Characterization of the Biological Effect of Biodentine™ on Primary Dental Pulp Stem Cells

Abstract

Background: Biodentine™ is relatively a new tricalcium silicate cement that has gained great attention of the researchers due to its biological potential in comparison with other materials. The aim of this study was to investigate the optimum concentrations of Biodentine in relation to its stimulatory or inhibitory effect on proliferation, migration and adhesion of stem cells of human exfoliated deciduous teeth (SHED). The cell cultures of SHED were treated with Biodentine™ extract at four different concentrations; 20mg/ml, 2mg/ml, 0.2mg/ml and 0.02mg/ml. Cells cultured without Biodentine™ were kept as a blank control. The proliferation potential of SHED cells was evaluated by MTT viability analysis for 6 days. Migration potential was investigated by wound healing and transwell migration assays. The growth, survival and communication potential of these cells was determined by Adhesion assay. Results: A significant increase was observed in the proliferation and migration of SHED at (2mg/ml, 0.2mg/ml and 0.02mg/ml) while higher concentration of Biodentine™ (20mg/ml) exhibited cytotoxic effect on the cells. However, three tested Biodentine™ concentrations were similar in effect (non-significant) to adhesion ability of cells when compared with blank control. Conclusion: Our findings suggest that lower concentrations of Biodentine™ can be considered as the optimum concentrations to enhance the stimulatory effect of Biodentine on SHED.

Keywords: 3-4,5-dimethylthiazole-2-yl]-2,5-diphenyldiazonium bromide, adhesion, BiodentineTM, migration, stem cells from human exfoliated deciduous teeth

Introduction

Identification of different sources of stem cells has facilitated their use in the field of cellular therapy. In particular, dental stem cells are considered as a suitable source of adult stem cells because of their accessibility by tooth extraction with local anesthesia or when a primary tooth is exfoliated.[1]

Dental stem cells are known for their neural crest origin during tooth development and have been classified into different groups according to the tissue from which these cells were isolated.[2] Stem cells from human exfoliated deciduous teeth (SHED) are a useful source for repair of craniofacial defects, regeneration of bone, treatment of several diseases including motor neurons injury, as well as for a wide range of applications in regenerative endodontic procedures.[3] SHED have also been successfully used for many clinical implications such as repairing heart muscle, transplantation of bone and cartilage, Crohn’s disease, spinal defect, and plastic surgery.[4-6]

In addition, they may also be attributed to the treatment of diabetes[7,8] and liver diseases.[9]

Biodentine™ (Septodont, Paris, France) is a tricalcium silicate material that had been launched in 2009 to be used as a dentine substitute material. Biodentine™ showed better cementing properties as compared to mineral trioxide aggregate, such as good handling features, reduction in settling time, and improvement in mechanical strength.[10] Wide ranges of Biodentine™’s clinical applications included apexification, perforation repair, resorptive lesion, as a retrograde filling material, dentine replacement in restorative dentistry, and a promising alternative for existing pulpotomy medications.[11-16]

Very few studies have been conducted to determine the optimum concentration of Biodentine™ to regenerate pulp–dentine complex for clinical applications.[17-20] The effect of Biodentine™ on proliferation, migration, and adhesive properties of human dental pulp stem cells (hDPSCs) extracted from permanent teeth has been evaluated.[21] Similarly, in periodontal

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ligament, there was an increased cell adhesion through the expression of focal contacts when treated with Biodentine™,[26]

However, there is no previous study which has investigated the stimulatory or inhibitory effect of Biodentine™ on the biological properties of SHED. Therefore, we hypothesize that Biodentine™ exerts a stimulatory effect on primary dental pulp cells, in vitro, and aim to evaluate an optimum concentration for regeneration of pulp–dentine complex for clinical application.

Materials and Methods

Sample collection

The samples (exfoliated deciduous teeth) were collected from the patients of Jordan University Hospital while the blood collection to prepare platelet lysate (PL) was completed at the Cell Therapy Center-University of Jordan. All the experiments in this study were conducted at the Cell Therapy Center-University of Jordan. All donors signed an informed consent in accordance with Helsinki declaration prior to sample collection.

For PL preparation, human peripheral blood was collected from five donors. All donors were healthy without any known disease, with no medication, nonsmokers, and nonalcoholic. The collection of samples was performed according to the guidelines of the Institutional Review Board. Peripheral blood was drawn in 4 ml citrated tubes (Vacuette, Greiner Bio-One, Austria) and stored at 4°C for a maximum of 1 h before further processing.

