Original Article

The Impact of Calcium Hydroxide on the Osteoinductive Capacity of Demineralized Freeze-Dried Bone Allograft: an *In-Vitro* Study

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ABSTRACT

Statement of the Problem: A great challenge in periodontal therapy is the regeneration enhancement of osseous defects through applying osteoinductive materials. Demineralized freeze-dried bone allograft (DFDBA) has already been introduced as an allograft with osteoconductive and variable osteoinductive properties. Calcium hydroxide [$Ca(OH)_2$] is an available well-known material in dentistry, which induces hard tissue formation.

Purpose: This study evaluated the efficiency of combination of DFDBA and $Ca(OH)_2$ in improving the quality of osteoinduction of DFDBA.

Materials and Method: Human bone marrow-derived mesenchymal stem cells were taken from volunteers' iliac crest. Cell proliferation was determined by MTT test at 18, 24 and 48 hours post-culture in 10 groups. The employed material were 0.5, 1.0 mg/ml $Ca(OH)_2$ in two forms of suspension and pH-adjusted solution, 10mg/ml DFDBA per se and in combination with 0.5 and 1.0 mg/ml $Ca(OH)_2$. Mineralization was assessed by Alizarin red staining in 10 mg/ml DFDBA, DFDBA+ 0.5 and 1 mg/ml $Ca(OH)_2$ in solution and suspension forms. The data were statistically analyzed by using one-way ANOVA and Tukey's post-hoc test ($p < 0.05$).

Results: The pH-adjusted solutions exhibited better cell proliferation compared with the suspension groups. The combination of 0.5mg/ml $Ca(OH)_2$ solution and DFDBA increased the cell proliferation and mineralization compared with DFDBA per se ($p= 0.033$).

Conclusion: The combination of $Ca(OH)_2$ with DFDBA improved the osteoinductivity of DFDBA.

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Introduction

Periodontitis is an inflammatory disease initiated by the presence of bacterial plaque, leading to the destruction of periodontium (periodontal ligament, alveolar bone and cementum) and eventually tooth loss. [1] Regenerating the periodontal defects as well as the osseous re-construction of edentulous ridge to place the implants is a major challenge in modern dentistry and can have great influence on the patient's aesthetics, chewing ability and quality of life. Several approaches have been introduced to improve the reconstruction of alveolar bone defects. [2-3]

The process of periodontal and bone regeneration is generally complicated by different types of present cells. Active regeneration is valuable through induction of purposed cell population to differentiate or migrate to the defect area, which is the migration of bone progenitor cells to the injured site and differentiation of stem cells to osteoblasts that leads to the regeneration of bone structure. [4] Also, in the reconstruction of large bony defects, there should be a graft material to provide both scaffold and active signaling to the bone marrow mesenchymal stem cells (MSCs) inducing them to migrate and differentiate. [4-5]

Among bone replacement grafts, autograft is considered the gold standard. [6] They are the only bone grafts that provide all the three properties of an ideal graft material including osteogenesis (to provide vital osteoblasts), osteoinduction (induction of osteoprogenitor cells to differentiate to osteoblasts) and osteoconduction (to provide scaffold). [6] However, owing to the low level of patient acceptance of autografts, allografts (grafts from the same species) have become the most popular bone grafts in oral surgery. Allografts are being processed as fresh-frozen or freeze-drying. [6]

Demineralized freeze-dried bone allograft (DFDBA) is a type of allograft claimed to be superior because of the osteoconductive and osteoinductive properties. The osteoinductivity is obtained through demineralization of the graft material and exposure of the bone morphogenetic proteins (BMPs). These are a group of growth factors inducing MSCs differentiation into bone-producing osteoblast cells in bone matrix. [7-8] Therefore, the demineralization is critical for osteoinductive properties of DFDBA, since it provides adequate levels of BMPs and does not allow the mineral content to drop to very low levels.

