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Proliferative activity of cells from remaining dental pulp in response to treatment with dental materials

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ABSTRACT

Background: The biological examination of pulp injury, repair events and response of dental pulp stem cells to dental restorative materials is important to accomplish restorative treatment, especially to commonly used dental materials in paediatric dentistry, such as glass ionomer cement (GIC) and calcium hydroxide ($Ca(OH)_2$) lining cement.
Methods: Healthy patients aged between 9 to 11 years with carious primary molars without pulp exposure were selected and divided into two groups: Group 1 (teeth restored with GIC) and Group 2 (teeth lined using $Ca(OH)_2$ and restored with GIC). The proliferative activity of stem cells of teeth between these two groups was compared using colourimetric cell proliferation reagent, alamarBlue. Immunocytochemistry and flow cytometry confirmation were performed using mesenchymal stem cell markers, CD105 and CD166.
Results: The proliferative activity using alamarBlue™ assay showed that cells derived from the remaining dental pulp of exfoliated deciduous teeth were positive for CD105 and CD166 and exhibited no difference between the two groups.
Conclusions: It can be concluded that the use of $Ca(OH)_2$ or GIC as a lining material in indirect pulp capping procedures has the same effect on cells derived from the remaining dental pulp of exfoliated deciduous teeth which have responded favourably to the restorative treatments.

Keywords: Stem cells, teeth, cell proliferation, dental materials.

Abbreviations and acronyms: ALCAM = activated leukocyte cell adhesion molecule; αMEM = alpha modified Eagle's medium; DPBS = Dulbecco's phosphate buffered saline; DPSC = dental pulp stem cells; FCS = foetal calf serum; GIC = glass ionomer cement; hMSC = human mesenchymal stem cell; PBS = phosphate buffered saline; PDLSCs = periodontal ligament stem cells; RDT = remaining dentine thickness; SHED = stem cells from human exfoliated deciduous teeth.

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INTRODUCTION

The advantages of using adhesive restorative materials such as glass ionomer cement (GIC) are liberation of long-term fluoride release, the ability to be recharged by exposure to fluoride solutions and gels,[1] its simplicity, a good cost-effectiveness ratio[2,3] and lack of formation of initial or recurrent carious lesions.[4] Also, calcium hydroxide ($Ca(OH)_2$) liners are often placed in deep cavities to protect the pulpal tissues from chemical insults, to stimulate dentinal bridge formation and serve as a protective barrier for pulp tissue not only by blocking patent dentinal tubules, but also by neutralizing the attack of inorganic acids and leached products from certain cements. Stem cells from human exfoliated deciduous teeth (SHED), isolated from pulp, have been found to divide continuously and differentiate into a variety of other cells types, including nerves, fat, tooth-generating cells[5] and into dentine-forming odontoblast.[6,7] Following physiological stimulation or injury, such as caries and operative procedures, stem cells in pulp may be mobilized to proliferate and differentiate into odontoblasts by morphogens released from the surrounding dentine matrix.[8] It is necessary to evaluate the biological changes which take place in teeth, such as tooth pulp reactions to the restorative procedure as well as to restorative materials in order to stimulate regenerative processes to accomplish the therapy by tissue engineering.[9] However, to our knowledge, there are limited data currently available regarding the biological response of stem cells to various dental restorative materials such as GIC and $Ca(OH)_2$. Therefore, this study is directed towards the following: to confirm the presence of mesenchymal stem cells in the remaining dental pulp exfoliated deciduous teeth by using surface antigenic markers CD105 and CD166; and to assess the proliferative activity of the cells derived from the remaining dental pulp of exfoliated

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deciduous teeth after placement of dental material that have direct contact with the floor of the prepared cavity.

MATERIALS AND METHODS

Selection of patients

Fifty healthy patients (9 to 11 years), with primary molar teeth having minor Class I cavities or caries with no pulp exposure were included in the study. Informed consent was obtained from the parent/guardian prior to study. This study was approved by the Human Ethical Committee of Universiti Sains Malaysia (vide reference USM 182.3(3) USMKK/PPSP®/JK P&E, PPSP dated 9 January 2007).

