Effect of Biodentine Coated with Emdogain on Proliferation and Differentiation of Stem Cells from the Apical Papilla

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Research Article

Keywords: Cell Survival, Stem Cells, Enamel Matrix Proteins, Biodentine

Posted Date: November 12th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-1004605/v1>

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Abstract

Background: This study assessed the effect of Biodentine coated with Emdogain (Biodentine/Emdogain) on proliferation and differentiation of stem cells from the apical papilla (SCAP).

Methods and Results: In this in vitro, experimental study, SCAP were isolated from two immature impacted third molars and cultured. After ensuring the stemness of the cells by assessing their cell surface markers, they were exposed to Biodentine, Emdogain, and Biodentine/Emdogain for 24 and 72 hours. The control cells did not receive any intervention. Cell viability was evaluated by the methyl thiazolyl tetrazolium (MTT) assay. Expression of odontogenic differentiation genes was analyzed by the quantitative reverse transcription polymerase chain reaction (qRT-PCR). Alkaline phosphatase (ALP) activity was quantified by the respective kit. Data were analyzed by one-way ANOVA, t-test, and Mann-Whitney test ($\alpha=0.05$). Cell viability did not change after 24 hours of exposure to biomaterials. At 72 hours, the viability of the cells exposed to Biodentine and Biodentine/Emdogain decreased compared with the control group. The expression of dentin sialophosphoprotein (DSPP), dentin matrix protein 1 (DMP1), and bone sialoprotein (BSP) genes, and ALP activity significantly increased in all three experimental groups, compared with the control group at both 24 and 72 hours; this increase was significantly greater in Biodentine/Emdogain group. The number of mineralized nodules significantly increased in all groups after 72 hours with a greater rate in Biodentine/Emdogain group.

Conclusions: All biomaterials increased the differentiation of SCAP, expression of odontogenic genes, and ALP activity, but Biodentine/Emdogain was significantly more effective for this purpose.

Introduction

Management of immature necrotic teeth is clinically challenging. Such teeth may require endodontic treatment due to trauma, severe anomalies, or extensive caries. Regenerative endodontic procedures (REPs) are the treatment of choice for permanent immature necrotic teeth with open apices to allow completion of root development and reinstate the normal physiological responses [1]. Mesenchymal stem cells required for REPs are isolated from the apical tissue of mature and immature teeth [2]. The apical papilla is the main source of these cells [3]. Evidence shows that the apical papilla remains viable in dental infections in both animal models [4] and humans [3]. Thus, stem cells from the apical papilla (SCAP) are commonly used for REPs. Bioceramic materials especially calcium silicate cements are commonly used for REPs due to favorable properties such as biocompatibility and antimicrobial activity [5–7].

A viable pulp tissue is imperative for root development in immature permanent teeth. Pulp infection or trauma can cease the process of root development and leave an open apex. REPs are increasingly used to allow completion of root development and apex closure by differentiation of stem cells and continuation of mineralization [8–10]. REPs are superior to the old apexification technique since they are faster and reinforce the root structure by increasing the root length and thickness [10–12].

Mineral trioxide aggregate (MTA) is a root-end filling material with optimal biocompatibility, which has been successfully used for single-session apexification. However, MTA is costly, and has a long setting time, difficult handling, and tooth discoloration potential [8, 13]. Also, despite the high success rate of single-session apexification treatment, the root structure remains weak in this procedure [5, 14].

Biodentine is a tricalcium silicate-based cement, which was introduced as a replacement for dentin in 2009. It has shown superior properties to MTA with regard to faster cementum deposition, and higher mechanical resistance [15]. Biodentine is commonly used for apexification and perforation repair, and as a pulp capping agent and regenerative biomaterial [16]. Studies on the optimal concentration of Biodentine for regeneration of the dentin-pulp complex in the clinical setting are limited [17]. However, its optimal efficacy for proliferation, migration, and adhesion of human dental pulp stem cells (DPSCs) has been documented [18].

Emdogain is an enamel matrix derivative derived from the developing porcine enamel matrix. Amelogenin is its main constituent. It also contains matrix metalloproteinases and several growth factors. Emdogain can enhance the migration, attachment, and proliferation of periodontal ligament cells [19], and has also been suggested for induction of pulp regeneration. Evidence shows that amelogenin particles participate in proliferation and maturation of DPSCs [20].

