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Effects of Alendronate on Human Alveolar Osteoblastic Cells: Interactions with Platelet-Derived Growth Factor

Laura Barres[1], Anna D.S. Mota[1], Melody Greenberg[1], Saud Almojaly[2], and Rosemary Dziak[1]*

[1]Department of Oral Biology, University at Buffalo School of Dental Medicine, USA
[2]Department of Dentistry, King Faisal Specialist Hospital and Research Centre, USA

***Corresponding author:** Rosemary Dziak, Department of Oral Biology, University at Buffalo, State University of New York, Buffalo, NY, USA, 14214; Tel:716-829-3827; Fax: 716-829-3942; **E-mail:** rdziak@buffalo.edu

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Abstract

Introduction: Bisphosphonates are widely used in the treatment of bone disease due to their inhibitory effects on bone remodeling. Although it is well established that bisphosphonates act by direct effects on osteoclastic cells, there has been increasing evidence suggesting that they may also work on osteoblast cells. The reported effects of these drugs on osteoblast cells are conflicting with increasing number of studies suggesting that at different concentrations, and with different types of bisphosphonates osteoblast differentiation and bone formation activities are varied. Side effects such as osteonecrosis of the jaw are seen with chronic use of bisphosphonates. To better develop approaches to minimize these adverse effects it is important tofurther understand the effects of bisphosphonates on osteoblasts and their modulation by endogenous regulatory factors.

Materials and Methods: Human alveolar osteoblastic cell cultures were treated with the bisphosphonate, alendronate, platelet derived growth factor and a combined treatment of alendronate and platelet derived growth factor. Cell activity was assessed with a mitochrondrial enzyme assay, and differentiation with spectrophotometric assays for alkaline phosphatase and mineralization over a period from 24 hours to 17 days.

Results and Conclusion: Treatment of the osteoblastic cells with alendronate (10^{-8} M) produced small, significant effects on cell activity and markers of differentiation that varied with the time of incubation. The effects of platelet derived growth factor on these same parameters were maintained with co-incubation with alendronate suggesting this growth factor may have a therapeutic role in the minimization of the negative side effects of the drug. These data are supportive of the emerging potential of the clinical use of platelet growth factor enriched plasma for bisphosphonate-induced osteonecrosis of the jaw.

Keywords: Alendronate; Bisphosphonates; Osteoblasts; Platelet-derived growth factor; Osteonecrosis

Abbreviations: BP: Bisphosphonate; ALD: Alendronate; PDGF: Platelet-derived growth factor; BRONJ: Bisphosphonate related osteonecrosis of the jaw

Introduction

Bisphosphonates (BPs) are drugs widely used in the treatment of bone diseases such as osteoporosis, Paget's disease, hypercalcemia associated with malignancy, bone metastasis and loss accompanying multiple myeloma and inflammatory conditions [1-6].

It is well established that BPshave inhibitory effects on bone remodeling via direct effects on osteoclasts, the main bone resorptive cell type [7]. However, since there is much evidence that osteoblasts, the main bone forming cell type, are also involved in the regulation of osteoclastogenesis, the effects of BPs may not be solely onosteoclastic cells. Osteoblasts are involved in osteoclast regulation via their production of nuclear factor (NF)-κB ligand (RANKL) [8]. Osteoblasts have also been widely shown to produce osteoprotegerin (OPG), a soluble receptor for RANKL, that acts as a decoy to competitively inhibit the binding of RANKL to RANK and inhibits RANK activation and thus decreases osteoclast activation [9]. Consistent with these studies are those that have shown that BPs can inhibit the expression of RANKL and increase the expression of OPG in osteoblastic cells [10,11].

Although these results implicate the involvement of osteoblastic cells in the antiresorptive action of BPs via RANKL signaling [10,11], other effects of these agents on osteoblastic cells have not been consistently observed as presented in a recent review [12]. It has been suggested that the conflicting results in osteoblastic cell studies might be due to the bisphosphonate being studied, its effective concentration, as well as the osteoblastic cell type [12]. For example, positive effects of BPs on markers of osteoblastogenesis have been noted with concentrations from 10^{-9} to 10^{-6}M but inhibitory effects at higher concentrations [13-22]. A recent study with alendronate (ALD) shows that this BP affects osteoblasts indirectly through the ephrinB1-EphB pathways involved in the cross talk between osteoclasts and osteoblasts. This study with rat bone marrow cells provides in vitro evidence that ALD acts directly on osteoclastic cell precursors, which then act on osteoblastic cell precursors to inhibit osteoblastic differentiation and related bone formative activities [23].