Restorative procedure

The carious dentine was removed according to standard procedure and GC cavity conditioner (GC Corporation, Tokyo, Japan) was applied. For Group 1, the prepared cavity was filled with GIC (Fuji IXGP capsule, GC, Japan). In Group 2, the floor of the prepared cavity was lined with a thin layer of $Ca(OH)_2$ (Dycal®, Dentsply, Germany), and filled with GIC. Patients were reviewed by the same operator once every month. The extraction procedure was made only for teeth that exhibited mobility and signs of exfoliation after six months of time lapse with alpha scale restoration according to the United States Public Health Service (USPHS) guidelines.

Isolation and culture

After transportation of teeth to the laboratory, the gingival and periodontal tissue was scraped from the tooth surface and cleaned with iodine and 70% ethanol, and then washed with phosphate buffered saline (PBS). The tooth was cut around the enamel-cementum junction to expose the pulp tissue in the pulp chamber. The sectioned tooth was briefly immersed in 70% ethanol followed by Dulbecco's phosphate buffered saline (DPBS) (Gibco™, Invitrogen, USA). The pulp tissue was picked up with sterile tissue forceps, minced and digested using 3 mg/ml of Collagenase Type 1 and 4 mg/ml of Dispase (Gibco™, Invitrogen, Germany). This process was performed in a CO_2 incubator (Thermo Forma, USA) for one hour. After digestion, 5 ml of culture medium containing alpha modified Eagle's medium (αMEM) (BioWhittaker™, Cambrex, USA) supplemented with 10% foetal calf serum (FCS) (Gibco™, Invitrogen, Germany), 100 μM L-ascorbic acid 2-phosphate (Stem Cell Technologies, Canada), 2 mM L-glutamine (Gibco™, Invitrogen, Japan) and penicillin (100 U/ml), and streptomycin (100 mg/ml) was added and centrifuged at 1200 rpm for 10 minutes. The cell pellet was suspended in culture medium and passed through a 70-μm pore size strainer to obtain single cell suspension. These cells were seeded into T25 culture flask with 5 ml of culture medium and incubated in CO_2 incubator. After 24 hours, non-adherent cells were removed. The adherent cells were washed vigorously twice with PBS and fresh complete medium was added. The cells were observed every day to check for culture condition and the medium was changed every three days until the fibroblast-like cells reached confluence.

Characterization of stem cells derived from the remaining dental pulp of exfoliated deciduous teeth using immunocytochemistry

Immunostaining was performed using biotin-strepavidin-horseradish peroxidase (HRP) complexed antibodies. Stem cells derived from the remaining dental pulp of exfoliated deciduous teeth and human mesenchymal stem cells (hMSCs) (positive control) and breast cancer cells (negative control), were subcultured into 2 four-chamber slides with density of 4×10^4 cells per well. After two days, the cells were fixed in absolute cold methanol and incubated at 4°C for 20 minutes. Blocking reagent was added for five minutes and the cells were incubated with one primary antibody in each four-well chamber slide either with primary antibodies of monoclonal anti-human endoglin (CD105) (Chemicon, USA) with dilution 1:25 or CD166 (DakoCytomation, Denmark) with dilution 1:50, overnight. Primary antibodies were then detected using a Chemicon IHC Select™ secondary detection system (Chemicon, USA) in which the samples were subsequently incubated with purified mouse secondary antibodies and strepavidin for 10 minutes, respectively, according to the manufacturer's protocol.

Characterization of stem cells derived from the remaining dental pulp of exfoliated deciduous teeth using flow cytometry

Fifty microlitres of single suspension of stem cells derived from the remaining dental pulp of exfoliated deciduous teeth were placed into the bottom of the round flow cytometry tube (BD Falcon, USA) and 20 μl of antibody was added. The tube was vortexed for 10 seconds and then incubated in the dark at room temperature for 30 minutes. After incubation, the cells were washed with 2 ml of PBS by vortexing at 1500 rpm for five minutes. The supernatant was discarded carefully leaving the cell pellet at the bottom of the tube. A total of 0.5 ml of PBS was added to resuspend the cells and gently vortexed. Cell fluorescence was evaluated by flow cytometry using a FacsCalibur instrument (Becton Dickinson, USA) and

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the data were analysed using CellQuest Pro software (Becton Dickinson, USA). The antibodies used were CD105 and CD166. Positive expansion was defined as the level of fluorescence greater than 95% of the corresponding isotype matched control antibodies. Anti-mouse IgG added to the stem cells from the remaining dental pulp formed the negative control in this study, the results of which are shown in Fig 2.

