Effect of Double Antibiotic Paste and Calcium Hydroxide on the Viability of the Stem Cells and Their Attachment to the Irrigated Dentin; An *In-vitro* Study

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Abstract

Successful regenerative endodontics in immature permanent teeth is highly dependent on proper disinfection. Intra-canal medicaments used for these procedures should be bactericidal and also provide a biological environment for the viability of stem cells and root maturation. This study was conducted to compare the effects of calcium hydroxide ($Ca(OH)_2$) and double antibiotic paste (DAP) on dental pulp stem cells (DPSCs) viability when seeded on irrigated radicular dentin. Thirty dentin samples were irrigated with 2.5% sodium hypochlorite followed by normal saline (NaCl) rinse. Group-1 samples (n=10) were treated with DAP (1mg/ml) while, group-2 (n=10) with conventional $Ca(OH)_2$ (500mg/ml) and group-3 (n=10) were controls treated with sterile water irrigation only. After 4-weeks of treatment, the samples were rinsed with NaCl. DPSCs from passage 7-9 were seeded onto the samples. Presto blue viability assay was performed on day 1, 3 and 7. Data was analysed using SPSS version 22 (IBM, USA) through one-way ANOVA test. Scanning electron microscope (SEM) imaging was performed on day 7 of cell seeding. $Ca(OH)_2$ group significantly increased DPSCs viability as compared to the DAP group ($p<0.05$). De-mineralisation of dentin and release of growth factors by $Ca(OH)_2$ promoted the viability of the DPSCs. Therefore, long-term treatment with $Ca(OH)_2$ promotes DPSCs survival and attachment after seeding on irrigated radicular dentin.

Keywords: regenerative endodontics, medicaments, stem cells, immature, permanent.

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Introduction

The management of pulp necrosis is one of the major challenges in paediatric dentistry. Challenge escalates with the involvement of immature permanent teeth that possess immature roots and open apices. Most common causes of pulp involvement in early adolescence are dental injuries, dental anomalies and dental caries [1]. Pulpal inflammation, if left untreated, can lead to multiple consequences including, abscess formation, gingival bleeding, pulp necrosis and eventually tooth loss. Conventional treatment therapies would ideally require further root maturation because conventional therapies result in thin and fragile roots which are more prone to fracture [2]. Regenerative endodontics (RE), is a new treatment modality, that has successfully replaced conventional treatment therapies utilising a triad of three components i.e. stem cells, growth factors, and scaffolds. RE uses hosts own vital cells to promote dentin-pulp complex formation and maturation of the roots [3]. As a result, the treatment gives better outcomes with complete root maturation and apex closure.

The success of the regenerative endodontic therapy depends mainly on the complete disinfection of the root canals. This is achieved by minimal or no instrumentation, chemical irrigation and intra-canal medicaments. This provides a perfect environment for the stem cell growth and proliferation. Induction of bleeding in irrigated and treated root canals results in the influx of fresh mesenchymal stem cells from the root apex [4]. The blood clot itself acts as a scaffold and the growth factors released through the blood help the stem cells in the complete maturation and revascularisation of the disinfected root canals.

Dental pulp stem cells (DPSCs) are the cells present in the root canals of the teeth, and are also known as the odontoblastoid cells since these cells are capable to differentiate into odontoblast-like cells [5]. The cells have a high capability for cellular and tissue repairs as compared to the other stem cells [6]. Therefore, RE commences on the possibility that the dental pulp tissue still has some viable DPSCs even with the pulp necrosis, particularly for immature permanent teeth. Hence, DPSCs play a significant role in RE along with proper disinfection of the root canals with irrigants, intracanal medicaments, and minimal or no instrumentation [7]

Different protocols applied in dental clinics over the last decade have produced different results. Most of the cases were successful with continued root maturation and apical closure [8]. But the *in-vitro* studies showed that although these intra-canal medicaments have excellent antimicrobial properties, higher concentrations of some medicaments during treatment are detrimental to the stem cell survival [9]. Irrigants are also used at different concentrations ranging from 0.5 to 6% [10]. The aim of this study was to compare the indirect effect of two medicaments, Calcium hydroxide ($Ca(OH)_2$) and 1 mg/ml Double antibiotic paste (DAP) on extracted teeth *in-vitro*. These medicaments were used in combination with 2.52 % sodium hypochlorite (NaOCl) and normal saline (NaCl) irrigation.