It is believed that a certain concentration of Ca^{2+} ions is needed for the osteoinductive properties of DFDBA; therefore, addition of Ca^{2+} ions might provide the necessary Ca^{2+} ions for the optimum function of these allografts. [6, 9] Moreover, other factors such as the donor's age make the osteoinductivity of DFDBA variable and unpredictable. [10] Therefore, the recent studies aimed at improving the active bone and periodontal regeneration investigated the addition of different growth factors such as emdogain or rh-PDGF to DFDBA. It was noted that although such additives im-

proved the regeneration, these growth factors are expensive and not readily accessible. [11-12]

In contrast, calcium hydroxide, $[Ca(OH)_2]$ with hard tissue formation and antimicrobial properties, is a readily accessible material and quite well known in dentistry, particularly in endodontics, for pulp capping or apexification. Furthermore, $Ca(OH)_2$ is a strong base with low solubility in water that forms suspension in aqueous environment and releases hydroxyl and calcium ions over time. Most properties of the $Ca(OH)_2$ come from this ionic dissociation. [13]

Based on *in vitro* studies, $Ca(OH)_2$ increases the recruitment, migration, and proliferation of periodontal ligament (PDL) stem cells and promotes the mineralization and cementogenesis. However, scholars do not have consensus over its efficiency mainly because it is dose-dependent. [14-15] Paula-Silva *et al*. [15] in their *in vitro* study found that low dosage of $Ca(OH)_2$ did not influence the induction of mineralization and high dosage was cytotoxic to cells. [15] Bone marrow-derived MSCs are a group of multipotent stem cells that are sensitive to their local environment and differentiate into different types of cells including periodontal ligament-like or alveolar bone cell types. [16]

The present study evaluated the effect of adding different doses of $Ca(OH)_2$ in both solution and suspension forms to DFDBA on viability, proliferation and differentiation of human bone marrow-derived mesenchymal stem cells (hBM-MSCs) into osteoblasts. The significance of this study is that $Ca(OH)_2$ is inexpensive, abundant and easy to obtain compared with other materials. To the best of the authors' knowledge, this is the first study carried out on this issue. Moreover, the controversies about the osteoinductivity of DFDBA encouraged the evaluation of *in vitro* behavior of hBM-MSCs in the presence of DFDBA (Cenobone, Demineralized Cortical Cancellous Powder; Tissue Regeneration Corporation Co., Kish, Iran) as a popular allograft material routinely used in periodontal and osseous reconstructive surgeries.

Materials and Method

Isolation and culture of human MSCs

Human MSCs were obtained from 5 ml bone marrow aspirated from iliac crest of normal donors within the age range of 19-45 years. They were donors of bone

marrow to relatives after obtaining approval of Ethic Committee. Written informed consent was also taken to permit the analysis of the clinical data. Bone marrow-derived MSCs were isolated from mononuclear cell (MNC) layer using our previously method [17] which is briefly mentioned.

Each aspirated sample was diluted (1:1) with Dulbecco's modified Eagle's medium (DMEM) (Invitrogen; Merelbeke, Belgium) containing 10% fetal bovine serum (FBS), 1% penicillin, 1% streptomycin, and 2 mM glutamine as the basal DMEM media. The cells were layered over about 5 ml of Ficoll (Lymphoprep; Oslo, Norway), then centrifuged at 338 g for 15 min to obtain MNC layer. The MNC layer was seeded on culture flasks, and maintained at 37°C in 5% CO_2 atmosphere. In order to obtain the MSCs cells, the adhered monolayer cells was detached and expanded for successive passages, and characterized.

Characterization of human MSCs

The cells viability was analyzed by using trypan blue staining. Flow cytometric analysis for detection of MSC-morphologic markers was also achieved following the method used by Ayatollahi *et al.* [18]

Proliferation assay

The two types of employed $Ca(OH)_2$ were suspension (non-pH-adjusted) and solution (pH-adjusted). For the suspension groups, 50 and 100mg $Ca(OH)_2$ (Merck; Germany) were added to 10 ml normal saline, yielding 5- and 10-mg/ml concentration of $Ca(OH)_2$. Then, they were diluted in the culture medium in a 1:10 ratio to obtain 0.5- and 1-mg/ml doses of $Ca(OH)_2$. In the solution group, the powder was first dissolved in HCl, then, NaOH was added and the pH was adjusted to 7.4 via a pH meter.

