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Effects of Two Calcium Silicate Cements on Transforming Growth Factor-β1 Secretion from Human Dental Pulp Stem Cells

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ABSTRACT

Introduction: The aims of this *in vitro* study were to evaluate the effects of two calcium silicate based cements, Calcum-enriched Mixture (CEM) and Biodentine on proliferation of human dental pulp stem cells (hDPSCs) and the effects of proposed cements on the secretion of Transforming Growth Factor β1 (TGF-β1). **Methods and materials**: The cell cultures of human Dental Pulp Stem Cells (hDPSCs) at passage 3-5 were treated with various dilutions (1/1, 1/2, 1/4, 1/8, 1/16, and 1/32) of CEM and Biodentine extracts to assess the cell proliferation using 3-(4, 5-dimethylthiazol-2-Y1)-2, 5-diphenyltetrazolium brovide (MTT) assay after 48 and 72 h. The amount of TGF-β 1 secretion were estimated after 72 h using an enzyme-linked immunosorbent assay. Data were analyzed using the one-way analysis of variance (ANOVA) followed by the Dunnett's test at the level of significance set at 0.05. **Result**: CEM showed the highest rates of cell proliferation compared to Biodentine after 72 h (P<0.05). A greater amount of TGF-β1 was secreted by hDPSCs treated with Biodentine compared to CEM (P<0.05). These differences were statistically significant (P<0.05). **Conclusion**: In this *in vitro* study hDPSCs showed more proliferation capacity with CEM rather than Biodentine and TGF-β1 secretion rate in Biodentine was higher.

Keyword: Biodentine; Calcium-Enriched Mixture; Human Dental Pulp Stem Cells; Proliferation; Transforming Growth Factor-β1

Introduction

Vital pulp therapy (VPT) is designed to preserve and maintain pulpal health in teeth that have pulp exposure due to trauma, caries, restorative procedures, and anatomic anomalies. The prime objective in VPT is to initiate the formation of tertiary reparative dentin or calcific bridge formation [1]. During treatment of exposed vital pulp, proliferation and differentiation of pulp cells are necessary. This process is affected by the interaction of human dental pulp stem cells (hDPSCs) and pulp capping material.

It is demonstrated that outcomes for VPT can vary, depending on the age of the patient, extent of bacterial contamination, degree of pulp inflammation and more importantly the type of pulp capping agent [2]. An ideal pulp capping agent should be biologically compatible and bioactive to stimulate DPSCs activity and hard tissue formation [3].

A variety of pulp capping materials have been investigated and used over the past century to encourage bridge formation and pulp preservation. With introduction of mineral trioxide aggregate (MTA), a calcium silicate-based cement as a pulp capping material in the mid-1990, significant success has been achieved in VPT [4]. Although MTA has excellent desirable properties for VPT, some disadvantages like long setting time, hard handling, discoloration of tooth and high price persuaded investors to develop new calcium silicate-based cements [5]. Recently, a variety of new bioactive calcium silicate-based cements without drawbacks of MTA have been developed such as calcium-enriched mixture (CEM) and Biodentine.

CEM cement was introduced to dentistry as an endodontic biomaterial. The sealing ability of CEM is similar to MTA and it promotes the process of differentiation in stem cells and induces hard tissue formation. It has been shown that CEM can induce osteo-/odontogenic like phenotype differentiation of hDPSC [6-9].

Biodentine is a tricalcium silicate-based cement that also demonstrates exceptional bioactive properties with potential for pulp capping procedures. It has a simple application, and its setting time is short. Recent data imply that Biodentine is a bioactive and biocompatible material capable of enhancing hDPSCs proliferation, migration and adhesion [3, 5, 10, 11]. The components of these two cements are listed in Table 1.

hDPSCs are multipotent mesenchymal stem cells (MSCs) reside within soft living tissue inside dental pulp. It has been shown that these cells could be beneficial for regenerative endodontic therapies [3, 12, 13]. Proliferation of hDPSCs is a principle phase in healing of dentin pulp complex, and survival studies with hDPSCs demonstrate a useful method for appraising the behavior of these cells in contact with new materials [3, 14]. To our knowledge, there is no study to compare the effect of two above-mentioned materials on proliferation of hDPSCs. Considering the gap of information, in the first part of this study we sought to evaluate the effects of CEM, and Biodentine on hDPSCs proliferation.