Proliferation assessment of stem cells derived from the remaining dental pulp of exfoliated deciduous teeth

After the cells reached confluence, they were subcultured in a 24-well plate with 2 ml of culture medium. The culture medium was changed and incubated in a CO_2 incubator for four hours. Subsequently, 10% of metabolic indicator dye, alamarBlue (Biosource International, Inc, USA) was added to each well containing cells as well as to the wells containing only medium without cells (negative control). One hundred microlitres of solution from each well plate was then taken and added to a 96-well plate and the absorbance of the media was measured spectrophotometrically using ELISA reader (Tecan, DKSH, Germany) at a wavelength of 570 nm and 600 nm at 0 minute, 60, 120 and 180 minutes. Subsequently, the medium was changed every day for seven days. The processes of alamarBlue staining and absorbance readings were repeated as before, every day. The reduction in alamarBlue assay was expressed as a percentage.

Histological sections

Sections were cut at 6 μm thickness from paraffin embedded teeth using a microtome and stained with hematoxylin and eosin. The remaining dentine thickness (RDT) area was measured in each slide using Image Pro Express software (Media Cypernetics Inc., USA).

RESULTS

The presence and characterization of stem cells derived from the remaining dental pulp of exfoliated deciduous teeth was confirmed using immunocytochemistry with hMSCs as the positive control and breast cancer cells as the negative control with mouse anti-human markers CD166 and CD105 monoclonal antibodies. Goat anti-mouse and anti-rabbit immunoglobulin G (IgG) as a secondary antibody (Chemicon, USA) were added and the expression of the cell surface markers were detected using the immunoperoxidase DAB secondary detection system (Chemicon, USA). The results showed the presence of brownish colour indicating positive reactivity for CD105 and CD166 primary antibodies on stem cells derived from the remaining dental pulp of exfoliated deciduous teeth and hMSCs and with no colour expression on the breast cancer cells (Fig 1). Flow cytometry results showed stem cells derived from the remaining dental pulp of exfoliated deciduous teeth were positive for CD105 and CD166 (Fig 2).

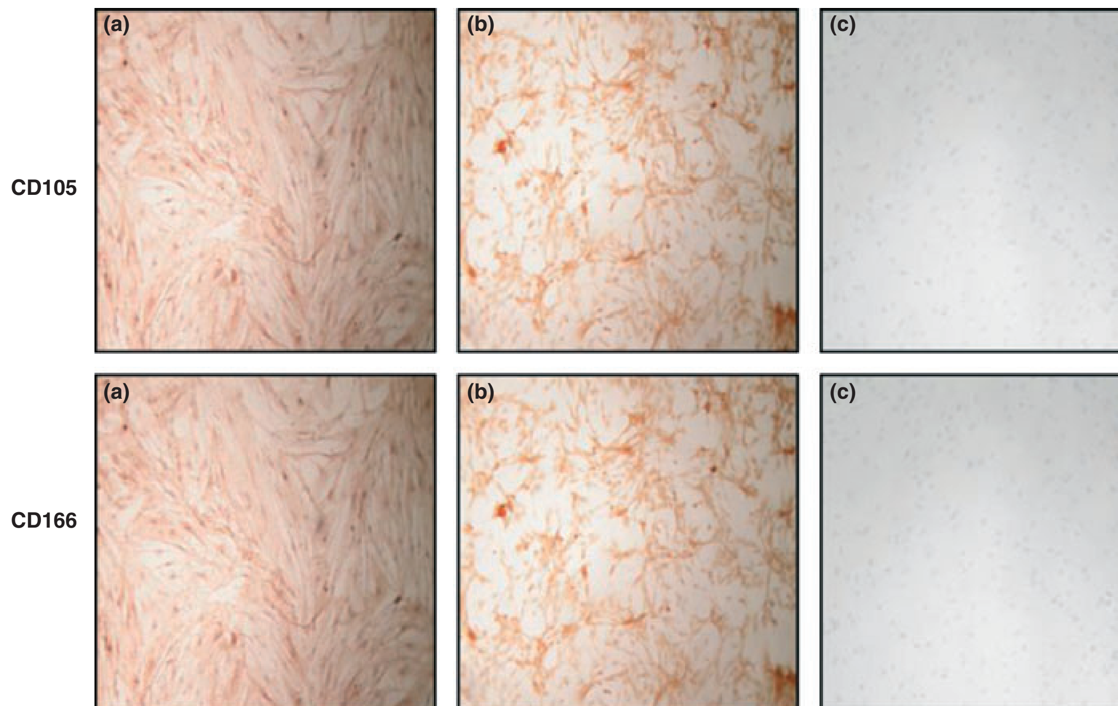

Fig 1. Characterization of stem cells derived from the remaining dental pulp of exfoliated deciduous teeth by immunocytochemistry staining using CD105 and CD166 antibodies viewed at 100×. The CD105 and CD166 expressions on stem cells derived from the remaining dental pulp of exfoliated deciduous teeth (a) and hMSC (b) – positive controls. No expression detected on breast cancer cells (c) – negative control.