Accordingly, 10 designed study groups were as follows; group 1: control group (MSCs in culture medium with no treatment), group 2: 0.5mg/ml $Ca(OH)_2$ solution, group 3: 0.5mg/ml $Ca(OH)_2$ suspension, group 4: 1mg/ml $Ca(OH)_2$ solution, group 5: 1mg/ml $Ca(OH)_2$ suspension, group 6: 10mg/ml DFDBA, group 7: 10mg/ml DFDBA+ 0.5mg/ml $Ca(OH)_2$ solution, group 8: 10 mg/ml DFDBA+ 0.5mg/ml $Ca(OH)_2$ suspension, group 9: 10mg/ml DFDBA+ 1mg/ml $Ca(OH)_2$ solution and group 10: 10mg/ml DFDBA+ 1mg/ml $Ca(OH)_2$ suspension. Each experiment was repeated 4 times.

Cell proliferation in each of the above groups was assessed by using Thiazolyl Blue Tetrazolium bromide (MTT) (Sigma-Aldrich Co.; St.Louis, MO, USA) assay. The hBM-MSCs (5.8×10^3) were cultured with 200μL of DMEM containing 10% FBS. After 3 days of culture, treatment in the mentioned groups was done for 18, 24 and 48 hours. Finally, MTT assay was conducted via 0.5 mg/ml 3-((4,5-dimethyl thiazole-2-yl)-2,5-diphe-nyl tetrazolium) bromide for 3 hours. Following the formation of formazan solution in DMSO (dimethyl sulfoxide), the absorbance of the solution was measured by an absorbance microplate reader at 570 and 630nm as the reference wavelength (BioTek Instruments Inc.;USA).

Osteogenic differentiation assay

The potential of human MSCs to differentiate into osteogenic lineages was examined in the presence of osteogenic media in four groups by using Alizarin red staining (Sigma-Aldrich Co., USA) within 5, 12 and 14 days after staining. The groups were group 1: MSCs in osteogenic media containing DFDBA (10mg/ml), group 2: MSCs in osteogenic media containing 0.5mg/ml $Ca(OH)_2$ (solution)+ DFDBA 10mg/ml, group 3: MSCs in osteogenic media containing 0.5mg/ml $Ca(OH)_2$ (suspension)+ DFDBA 10mg/ml, and group 4: MSCs in osteogenic media (positive control).

The osteogenic media consisted of basal DMEM media supplemented with 10 M/L dexamethasone, 10 mM/L glycerol phosphate, 3.7 g/L sodium bicarbonate and 0.05 g/L ascorbic acid (all from Sigma-Aldrich; St. Louis, USA). The osteogenesity was assessed by Alizarin red staining. The morphologic differentiation of cells and red mineralized nodule formation were observed by using microscope (Nikon) under 10×40 magnification.

Statistical analysis

The triplicate samples obtained from different cases were analyzed in duplicate. The statistical analyses were performed by using SPSS software, version 16 (IBM; USA). One-way ANOVA was used to analyze the data. Tukey's post-hoc test was used for pair-wise comparisons between the study groups. $p < 0.05$ was considered to be statistically significant. Alizarin red staining results were presented qualitatively.

Results

Characterization of human MSCs

The rapidly grown fibroblast-like cells, which exhibited homogeneous morphology, were selected for culture