Materials and Methods

Preparation of dental chip samples

This study was approved by the institutional Human Research Ethics Committee, Universiti Sains Malaysia (USM) Health Campus, Malaysia (USM/JEPeM/1406240). Fifteen human premolars from healthy adults (16 to 32-year-old) extracted for orthodontic treatment were collected after obtaining the patient's informed consent. The teeth were disinfected by immersing in NaOCl solution (2.5%) for 24 hours at 4℃ followed by washing with copious amount of sterile water. The disinfected teeth were stored in sterile water at 8℃ until further experimentation. Firstly, a sterile blade was used to remove any remnant of gingival or periodontal tissues on the root surface. The apical and coronal thirds of each root were removed using a hard tissue cutter leaving a 5-mm middle third of the root that was cut longitudinally into two halves. A great care was taken neither to touch nor encroach the root canal area of the dissected roots. The prepared root specimens were immersed in 30 ml of NaOCl solution (2.5%) for 24 hours to dissolve the dried pulp tissues and stored into 30 ml of distilled water at 4℃ [11].

Medicament placement

The present study used $Ca(OH)_2$ and DAP in solution form as medicaments for experimental groups 1 and 2 respectively. For the group-1, 500mg of $Ca(OH)_2$ powder (HmbG® Chemicals, Johor Bharu, Malaysia) was mixed in 1ml of sterile water to get a slurry paste and was then delivered onto the irrigated root slices using a sterilised stainless steel instrument. For the group-2, we used double antibiotic solution that is similar to the DAP commonly used in regenerative endodontic treatment and contained metronidazole (METRONOL® 0.5% w/v injection: Metronidazole BP 500mg/100ml in citrate-phosphate buffered isotonic solution) and ciprofloxacin (Ificipro Injection: 200mg/100ml Ciprofloxacin Injection USP). Low concentration of DAP solution (1mg/ml concentration) was prepared by diluting 2 ml (10mg) of metronidazole infusion solution and 5ml (10 mg) of ciprofloxacin injection solutions in corresponding amounts of sterile water (8ml and 5 ml respectively).

For the control group, no active medicament was added to the samples placed in 5 ml of sterile water to prevent drying. All samples were placed in an incubator at 37°C in a humidified atmosphere of 5% CO2. After 4 weeks of incubation, all samples were washed off with 2.52% NaOCl for 10 minutes, then immersed in normal saline before adding DPSCs.

Dental pulp stem cells (DPSCs) addition

The present study used DPSCs (All Cells, USA) as they had been extensively studied in the field of regenerative endodontic research [11–15]. The DPSCs were cultured in Mesenchymal Stem Cells (MSC) Basal Medium (AllCells, Buckingham, UK) supplemented with MSC stimulatory supplement (AllCells, Buckingham, UK) and 1% Penicillin-Streptomycin (Gibco, USA) maintaining at 37°C in a humidified atmosphere of 5% CO_2 with the culture media being replenished 72 hourly. Cells were allowed to expand in culture to confluence of 70 % - 80 % and were used in 7th passage. All treated root samples (n = 30) were soaked in the neutral PBS and stored at 37°C for 1 hour before adding DPSCs. Then each root sample was placed individually inside the 24 well-plates with the root canal surface facing upward. DPSCs (10,000 cells) were seeded on the irrigated dentin chips and allowed to attach the root canal surface of the samples for 30 minutes prior to topping up with 1 ml of culture media (37°C in a humidified atmosphere of 5% CO_2).

Cell viability tests

Cell viability assay was performed at day 1, 3 and 7 using Presto Blue® ™ cell viability reagent according to the manufacturer's instructions. On each test day, fresh culture medium with the reagent was added to the cell seeded on treated root samples in 24-well plate. Following an hour of incubation of assay plate, the absorbance was read at 600 nm wavelengths using Elisa. The viability of the cells in each treatment/group was normalized with the absorbance reading of culture media without DPSCs under the similar conditions.

SEM preparation

The surface morphology of samples was analysed using the scanning electron microscopy (SEM) following 7 days of stem cells attachment. The samples were fixed in paraformaldehyde solution (4%) at room temperature for 2 hours, followed by formaldehyde (8%) at 4°C for 2 days. All samples were dehydrated using a gradient series of ethanol solutions (20%, 50%, 70%, 90% and 100% for 10 minutes each). For fixation of dehydrated specimens, hexamethyldisilazane was used for 10 minutes at room temperature and dried using an oven at 40℃. The samples were mounted on stubs and gold sputtered and analysed under scanning electron microscope (Fie, Quanta FEG 450, Netherlands) at 1000x magnification and using digital image analysis software.

Data Analysis

Data were analysed using SPSS software version 22 (Graph Pad Software, San Diego, CA). The results were analysed statistically using one-way analysis of variance (ANOVA), and pairwise comparisons were made using the Bonferroni post hoc test. All data have been presented as the mean ± SD values with statistical significance set at $p < 0.05$.

