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**ORIGINAL ARTICLE**

# ABM/P-15 modulates proliferation and mRNA synthesis of growth factors of periodontal ligament cells

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**Abstract**

***Objective.*** Periodontal regeneration is histologically defined as regeneration of the tooth supporting structures, including alveolar bone, periodontal ligament, and cementum. Cells in the remaining periodontal tissues need optimal conditions if they are to perform their functions in the regeneration process. The present study is an investigation of the molecular effects of ABM/P-15 on human periodontal ligament cells (PDL) *in vitro*. ***Material and methods.*** PDL cells obtained from healthy subjects were used for *in vitro* experiments. Cell proliferation, morphology, and mineralization using Von kossa staining were evaluated. mRNA expressions for transforming growth factor-β (TGF-β), insulin-like growth factor-I (IGF-I), basic fibroblast growth factor (b-FGF), vascular endothelial growth factor (VEGF), bone morphogenic protein-2 (BMP-2), platelet-derived growth factor (PDGF), and type 1 collagen (COL1) were assessed on days 3 and 7 using RT-PCR. ***Results.*** ABM/P-15 enhanced proliferation of cultured PDL cells. It increased the mRNA expression of TGF-β and BMP-2 in cultured PDL cells on days 3 and 7. IGF-I and b-FGF mRNA expressions showed a slight decrease, while PDGF expression was observed to have increased on day 3. VEGF and COL1 mRNA expressions were found not to be different on days 3 and 7. No differences were observed in the mineralization properties of cultured PDL cells treated with or without ABM/P-15. ***Conclusions.*** Based on the results of this *in vitro* study, it may be concluded that ABM/P-15 enhanced the regenerative capacity of PDL by regulating specific gene expressions of cells during early wound healing.

**Key Words:** *ABM/P-15, bone-grafting material, growth and differentiation factors, periodontal ligament cells, periodontal regeneration*

## Introduction

The ultimate goal of periodontal therapy is to restore periodontal tissues affected by disease to their original architectural form and function. This requires regeneration of the gingival connective tissues, formation of the cementum, restoration of the lost bone, and establishment of connective tissue fibers in previously diseased root surfaces. Periodontal regeneration is a possible objective of several periodontal therapeutic approaches; however, outcomes of these therapies are not always predictable [1]. Although the exact role of specific cells in the regeneration process is yet to be established, some studies show that periodontal ligament cells have the capacity to function as osteoblasts and/or cementoblasts under regenerative conditions [2–5].

Freshly harvested autologous bone is a widely accepted bone substitute in periodontal surgical procedures [6]. However, the limited amount of bone that can be harvested and morbidity in the donor site are considered as drawbacks of using autologous bone [7]. As a consequence, alternative materials to autologous bone have been investigated [8]. Bone replacement grafts (BRG), including autografts, allografts, xenografts, and alloplasts, are widely used treatment options for the correction of periodontal osseous defects [9].

ABM/P-15 is a commercial product developed by combining 1 g of anorganic bovine-derived

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hydroxyapatite matrix (ABM) with 200 ng of synthetic cell binding peptide (P-15). P-15 is a synthetic linear polypeptide that mimics the sequence contained in residues 766–780 of the α1 chain of Type 1 collagen [10]. The matrix with the immol/Lobilized peptide is assumed to mimic the properties of collagen in promoting cell attachment, migration, and differentiation [11]. It has been reported that ABM/P-15 promotes concentration-dependent binding of cultured human dermal fibroblasts [10] and, in addition, the attachment of periodontal ligament cells on ABM [12]. *In vitro* studies have demonstrated that ABM/P-15 is superior to ABM alone in inducing more DNA synthesis and in enhancing alkaline phosphatase activity in cultured dermal fibroblasts [13]. These studies have revealed greater osteogenic properties in association with a mineralization-related membrane protein [14] and stimulated more TGF-β1 expression of rat osteoblast cells compared to other bovine-derived bone grafts [15]. In addition, Carinci et al. [16] have provided molecular information regarding ABM/P-15 by demonstrating that a limited number of genes involved in cytoskeleton, extracellular matrix, and apoptosis are regulated by ABM/P-15.