Platelet lysate preparation

The blood was centrifuged at 192 × g for 13 min at 4°C. The supernatant, platelet-rich plasma (PRP), was pooled from multiple donors and kept frozen at −80°C until use. Further PRP preparation included centrifugation at (192 × g for 10 min), and two rapid freeze-thaw cycles (−80°C and 37°C). The resulting human PL was centrifuged at 3894 × g for 10 min at 4°C to remove platelet bodies. The PL was filtered through 0.22 μ filters to remove platelet membranes.[27]

Isolation and expansion of primary dental pulp stem cells (stem cells from human exfoliated deciduous teeth)

The following inclusion and exclusion criteria were used for isolation of SHED.

Children aged 5–6 years old, or any children in the mixed dentition state.

Inclusion criteria

• The teeth should be sound (not deep carious teeth or infected teeth)
• The exfoliated teeth should have pulp in red color, indicating that the pulp received blood flow up until the time of removal, which is indicative of cell viability
• The teeth are not very mobile.

Exclusion criteria

• The teeth, extensively carious or infected. If the pulp was gray in color, it was likely that the blood flow to the pulp has been compromised, and thus, the stem cells are likely necrotic and are no longer viable for recovery
• Teeth that become very mobile, either through trauma or disease, were not candidates for stem cell recovery
• Teeth which were extracted before 48 h of isolation are no longer considered as part of study.

The teeth were submerged in transport medium, consisting of alpha-modification of Eagle’s medium (α-MEM; Gibco, Life Technologies, USA), supplemented with 10% penicillin/streptomycin and amphotericin B (Invitrogen, Life Technologies, USA) and stored at 4°C until use.

Isolation of stem cells from human exfoliated deciduous teeth

Isolation of SHED was done as previously described,[28] within 24 h of receiving the sample. Cell cultures were established using the enzymatic dissociation method (3 mg/ml collagenase type I [Gibco, Life Technologies, USA] and 4 mg/ml dispase II [Gibco, Life Technologies, USA]). Single-cell suspensions were obtained by passing the cells through a 70 μm strainer (BD biosciences, USA). These cells were seeded in 6-well tissue culture plate (TPP, Switzerland) using α-Minimal essential medium (MEM) (Gibco, Life Technologies, USA), supplemented with 2 mM L-glutamine (Glutamax, Invitrogen, USA), 100 units/ml penicillin (Invitrogen, USA), and 5% PL and incubated in a humidified incubator (Memmert, Germany) at 37°C in 5% CO₂ atmosphere. For subculturing, cells were seeded at a density of 1 × 10⁴ cell/cm² and maintained at the same conditions as mentioned above. For all the following experiments, cells at passage 3 were used.

Phenotypic characterization

To identify mesenchymal stem cell (MSC) surface markers, cells at passage 3 were trypsinized and 2 × 10⁶ cells were incubated for ½ h at room temperature with the MSC characterization kit (stem flow kit, BD Biosciences, USA Cat # 562245) containing different fluoresceinated antibodies. The kit contains the following MSC markers; PerCP Cy5.5- CD105, FITC-CD90 and PE-CD44, and APC-CD73 and hematopoietic stem cell markers such as the PE-Negative cocktail (CD34, CD45, Cd11b, and Human leukocyte antigen – antigen D related (HLA-DR)), with their isotype controls. Incubated cells were centrifuged at 300 × g for 5 min and re-suspended in phosphate-buffered saline (PBS). Antibodies were used at concentrations based on manufacturer’s suggestion (BD, USA). The expression
profile was analyzed by fluorescein-activated cell sorter Canto II (BD, USA).

**Preparation of Biodentine™**

Biodentine™ was obtained from Septodont (St. Maur des Fosses, France) and prepared according to a previously described method[22-24] with some modifications. One capsule of Biodentine™ had been used, by mixing the powder and liquid together manually (one capsule contains 1 g). Then, the mixture was placed in the oven at 50°C for 15 min. For preparation, 20 mg/ml, 1 g of the dried powder of the prepared Biodentine™ was added to 50 ml α-MEM supplemented with 5% PL. The supernatant from the preparation was filtered twice by using cell strainer (Falcon, 70 μm, USA).

After preparation of 20 mg/ml Biodentine™, serial dilution (10 folds) was done to prepare the other Biodentine™ concentrations (2 mg/ml, 0.2 mg/ml, and 0.02 mg/ml).