Although chronic use of BPs has been associated with side effects such as atypical femoral fractures and osteonecrosis of the jaw, these drugs are still widely used because of their generally positive therapeutic effects on management of fractures and associated bone pain.ALD is often the first-line therapy choice for prevention of osteoporotic fractures but it might impart a higher risk of atypical femoral fractures and osteonecrosis of the jaw than other orally administered BPs [24]. In order to develop strategies to minimize the negative effects of BPs it is imperative to have a better understanding of the mechanism of action of these drugs. Toward this end, the effects of ALD on primary cultures of osteoblastic cells from alveolar bone specimens were studied here with a focus on modifications of the responses in the presence of platelet derived growth factor, (PDGF). The

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therapeutic implications of these responses in BP induced osteonecrosis of the jaw (BRONJ) are discussed.

Materials and Methods

Alendronate (ALD) was purchased as alendronate sodium from Sigma -Aldrich (St Louis, MO). The platelet-derived growth factor (PDGF) used here was human recombinant PDGF-BB. This and all other reagents were also purchased from Sigma-Aldrich unless otherwise stated.

Human osteoblastic cells were obtained from alveolar bone specimens using conditions previously described [25]. The use of these specimens, that would have been otherwise discarded, was according to guidelines of the University at Buffalo's Human Subjects Institutional Review Board.

Mineralization assay

The osteoblastic cells were seeded at a concentration of 30,000 cells per well, in a 24 well tissue culture plate. The cells were cultured with ALD (10^{-8} M), PDGF (10^{-8} M) or a combination of the two agents with controls having no added agent in alpha-minimum essential medium (MEM, Gibco, Life Technologies, Grand Island, NY) supplemented with heat activated 10% fetal calf serum, 1% L-glutamine, penicillin G, streptomycin sulfate, and amphotericin B at 37°C with 5% CO_2. In most experiments, the cultures were incubated with replacement of media and treatment solutions every two days. Total incubation periods varied from 10-21 days.

Mineralization was quantified with a colorimetric Alizarin red assay that measures the calcium mineral content associated with the cell cultures using an adaptation of the protocol of Gregory et al [26]. Cell monolayers were washed with phosphate-buffered saline (PBS) and fixed with cold 70% ethanol for one hour. The monolayers were rinsed with highly purified water before the addition of 40mM Alizarin red S (ARS), at pH 4.2. After all the dye was absorbed into the monolayers the wells were each washed 5 times with purified water and once with PBS. The plates were stored at -20^0 prior to dye extraction. Cetylpyridinium chloride (CPC) extraction was used for the destaining. ARS was removed from the monolayers by the addition of CPC (10% w/v, pH 7.0). The plates then were incubated at room temperature with gentle shaking for 1 hour. The absorbance of the CPC extractions was measured at 550 nm [26].

Alkaline phosphatase activity

The human osteoblastic cells were incubated with ALD and PDGFas described above for time periods from 24-72 hours, and analyzed for ALP activity as an indicator of osteoblastic cell differentiation using the para-nitrophenol phosphate assay previously described in detail with normalization of the data on the basis of associated total cell protein [27].

Tetrazolium salt (MTT) assay: This assay was used to assess osteoblastic cell activity. The primary human osteoblastic cells were incubated with ALD, PDGF or combinations of these agents as described above for various time periods. At the end of the experimental period (24, 48 or 72 hrs), MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reagent was added to the cells for 4 hrs and the assay conducted as previously described in detail [28].

Results and Discussion

Figure 1 shows that the effects of ALD (10^{-8} M) on osteoblastic cell activity varied over the course of time of incubation with small, but significant decreases compared to controls after 24 hours of incubation,