Considering the positive role of Emdogain and Biodentine in REPs, this study sought to assess the effects of Biodentine, Emdogain and Biodentine coated with Emdogain (Biodentine/Emdogain) on proliferation and differentiation of SCAP.

Materials And Methods

This in vitro experimental study was conducted on SCAP isolated from two sound immature impacted mandibular third molars scheduled for extraction. The patients signed informed consent forms and consented to the use of their extracted teeth for research purposes. The sample size was calculated according to previous studies, and three repetitions were considered for each test at each assessment time point [21, 22]. The study was approved by the Ethics Committee of Hamadan University of Medical Sciences (IR.UMSHA.REC.1398.787).

Cell culture:

The extracted teeth were immediately rinsed with sterile phosphate buffered saline (PBS; Gibco BRL, Grand Island, NY, USA) and stored in it. Stem cells were isolated from the apical papilla by enzymatic digestion using 2 mg/mL of type I collagenase (Worthington Biomedical, Lakewood, NJ, USA) and placed in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY, USA). The cells were then re-cultured in the culture medium supplemented with 15% fetal bovine serum (Gibco, Grand Island, NY, USA) and then in alpha-minimum essential medium supplemented with 10% fetal bovine serum in sterile cell culture flasks (SPL Life Science, Gyeonggi-do, South Korea). The culture medium was refreshed every 2-3 days, and the cells were passaged after 1 week. After 4 passages, the cells reached adequate confluence,

and 4- μ m insert plates (SPL Life Science, Gyeonggi-do, South Korea) were used for treatment of the cells with the respective biomaterials.

Assessment of stemness:

After the cells reached 80% confluence, the culture medium was removed from the flask, and the cells were rinsed with PBS twice. The cells were detached from the bottom of the flask using trypsin/EDTA, and the culture medium was added to the flask. The cell suspension was then transferred into 15 mL Falcon tubes and centrifuged at 1200 g for 6 minutes. The cell sediment was rinsed with PBS twice, and the cells were then evaluated for stem cell specific markers (CD105, and CD90) and hematopoietic cell markers (CD45 and CD34). SCAP expressed the mesenchymal cell markers and were negative for the hematopoietic cell markers.

Grouping:

The cells in the control group did not receive any treatment. The cells in the three experimental groups were treated with Emdogain (Biora AB, Malmo, Sweden), Biodentine (Septodont, Saint-Maur-des-Fosses, France), and a combination of Emdogain and Biodentine. Each test was repeated in triplicate for each group [22, 23].

Assessment of cell viability:

The methyl thiazolyl tetrazolium (MTT) assay was used for assessment of cell viability. For this purpose, the cells were cultured in a 96-well plate and treated with Emdogain, Biodentine, and a combination of the two. After 24 and 72 hours, 10 λ of the MTT solution was added to all wells, and the plate was incubated at 37°C. After 2 hours, the overlaying medium was removed and replaced with 100 λ of dimethyl sulfoxide. After 20 minutes, the percentage of cell viability was determined by reading the optical density of the solution at 570 nm wavelength.

Assessment of the expression of odontogenic genes:

After treatment of the cells with the biomaterials, RNA was extracted by Trizol, and the amount of extracted total RNA was quantified by NanoDrop at 260 and 280 nm wavelengths. The cDNA was then synthesized by superscript II first-strand cDNA synthesis kit (Invitrogen, CA, USA) according to the manufacturer's instructions. The expression of DMP1, BSP, and DSPP genes was quantified by using the beta-actin gene as the housekeeping gene. The cDNA was quantified by real-time polymerase chain reaction using specific primers.

Assessment of alkaline phosphatase (ALP) activity:

The ALP activity was assessed by the ALP staining kit (Sigma–Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. The cells were rinsed with PBS twice and incubated overnight with 0.2% TritonX-100 (Jiancheng, Nanjing, China) at 37°C. The working solution was then added, and the absorbance was read by an automatic microplate reader (BioTek, Winooski, VT, USA) at 520 nm wavelength.