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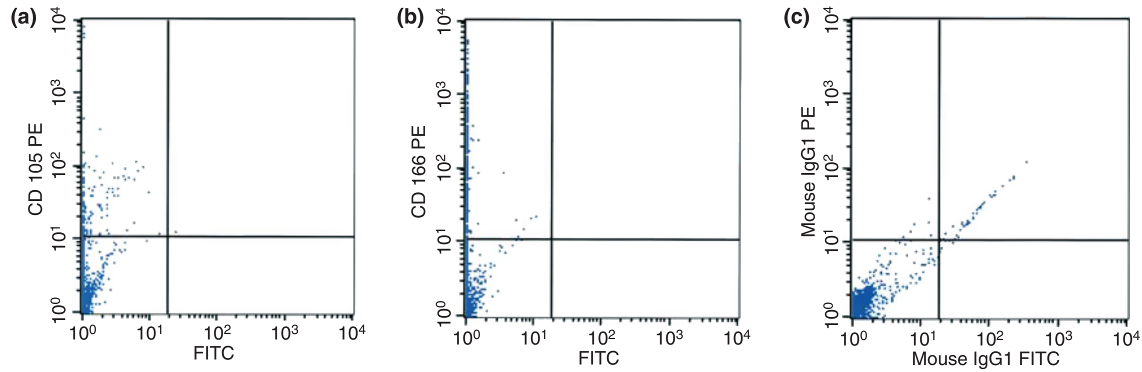

Fig 2. Characterization of stem cells derived from the remaining dental pulp of exfoliated deciduous teeth by flow cytometry showed positive for CD105 (a) and CD166 (b). Negative control (c).

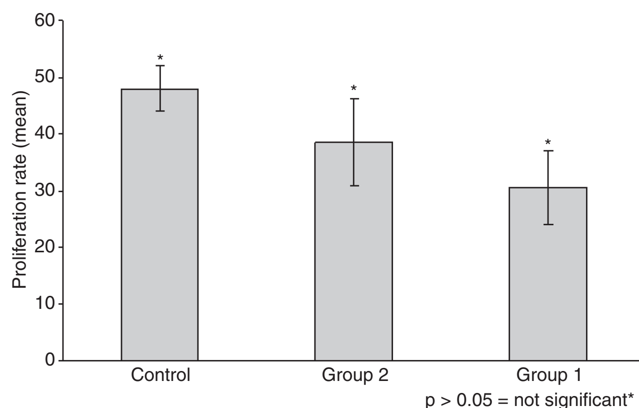

Fig 3. Mean cell proliferation rate of control, Group 1 (teeth filled with GIC) and Group 2 (teeth lined with $Ca(OH)_2$ and filled with GIC).

Statistical analysis of the alamarBlue reduction percentage between stem cells derived from the remaining dental pulp of exfoliated deciduous teeth without treatment with restorative material (control), stem cells derived from the remaining dental pulp of exfoliated deciduous teeth obtained from teeth in Group 1 and stem cells derived from the remaining dental pulp of exfoliated deciduous teeth obtained from teeth in Group 2 was not significant (Fig 3). From the histological samples that were used in this study, the mean RDT was 0.3 mm in Group 1 and 0.31 mm in Group 2, in which reactionary dentine formation (Fig 4) takes place due to survival of odontoblasts and the buffering activity of the remaining dentine protects the pulp tissues from the injuries that may be associated with the cavity preparation procedure and chemical irritant of restorative materials that have been used in this study.