**Cell proliferation assay (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide assay)**

A total of 2500 cells at passage 3 were seeded into a 96-well plate (TPP, Switzerland Cat# Z707910) and cultured in 200 μl of different concentrations of Biodentine™; 20 mg/ml, 2 mg/ml, 0.2 mg/ml, 0.02 mg/ml, and a blank control (α-MEM supplemented with 5% PL only) at 37°C. Cells were incubated for a total of 6 days and culture medium was changed every 3 days. Proliferation rate was measured by using a 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay kit (Promega, USA, Cat # G4000).

**Migration assay**

**Wound healing assay (Scratch assay)**

Approximately 1 × 10^5 cells (at P3) were seeded into 6-well culture plates (Thermo Scientific, Nunclon Delta Surface, Denmark) and allowed to reach confluent monolayer. Cells were incubated for 24 h with serum-free medium. After 24 h, a wound was inflicted by making a vertical scratch on the confluent layer of cells in every well, using 200 μl pipettes (Gilson, Diamond Tipack, 20–200 μl). Serum-free medium was removed and the cells were washed with PBS to remove cell debris, and then incubated with α-MEM medium in the presence of different Biodentine™ concentrations, in addition to blank control for 24 h. Microphotographs of the scratch were taken at day 0 and after 24 h of wound infliction. The cultured cells were observed using inverted microscope (Axiovert, Zeiss, Germany) to detect the differences in closure pattern.

**Transwell migration assay**

The second method for evaluating cell migration was by using 2-chamber transwell systems (8 μm pore size and 6.5 mm diameter inserts) (Costar, USA). 5 × 10^4 cells were re-suspended in 250 μl α-MEM and seeded in the upper chamber. On the other hand, 700 μl of Biodentine™ media were added to the lower chamber according to different Biodentine™ concentrations. After 24 h incubation at 37°C in 5% CO_2_, cells remaining on the top surface of the filter were removed with transport swab (Gamma sterile, AFCO, Jordan), and cells that traversed the membrane to the bottom chamber were fixed for 10 min with 4% paraformaldehyde (Sigma, USA). Then, glass slabs were used to add one point of molecular probe DAPI (Gibco, USA) in the membrane that was removed and attached to the drop of molecular probe and then it was covered with other cover slip. To quantify the migrated cells, 10 random microscopic fields per filter at 200X magnification were selected for a cell count using fluorescent microscope (Axio Imager z2, Zeiss, Germany). Measurements were done in triplicate and results were calculated as mean in each experiment.

**Adhesion assay**

96-well plates (TPP, Switzerland) were used for evaluating the adhesion ability of SHED. Each well of the plate was coated with collagen type I (Gibco, USA) (40 mg/l in PBS solution) for 24 h at 4°C. Cells were trypsinized and resuspended in serum-free medium at a cell density of 1 × 10^5 cells/ml and 100 μl of the suspension was added for each well and cells were incubated for 1 h at 37°C.

Nonadherent cells were removed by PBS and the adherent ones were fixed in 4% paraformaldehyde (Sigma, USA) for 20 min. The cells were stained with 0.1% crystal violet (Sigma, USA) for 30 min. After a thorough washing with PBS, a solubilization solution of 10% acetic acid (Surechem, England) was added to each well. The colored solution was quantified at 595 nm on a multiple reader (Glutamax, Promega, USA). The experiment was performed in triplicate and the results were calculated as mean in each experiment.

**Statistical analysis**

Data were analyzed using GraphPad Prism software Version 6.01 (USA) and Microsoft Excel for Windows. All assays were performed in three independent experiments (n = 3), with triplicates of each, results were expressed as means with standard deviations. Both two-way and one-way ANOVA tests were used to determine the significant effect of the variables followed by Tukey’s multiple comparisons to determine the differences in the proliferation, migration, and adhesion rates of SHED in the presence of different concentrations of Biodentine™ at variable time (significance assumed for P < 0.05).
The percentage of cell viability for MTT assay was calculated according to the following equation:

\[
\% \text{ viable cells} = \frac{\text{Absorbance value from well}}{\text{Mean absorbance of the control}} \times 100\%
\]

**Results**

**Morphological and phenotypical characteristics of stem cells from human exfoliated deciduous teeth**

The extracted cells revealed a heterogeneous morphological appearance of MSC, ranging from spindle shaped to elongated, neuron like and fibroblastic [Figure 1]. Flow cytometric analysis revealed that SHED were positive for CD90 (99%), CD105 (92%), CD73 (92%), and CD44 (87%) and negative for hematopoietic stem cell markers CD34, CD45, HLA-DR, and CD11b (4%) [Figure 2].