Transforming Growth Factor β1 (TGF-β1) is a multifunctional cytokine that can induce MSCs differentiation to odontoblasts and is essential for dentinogenesis [15]. Moreover, TGF-β1 has immunosuppressive and immunomodulatory effects [16]. TGF-β1 is also an important cytokine in cell proliferation, survival and migration [17, 18].

So far, separate studies have shown positive effects of CEM and Biodentine on the secretion of TGF-β1 but this effect has not been compared yet. Accordingly, in an attempt to provide more information to introduce a more biocompatible endodontic material, in this study the effect of CEM and Biodentine on hDPSCs proliferation and also the level of TGF-β1 secretion were investigated.

Materials and Methods

Preparation of pulp-capping material extracts

The materials tested in this study were CEM (Bioniquedent, Tehran, Iran) and Biodentine (Septodont, Saint Maurdes Fosses, France). The materials were mixed according to the manufacturers' instructions. Discs of each pulp-capping material were shaped under aseptic conditions in 6-plates measuring 35×2 mm, sterilized using ultraviolet irradiation for 15 min, and stored in an incubator at 37°C for 48 h to achieve complete setting. The materials were stored in the culture medium for 24 h at 37°C in a humid atmosphere containing 5% CO_2. The ratio of material surface area to medium volume was set at approximately 1.5 cm^2/mL in accordance with the guidelines of the International Organization for Standardization 10993-5. The extraction medium was filtered with sterile filters of 0.22 µm of diameter of the porous.

Cell culture

hDPSCs were obtained from Iranian Biological Resource Center (Tehran, Iran). Cells were cultured in media containing Dulbecco's Modified Eagle Medium (DMEM) supplemented with 15% FBS and 1% penicillin-streptomycin (10000 units/mL) as antibiotics. Cells were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Primary dental pulp stem cells at passage 3-5 were used for the experiments.

3-(4, 5-dimethylthiazol-2-Y1)-2, 5-diphenyltetrazolium brovide (MTT)

The effect of the different pulp-capping elutes on cell proliferation and viability was evaluated using the MTT assay, after 48 and 72 h of culture, using hDPSCs cultured in complete medium as control. Several concentrations (undiluted, 1/2, 1/4, 1/8, 1/16, 1/32) were prepared. Cells were seeded at density equal to 4000 cells/cm2 on a 96-well plate in 200 µL DMEM. After 24 h, the cultured cells were exposed to pulp capping extracts. Complete culture medium was replaced by serum-free culture medium, at the time points of 48 and 72 h. MTT was added at a final concentration equal to 1 mg/mL and cells were incubated for 4 h. Then, the culture medium containing MTT was removed and 100 mL dimethyl sulfoxide was added to release formazan. The absorbance at 570 nm was measured using an automatic microplate reader (Stat Fax 2100, Palm City, FL, USA). Each condition was analyzed in quintuplicate. The percentage of the viable cells was calculated using the following equation: (mean OD of treated cells/mean OD of control cells) 100×.

Table 1. Composition of tested materials

Materials	Manufacturer	Composition
CEM	Bioniquedent, Tehran, Iran	Powder: calcium oxide (CaO), sulfur trioxide (SO_3), phosphorous pentoxide (P2O5), and silicon dioxide (SiO_2); Liquid: distilled water
Biodentine	Septodont, St. Maurdes-Fosses, France	Powder: tricalcium silicate (Ca_3SiO_5), dicalcium silicate (Ca_2SiO_4), calcium carbonate ($CaCO_3$), iron oxide (Fe_2O_3), and zirconium oxide (ZrO_2) Liquid: water (H_2O) with calcium chloride ($CaCl_2$) and soluble polymer (polycarboxylate)