Use of ABM/P-15 in periodontal infrabony defects gives rise to better clinical results than using anorganic bovine mineral alone, demineralized freeze-dried bone allografts or open-flap debridement [17]. Clinical studies have shown the positive effects of ABM/P-15 on the healing of periodontal defects and experimental cranial bone defects and sinus floor augmentations [11,18,19]. Yukna et al., too, have shown the benefits of ABM/P-15 in periodontal regeneration in a human clinical trial study by histological evaluation [20]. However, the number of studies evaluating mechanisms of how ABM/P-15 modifies functions of periodontal ligament cells and promotes regeneration is limited.

The present study investigates the effects of ABM/P-15 on (1) the proliferation and (2) the mineralization of periodontal ligament cells. An additional aim is to improve our understanding of how AMB/P-15 regulates growth factors, which are markers of periodontal regeneration *in vitro*.

## Material and methods

### *Isolation of PDL cells*

Periodontal ligament cells were obtained from the root surfaces of healthy premolar teeth extracted for orthodontic reasons [21]. Extracted teeth were placed in a tube containing biopsy media [Dulbecco's Modified Eagle's Media (DMEM) with 10% fetal bovine serum, 250 μg/ml gentamycin sulphate, 5 μg/ml amphoterycin B, 100 units/ml penicillin and 100 μg/ml streptomycin (Sigma-Aldrich, St. Louis, Mo., USA)]. Periodontal ligament from the middle third of the roots was curetted with a sterile Gracey curette (Hu-Friedy, Chicago, Ill., USA). The obtained tissue was cut into small pieces, rinsed three times in biopsy media, and cultured in petri dishes. PDL cells were incubated overnight in biopsy medium in a humidified atmosphere of 95% air and 5% $CO_2$ at 37°C. On the next day, biopsy media were replaced with culture media (DMEM with 10% fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin). Cells were passaged with 0.25% trypsin – 1% ethylene diaminotetraacetic acid (EDTA) (Sigma-Aldrich, St. Louis, Mo., USA). Periodontal ligament cells from the third to fifth passages were used for further experiments. All experiments were done in triplicate.

### *Cell morphology*

Images of PDL cells treated with DMEM containing 5% FBS and 5% FBS + ABM/P-15 (50 mg/ml) were examined visually on day 7 using a phase contrast microscope. Morphology of the PDL cells and their relationship with ABM-P/15 particles were also investigated.

### *Proliferation assay*

Proliferation assay was performed using 24 well tissue culture plates. $5 \times 10^4$ PDL cells were seeded into each well containing 50 mg/well of ABM/P-15 (PepGen P-15, DENTSPLY Friadent, Cologne, Germany). Cells seeded into empty wells were used as the control group. After seeding of the cells, 2 ml of culture media was added to the wells and these incubated for up to 10 days. Cells were stained with trypan blue (Sigma-Aldrich, St. Louis, Mo., USA) in order to determine cell viability, and viable cells were counted using a hemocytometer on days 1, 6, 8, and 10.

### *Mineralization assay*

For mineralization assay, $1 \times 10^4$ PDL cells were seeded into each well of 24 well plates containing 50 mg of ABM/P-15. Mineralization media were prepared by adding 10 mmol/l β-glycerophosphate and 50 μl/ml ascorbic acid (AA) (Sigma-Aldrich, St. Louis, Mo., USA) to the culture media. The Von Kossa staining method [22] was used to evaluate mineralized nodule formation. PDL cells in the wells were fixed with 95% ethanol; after fixation, 5% $AgNO_3$ was added to the wells to render the newly formed mineralized nodules visible on the first, second, third, and fourth weeks.

### *RNA isolation*

Total RNA from PDL cells was isolated on days 3 and 7 using guanidine isothiocyanate (Sigma-Aldrich, St. Louis, Mo., USA). Medium was aspirated

from 100 mmol/l petri dishes (Corning, N.Y., USA) and cells were washed in PBS (Biological Industries, Migdal HaEmek, Israel). After the addition of guanidine isothiocyanate, the cells were scraped with a cell scraper (Corning, N.Y., USA) that had been cleaned with RNAse Zap and rinsed in diethyl pyrocarbonate-treated distilled water (DEPC-dd $H_2O$). One-fifth volume of chloroform was added to the homogenate, and the samples were incubated at room temperature for 2–3 min. The suspension was spun at 10,400*g* for 15 min at 4°C. The RNA containing aqueous phase was removed and isopropanol was added (half of the volume of aqueous phase). Samples were incubated at room temperature for 10 min and spun at 10,400*g* for 15 min. The supernatant was removed and the RNA pellet washed in 75% ethanol. The precipitated RNA was dissolved in DEPC-treated $H_2O$. Total RNA was quantified at 260 nm using a spectrophotometer. RNA samples were stored at −70°C.