expansion after inverted microscopic evaluation (Figure 1a). The viability assessed by trypan blue staining analysis showed 98-100% viability in human MSCs. The flow cytometry determined the surface phenotype to be positive for CD90 and CD73. Additionally, no cell expressed the hematopoietic markers CD45 and CD34 (Figure 1).
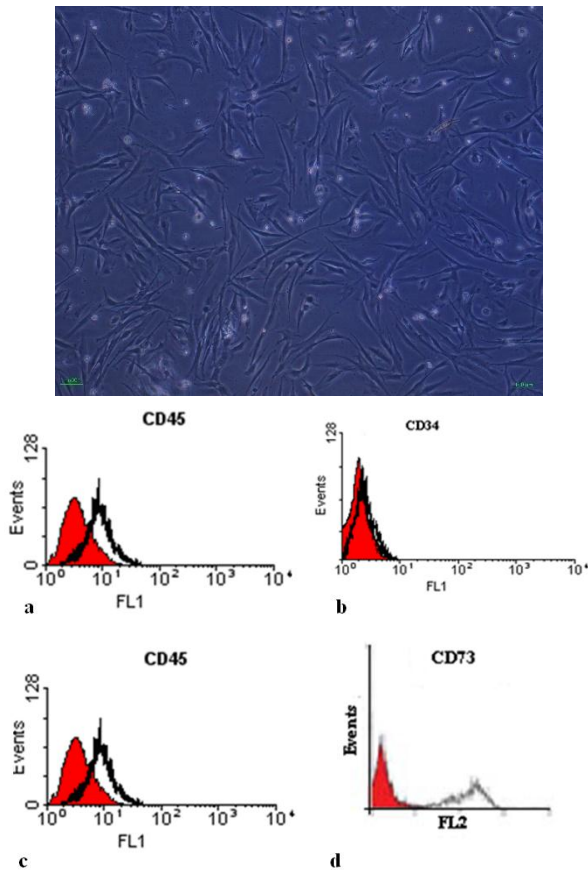
Figure 1: Characterization of human mesenchymal stem cells. Relatively homogenous fibroblast-like cells were obtained within 9-10 days after cell culture [A]. The surface phenotypic markers were positive for CD90, and CD73. Additionally, no cells expressed the hematopoietic markers CD45, CD34. The shaded area shows the profile of the negative control.

Cell proliferation analysis

There was a significant interaction effect between the time and group, i.e., the culture duration and material concentrations significantly affected the cellular proliferation ($p < 0.001$). The cell proliferation was compared among the groups at each time point of 18, 24 and 48 hours as demonstrated in Table 1.

After 18 hours treatment

After 18 hours of treatment, the cell proliferation was significantly lower in the group 10 compared with the other groups, which were not significantly different from the control group.

Table 1: Bone morrow-derived mesenchymal stem cells viability in different study groups (significant differences have been marked)

Group	Time (hr) 18h	24h	48h
1	0.98 ± 0.10^{a}	$0.96 \pm 0.07^{a,b}$	$0.96 \pm 0.05^{a,b,c}$
2	0.8 ± 0.06^{a}	$0.91 \pm 0.06^{a,b}$	$0.93 \pm 0.04^{a,b,c}$
3	0.88 ± 0.07^{a}	0.74 ± 0.13^{b}	$0.76 \pm 0.05^{b,d,f}$
4	0.78 ± 0.02^{a}	$0.90 \pm 0.05^{a,b}$	$0.93 \pm 0.01^{a,b,c}$
5	0.91 ± 0.05^{a}	0.17 ± 0.02^{a}	$0.14 \pm .03^{a,c}$
6	0.86 ± 0.08^{a}	$0.92 \pm 0.02^{a,b}$	$0.90 \pm 0.04^{a,b,d}$
7	0.84 ± 0.17^{a}	$0.86 \pm 0.13^{a,b}$	$1.04 \pm 0.05^{a,b,c,e}$
8	0.78 ± 0.04^{a}	0.73 ± 0.22^{b}	$0.82 \pm 0.06^{a,b,c,d,f}$
9	0.86 ± 0.09^{a}	$0.98 \pm 0.04^{a,b}$	$1.00 \pm 0.10^{a,b,c,e}$
10	0.39 ± 0.08	0.53 ± 0.16^{b}	$0.66 \pm 0.05^{b,c,d,e,f}$

$^{a,b,c,d,e,f} p < 0.05$ as compared with group 10, 5, 3, 7, 8 and 9 respectively

After 24 hours treatment

The cell proliferation in groups 5 and 10 was significantly lower than the control group. 1-mg/ml $Ca(OH)_2$ solution yielded significantly higher cell proliferation compared with the suspension form. Furthermore, cell proliferation was significantly lower in 1 mg/ml $Ca(OH)_2$ suspension than in 0.5 mg/ml $Ca(OH)_2$ suspension. Compared with 1 mg/ml $Ca(OH)_2$ suspension per se, the combination of 1 mg/ml $Ca(OH)_2$ suspension and DFDBA significantly increased the cell proliferation. However, the cell proliferation in DFDBA was higher than its combination with 1 mg/ml $Ca(OH)_2$ suspension.