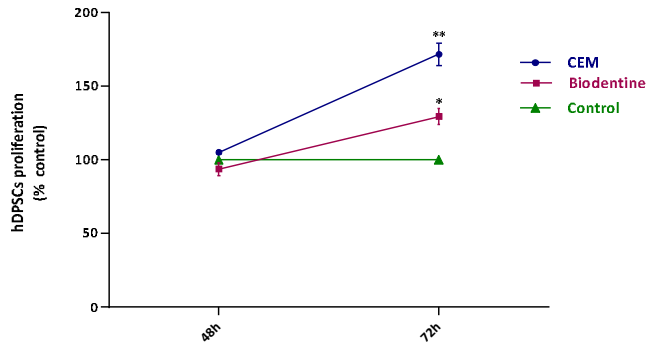

Figure 1. The proliferation of hDPSCs after exposure to CEM and Biodentine extracts at 48 and 72 h. There is no significant difference between either of the tested biomaterials with the control group after 48 h. However, a significant difference was noted in this regard at 72 h between CEM and control group ($P<0.01$) and also between Biodentine and control group ($P<0.05$) * and ** donate $P<0.05$ and $P<0.01$, respectively, as compared to the control

Enzyme-linked immunosorbent assay (ELISA) for TGF-β1

hDPSCs were seeded in 96-well plates in the conditioned medium for 72 h. The medium was collected and the levels of secreted TGF-β1 were determined using immunoassay kits (IBL GmbH; Hamburg, Germany) according to the manufacturer's protocol. The optical density of each well was determined using a microplate reader (Stat Fax 2100, Palm City, FL, USA) at 540 nm.

Statistical analysis

The data were analyzed using one-way ANOVA followed by the Dunnett's test. GraphPad Prism 5.0 statistical and graphing software was used for the statistical analyses. The values are presented as the mean ± SD. *P*-values less than 0.05 were considered statistically significant.

Results

Proliferation of hDPSCs after 48 and 72 h exposure to CEM and Biodentine

Assessment of cell proliferation in CEM and Biodentine groups after 48 h revealed no significant difference with the control group. However, after 72 h, there was significant difference between CEM ($P<0.01$) and Biodentine ($P<0.05$) with control group (Figure 1).

Proliferation of hDPSCs in different dilutions of CEM and Biodentine after 48 h

The percentage of proliferation rate of hDPSCs in different dilutions of CEM and Biodentine after 48 h is shown in Figure 2A. The proliferation of hDPSCs after exposure to CEM in dilutions of 1/1, 1/2 and 1/4 were significantly higher than Biodentine.

Proliferation of hDPSCs in different dilutions of CEM and Biodentine after 72 h

The percentage of proliferation rate of hDPSCs in different dilutions of CEM and Biodentine after 72 h is shown in Figure 2B. Biodentine was found to have significantly lower cell proliferation compared to CEM at dilutions of 1/1, 1/2, and 1/4 ($P<0.01$). However, no such difference is observed at concentrations of 1/8, 1/16 and 1/32 after 48 and 72 h exposure to CEM and Biodentine.

TGF-β1 secretion by hDPSCs after exposure to CEM and Biodentine

The concentration of released TGF-β1 was significantly greater in Biodentine group compared to CEM ($P<0.05$) (Figure 3).

Discussion

The results of our study revealed that there was no difference between tested materials and control group in cell proliferation after 48 h. However, the ability of CEM and Biodentine to induce proliferation increased over time. After 72 h, cell proliferation was significantly greater in the groups exposed to materials than control indicating that CEM and Biodentine have optimal biocompatibility for use in VPT. This finding is in line with the results of previous studies that separately assessed the biocompatibility of these materials [3, 5, 19-21]. Moreover, our finding revealed that this increase was more in CEM compared to Biodentine.

In our study the effects of CEM and Biodentine on cell proliferation was also compared with one another. The survival rate of CEM in dilutions of 1/1, 1/2 and 1/4 was significantly higher than Biodentine.