Results and discussions

The DAP treated group showed no remarkable effects on DPSCs survival compared to the control group while cultured on the irrigated dentin for 4 weeks ($p < 0.05$). In contrast, the $Ca(OH)_2$ treated group had no toxic effects and resulted in a significant increase in DPSCs survival and proliferation compared to the control group and DAP treated group ($p < 0.05$). The PrestoBlue assay showed that there was a significant increase in cell viability from day 1 to 7 while the cell viability decreased in DAP treated root samples simultaneously (Figure. 1).

The SEM analysis evaluated the morphological attachment of stem cells on the irrigated dentin. The cells were significantly attached to the $Ca(OH)_2$ treated root samples (Figure. 2.b) as compared to DAP treated (Figure. 2.c) and the control root samples (Figure. 2.a).

Cells were attached to the dentinal tubules even in the presence of smear layer in all the groups. A few root samples showed no cellular attachment despite exposed dentinal tubules as a result of complete smear layer removal. The cell morphology varied widely ranging from round to oblong to totally flattened cells. In the $Ca(OH)_2$ treated group, the cells had a different morphology than the cells attachment in case of DAP treated group.

The morphology of the DPSCs attachment was different for the treatment groups i.e. control, DAP treated and $Ca(OH)_2$ treated group. The cell morphology ranged from round to oblong to totally flattened cells. The morphology of the cells attached points to the stage of the cell attachment to the dentin. Initially, the cells are round when they are attaching to the substratum. Then with the growth of the filopodia and the progress to cytoplasmic webbing, they become oblong in shape. Finally, they are flat when they attain full attachment to the dentin.

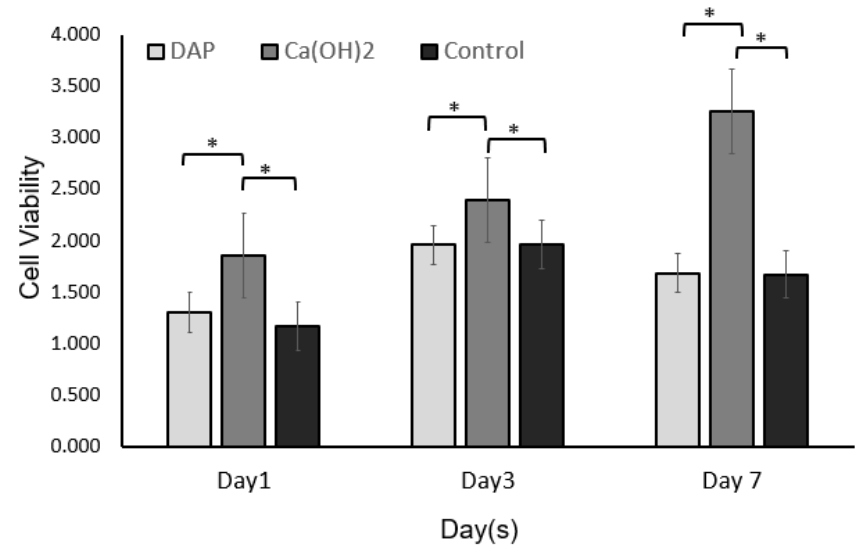

Figure. 1 Effect of intra-canal medicaments on the viability of DPSCs using PrestoBlue assay. The medicaments used were DAP 1mg/ml and $Ca(OH)_2$ 500mg/ml. The cell viability was analysed at day 1, 3 and 7. Data are presented as the mean ± standard error of the mean of the optical density at the absorbance of 570nm wavelength normalised to 600nm wavelength. *, indicates a significant difference ($p < 0.05$) between the groups. Control, cells seeded on irrigated dental chip; DAP, cells seeded on irrigated dental chip treated with DAP; $Ca(OH)_2$, cells seeded on irrigated dental chip treated with $Ca(OH)_2$.