DISCUSSION

The characterization of stem cells derived from the remaining dental pulp of exfoliated deciduous teeth was demonstrated by immunocytochemistry staining because of its stability, sensitivity, clear cytomorphological details, ease of use, cost-effectiveness and low technology need.[10] Flow cytometry is a powerful technique for correlating multiple characteristics on single cells, which allows the identification of the presence of antigens either on the surface of or within cells. Moreover, the information obtained is both qualitative and quantitative. CD105 is also known as human endoglin (ancillary TGF-beta receptor), a transmembrane glycoprotein expressed by vascular endothelial cells and activated macrophages. CD166 (activated leukocyte cell adhesion molecule (ALCAM)), is a member of the Ig superfamily and is expressed primarily in the spleen, placenta, liver (weakly) and activated T-cells, B-cells, thymic epithelial cells, fibroblasts, keratinocytes and neurons. In this study, stem cells derived from the remaining dental pulp of exfoliated deciduous teeth were found to be positive for both antibodies against human antigens CD105 and CD166. Similar results have been reported by many researchers, where MSCs isolated from placenta/umbilical cord cells population were positive for CD54, CD29, CD73, CD13, CD44, CD105 and CD166. These results suggested that the immunophenotypical and morphological profiles of these cells are the same as those of MSCs isolated from bone marrow.[11–13] Moreover, CD13, CD29, CD44, CD73 and CD105 were expressed in human dental pulp stem cells (DPSCs), when compared to hMSCs.[14] It was also found that CD29, CD44, CD105, CD106 and CD166 were positive surface marker antigens for periodontal ligament stem cells (PDLSCs).[15,16]

In stem cell research, there is no standard method for investigating the proliferation activity. Although several methods are available, they are limited by the complexity of the tests and expensive reagents or equipments, such as bromodeoxyuridine, used to measure the proliferative activity of SHED[17] and WST-1, used to demonstrate the proliferation activity of dental pulp

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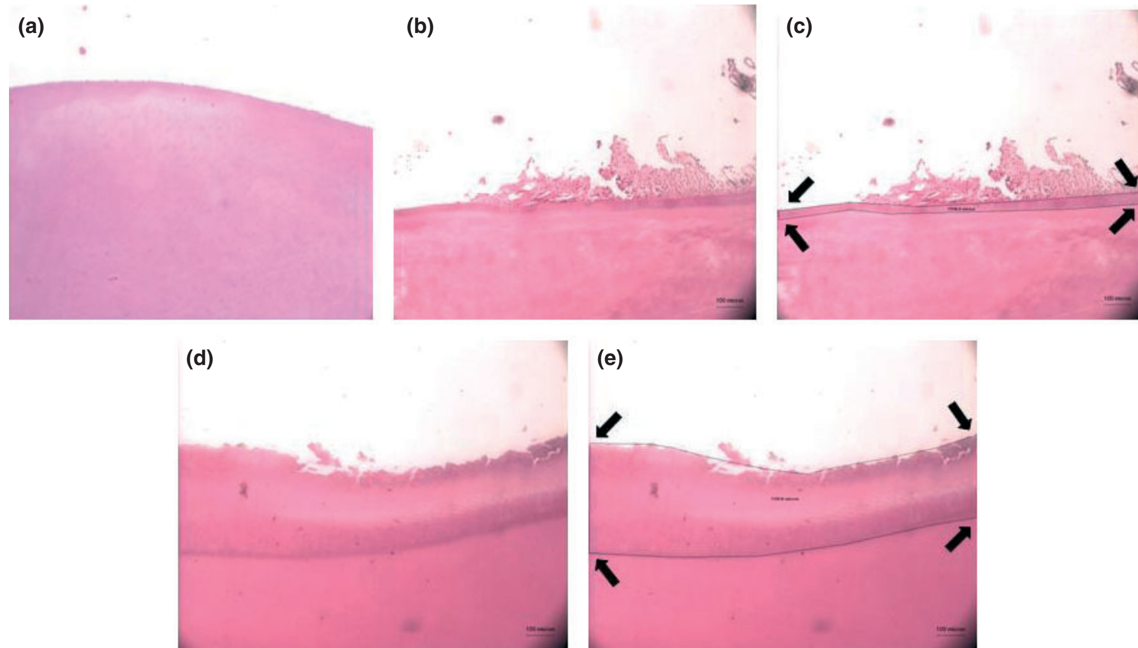