The effects of these materials on proliferation rate of different types of cells including periodontal ligament (PDL) fibroblasts [22], human gingival fibroblasts [23] and apical papilla stem cells [24] were examined. To our knowledge, the effects of CEM and Biodentine on the proliferation ability of hDPSCs has not yet been compared. Saberi *et al.* [23] evaluated the effect of CEM and Biodentine on viability of gingival fibroblasts. Their findings showed that there is no significant difference at 24 and 48 h compared to the control group which was in accordance with our results. In another similar study, the effects of CEM and Biodentine on the proliferation of apical papilla stem cells after different time points were evaluated and no significant difference was reported [24].

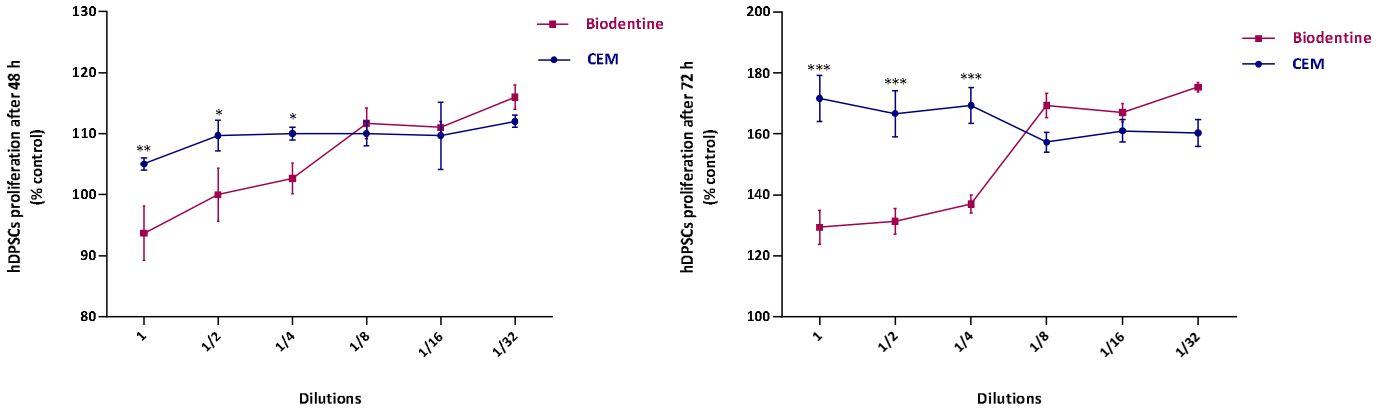

Figure 2. Percentage of hPDSCs proliferation following exposure to serial dilutions of CEM and Biodentine after 48 and 72 h was assessed by MTT test; *A)* Comparison of the CEM and Biodentine groups with one another revealed significant differences between them at dilutions of 1/1, 1/2, and 1/4; *B)* Similar results were observed after 72 h* and ** donate $P<0.05$ and $P<0.01$, respectively, as compared to the control

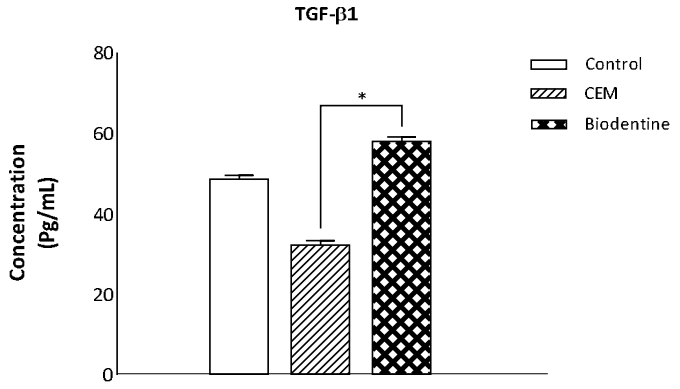

Figure 3. The concentration of TGF-β1 measured by ELISA after 72 h; the concentration of TGF-β1 in Biodentine group was significantly higher compared with the CEM group ($P<0.05$)

Küçükkaya *et al.* [22] reported no significant difference in cell viability between CEM and Biodentine after 24, 48, and 72h in human PDL fibroblasts. Moreover, their findings showed that CEM induced more than 90% cell viability after 24 and 48 h of incubation, while significantly less cell viability was observed in CEM group after 72 h of incubation. In contrast, Biodentine showed significantly less cell viability after 24 h of incubation, whereas it was increased significantly after 48 and 72 h of incubation.