*cDNA synthesis for RT-PCR*

cDNA was synthesized in accordance with the reverse transcriptase enzyme (Promega, Madison, Wisc., USA) procedure using a thermal cycler (Eppendorf AG, Hamburg, Germany). Reactions were carried out in 20 μl synthesis mixtures containing 1 × reaction buffer, 3 mmol/l $MgCl_2$, 0.5 mmol/L dNTP, 1 μl (500 μg/ml) random hexamer, 1 unit/μl recombinant rnasin (ribonuclease inhibitor) (Promega, Madison, Wisc., USA), 1 μl RT enzyme and 1 μg total RNA. RNA samples and primers were first combined in a separate tube in 5 μl volume, incubated at 70°C for 5 min, and held on ice until their addition to the reaction mixture, which contained the other reaction components. When combined, primers were annealed at 25°C for 5 min and then incubated at 42°C for 60 min before heating at 70°C for 15 min. cDNA samples were stored at –20°C until used for reverse transcriptase-polymerase chain reaction (RT-PCR).

## RT- PCR

Semiquantitive RT-PCR was performed for collagen Type I (COL1), insulin-like growth factor-I (IGF-I), basic-fibroblast growth factor (b-FGF), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), bone morphogenic growth factor-2 (BMP-2), and transforming growth factor-beta (TGF-β). Amplification reactions were performed in a final volume of 25 μl containing 0.5 μl cDNA, 25 mmol/l of each dNTP (Larova, Biochemie GmbH, Germany), 50 pmol of each forward and reverse primer, 1.5 mmol/l $MgCl_2$ (Bioron GmbH, Germany), 1 × reaction buffer and 1U of Taq DNA polymerase (Bioron GmbH, Germany). The RT-PCR products (18 μl) resolved by electrophoresis in 1% agarose gels were stained with ethidium bromide. Gel photographs were taken using a gel documentation system (UVP Gel Documentation System-8000; Calif., USA).

The intensities of the photographed bands were analysed using SCION IMAGE analysis software (Scion Image Beta 4.0.2; Scion Corporation, Frederick, Md., USA). The level of mRNA expression of each gene was calculated as the ratio of band intensity of the gene to 15S mRNA band intensity. This intensity ratio of the target mRNA to 15S mRNA was expressed as the relative rate of that in the control cells.

## Statistical analysis

Proliferation and mineralization assays were carried out in triplicate. Statistical analysis was by Student's *t*-test and the Graph-Pad Instat (GraphPad Software San Diego, Calif., USA) statististics program with significance at $p<0.05$.

## Results

*Cell morphology*

The PDL cells treated with ABM/P-15 showed spindle-shaped, densely packaged and well-oriented morphology microscopically. Morphologically, ABM/P-15 did not affect the fibroblastic morphology of PDL cells, and the cells were well oriented with the ABM/P-15, as can be seen in Figure 1A–D.

*Proliferation assay*

To evaluate proliferation of the PDL, $5 \times 10^4$ cells were plated in each well and the culture medium was changed at 2-day intervals. Cells were counted on days 1, 6, 8, and 10. PDL cell counts and proliferation results are shown in Figure 2. Statistical analysis showed a statistically significant increase in cell counts ($p<0.05$) in the cultures treated with ABM/P-15 on days 6, 8, and 10 when compared to the control group.

*Mineralization assay*

The Von Kossa staining method was used to show the mineralized nodule formation at the first, second, third, and fourth weeks. Mineral-like nodule formation was not observed on PDL cells treated with or without ABM/P-15 when stained with $AgNO_3$ (Figure 3).

