

# Osteoblast proliferation and apoptosis in platelet-derived growth factor incubated allograft and bioceramic bone grafts in human osteoblast cell culture

## Abstract

**Background:** Several methods exist for tissue regeneration following bone loss in oral cavity. Bioceramic graft materials and allografts are often preferred given their biocompatibility and ease of use in clinical settings. Increasing osteoblastic activity by adding different substances to bone graft materials is a commonly studied topic in medicine. Growth factors play an important role in bone regeneration. The purpose of this study was to investigate osteoblast proliferation and apoptosis in platelet-derived growth factor incubated allograft and tricalcium phosphate bone grafts. We examined the effect of growth factors on human cells *in vitro* at 24, 48 and 72hours. We analyzed 6 main groups and 18 subgroups using MTT and ALP tests. Our results show higher osteoblast proliferation in the control group compared with all other groups. PDGF increased proliferation of osteoblasts in the first 24 h, with no effect after 24h. Tricalcium phosphate graft material increased proliferation in the early period, but reduced proliferation after 24 hours due to phosphate ion release. Our results suggest PDGF, in combination with allograft, is more effective for osteoblast proliferation compared with other materials. Therefore, a carrier material should be used with rhPDGF for most effective treatment outcomes.

**Keywords:** PDGF, allograft, bioceramic, osteoblast, cell culture

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## Introduction

Bone defects resulting from tumors, diseases, infections, trauma, biochemical disorders, and abnormal skeletal development pose a significant health problem.<sup>1,2</sup> Allografts are derived from same species with different genotypes. They are found in many forms, including dust, cortical and cancellous chips, gel, collagen sponge, cancellous and cortical blocks, and demineralized bone matrix.<sup>3,4</sup> Allografts generally have osteoconductive properties.<sup>5</sup> Tricalcium phosphates (TP) is a natural component of hard tissue and constitutes 60-70% of bone.<sup>6</sup> Given that TP has a more porous structure than hydroxyapatite, it undergoes biological degradation 10-20 times more rapidly.<sup>7</sup> Growth and differentiation factors are partially, or completely, inactivate precursor molecules that are activated by a proteolytic mechanism. Growth factors affect cell growth and/or differentiation with local or systemic routes. They play a critical role in tissue repair during embryonic development. Growth factors in bone development include platelet-derived growth factor (PDGF), transforming growth factor (TGF), insulin growth factor (IGF) and bone morphogenetic protein (BMP).<sup>8,9</sup>

PDGF plays a mitogen role for mesenchymal cells by stimulating DNA and protein synthesis in the bone.<sup>10,11</sup> PDGF is a powerful chemotactic factor for osteoblasts, fibroblasts, macrophages, leukocytes and mesenchymal progenitor cells.<sup>12,13</sup> According to experimental research, PDGF plays a role in wound healing and stimulates the formation of new blood vessels.<sup>11</sup> Growth factors within demineralized freeze-dried bone allografts (DFDBA) are extracted during the demineralization process, rendering the allograft incapable of spontaneous osteogenesis. However, adding exogenous

growth factors to DFDBA enhances the osteogenic capacity of native osteoblasts.<sup>1</sup>

The purpose of this study was to analyze osteoblast proliferation and apoptosis in platelet-derived growth factor incubated allograft and tricalcium phosphate bone grafts.

## Methods

This research was conducted in collaboration with Istanbul University, Faculty of Dentistry, Department of Oral and Maxillofacial Surgery and Istanbul University, Institute of Experimental Medicine Research, Department of Genetics. *In vitro* human origin cell cultures were examined at 24, 48, and 72h. Six main groups and 18 subgroups were analyzed with MTT and ALP tests. Friedman test was used for repeat measurements of multiple group comparisons. Kruskal-Wallis test was used for inter-group comparisons. Post Hoc Dunn's multiple comparison test was used for subgroup comparisons. MinerOss (USA)(Size: 0.60-1.25 mm)(1.0 cc) was used as allograft material and CompactBone S (Germany)(Size: 0.50-1.0 mm)(1.0 cc) as tricalcium phosphate graft material. rhPDGF-AA (5µg) was obtained from NovaTeinBio Company. rhPDGF-AA was dissolved in 1 ml sterile distilled water before use according to manufacturers instructions. Sustained human-induced osteoblast cell cultures (hFOB 1.19) were obtained from American Type Culture Collection Company(ATCC) (Rockville, MD, USA).

