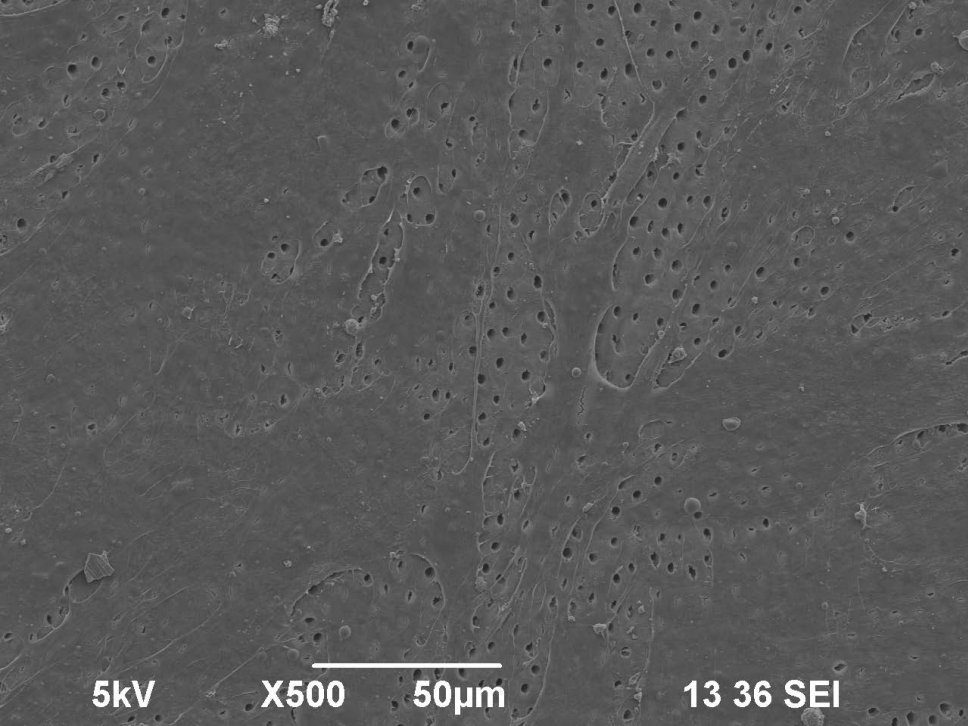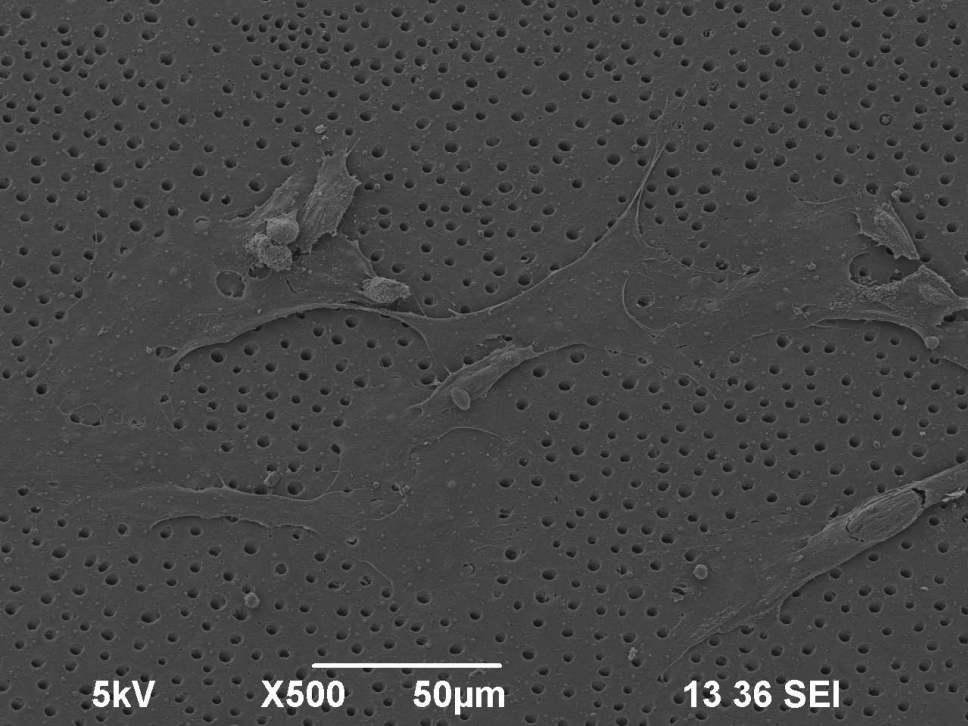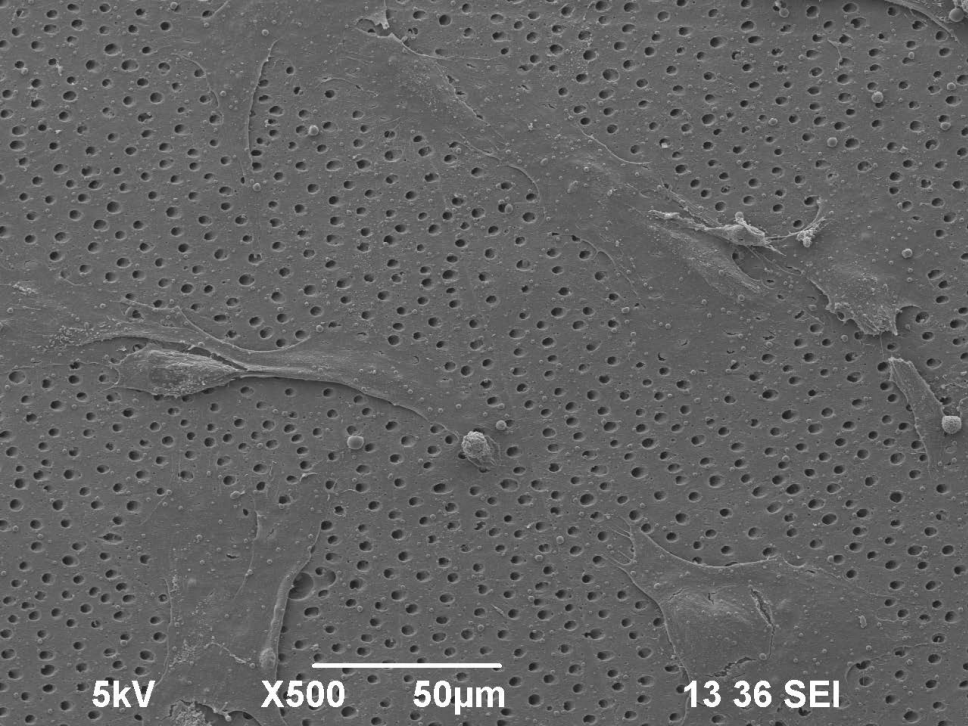5kV　　X500　　50μm　　13 36 SEI

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