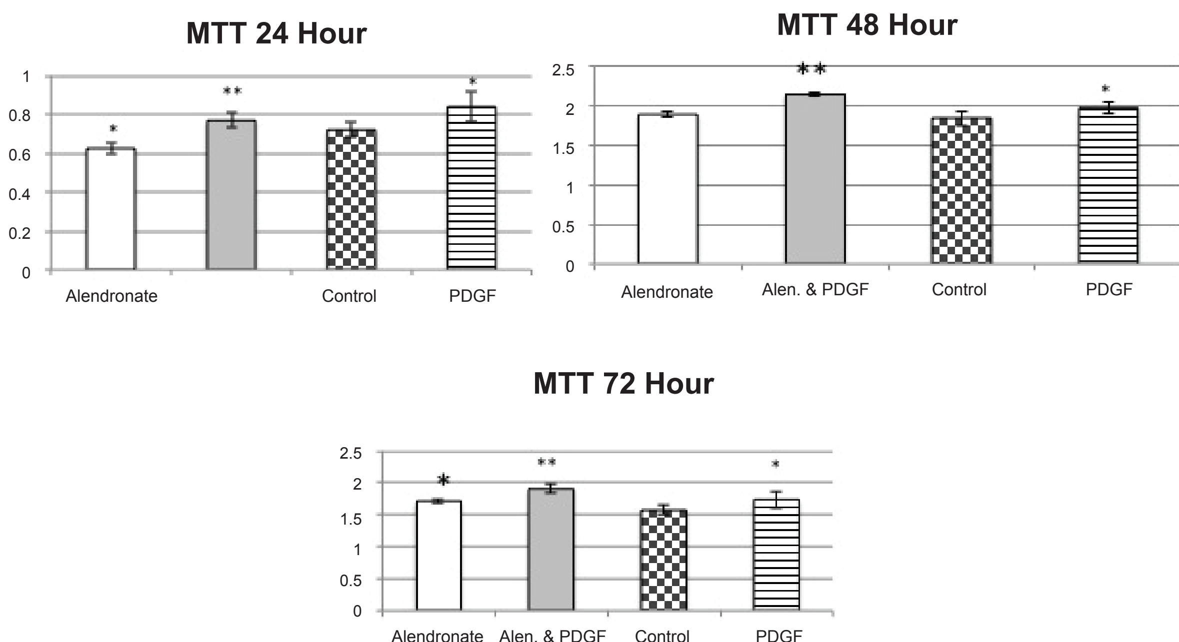

Figure 1: MTT Cell Activity
After 24 hours of incubation, alendronate produced small, significant decreases in cell activity compared to controls and coincubation of alendronate and PDGF resulted in levels significantly greater than with alendronate alone and not significantly different than PDGF alone. After 48 hours there was no effect of alendronate alone compared to controls with PDGF and alendronate together still exhibiting the PDGF induced increases. Small, but significant increases were observed with alendronate after 72 hours with the PDGF induced increase not significantly altered by coincubation with alendronate. Values are the mean +/- SEM with n=4 samples per group: * = significantly different from control; ** = significantly different from alendronate alone; = p<0.05 ANOVA.