After 48 hours treatment

The above-mentioned differences were still present among the groups. Both 0.5 and 1 mg/ml $Ca(OH)_2$ suspensions and DFDBA+ 1mg/ml $Ca(OH)_2$ suspension exhibited significantly lower proliferation in comparison with the control group. Both 0.5 and 1mg/ml $Ca(OH)_2$ solutions exhibited significantly higher cell proliferation than the same doses in suspension form. Furthermore, the combination of DFDBA with 0.5 mg/ml, $Ca(OH)_2$ solution significantly increased the cell proliferation in comparison with DFDBA per se. The cell proliferation in groups 7 and 9 showed an ascending trend from 18 to 48 hours to reach to a level higher than the control group but the difference was not statistically significant (Figures 2 and 3).

Osteogenic differentiation assay

Qualitatively, Alizarin red staining results showed osteogenic differentiation in BM-MSCs in four designed groups. The morphologic differentiation of cells and red mineralized nodule formation were observed by micros-
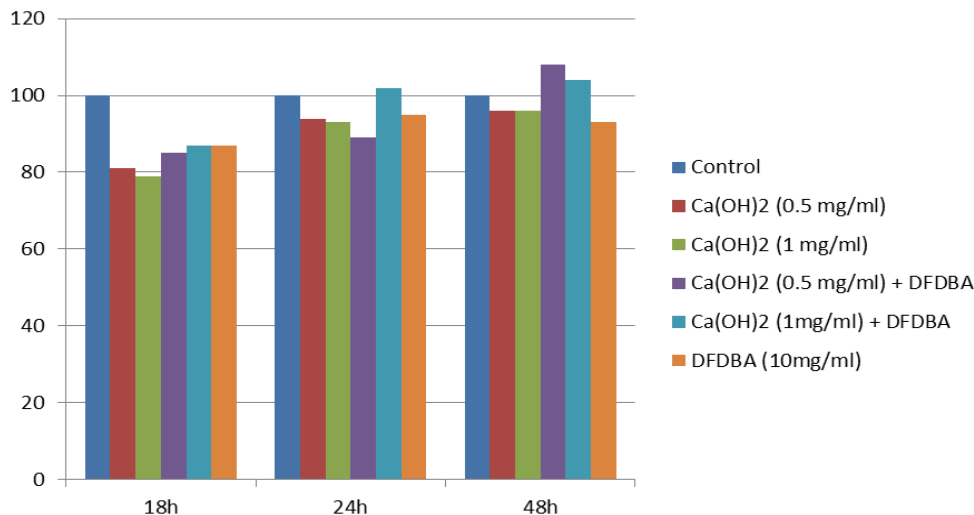
Figure 2: percentage change of proliferation and viability in different groups as compared with the control in different time points. DFDBA per se, Ca(OH)$_2$ solution per se, and in combination with DFDBA

cope. It was noted that only MSCs in osteogenic media containing Ca(OH)$_2$ in the solution group qualitatively exhibited mineralization in comparison with Ca(OH)$_2$ in suspension form and DFDBA groups (Figure 4).

Discussion

Results of the current study revealed that all the Ca(OH)$_2$ groups in solution form with an adjusted pH (7.4) had a better proliferation viability in comparison with the suspension groups (non-pH-adjusted), indicating an improved cellular function in the presence of a proper pH.

Ca(OH)$_2$ suspension in 1.0 mg/ml dose was cytotoxic to the cells and significantly decreased the cell proliferation compared with the baseline. Combination

of 1 mg/ml Ca(OH)$_2$ suspension with DFDBA increased the viability of cell proliferation. This was probably due to the buffering ability of DFDBA through a reduction in Ca(OH)$_2$ pH.