Assessment of odontogenic-osteogenic differentiation:

The cells were cultured in a 24-well plate and after exposure to biomaterials, osteogenic-odontogenic medium including regular medium containing 10 mM beta-glycerophosphate (Sigma–Aldrich, St. Louis, MO, USA), 10 nM dexamethasone (Sigma–Aldrich, St. Louis, MO, USA) and 50 mg/mL ascorbic acid was added to the cells. The culture medium was refreshed every 72 hours. After 21 days, the cells were fixed with 4% formaldehyde, rinsed with PBS, and incubated with Alizarin Red stain at room temperature for 15 minutes. The differentiated cells were stained red due to the presence of calcium deposits [24].

Statistical analysis:

The four groups were compared regarding cell viability and expression of odontogenic genes by t-test in case of normal distribution of data and by the Mann-Whitney test for non-normally distributed data.

Results

Cell viability:

At 24 and 72 hours, Emdogain had no significant difference with the control group with respect to cell viability; however, treatment with Biodentine and Biodentine/Emdogain significantly decreased the cell viability compared with the control group. At 24 and 72 hours, Emdogain showed significantly higher cell viability than the Biodentine and Biodentine/Emdogain groups. Also, at both 24 and 72 hours, the Biodentine/Emdogain group showed significantly higher cell viability than the Biodentine group.

One-way ANOVA revealed a significant difference among the groups in cell viability at both 24 and 72 hours. Pairwise comparisons by the Tukey's test revealed significant differences between the control and Emdogain/Biodentine groups at both 24 and 72 hours, and Biodentine and control groups at both 24 and 72 hours. At both 24 and 72 hours, the biocompatibility of Emdogain was higher than Biodentine. Also, the biocompatibility of Biodentine/Emdogain was significantly lower than the control group at both time points. No other significant differences were noted (Figure 1).

Expression of odontogenic differentiation genes:

BSP: Greater expression of BSP gene was noted in the experimental groups compared with the control group at both 24 and 72 hours (Figure 2). The maximum expression of BSP gene was noted in the Emdogain/Biodentine group followed by the Emdogain, Biodentine, and control group. One-way ANOVA revealed a significant difference in BSP gene expression among the groups at both 24 and 72 hours. Tukey's test revealed significantly higher expression of BSP gene in the Emdogain/Biodentine, Emdogain, and Biodentine groups compared with the control group. Also, BSP gene expression in the Emdogain group was higher than the Biodentine group.

DMP1: Greater expression of DMP1 was noted in the experimental groups compared with the control group at both 24 and 72 hours (Figure 3). Maximum expression of DMP1 gene was noted in the Emdogain/Biodentine group followed by the Emdogain, Biodentine, and control group.

One-way ANOVA revealed a significant difference in expression of DMP1 among the groups at both 24 and 72 hours. Tukey's test revealed significantly higher expression of DMP1 in Emdogain/Biodentine, Emdogain, and Biodentine groups compared with the control group at both time points. Also, the expression of DMP1 was significantly greater in the Emdogain than Biodentine group.

DSPP: Greater expression of DSPP was noted in the experimental groups compared with the control group at both 24 and 72 hours (Figure 4). Maximum expression of DSPP gene was noted in the Emdogain/Biodentine group followed by the Emdogain, Biodentine, and control group.

One-way ANOVA revealed a significant difference in expression of DSPP among the groups at both 24 and 72 hours. Tukey's test revealed significantly higher expression of DMP1 in Emdogain/Biodentine, Emdogain, and Biodentine groups compared with the control group at both time points. Also, the expression of DMP1 was significantly greater in the Emdogain than Biodentine group.

ALP activity:

An increase in ALP activity was noted in the experimental groups at both 24 and 72 hours, (Figure 5). The maximum ALP activity was noted in the Emdogain/Biodentine group followed by the Emdogain, Biodentine, and control group.

One-way ANOVA revealed a significant difference in ALP activity among the groups at both 24 and 72 hours. Tukey's test revealed significantly higher ALP activity in Emdogain/Biodentine, Emdogain, and Biodentine groups compared with the control group at both time points. Also, ALP activity was significantly greater in the Emdogain than Biodentine group.

Odontogenic-osteogenic differentiation:

Alizarin Red staining revealed an increase in the number of calcified nodules in all experimental groups at 24 and 72 hours. The number of calcified nodules in the Biodentine/Emdogain group was greater than other groups at both time points (Figure 6).