*mRNA expression of growth factors*

Increased TGF-β and BMP-2 mRNA expressions were noted, compared to the control group, in PDL cells treated with ABM/P-15 on day 3 and day 7 (Figures 4, 5). A slight decrease in IGF-I and

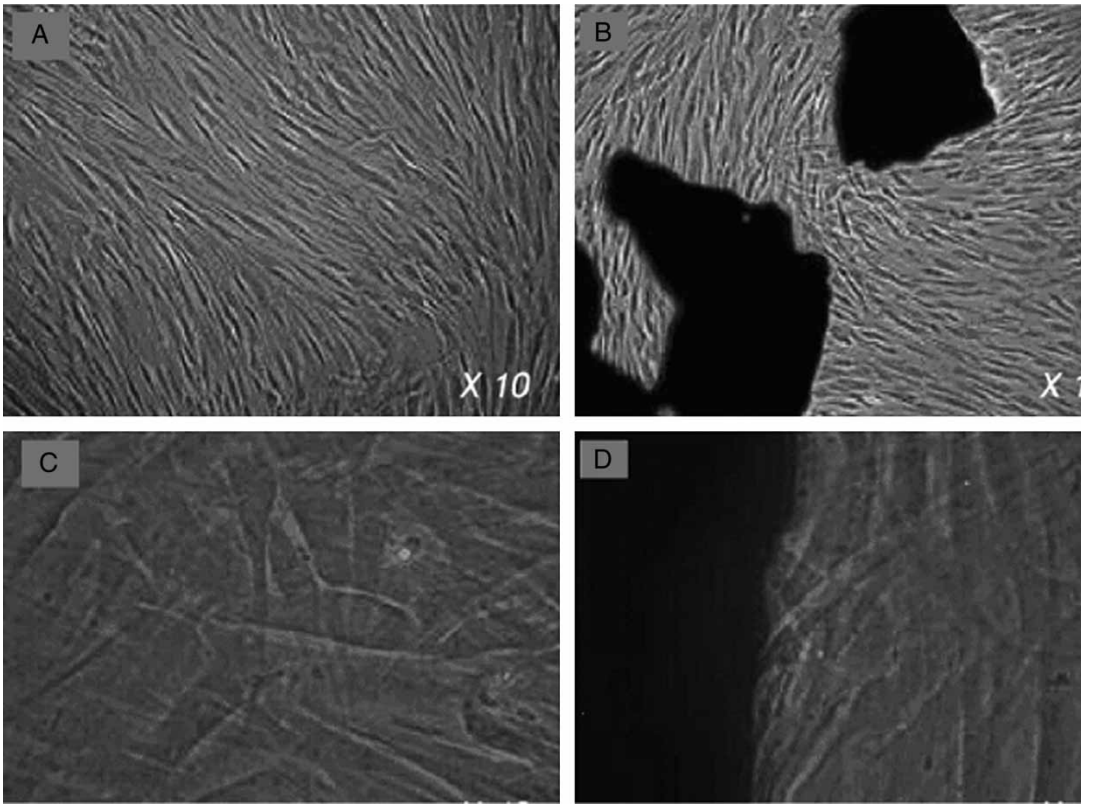



Figure 1. On day 7, the PDL cells treated with ABM/P-15 showed spindle-shaped, densely packaged and well-oriented morphology, where a close contact with ABM/P-15 particles was observed (B, D). A and C show PDL cells in the control group on day 7.

b-FGF expressions was observed on day 3, while PDGF showed an increased expression on the same day in the ABM/P-15 treated group compared to control. There were no differences detected between ABM/P-15 treated and control cultures on day 7 regarding expressions of I-IGF, b-FGF, and PDGF (Figures 5, 6). COL1 and VEGF mRNA expressions did not indicate any differences between groups on days 3 or 7 (Figures 4, 6). The expression rates of growth factors in the control and ABM/P-15 treated groups on days 3 and 7 are illustrated in Figure 7. The mRNA expression changes in the ABM/P-15 treated group relative to the control group were analyzed and relative rates were recorded as 1.37, 1.18, 1.11, and 0.85 on day 3, and 1.07, 1.18, 0.99, and 0.96 for BMP-2, TGF-β, PDGF, and IGF-I, respectively, on day 7 (Figure 8).

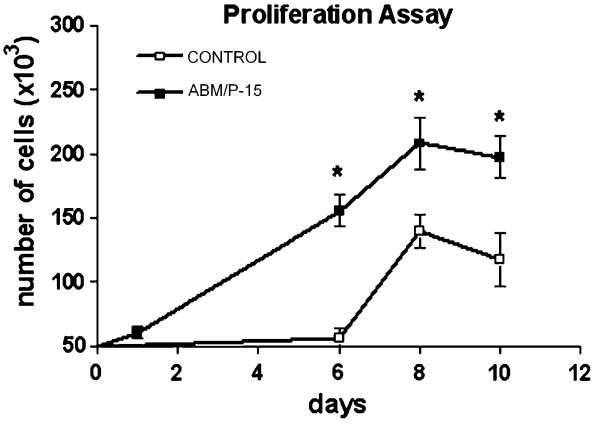



Figure 2. Significant differences between the test and control group cell counts on day 6, 8, and 10 (*$p < 0.05$).