Narita *et al*. [19] demonstrated that dentine powder and hydroxyapatite (both comprised of mineralized compounds similar to DFDBA) had buffering effect on Ca(OH)$_2$; this is beneficial when using a mixture of DFDBA and Ca(OH)$_2$.

The results of the present study were consistent with some previous studies. Narita *et al*. [19] used different doses of Ca(OH)$_2$ at different pH levels. They concluded that high dose of Ca(OH)$_2$ (2.5 mg/ml) was cytotoxic to the primary osteoblast cells (in both pH-adjusted and non-pH-adjusted groups); whereas, in low-
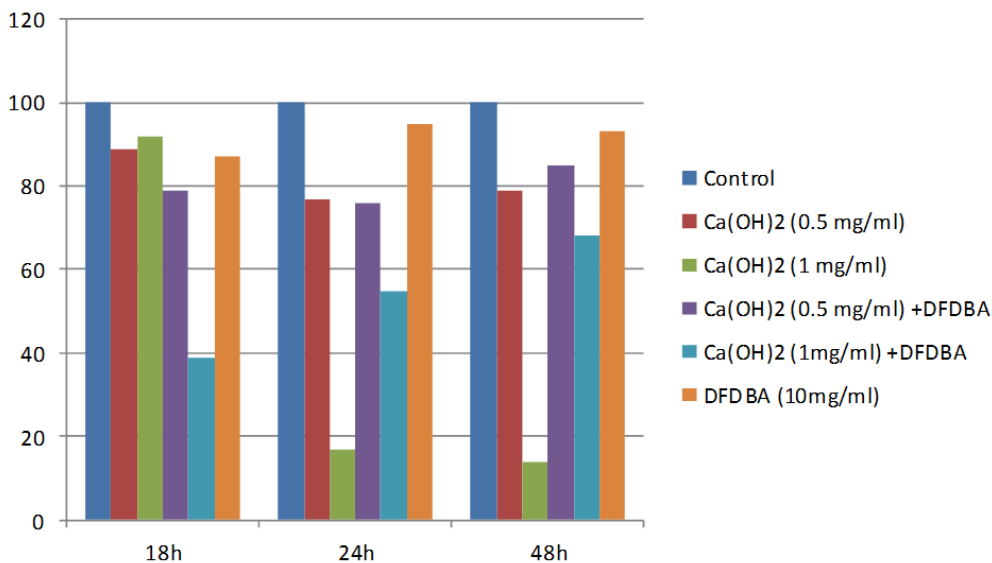
Figure 3: percentage of cell proliferation and viability in different groups in comparison with the control in different time points. DFDBA per se, Ca(OH)$_2$ suspension per se and in combination with DFDBA
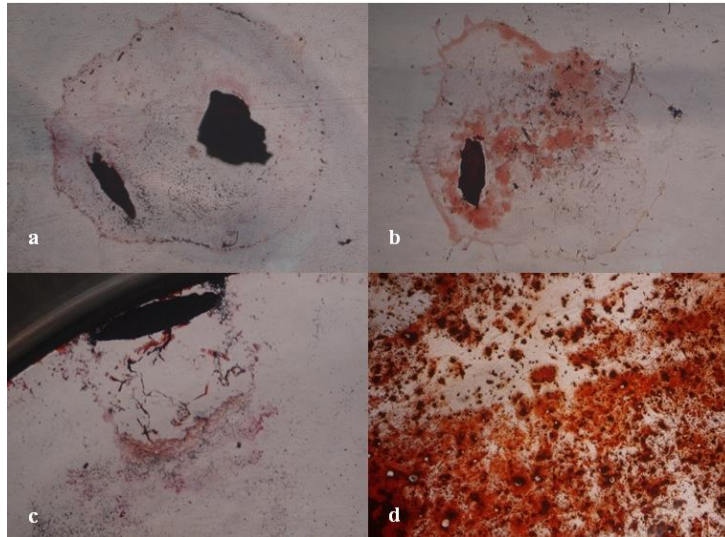
Figure 4: Osteogenic differentiation assay. Alizarin red staining results qualitatively showed osteogenic differentiation in marrow-derived mesenchymal stem cells in four designed groups. **a:** The red mineralized nodule formation were observed in osteogenic media containing DFDBA, **b:** $Ca(OH)_2$ solution $_+$ DFDBA, **c:** Ca (OH)2 suspension $_+$ DFDBA. **d:** The MSCs in osteogenic media was cultured as positive control.