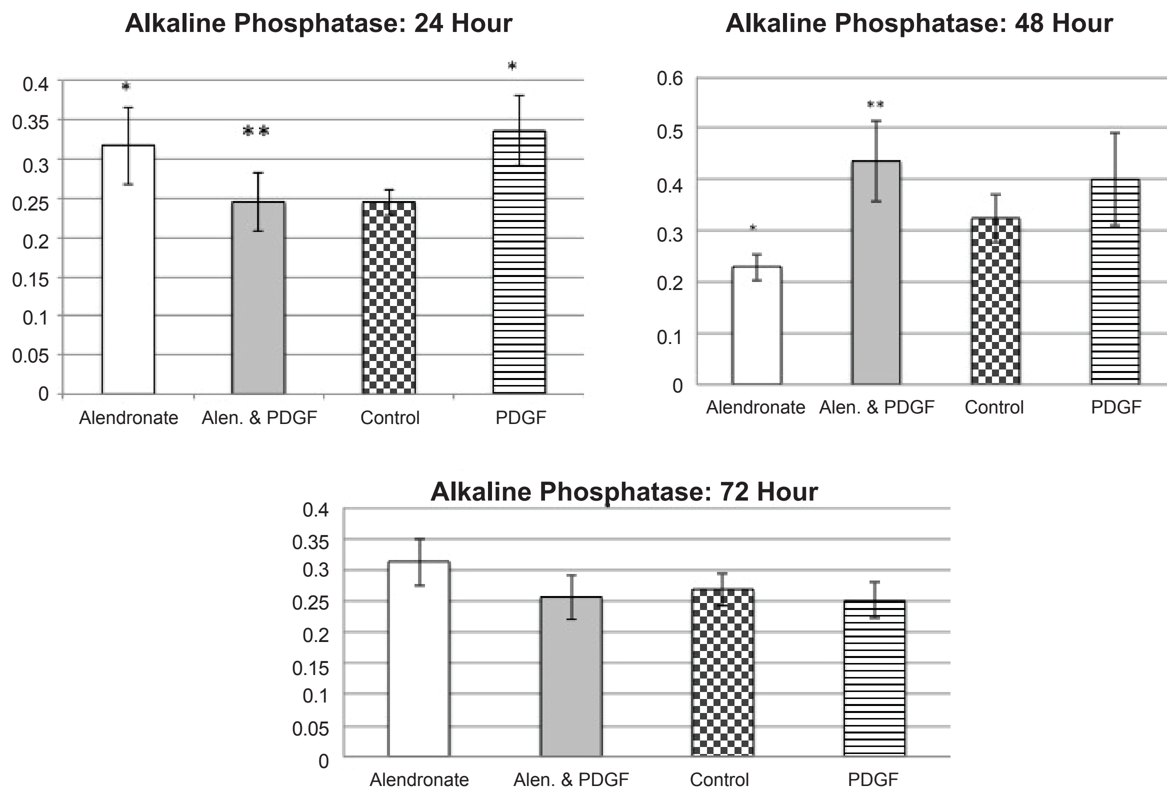

Figure 2: Alkaline Phosphatase Studies
After 24 hours, alendronate produced significant increases in alkaline phosphatase activity compared to controls. PDGF alone produced similar effects but in combination with alendronate there was a significant reduction in this effect to control values. After 48 hours, alendronate significantly decreased alkaline phosphatase levels compared to all groups. The combination of PDGF and alendronate significantly increased alkaline phosphatase compared to alendronate alone. After 72 hours. No significant effects of either alendronate or PDGF were observed. Values are the mean +/- SEM with n=4 samples per group: * = significantly different from control; ** = significantly different from alendronate alone; = p<0.05 ANOVA

no effects after 48 hours and small, but significant increases after 72 hours. At all of these time periods, PDGF (10^{-8} M), produced increases in activity compared to controls and these PDGF-induced increases were not altered in cells cultured in combination with ALD (10^{-8} M).

The effects of ALD and PDGF on ALP activity were also time dependent. Figure 2 shows that after 24 hours of incubation with ALD (10^{-8} M) there was a small, but significant increase in this early marker of osteoblastic cell differentiation. PDGF (10^{-8} M) produced a similar increase over controls. However, at this time period, incubation with the combination of both of these agents, each at 10^{-8} M, resulted in no effect on ALP activity compared to controls. After 48 of incubation with ALD there was a significant decrease in ALP compared to controls. Although the effects of PDGF alone at 10^{-8} M or in combination with ALP were not significant increases compared to controls, they were significantly greater than ALD alone. After 72 hours, there were no significant effects on ALP activitywith any treatment group compared to controls.

Figure 3 shows that a 10-day incubation with ALD (10^{-8}M) resulted in significant decreases in mineralization in the human osteoblastic cell cultures compared to controls. During this same time period, PDGF (10^{-8}M)-treated cells had significant increases in mineralization. When cells were incubated with both agents each at a concentration of 10^{-8} M for the 10-day period, the PDGF-induced increases in mineralization were not significantly altered by the ALD treatment. ALD-induced decreases in osteoblastic cell mineralization were noted when incubations were conducted up to 17 days (data not shown). The results shown in Figure 4 suggest that ALD may not need to be present for the entire duration to achieve a significant decrease in mineralization in this cell system. Incubation with ALD (10^{-8}M) for 7 days, followed by removal of the drug containing media and replacement with fresh media and incubation for an additional 5 days resulted in a similar decrease compared to controls as when the cells were incubated with ALD for the entire 12-day period. When PDGF (10^{-8}M) was added to cultures in which the ALD was removed, there was a significant increase in mineralization during the subsequent 7-day incubation period.

Although there are several BPs presently used for therapeutic management of skeleton related conditions, in this present study the effects of ALD on human alveolar osteoblastic cells was the focus for several reasons. ALD is often the main line choice for oral antiosteoporotic therapy [24]and there are data that suggest that adverse skeletal side effects such as osteonecrosis of the jaw and atypical femoral fractures may be higher with ALD compared to other orally administered BPs such as risedronate, ibandronate, etidronate and clodronate [24].