## Discussion

The results of the present study indicate that PDL cells treated with ABM/P-15 show spindle-shaped, densely packaged and well-oriented morphology microscopically. This finding is in agreement with studies reporting that ABM/P-15 is biocompatible [23] and has the potential to promote fibroblast attachment [24]. A significant increase in proliferation was detected on days 6, 8, and 10 ($p < 0.05$) in ABM/P-15 treated cultures compared to the control group. In general, the promotion of PDL cell proliferation can enhance periodontal regeneration by inhibiting the apical migration of epithelial and fibroblast cells from the gingiva. Thus, periodontal defects will be repopulated with cells that have regenerative capacity. Previously, Lallier et al. reported that ABM/P-15 promoted a rapid dynamic attachment to PDL, but not proliferation within 4 days [12]. Cell counts were determined on days 1, 6, 8, and 10 in the ABM/P-15 and control groups. Although our proliferation assay demonstrated that PDL cell counts in both groups were similar on day 1, the ABM/P-15 group had higher cell counts compared to the control group and the differences were statistically significant on days 6, 8, and 10.

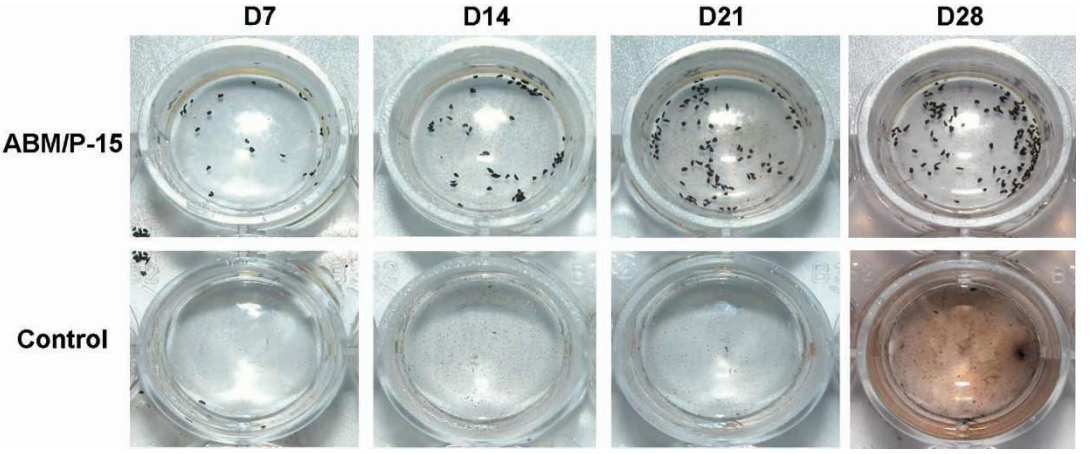



Figure 3. No mineralization nodules were observed in the ABM/P-15 group. Only the ABM/P-15 particles have been stained in the test group. In the control group, the PDL cells started to have mineral-like nodules on day 14. Note the more evident staining on days 21 and 28 in the control group.

The difference in cell counts between the groups could have started any time between the 1st and 6th days. Increased proliferation with ABM/P-15 treatment is further supported by Kubler et al., who demonstrated that the ABM/P-15 reflects the highest proliferation and differentiation rate when compared to other graft substitutes on human osteoblasts *in vitro* [25].

To understand how ABM/P-15 triggers proliferation and differentiation on PDL cells, the expressions of TGF-β, b-FGF, IGF, PDGF, VEGF, and BMP-2 were investigated. These growth factors are also known to affect the osteogenic cells and are considered crucial in periodontal regeneration [26].