Fig 4. Tertiary dentine formation as a response to dental materials placement and measurement of tertiary dentine thickness area, viewed at 100× (H&E). Absence of tertiary dentine formation in control teeth (a); tertiary dentine formation as a response to GIC placement (b, c); tertiary dentine area before measurement using the software (b); tertiary dentine area after measurement by software (c) – the arrows show the measurement points for the tertiary dentine area; tertiary dentine formation as a response to $Ca(OH)_2$ placement (d, e); tertiary dentine area before measurement using the software (d); tertiary dentine area after measurement by software (e) – the arrows show the measurement points for the tertiary dentine area.

stem cells and PDLSCs.[16] Determination of proliferative activity enables researchers to optimize cell culture conditions, quantitate the activity of cell growth factors including cytokines, facilitate the discovery of new therapeutic agents, assess the efficacy of therapeutic agents, assess the toxicity of environmental pollutants, assess cell mediated toxicity and quantitate apoptosis (alamarBlue^{TM}, Biosource, USA). In this study, proliferation assessment offers a demonstration of the efficacy of dental materials that have been used. An indirect pulp capping procedure was adopted in order to compare the proliferative activity of dental pulp stem cells between the materials that have been used in this study. Colourimetric alamarBlue assay is simple to perform, since it is water soluble, thus eliminating the washing/fixing and extraction steps, stable in culture medium, non-toxic and does not alter the viability of cells cultured for various times. Therefore, the cells under study can be returned to the culture or used for other purposes. It has also been found that the cultured cells activity such as hybridoma cells to secrete antibody cells does not interfere with alamarBlue as well as cell growth and doubling has been found to be similar as non-alamarBlue exposed cells.[18]

In restorative dentistry, dentinal repair occurs through the activity of specialized cells called odontoblasts. Shallow or mild injury to the pulp stimulates the secretory activity of the odontoblasts to elaborate reactionary dentine, while in deep cavities or severe injury to the pulp, may lead to partial or total destruction of the odontoblasts layer. These conditions attract cells to the injury site and differentiate into odontoblast-like cells that can replace the necrotic odontoblasts and secrete a reparative dentine matrix.[19] Moreover, in the case of exposed cavities, a reparative dentinogenesis response was observed, while in non-exposed cavities, a reactionary dentinogenesis response was stimulated.[20] A study of pulpal injury and dentine repair activity with regard to the variables of cavity cutting, restoration materials and patients, showed that the cavity RDT is considered the most important variable to influence pulp activity and plays a central role in determining the extent of pulp injury. Reductions in RDT increasingly sensitize the pulp to cavity preparation injury and the possible cytotoxicity of dental materials. However, maximizing the RDT could have a beneficial effect to limit pulp tissue destruction with buffering properties of dentine which could modify the possible cytotoxic properties of dental materials.[21] In very deep cavities, RDT between 0.25 to 0.01 mm or in pulp exposure cavities, dental pulp stem cells proliferate, migrate to the site of injury and differentiate to form odontoblastoid cells. These odontoblastoid cells replace the severely damaged odontoblasts that have been reduced 100% and secrete reparative dentine, which is considered to be the most visible repair response to pulp injury.[22] This study showed that placement of lining materials for pulpal protection has generated increased tertiary dentine formation as compared to the teeth without any lining material.

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Stem cells are thought to reside in a specific area of each tissue where they may remain quiescent until they are activated by disease or tissue injury.[23] Proliferation, migration and differentiation activities of the dental pulp stem cells seem to be inactive when there is no pulp exposure or there is no severe loss of odontoblast cells related to increased dentine loss and decrease the cavity preparation RDT.[9] This theory was approved by a study which was directed to investigate the activation and migration of stem cells in response to pulpal injury. It has been found that proliferation, migration and differentiation of dental pulp stem cells take place when there is pulpal injury and not dentine injury.[24]

The indirect pulp capping that was used with RDT was preserved to avoid pulp exposure and to protect the pulp tissues from chemical and mechanical irritants.[22,25] The restorative materials that have been used on the pulp-dentine complex tissues have good biocompatibility and physical properties.[26,27] These could be the reasons that reflect the similarity between these materials in proliferation activity, in which signals for induction of proliferation, migration and differentiation of the new generation of odontoblast-like cells were prohibited, since the injury was at the dentinal level and did not reach the pulpal level. The tertiary dentine (reactionary dentine) was seen in the present study, which is in agreement with the previous studies.[28] However, it was not possible to distinguish whether the presence of tertiary dentine that was secreted in response to a variety of stimuli in this study was either reactionary or reparative in nature.

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