## The production of continuous cell culture

Osteoblast culture conditions were prepared according to the manufacturer's protocol. Osteoblast cells in 2ml tubes were removed

from liquid nitrogen and placed in 37°C water bath. Cells were cultured in DMEM and F12 medium (1: 1 mixture of the medium) (Lonza, Belgium) (10% fetal bovine serum, 2.5 mM L-glutamine, 0.3 mg/ml G418) and seeded onto tissue culture flasks (25 cm<sup>2</sup>) placed in 5% CO<sub>2</sub> and 95% normal air in a 37°C saturated steam CO<sub>2</sub> incubator (Therma Form Direct Heat Incubator, USA). Cells were divided into 6 main groups, with 18 sub-groups, organized according to 24h, 48h, and 72h *in vitro* examinations.

- i. Group: Control group
- ii. Group: rhPDGF-AA group (only growth factor was applied)
- iii. Group: Allograft group (only allograft was applied)
- iv. Group: Bioceramic group (only tricalcium phosphates was applied)
- v. Group: rhPDGF-AA incubated allograft group
- vi. Group: rhPDGF-AA incubated tricalcium phosphates group

### Osteoblast cells

Developmental stage completed culture flasks were removed from incubation and washed with PBS. The cells were separated from the surface with Trypsin-EDTA solution mixed with 20ml medium, centrifuged at 1500rpm for 3 minutes. One ml of this solution was used for cell viability assay and counted with a Vi-Cell device (Vi-Cell XR, Beckman Coulter, USA). After 2 weeks of passages, cell count was measured at 13.6 x10<sup>6</sup>. The survival rate was 96.7%.

### Osteoblast cell culture

5 x 10<sup>4</sup> cells were added to each well of a 96 well cell culture plate (TPP, Switzerland) in a volume of 200µl. Allograft and tricalcium phosphates grafts were incubated separately in a CO<sub>2</sub> incubator at 37°C for 3hours. Graft materials were incubated with 2ng/ml in concentrated rhPDGF-AA. According to the specified groups, 3mg grafts were placed in each well. The graft materials were measured with a sensitive electronic scale and added to wells with a sterile surgical curette. Each experiment was repeated with 5 samples.

### MTT Test

100µl of media was extracted from from 96 well osteoblast cell culture using mini-pipettes. 10µl of MTT reagent was added, gently mixed in an orbital shaker for 1 minute and incubated for 3-4hours

in a CO<sub>2</sub> incubator at 37°C. Remainder of medium was discarded and 100µl Cristal Dissolving Solution was added to the wells. The wells were measured in ELISA microplate reader (ELX800 Universal Microplate Reader, BIO-TEK Instruments, USA) at 570nm. MTT kit (Cell Proliferation Assay Kit, Cayman, USA).

### ALP Assay

When cell density reached 1x10<sup>5</sup> cells in each well, cells were washed with PBS and homogenized in Assay Buffer. Samples were taken from each 96 well and transferred to 0.2mL eppendorf tubes. Cells were centrifuged for 3min at 13,000g, supernatant discarded and assay buffer added above for a total of 80µl. 50µl of 5mMppNPP solution was added to each well and incubated 60min at 25°C in dark conditions. 20ml of Stop Solution was added to stop reactions. The plate was placed in an orbital shaker for 1minute. Samples were measured on ELISA microplate reader at 405nm. ALP kit (Biovision, USA).

### Statistical methods

Friedman test was used for multiple group comparisons, Kruskal-Wallis test was used for inter-group comparisons and Post Hoc Dunn's multiple comparison tests were used for sub-group comparisons.

## Results

### MTT Assay

The MTT assay measures cell viability. We found the average cell viability in the 24.h control group was significantly higher compared with bioceramic and bioceramic + rhPDGF-AA groups (p=0,016, p=0,009).The 48h control group was significantly higher than all other groups. The 48 h bioceramic+rhPDGF-AA group was significantly lower compared with rhPDGF-AA and Allograft + rhPDGF-AA groups (p=0.009, p=0.047). The 72h rhPDGF-AA group was significantly lower than all other groups (p=0.009, p=0.028) (Table 1). The 24h rhPDGF-AA group was significantly lower than allograft +rhPDGF-AA group (p=0,028), and higher than bioceramic +rhPDGF-AA group (p=0,028). The 72h bioceramic+ rhPDGF-AA group was significantly higher than bioceramic +rhPDGF-AA group (p=0,047). Our results show the 72h bioceramic+rhPDGF-AA group was significantly lower than rhPDGF-AA and Allograft + rhPDGF-AA groups (p=0.009, p=0.047). The 72h rhPDGF-AA group was significantly lower than all other groups (p=0.009, p=0.028). These results show PDGF is least toxic to cells.