TGF-β is a growth factor that enhances fibroblast and osteoblast proliferation while suppressing epithelial proliferation. During the early stages of bone formation, the action of TGF-β is to recruit and stimulate osteoprogenitor cells to proliferate, providing a pool of early osteoblasts [27]. It has been demonstrated that TGF-β inhibited the mitosis of epithelial and endothelial cells, which indicates that this growth factor is fairly specific on its proliferative effects [28]. Reportedly, TGF-β increased cell proliferation in a time- and dose-dependent manner [29]. Belonging to the same growth factor family as TGF-β, the primary effect of BMPs is on the pluripotent cells that are capable of differentiating into other mesenchymal cell types [30], and BMP-2 can direct these cells to commit to an osteoblastic pathway, as can BMP-4 and BMP-6 [31]. In the present study, we noted that ABM/P-15 induced an

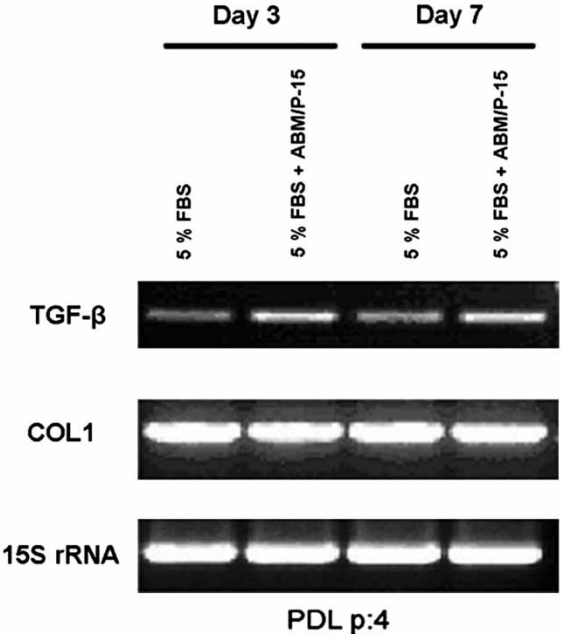



Figure 4. Increased TGF-β1 mRNA expression was observed in PDL cell cultures treated with ABM/P-15 on days 3 and 7. No change in COL1 expression on either of these days.

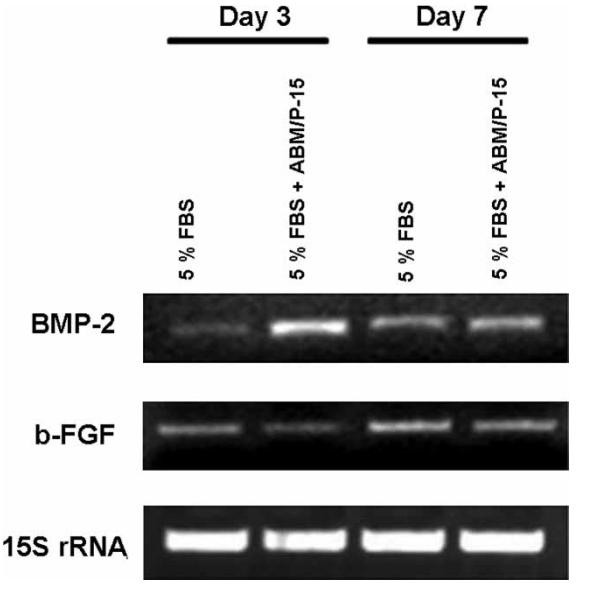



Figure 5. Increased BMP-2 mRNA expression was observed on cultured PDL cells treated with ABM/P-15 on days 3 and 7. b-FGF expression was decreased on day 3, while no changes were observed on day 7 between ABM/P-15 and control cultures.

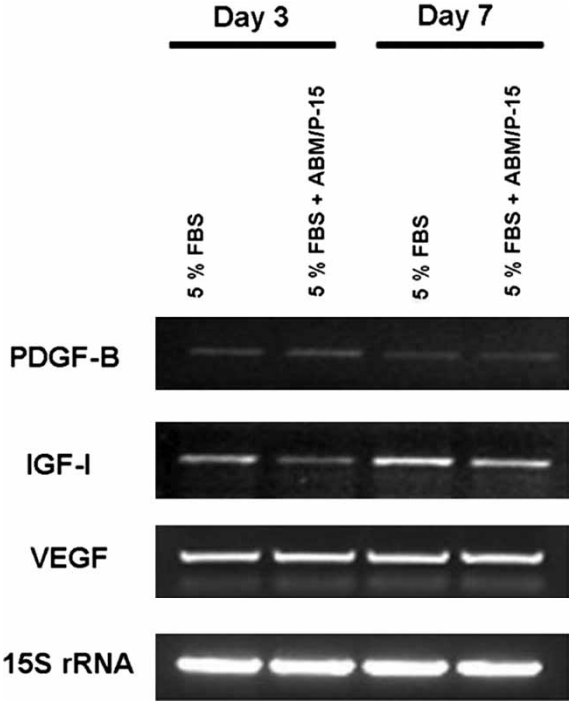
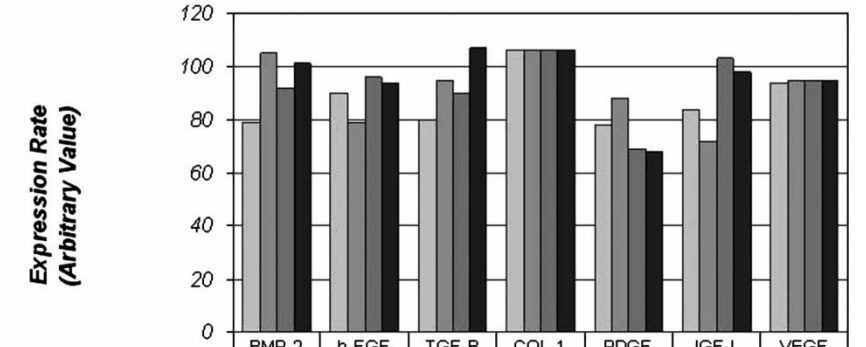