The underlying mechanisms involved in the adverse side effects on bone are not understood, but it has been reported that ALD has a greater affinity for the tissue along with more significantdecreased bone turnover as well as greater anti-angiogeniceffects in comparison with other BPs that are given orally [29,30].

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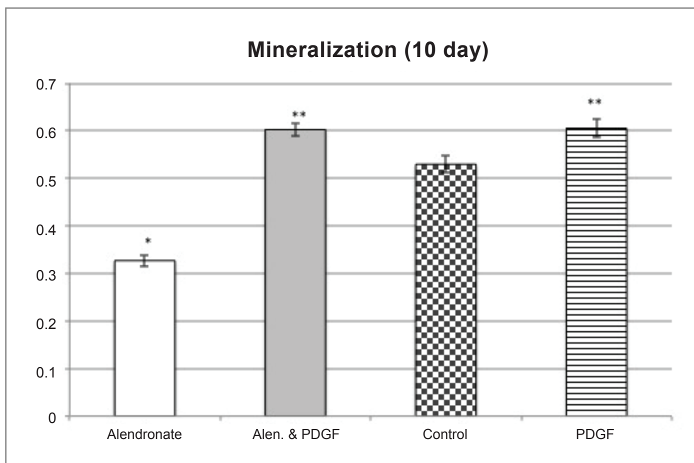

Figure 3: Mineralization Studies
After 10 days of incubation, alendronate produced significant decreases in mineralization compared to controls. Incubation with PDGF for this same period produced significant increases that were not altered with coincubation with alendronate. Values are the mean +/- SEM with n= 4 samples per group: * = decreases compared to controls; **= increases compared to controls as well as alendronate alone; $p<0.05$ ANOVA

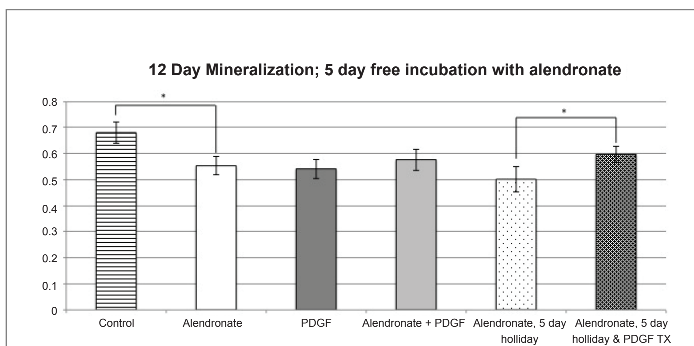

Figure 4: Mineralization Studies with an Alendronate free period
After 12 days of incubation, the alendronate group showed a significant decrease in mineralization. A five-day drug free period (holiday) from the alendronate had no significant effect on mineralization compared to alendronate present for the entire period. The treatment of PDGF during the alendronate free (holiday) period increased mineralization significantly compared to the alendronate 5 day free treated group without the added PDGF. Values are the mean +/- SEM with n= 4 samples per group: * = significant differences; $p<0.05$ ANOVA

The results reported here are consistent with the growing body of studiesthat suggest that ALD can have direct effects on osteoblastic cells and that these effects can vary with the time of incubation and various associated factors [12]. The studies presented here focused on the possible interaction between ALD and PDGF primarily because there is some evidence that PDGF may have some therapeutic value in the healing process in BP- induced osteonecrosis of the jaw [31].

PDGF is present in bone matrix, synthesized not only by platelets, but also monocytes, macrophages, endothelial cells and osteoblasts [32]. The growth factor molecule is a dimer that can be made from four difference polypeptide chains (A,B, C, D). From the different possible combinations of these chains, PDGF-BB appears to be the most biologically potent in the skeleton and has been shown to bind to osteoblasts with the highest affinity [33, 34].It has been shown to be produced at fracture sites and to be present during the early stages of fracture repair [35]. In a rat model, systemic administration of PDGF not only prevented the loss of bone normally induced by ovariectomy, it also maintained bone strength throughout the skeleton. Co administration of PDGF and alendronate in this animal model resulted in bone density levels greater than that seen with either agent alone. These data suggest that PDGF may be effective in producing anabolic effects on bone even in the presence of the bisphosphonate and potentially inhibited bone remodeling activity [36].