**Table 1** MTT assay findings according to Post HocDunn'smultiple comparison test for each experimental group

Post HocDunn's multiple comparison test	24.hours	48.hours	72.hours
control group/rhPDGF-AAgroup	0,175	0,016	0,016
control group/Allograft group	0,076	0,009	0,917
control group/Bioceramicgroup	0,016	0,009	0,465
control group/Allograft +rhPDGF-AAgroup	0,602	0,009	0,754
control group/Bioceramic + rhPDGF-AAgroup	0,009	0,009	0,175
rhPDGF-AAgroup/Allograft group	0,917	0,076	0,009
rhPDGF-AAgroup/Bioceramicgroup	0,175	0,076	0,028
rhPDGF-AAgroup/Allograft +rhPDGF-AAgroup	0,028	0,602	0,009
rhPDGF-AAgroup/Bioceramic	0,028	0,009	0,028
Allograft group/Bioceramicgroup	0,917	0,754	0,175

Table Continued...

Post HocDunn's multiple comparison test	24.hours	48.hours	72.hours
Allograft group/Allograft +rhPDGF-AAgroup	0,117	0,251	0,754
Allograft group/Bioceramic + rhPDGF-AAgroup	0,347	0,347	0,076
Bioceramicgroup/Allograft +rhPDGF-AAgroup	0,009	0,251	0,175
Bioceramicgroup/Bioceramic + rhPDGF-AAgroup	0,076	0,465	0,917
Allograft +rhPDGF-AAgroup/Bioceramic + rhPDGF-AAgroup	0,009	0,047	0,047

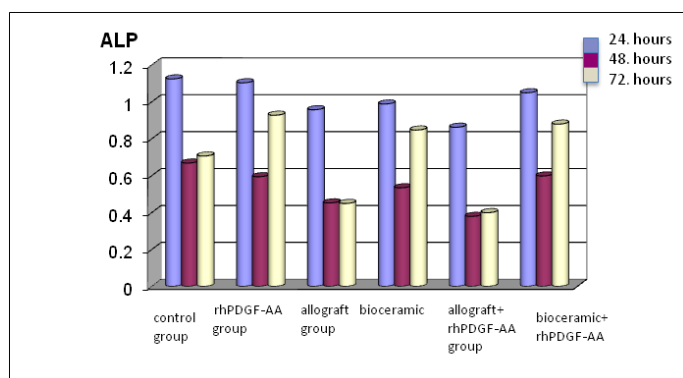
### ALP Assay

We found the 24h allograft +rhPDGF-AA group was significantly lower compared with control and rhPDGF-AA and Bioceramic +rhPDGF-AA groups (p=0.016, p=0.028). Results show the 24h rhPDGF-AA group was significantly higher compared with allograft group (p=0.016). We found no significant differences among other groups (p> 0.05)(Table 2). We found the 48h control group was significantly higher than Allograft, Bioceramic, Allograft + rhPDGF-AA groups (p=0.048, p=0.009). And, the 48h rhPDGF-AA group was significantly higher compared with both allograft and allograft +

rhPDGF-AA groups (p=0.009, p=0.028) (Figure 1). The 48h allograft group was significantly lower than bioceramic and bioceramic + rhPDGF-AA groups (p=0,009). The 72h control group was significantly higher compared with all other groups (p=0,047, p=0,009). The 72h rhPDGF-AA group was significantly higher than allograft and allograft + rhPDGF-AA groups (p=0,009). The 72h allograft group was significantly lower than bioceramic and bioceramic + rhPDGF-AA groups (p=0,009). The 72h allograft + rhPDGF-AA group was significantly lower than bioceramic and bioceramic + rhPDGF-AA groups (p=0,009).

**Table 2** ALP assay findings according to Post HocDunn's multiple comparison test for each experimental group

Post HocDunn's multiple comparison test	24.hours	48.hours	72.hours
control group/rhPDGF-AAgroup	0,465	0,173	0,016
control group/Allograft group	0,117	0,009	0,009
control group/Bioceramicgroup	0,117	0,047	0,047
control group/Allograft +rhPDGF-AAgroup	0,028	0,009	0,016
control group/Bioceramic + rhPDGF-AAgroup	0,251	0,293	0,028
rhPDGF-AAgroup/Allograft group	0,016	0,028	0,009
rhPDGF-AAgroup/Bioceramicgroup	0,117	0,465	0,754
rhPDGF-AA group/Allograft +rhPDGF-AAgroup	0,028	0,009	0,009
rhPDGF-AAgroup/Bioceramic + rhPDGF-AAgroup	0,465	0,754	0,834
Allograft group/Bioceramicgroup	0,754	0,117	0,009
Allograft group/Allograft +rhPDGF-AAgroup	0,347	0,009	0,602
Allograft group/Bioceramic + rhPDGF-AAgroup	0,175	0,047	0,009
Bioceramicgroup/Allograft +rhPDGF-AAgroup	0,076	0,009	0,009
Bioceramicgroup/Bioceramic + rhPDGF-AAgroup	0,251	0,602	0,754
Allograft +rhPDGF-AAgroup/Bioceramic + rhPDGF-AAgroup	0,028	0,009	0,009