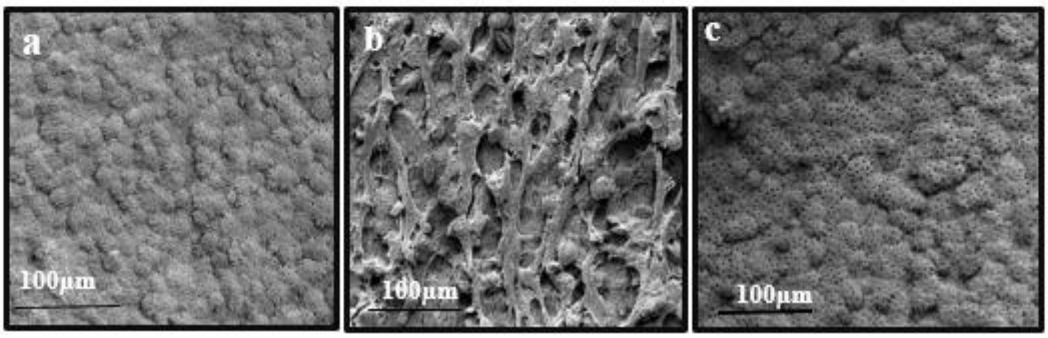

Figure 2. SEM observation of DPSCs attachment to dentine surface. (a) No flat cells on the control group root samples indicate that the DPSCs were unable to survive and attach to the dentine, (b) Overlapping DPSCs forming a meshwork with calcium crystals on $Ca(OH)_2$ treated root samples, (c) Few round cells on the DAP treated root samples indicate failure of cells to flatten and fully attach the treated surface.

In $Ca(OH)_2$ treated root samples (Figure 2.b), the dentin surface was overlapped with widespread fibrous layer of elongated flattened cells. The cells had long cytoplasmic processes extended into the open dentinal tubules. Some $Ca(OH)_2$ crystals could be seen indicating the incomplete removal of the medicament. But this did not interfere with the attachment of the cells to the dentin surface. DAP treated samples showed few small rounded cells with diameter below 10μm (Figure 2.c) The dentin surface was heavily cracked and nodular with some round structures. Although there was no smear layer on the DAP treated samples, yet, majority of the cells failed to attain the flat shape. Samples without any treatment were used in control. The average number of cells attached to the dentin from highest to the lowest were in order of $Ca(OH)_2$, control and in the DAP.

This study investigated the effect of NaOCl in conjunction with $Ca(OH)_2$ or DAP on the stem cell viability and attachment to the dentin. NaOCl, was used in a lower concentration of 2.52 % as higher concentration are not biocompatible [16]. PrestoBlue assay revealed that the samples treated with 1mg/ml DAP showed significant decrease in the number of viable stem cells in comparison to $Ca(OH)_2$. Same results were reported with the SEM analysis. These results suggested that in DAP, the survival of the DPSCs is limited.

Ideally, intracanal medicament should have antibacterial property as well as be non-toxic to the stem cells [14]. This is important for the further root maturation after the regenerative procedure especially in immature permanent teeth with open apices. DAP at concentration 1mg/ml and higher are toxic to the stem cells [18]. This could be due to the concentration dependent toxicity to stem cells. The use of lower concentrations of DAP for 4 weeks resulted in no difference in DPSCs survival in comparison to the control group. This is consistent with a study that previously reported that the direct toxic effect of these drugs was also dependent on concentration [19]. As a result, the adverse effects of DAP on the survival of DPSCs can be largely prevented when used at a concentration lower than 1 mg/ml. In contrast, 500mg/ml $Ca(OH)_2$ promotes the attachment and proliferation of the stem cells. This proves the capability of $Ca(OH)_2$ not only to control the infection but also to promote the survival and proliferation of stem cells at this concentration. This favorable change could be due to the high alkaline pH of $Ca(OH)_2$, which makes the environment toxic for the microbes to survive [7,20,21]. As a result, the surface is favorable for the attachment of the stem cells. Also, during dentinogenesis, certain growth factors like transforming growth factor-beta (TGF-beta), vascular epithelial growth factors (VEGF), bone morphogenic protein (BMP), dentin sialoprotein (DSP) and other factors are retained within the dentin [22]. These factors once released again have the capability to induce cellular changes [23]. $Ca(OH)_2$ solubilises dentin, and this solubilisation results in the release of these retained growth factors which help in the promotion of odontogenesis [24,25]. Thus, $Ca(OH)_2$ dentin conditioning indirectly promotes the survival and proliferation of DPSCs, possibly by releasing bioactive growth factors from dentin or some mechanism which is still unknown.

Conclusion

Dentin conditioning with $Ca(OH)_2$ significantly promotes DPSCs survival, proliferation and attachment to the dentin. This study used 500mg/ml of conventional $Ca(OH)_2$. Further studies should be conducted to evaluate the cytotoxic effects at much higher concentrations of 1g/ml which are consistent with the concentrations used clinically.

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Author contributions

All authors contributed toward data analysis, drafting and critically revising the paper and agree to be accountable for all aspects of the work.

Disclosure of conflict of interest

The authors have no disclosures to declare.

Compliance with ethical standards

The work is compliant with ethical standards.

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