Studies have shown that PDGF-BB stimulates chemotaxis and proliferation in osteoblasts and increases collagen synthesis by this cell type [33]. The direct effects of PDGF on differentiation parameters such as alkaline phosphatase and mineralization appear to be more variable depending upon exposure conditions. A study focusing on the expression of these parameters in vitro showed thatshorter-term exposure to PDGF produces increases where asin longer-term incubation there are decreases in these parameters [34]. Based on these observations, it appears that increases in bone formation seen in several in vivo studies are largely due to the increased proliferative effects on osteoblastic cells [34].The studies presented here support the temporal effect of PDGF on alkaline phosphatasewith increases at the earliest measured time period of 24 hours and decreases or no significant effects after longer periods of 48 or 72 hrs. Likewise, PDGF's effects on mineralization were increases after 10 days of incubation, but after 12 days, decreases were observed. The stimulatory effects of PDGF on cell activity observed here are consistent with increases in proliferation over sustained periods of time. Of particular interest to the potential use of PDGF as a therapeutic agent to restore bone healing in BRONJ is that the combination of ALD and PDGF in the present study restores the ALP induced decrease in osteoblastic cell activity after 24 and 48 hours of incubation. In the mineralization study it is particularly interesting to note that after ALD is removed from the osteoblastic cell cultures for 5 days after a 7-day incubation, the addition of PDGF restores the decreased mineralization marker levels to control levels at the end of the total 12-dayperiod. These results are consistent with a previous report that osteoblastic cells isolated from patients with BP-induced osteonecrosis responded to PDGF in a positive manner similar to cells isolated from alveolar bone of persons not treated with BP [37].

As recently reviewed [38] there have been a number of reports on the therapeutic effects of PDGF on the regeneration of alveolar bone, periodontal tissues as well as wound healing in general[39-43]. Local applications of PDGF-BB have been shown to destabilize blood vesselsand result in growth of new vasculature at the site of the healing wound [38].

Since PDGF has been documented to possess amultitude of effects that promote bone and periodontal tissuerepair and regeneration it should be a natural candidate for therapy inoral necrotic conditions although it does not appear that it has been tested directly in this regard. There have, however, been several reports of successful use of platelet rich plasma (PRP) containing relatively high levels of PDGF in addition to other growth factors for the treatment of BRONJ. Adornato et al. [44] treated 12 patients with refractory BRONJ with a combination of bone resection and autologous platelet-derived growth actors.After six months, 10 of the patients had complete recovery of mucosal and bony defects and the remaining 2 showed some improvement in healing. Subsequently, Mozzati et al. [45] reported successful treatment of 32 cases of BRONJ (Marx IIB classification [46]) by application of PRP over the bony defect after resection of the necrotic tissue. An update paper from this group documented freedom from complications and need of reintervention to be 100% in these patients after a 7-year follow up [47]. In addition, another report of 32 successful cases of treatment of BRONJ with PRP is found in the clinical review of Long et al. [48].

It is recognized that clinical use of platelet rich plasma (PRGF) can offer advantages over the use of PDGF alone. PRP contains many growth factors released from activated platelets in addition to PDGF such as transforming growth factor-beta, endothelial growth factor, vascular

endothelial growth factor, insulin-like growth factor-1, basic fibroblast growth factor and hepatocyte growth factor [49].

Studies have reported that bisphosphonates such as pamidronate and zolendronic acid, given to cancerpatients before chemotherapy, can produce significantdecreases in PDGF as well as angiogenic factors such as vascular endothelial growth factor (VEGF) [50,51]. Decreases of this nature in the concentrationsof factors that have significant effects on osteoblastic andosteoclastic cells can influence the overall effects of thebisphosphonates on bone remodeling and lead to anosteonecrotic condition. Local application of PRP on BP induced osteonecrotic wounds, may therefore have significant positive effects of healing of bone and surrounding tissues via increased concentrations of PDGF as well as angiogenic factors such as a VEGF at the compromised site as suggested by the case reports of successful management of this condition reported by an growing number of clinical investigators [44-49,52,53]. Although the number of such cases reported in the literature has rapidly increased, case- control randomized studies to support the use of PRP therapy for BRONJ are still lacking [48].

Conclusion

Direct effects of alendronate on human alveolar osteoblastic cells activity, ALP and mineralization were observed with both increases and decreases depending upon the incubation conditions. hrPDGFmodulated these effects in a manner consistent with what has been observed in clinical reports on therapeutic effects of platelet rich plasma in BRONJ.

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