**Cell proliferation assay (3-[4,5-dimethylthiazole-2-yl]-2',5-diphenyltetrazolium bromide assay)**

MTT assay was performed at different concentrations of Biodentine™ for 6 days [Figure 3]. The figure shows that at concentrations of 2, 0.2, and 0.02 mg/ml of Biodentine™, the cell viability ranged from 81% to 114% compared to the control, while a sudden increase was observed from 5th to 6th day. However, at the concentration of 20 mg/ml, there was a decrease in cell viability on 2nd day (85%), 4th day (18%), and a slight increase was observed (31%) at 6th day of the treatment.

| Days | Day 1 | Day 2 | Day 3 | Day 4 | Day 5 | Day 6 |
|------|-------|-------|-------|-------|-------|-------|
| 2 mg/ml | 0.109 | 0.010 | <0.0001* | 0.001* | 0.031* | 0.005* |
| 0.2 mg/ml | 0.716 | 0.567 | 0.957 | 0.979 | 0.107 | <0.0001* |
| 0.02 mg/ml | 0.241 | 0.353 | 0.241 | 0.241 | 0.241 | 0.241 |

*Mean significantly different
Two-way ANOVA revealed highly significant effect of both variables, the concentration of the Biodentine™ ($P < 0.0001$), with the day of treatment ($P < 0.0001$), and the interaction between the concentration and the day of treatment ($P < 0.0001$).

Follow-up comparison was carried out using Turkey’s multiple comparisons. Overall, the concentration of 20 mg/ml of Biodentine™ had a highly significant cytotoxic effect which reduced the cell viability far more than the other concentration of 2, 0.2, and 0.02 mg/ml ($P < 0.0001$), while the difference in cell viability between the concentrations of 2, 0.2, and 0.02 mg/ml was not statistically significant [Tables 1 and 2].

Furthermore, it was clear that at high concentration of Biodentine™ (20 mg/ml), a significant decrease in SHED proliferation was observed, suggesting cytotoxic effect of Biodentine™ at higher concentration. Therefore, 20 mg/ml had been excluded for the further assays in the current study.

**Migration assays**

*Wound healing assay (Scratch assay)*

Wound closure was observed after 24 h of wound infliction.

Migration mobility of cells treated with Biodentine™ was determined based on the pattern of wound closure. Cells treated with the following concentrations of Biodentine™ (2 mg/ml, 0.2 mg/ml, and 0.02 mg/ml) showed variable migration mobility. However, Biodentine™ concentrations at 0.2 mg/ml and 0.02 mg/ml showed the best migration mobility and the best wound closure after 24 h in comparison with the blank control [Figure 4].

**Transwell assay**

The second method of evaluation of migration ability was done by using two-chamber transwell system, by culturing SHED with various Biodentine™ concentrations, as well as blank control, for 24 h. A significant difference was observed among three different treatments compared to blank control ($P < 0.0037$) using one-way ANOVA analysis [Figures 5 and 6].

![Figure 4: Wound healing assay. SHED cells were incubated with different concentrations of biodentine to assess the effect on the migration mobility at different time point 0 and 24 hours. At 0 hr: (a) 2 mg/ml BD, (b) 0.2 mg/ml BD, (c) 0.2 mg/ml BD, (d) Blank control. At 24 hours: (e) 2 mg/ml BD, (f) 0.2 mg/ml BD, (g) 0.2 mg/ml BD, (h) Blank control. BD = Biodentine](image)

![Figure 5: The effect of different concentrations of BD™ on the migration motility by using a two-chamber transwell system. Cells were treated with BD™ for 24 h and then the migrated cells were fixed and stained. Data are the mean ± standard deviation of the three independent experiments. $P<0.0037$ using one-way ANOVA analysis. BD™ = Biodentine™](image)

![Figure 6: The effect of different concentrations of BD™ on the migration motility by using a two-chamber transwell system. Representative photographs of migrated stem cells from human exfoliated deciduous teeth were observed under fluorescent microscope. (a) Stem cells from human exfoliated deciduous teeth cells treated with the blank control; cell culture medium without BD™. (b) Stem cells from human exfoliated deciduous teeth cells treated with 2 mg/ml BD™. (c) Stem cells from human exfoliated deciduous teeth cells treated with 0.2 mg/ml. (d) Stem cells from human exfoliated deciduous teeth cells treated with 0.02 mg/ml. BD™ = Biodentine™](image)
In addition, there was a significant increase in the migration of SHED treated with Biodentine™ (2 mg/ml) compared with other concentrations (0.2 mg/ml and 0.02 mg/ml) and blank control cells [Table 3].