Discussion

This study assessed the effects of Biodentine, Emdogain and Biodentine coated with Emdogain (Biodentine/Emdogain) on proliferation and differentiation of SCAP. Assessment of cell viability by the MTT assay, which is highly reliable for this purpose [25], revealed that after the control group, Emdogain had the highest biocompatibility followed by Emdogain/Biodentine. The Emdogain group had no significant difference with the control group in this regard. However, the Emdogain/Biodentine and Biodentine groups had significantly lower biocompatibility than the control group. Also, Biodentine had significantly lower biocompatibility than Emdogain. Thus, it appears that Biodentine is cytotoxic early

after use; however, this effect seems to be alleviated by addition of Emdogain due to its proliferative effect. The biocompatibility of Emdogain at 24 hours was lower than that at 72 hours, which indicates that Emdogain has some cytotoxic effects at first, which are neutralized after 72 hours due to chemical stabilization of the cement.

Emdogain has properties similar to that of extracellular matrix, and regulates the proliferation, migration and differentiation of osteoblasts [26]. Wang et al. [27] demonstrated that Emdogain enhanced the mineralization of pulp cells [27]. The effects of Emdogain in combination with Biodentine are probably due to the possible molecular mechanisms and release of growth factors [28]. Karkehabadi et al. [29] indicated that addition of Emdogain to different biomaterials did not affect the cell viability after 24 and 48 hours; however, it significantly enhanced the cell viability at 7 days. Their results were different from the present findings since a reduction in cell viability occurred at 72 hours in the Emdogain/Biodentine group in the present study. It appears that cell proliferation neutralized the cytotoxic effects at 7 days. Difference between the two studies may be due to the fact that Karkehabadi et al. [29] evaluated DPSCs; while, SCAP were assessed in the present study. The present results regarding no cytotoxicity of biomaterials at 24 hours were in accordance with the findings of Saberi et al, [30] who found no significant difference in cytotoxicity of different biomaterials and the control group at 24, 48 and 168 hours. However, in the present study, Biodentine and Biodentine/Emdogain showed cytotoxicity at 72 hours, which was different from their results at 168 hours, and may be due to the fact that cell proliferation and release of calcium ions are considerably lower at 72 hours compared with 168 hours. The present results were also different from the findings of Mohamed and Fayyad [31] who reported a reduction in cell viability at 24 hours, which was compensated by cell proliferation on the next day. This difference may be due to the use of different cell types (DPSCs).

The rate of release of calcium ions at different time points may explain the variations in cell viability in presence of different cements. Calcium silicate-based cements (such as Biodentine) continuously release calcium ions [32]. Calcium silicate hydrate is then formed, and calcium carbonate phosphate deposits. Release of calcium ions can induce inflammatory toxic reactions [33]; however, it is also critical for the viability of mesenchymal stem cells [34]. Calcium ions play a fundamental role in signaling pathways and regulation of cellular activities such as cell migration [35].

ALP activity was also evaluated in this study since ALP is the primary marker of osteogenic differentiation [36]. The present results indicated a significant increase in ALP activity in all experimental groups at 24 and 72 hours. The maximum ALP activity was noted in the Emdogain/Biodentine group followed by Emdogain, Biodentine and finally the control group. These findings highlight the role of Emdogain and Biodentine (alone or in combination) in enhancement of pulp and dentin regeneration. Similar to the present study, Li et al. [37] indicated a significant increase in ALP activity of DPSCs after 7 days of incubation with Emdogain compared with the control group, and Miller et al. [38] reported higher ALP activity of SCAP induced by some bioceramic materials. Moreover, Wu et al. [36] reported significant enhancement of ALP activity for 3 hours caused by Emdogain. Min et al. [39] demonstrated that

Emdogain + MTA significantly increased the ALP activity, which was in line with the present findings regarding the use of Biodentine/Emdogain.