er doses (0.25 and 0.025mg/ml), proliferation was comparable to the control group. In addition, 0.25mg/ml $Ca(OH)_2$ had better mineralization potency and this was significantly higher for 7.4 pH in comparison to 8.5 pH. [19]

Paula-Silva *et al*. [15] evaluated the effect of different concentrations of $Ca(OH)_2$ on cementoblastic differentiation of PDL mesenchymal cells, observed that $Ca(OH)_2$ with the concentration below 15mM (1.1mg/ml) led to cell viability of more than 90%. A 10-mM (0.74mg/ml) suspension of $Ca(OH)_2$ decreased the cell proliferation at post-culture 48-72 hours; meanwhile, it led to significant cementoblastic differentiation of PDL cells, increasing mineralization. In their study, the pH was adjusted to 7.4 by the buffering agents of culture environment and 5% CO2 incubation. [15]

Ji *et al*. [20] evaluated the effect of low doses of $Ca(OH)_2$ (1-10 µg/ml) on cell proliferation of periodontal stem cells. They concluded that low doses of $Ca(OH)_2$ positively affected the proliferation of pulp and PDL-derived stem cells. They also found that the combination of 10µg/ml $Ca(OH)_2$ and osteogenic media increased the mineralization *in vitro* in comparison with each of them per se. [20] This was not consistent with the study conducted by Narita *et al*. [19] who found that low dose of $Ca(OH)_2$ (0.025 mg/ml) did not influence the mineralization. This could be due to applying $Ca(OH)_2$ with osteogenic media, as well as different *in vitro* conditions in different studies.

The present study, in line with Narita *et al*. [19] and Paula-Silva *et al*., [15] showed that the pH-adjusted solution of $Ca(OH)_2$ yielded better outcomes compared with the suspension form. They found that the $Ca(OH)_2$-induced mineralization was strictly dose-dependent and responsible for osteoblast differentiation through providing extracellular and intracellular Ca^{2+} ions which led to the activation of calcium/ calmodulin pathway. [15, 19] However, previously the induction of hard tissue formation by $Ca(OH)_2$ was mostly attributed to its alkaline pH (hydroxyl ions).

The elevated pH by $Ca(OH)_2$ may not be a problem in pulp-capping or apexification, so that applying $Ca(OH)_2$ adjacent to the pulpal structure leads to the necrosis (2mm), followed by an inflammation zone and eventually hard-tissue formation. Yet, the elevated pH should be considered in periodontal regeneration in order not to disturb the cell viability, migration, and differentiation. [13] The main and interesting result of the study was that combination of 0.5 mg/ml pH-adjusted solution of $Ca(OH)_2$ and DFDBA significantly increased the proliferation and mineralization compared with the DFDBA per se. Further studies are suggested to test it *in vivo*.

Moghadam *et al*. [21] evaluated the effect of adding 0.15% by weight $Ca(OH)_2$ to demineralized bone material (DBM) gel on bone defects in rabbit calvaria. They found no beneficial effect in adding $Ca(OH)_2$ to DBM gel. [21] This could be because of the improper

dose or form of $Ca(OH)_2$, which is highly dose-dependent regarding both cell proliferation and induction of hard tissue formation.