Such controversy in results may be related to the type of target cells, method of cell proliferation assessment, direct contact of cells with the materials, concentration of materials and assessment time points.

Lee *et al.* [25] showed that cell viability of Biodentine in concentrations of 1, 1/2, and 1/4 was significantly lower than MTA and Bioaggregate in MSCs.

The biocompatibility of calcium silicate-based cements is related to releasing of calcium ions during their setting time and hydroxyapatite formation *via* calcium binding to phosphorus [19, 26].

Although release of calcium ions can cause inflammatory toxic reactions for some types of the cells [27], release of this ion from silicate cements is important for the survival of MSCs [25]. The proliferation of human dental pulp cells increase when exposed to media containing exogenous calcium ion [28]. This ion has signaling ability and plays an important role in up-regulation of different cell activities [24, 29]. Thus, the excellent biocompatibility of calcium silicate-based cements such as CEM cement and Biodentine can be attributed to the release of calcium ion. Difference in the percentage of cell proliferation and viability in CEM cement and Biodentine groups at different time points in our study may be due to the different release of calcium ions. The chemical composition of Biodentine is different from that of CEM cement, which could be another reason for the difference in cellular proliferation.

Over time, the components released from materials can induce cytotoxicity [22]. Biodentine is claimed by the manufacturer as a high purity dental material. However, a recent study has found traces of arsenic, chromium, and lead in elutes from a mixed Biodentine solution [30].

The biocompatibility of radiopacifier in materials can be another important issue since they have been observed in high levels in tissues adjacent to the capping materials [22]. Biodentine contains zirconium oxide as a radiopacifier which presents a lower toxicity profile than bismuth oxide, another radiopacifier in the MTA structure [31, 32]; however, in the CEM structure, there are not any of these elements [33].

In the second part of this study, we evaluated the level of TGF-β1 secreted by hDPSCs. Until now, no study has compared the effect of CEM and Biodentine on the level of TGF-β secretion by hDPSCs. Our findings indicate that after 72 h, CEM induced a decrease in TGF-β1 secretion as compared to the baseline values but the level of secretion of TGF-β1 was significantly higher in Biodentine. In accordance with our results, Laurent *et al.* [15, 34] reported that Biodentine significantly increased TGF-β1 secretion from human pulp cells. Similarly, Asgary *et al.* [6] showed the secretion of TGF-β1 from hDPSCs after incubation with MTA and CEM; however, this secretion was significantly lower in CEM group.

TGF-β1 acts as a regulator of many reparative processes in various tissues. In dental pulp, TGF-β1 promotes progenitor cell migration [35] and odontoblast differentiation [36]. Additionally, previous work has shown that dentine matrix contains sequestered TGF-β [37]. During dentine demineralization through carious lesions or acidic etchant application, this growth factor can be released to the pulp tissue where they could mediate dentine regeneration processes. Additionally, pulp capping materials such as MTA and Biodentine have been shown to stimulate human dental pulp and fibroblasts to secret TGF-β1 [15, 34, 38], but less studies have been done on the inductive effect of CEM on TGF-β1 secretion by pulpal cells. The results of our study in line with previous studies revealed that Biodentine has a prominent induction effect on TGF-β1 secretion.

The reason for difference in the secretion value in the tested materials can be attributed to difference in their chemical component. Moreover, our findings revealed the high secretion of TGF-β1 in Biodentine group compared with CEM and it may be attributed to high similarity of Biodentine structure to the dentine.

Conclusion

Human dental pulp cells increase proliferation when exposed to media containing CEM and Biodentine, however the effect of CEM is more. Moreover, Biodentine promotes TGF-β1 secretion by hDPSCs.

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Conflict of Interest: 'None declared'.

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