Osteogenic-odontogenic differentiation of SCAP was also evaluated in this study by assessment of the expression of DSPP, BSP, and DMP genes, which play a fundamental role in odontoblastic differentiation and dentin mineralization [40]. The results indicated a significant increase in BSP expression at both 24 and 72 hours in all experimental groups, compared with the control group. This up-regulation was significantly greater in the Biodentine/Emdogain group followed by the Emdogain group. Min et al. [39] reported up-regulation of BSP gene in the MTA and MTA/Emdogain groups, which reached its maximum level after 3 days. Wang et al. [27] reported the up-regulation of odontoblast-like and osteoblast-like cell markers by Emdogain. Also, Jue et al. [41] demonstrated over-expression of BSP by human mesenchymal stem cells exposed to Emdogain. The abovementioned findings all support the present results.

The current results also revealed up-regulation of DMP1 at both time points in all experimental groups compared with the control group. The expression of DMP1 was maximum in the Emdogain/Biodentine group followed by the Emdogain group. DMP1 has a regulatory role in the mineralization process of reparative dentin, and is an odontoblastic marker [42]. The present results regarding the over-expression of DMP1 was in agreement with the findings of Asgary et al, [43] although they evaluated DPSCs.

Expression of DSPP indicates the presence of mature osteoblasts, and is correlated with dentinogenesis [44, 45]. Up-regulation of DSPP was also noted in the experimental groups, compared with the control group at both time points, which was maximum in the Biodentine/Emdogain group followed by the Emdogain group. This finding was in accordance with the results of Miller et al, [38] who reported the over-expression of DSPP by SCAP in presence of Biodentine and EndoSequence, Hajizadeh et al, [46] who reported the over-expression of DSPP by SCAP after 3 weeks of using different biomaterials, and Saberi et al, [47] who showed the up-regulation of osteogenic markers by SCAP due to exposure to Biodentine and MTA.

The mechanism of action of Emdogain in odontoblastic-osteoblastic differentiation has not been well elucidated. It may directly stimulate the odontoblasts or pulp cells to produce collagen matrix [48]. Alternatively, presence of transforming growth factor B1 or amelogenin peptides in Emdogain may induce cell signaling and matrix formation, and lead to subsequent mineralization [49].

Alizarin Red staining of odontoblast-like cells was also performed in this study. This test reveals calcium deposits in the extracellular matrix [50]. The results indicated staining of all experimental groups.

Overall, Biodentine has some toxic effects on the stem cells, which subsides over time. In the long-term, use of Emdogain can help alleviate this effect by induction of cell proliferation. On the other hand, combined use of Emdogain and Biodentine can have a synergistic effect on expression of odontoblastic markers and formation of calcified nodules, which is probably due to the release of calcium ions from Biodentine and growth factors from Emdogain.

This study evaluated cell viability, gene expression, and mineralization after 24 and 72 hours of exposure of SCAP to biomaterials. Future studies are required to assess the effects of biomaterials over longer periods of time. Also, the possible synergistic effects of other biomaterials should be investigated in future studies on different types of stem cells.

Conclusion

All biomaterials increased the differentiation of SCAP, expression of odontogenic genes, and ALP activity but Biodentine/Emdogain was significantly more effective than each biomaterial alone in the latter two parameters.

Declarations

Conflict of interest

Hamed Karkehabadi, Erfan Ahmadyani, Rezvan Najafi, Elham Khoshbin declare that they have no conflict of interest.

Funding Statement

This study was financially supported from the School of Dentistry, Hamadan University of Medical Sciences, Hamadan, Iran

Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent

Informed consent was obtained from all individual participants included in the study.

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Figures

Figure 1

Viability of SCAP exposed to different biomaterials for 24 and 72 hours. *** $P < 0.001$ compared with the control group. ** $P < 0.01$ compared with the control group. * $P < 0.05$ compared with the control group.

Figure 2

Mean expression of BSP gene in the study groups at 24 and 72 hours. *** $P < 0.001$ compared with the control group. ** $P < 0.01$ compared with the control group. * $P < 0.05$ compared with the control group

Figure 3

Mean expression of DMP1 gene in the study groups at 24 and 72 hours. *** $P < 0.001$ compared with the control group. ** $P < 0.01$ compared with the control group. * $P < 0.05$ compared with the control group

Figure 4

Mean expression of DSPP gene in the study groups at 24 and 72 hours. *** $P < 0.001$ compared with the control group. ** $P < 0.01$ compared with the control group.

Figure 5

Mean ALP activity of SCAP in the study groups at 24 and 72 hours. ***P < 0.001 compared with the control group.

Figure 6

Results of Alizarin Red staining

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