In the present study, the results of cell proliferation in DFDBA group revealed that 10mg/ml concentration of DFDBA caused lower cell proliferation than the control group, and did not induce mineralization. This was in agreement with Carnes *et al.*'s study [22] on 2T9 cells (preosteoblastic cell line derived from transgenic mice calvariae). They reported that the exposure of these cells to DFDBA did not result in mineralization. However, exposure of these cells to soluble BMP-2 led to increased mineralization and alkaline phosphatase activity. Accordingly, they concluded that DFDBA was not likely to liberate soluble BMPs towards osteoinduction, or *in vivo* studies might be more appropriate to determine the osteoinductive properties. [22]

Vaziri *et al.* [23] in a study on the evaluation of osteoinductive property in three different commercial brands of DFDBA (one of them Cenobone- the same brand as the present study) reported that 8 and 16 mg/ml DFDBA decreased the proliferation of osteoblast-like cells. However, in contrast to the present study, they observed increased mineralization in 16mg/ml DFDBA. [23] These differences could be due to different cell lines and different doses administered.

In order to adopt the appropriate dose of DFDBA in *in vitro* assessment, the present study followed Vaziri *et al.* [23] and adopted 16mg/ml at the beginning. However, this dose resulted in very low cell viability and proliferation in hBM-MSCs. Hence, 10mg/ml was chosen according Dereka *et al.*'s study. [24] Other factors likely to be involved in the variability of osteoinductive property of DFDBA were the donor's age, improper processing of DFDBA, and not exposing adequate amount of osteoinductive portions such as BMPs or inadequate Ca^{2+} ions remained in its structure. [9, 25]

This study observed that the low solubility of $Ca(OH)_2$ led to the formation of a suspension when mixing $Ca(OH)_2$ with culture medium. Therefore, $Ca(OH)_2$ was used in two forms of suspension and solution. The amount of ionic dissociation in the suspension form was different from the solution form. The ions in the suspension form were released into the environment over time in a non-linear manner; while, in the solution form, $Ca(OH)_2$ was solved by applying acid, yielding a consistent solution with a higher level of Ca^{2+} ions available.

The present study showed that combination of $Ca(OH)_2$ solution and DFDBA enhanced the osteoinductive activity. However, regarding its use in clinical conditions, especially in regeneration of osseous defects, one should consider the following issues. First, an appropriate combination is required to provide adequate Ca^{2+} ions during the active phase of osseous regeneration *in vivo* in the presence of blood and inflammatory exudate. Second, proper pH for regeneration is necessary as alterations in pH may impair the regeneration process. [2] There is a specific kind of oil-based $Ca(OH)_2$ called Osteora (previously Osteoinductal), which is introduced for periodontal purposes considered to have a milder pH increment. There are controversies about the impact of this product on improving the periodontal regeneration and whether it could release the appropriate content of Ca^{2+} ions in favor of increasing mineralization or not. [14, 26] Therefore, the impact of adding this product to DFDBA on osteoinduction could be assessed in future studies.

Despite the promising findings of this study, it still has some limitations including the undetermined content of Ca^{2+} and OH^- ions in different time spans in $Ca(OH)_2$ suspension groups as well as the Ca^{2+} content in DFDBA group to verify the hypothesis of the study.

Despite the previously conducted studies on the properties of $Ca(OH)_2$ in cell proliferation and osteoblast differentiation, the strength of the present study lied in evaluation of the most effective dosages of $Ca(OH)_2$ with respect to the previous studies. Furthermore, the current study evaluated both solution and suspension forms in order to investigate different aspects of this issue and to examine the previously proposed hypotheses regarding the mechanism of mineralization induction by $Ca(OH)_2$. Besides, the recruited cell line was hBM-MSCs, which are one of the most important cells involved in the process of bone regeneration.

Conclusion

Within the limitations of this study, it seems that the addition of 0.5mg/ml $Ca(OH)_2$ solution to DFDBA is beneficial for improving cell proliferation as well as osteoblast differentiation compared with DFDBA per se.

Owing to the availability and low price of $Ca(OH)_2$, it is recommended that the manufacturers consider Ca^{2+} content of DFDBA and apply DFDBA in intraosseous defects or bone reconstruction surgeries in the combination form to provide appropriate content of Ca^{2+} ions.

Acknowledgements

Conflict of Interest

The authors of this manuscript certify that they have no conflict of interest.

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