Figure 6. An increase on PDGF and a decrease in IGF-I were observed on day 3, while there were no differences between ABM/P-15 and control cultures on day 7. No changes were observed on either of the days between groups in VEGF expression.

increase in the levels of TGF-β1 and BMP-2 mRNA expressions of PDL cells on days 3 and 7 compared to control cultures. This finding was consistent with another study where ABM/P-15 induced TGF-β1 expression in rat osteoblast cells compared to other bovine-derived bone grafts [15]. Therefore, it may be speculated that ABM/P-15 can promote the proliferation, differentiation, and regenerative capacity of PDL cells by increasing the TGF-β1 and BMP-2 gene expression in the wound environment.

Type-1 collagen is the main protein synthesized by mesenchymal cells and it is the major collagen type constituting periodontal ligament fibers [32]. The present study did not demonstrate any difference in COL1 expression between groups treated with and without ABM/P-15. One explanation could be that ABM/P-15, which upregulates TGF-β1 expression, may still enhance the synthesis of extracellular matrix components other than Type-1 collagen of PDL cells or promote expression of Type 1 collagen from cells other than PDL cells of the periodontium, such as osteoblasts. On the other hand, COL1 expression was observed to reach a plateau as early as day 3 and no change was seen on day 7. It is possible that ABM/P-15 induced COL1 synthesis before day 3 and reached a plateau. We speculate that it would be reasonable to investigate the effect of ABM/P-15 on COL1 expression earlier than day 3.

The differences in expression of b-FGF, IGF-I, and PDGF were more pronounced on day 3, while we did not observe any differences in VEGF expressions on any of the days in cultured PDL treated with and without ABM/P-15. It can be speculated that there was a possible association between the increase in PDGF expression on day 3 and the increase in proliferation on the following days, since PDGF is well known for its mitogenic effect on PDL cells *in vitro* [33]. However, ABM/P-15 revealed a slight decrease in the expression of IGF-I and b-FGF. When the different expression patterns of investigated growth factors are considered, it is important to note that ABM/P-15 has the potential to trigger PDL cell proliferation and differentiation *in vitro*. The effect of ABM/P-15 is more prominent within the earlier days of wound healing. Support for our findings also comes from two studies investigating the effects of ABM/P-15 in osteoblast-like cells




| | BMP-2 | b-FGF | TGF-B | COL-1 | PDGF | IGF-I | VEGF |
|---|---|---|---|---|---|---|---|
| Day 3 %5 FBS | 79 | 90 | 80 | 106 | 78 | 84 | 94 |
| Day 3 %5 FBS+ABM/P-15 | 105 | 79 | 95 | 106 | 88 | 72 | 95 |
| Day 7 %5 FBS | 92 | 96 | 90 | 106 | 69 | 103 | 95 |
| Day 7 %5 FBS+ABM/P-15 | 101 | 94 | 107 | 106 | 68 | 98 | 95 |

Figure 7. Expression rate of growth factors in the control and ABM/P-15 treated groups on days 3 and 7. Expression rates were detected by analyzing the intensity of the mRNA bands and illustrated with arbitrary values obtained using the Scion Image Program.