Adhesion assay

No significant difference was observed among the three different concentrations of Biodentine™ on the adhesion ability of SHED, when compared to the blank control ($P > 0.05$) [Table 4] using one-way ANOVA [Figure 7].

![Figure 7: The adhesion ability of stem cells from human exfoliated deciduous teeth cells under the effect of various BD™ concentrations. No significant differences were observed among the three BD™ concentrations ($P = 0.1219$ for one-way ANOVA test). "OD: Optical density, BD™ = Biodentine™.](image)

### Table 3: Tukey’s multiple comparison test of SHED migration under different concentrations of Biodentine in comparison with blank control and among different Biodentine concentrations

| Tukey’s multiple comparison test (migration) | $P$  |
|---------------------------------------------|------|
| 2 mg/ml vs. 0.2 mg/ml                       | $<0.05$ |
| 2 mg/ml vs. 0.02 mg/ml                      | $<0.01$ |
| 2 mg/ml vs. control                         | $<0.01$ |
| 0.2 mg/ml vs. 0.02 mg/ml                    | $>0.05$ |
| 0.2 mg/ml vs. Control                       | $>0.05$ |
| 0.02 mg/ml vs. Control                      | $>0.05$ |

### Table 4: Tukey’s multiple comparison test of SHED adhesion under different concentrations of Biodentine in comparison with blank control and among different Biodentine concentrations. No significant differences were observed among different Biodentine concentrations

| Tukey’s multiple comparison test (adhesion) | $P$  |
|---------------------------------------------|------|
| Control vs. 2 mg/ml                         | $>0.05$ |
| Control vs. 0.2 mg/ml                       | $>0.05$ |
| Control vs. 0.02 mg/ml                      | $>0.05$ |
| 2 mg/ml vs. 0.2 mg/ml                       | $>0.05$ |
| 2 mg/ml vs. 0.02 mg/ml                      | $>0.05$ |
| 0.2 mg/ml vs. 0.02 mg/ml                    | $>0.05$ |

### Discussion

SHED cells located in the pulp are responsible to respond to tooth injury through the process of proliferation, migration, and adhesion to synthesize and secrete tertiary dentine. Biodentine™, a biocompatible dentine replacement and repair material, has favorably enhanced the healing effect of pulp by stimulating the biological properties of DPSCs. Hence, we planned to evaluate an optimum concentration of Biodentine™ to stimulate the cellular activities of primary DPSCs (SHED) to assess their ability to regenerate pulp–dentine complex, *in vitro*.

We found that the morphological appearance and surface expression pattern of SHED were according to the standard characterization of MSCs. They were spindle shaped, elongated, and fibroblastic in appearance. SHED were positive for CD90, CD105, CD73, and CD44 and negative for hematopoietic stem cell markers such as CD34, CD45, HLA-DR, and CD11b.

MTT assay revealed that the lower concentrations of Biodentine™ (2 mg/ml, 0.2 mg/ml, and 0.02 mg/ml) stimulated the proliferation capacity of SHED, while higher concentration (20 mg/ml) inhibited the growth of cells by exerting a cytotoxic effect. Previously, it has been shown that there was a significant increase in the migration and adhesion ability of hDPSCs at Biodentine™ concentration 0.2 mg/ml as compared to 0.02 mg/ml and blank control only. Our results from wound healing assay also coincide with the abovementioned results. However, we found that Biodentine™ concentrations at 0.2 mg/ml and 0.02 mg/ml have shown preferable closure pattern after 24 h. Moreover, transwell assay showed a significant increase in the migration ability of SHED, at 2 mg/ml. However, adhesion assay revealed no significant difference in the adhesion potential of SHED when treated with three different concentrations of Biodentine™.

According to these findings, higher concentrations of Biodentine™ (20 mg/ml) exert cytotoxic effect on cells, while lower concentrations of Biodentine™ (2 mg/ml, 0.2 mg/ml, and 0.02 mg/ml) can be considered as the optimal concentrations for enhancing the regenerative potential of SHED.

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### Conflicts of interest

There are no conflicts of interest.

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