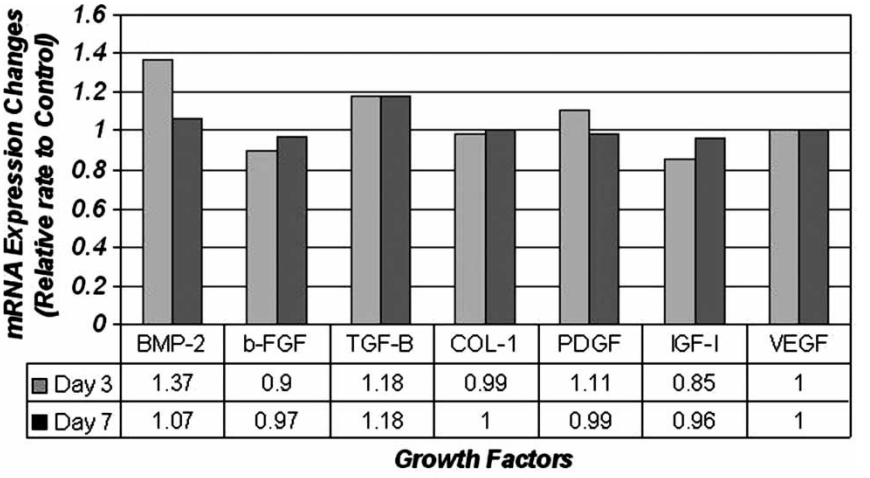



Figure 8. Changes in mRNA expression of growth factors in the ABM/P-15 treated group relative to control on days 3 and 7.

by microarray techniques [16,34]. These studies indicate that ABM/P-15 regulated the expressions of some genes related to bone formation [34] and also genes that have functional activities in signaling transduction, differentiation, apoptosis, and cell cycle regulation [16].

Although mineralization is not observed by PDL cells in normal conditions, in the available circumstances it is reported that PDL cells have the potential to show osteoblastic characteristics and expose mineralization [22]. In the present study, ABM/P-15 treatment did not reveal mineralization in cultured PDL cells. This was not surprising, since it is well known that the factors which induce proliferation of cells generally inhibit the bio-mineralization capacity of cells [35–37]. Recent studies have reported that ABM/P-15 treatment helps to express higher levels of alkaline phosphatase activity on cultured osteoblast cells [25,38] and on cultured PDL cells, which also accompanied an increase in a mineral bound protein called annexin II [14]. Within the mineralization pathway, alkaline phosphatase expression is considered an early differentiation marker of the osteoblastic phenotype, while the von Kossa stain of mineralized nodule formation represents the end differentiation result of the osteoblasts [39]. On the other hand, high levels of ALP activity are needed for the mineralization process [40], and whether the binding *per se* was activated by the calcium channel and led to mineralization or increased mineralization occurred using indirect pathways was unclear [14]. Therefore, it may be concluded that the effects of ABM/P-15 might not be enough for mineralized nodule formation to be observed, or the mineralization effect of ABM/P-15 might be more pronounceable on cells other than PDL fibroblasts.

The *in vitro* events do not mimic the exact actual events *in vivo* but they provide information regarding the diverse responses of cells and cascade of events within the periodontium. Our findings in this *in vitro* study are supported by both experimental animal and human clinical studies which used histological and histomorphometrical parameters to demonstrate that ABM/P-15 particles were in very close contact with newly formed bone tissue. These studies show that ABM/P-15 particles were biocompatible and enhanced initial cell interaction, which may further promote periodontal regeneration [23,41–46]. To the best of our knowledge, given the results of the present study we may speculate that ABM/P-15 induces the regenerative capacity of PDL cells by promoting proliferation and regulating expressions of growth factors, especially in the early days of wound healing.

Previous clinical studies have demonstrated that ABM/P-15 is a suitable BRG material for various regenerative applications. However, very few studies have investigated the effects of ABM/P-15 at cellular level. The results of our *in vitro* studies demonstrate that ABM/P-15 enhances the proliferation of PDL cells. ABM/P-15 also increased TGF-β and BMP-2 mRNA expressions of the PDL cells. These results suggest that ABM/P-15 might enhance the regenerative capacity of the PDL cells by increasing proliferation and inducing their mRNA expressions for growth factors. Further studies investigating the effects of ABM/P-15 on the cellular behavior of cells residing in periodontium other than PDL cells, such as cementoblasts and osteoblasts, can be useful in our understanding of the exact role of ABM/P-15 in regeneration of the periodontium and other sites.

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***Declaration of interest:*** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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