**Figure 1** ALP Findings.

### Discussion

Many methods are used for tissue regeneration following bone loss in the oral cavity. Bioceramic graft materials and allografts are often preferred given their biocompatibility and ease of use in clinical settings. Increasing osteoblastic activity by adding substances in bone graft materials is commonly studied in various fields in medicine and biology. Growth factors play an effective role in bone regeneration. Bone tissue regenerates itself. However, in conditions of excessive tissue loss such as traumatic, degenerative, inflammatory, infectious, cystic and neoplastic conditions, the regeneration ability is not sufficient for optimal recovery. Primary cells mimic *in vivo* conditions. However, limited life span, slow growth and genetic differences create problems in the use of primary cells.<sup>14</sup> In this study, we used human-induced permanent osteoblast cell cultures (ATCC, American Type

Culture Collection, Rockville, MD, USA- hFoB 1.19). They have the advantage of having similar metabolic potential to original cells, reproducing very quickly with no genetic differences.

A key component of successful *in vitro* cell culture is cell-material contact. Cell-material contact can be created by solution, direct or indirect methods. Shaini et al.<sup>15</sup> reported that direct contact of cells with the material is a more advantageous strategy of reflecting *in vivo* conditions. Thus, we used the direct contact method in our study to assess the biocompatibility of experimental models with target cells. PDGF is stored in the bone matrix<sup>1,2</sup> and released upon activation of osteoblasts, resulting in new bone formation.<sup>16,17</sup> *In vitro* studies have demonstrated that PDGF initially stimulates bone resorption and stimulates the proliferation and chemotaxis of osteoblasts.<sup>18,19</sup> PDGF is found in high concentrations in the early phase of wound healing and increases the synthesis and release of other supporting growth factors such as IGF. Recombinant PDGF increases resistance to tensile forces on surgery wounds and accelerates healing of chronic wounds.<sup>10,20</sup>

The clinical effectiveness of rhBMP-2 and PDGF vary according to the time of application and duration of release. In the initial hours after wounds, the demolition phase, conditions are hypoxic and acidic. Our results demonstrate that PDGF destruction suffered in the early days. Uchida et al.<sup>21</sup> showed the porosity and grain size of graft materials affects proliferation of osteoblast cells. Pioletti et al.<sup>22</sup> have shown that Calcium phosphate bone graft particles, smaller than 100µ, reduced osteoblast proliferation and alkaline phosphatase activity. In addition, the small particles have a negative impact on the viability of osteoblasts. In our study, Compact Bone S with 500-1000µ particle size and Mineross® with 600-1250µ particle size were used. Bloemers et al.<sup>23</sup> studied the use of calcium phosphate grafts in bone defects from trauma. They reported calcium phosphate is the most preferred graft group because of higher bioavailability and successful osteogenesis. Tricalcium phosphate's porous structure mimicks cancellous bone trabecular structures. The small pores in this material structure allow shape bone cells, nutrients, growth factors, capillary vessels and phagocytic cells to interact.

Knabe et al.<sup>24</sup> studied cell proliferation in two separate studies by generating primary rat bone marrow cells over calcium phosphate graft materials in different compositions for 14days. At the end of the study they found a high rate of phosphate release from all calcium phosphate bone grafts. Tricalcium phosphate graft material induced bone formation. Previous reports show the control group has maximum cell coated surface. Our results are consistent with their findings. In our MTT study, we found the control group had significantly higher viability compared with other groups ( $p=0.016$ ,  $p=0.009$ ). Sun et al.<sup>25</sup> evaluated the biocompatibility of HA and  $\beta$ -TKF in myoblasts and fibroblasts cell cultures. According to ALP assays, the number of cells increased in the first 3days, then decreased. As a result,  $\beta$ -TKF and HA showed inhibitory effects on cell proliferation. They explained the cause of this inhibition in the cells with high rate secretion of Prostaglandin E2. In our study, the ALP average of bioceramic group was significantly higher in the first 3days compared with the allograft group. Zambonin et al.<sup>26</sup> reported tricalcium phosphate, in combination with hydroxyapatite grafts, had phosphate ion oscillation, which inhibited osteoblast proliferation. In our study, osteoblast cell proliferation in bioceramic groups were significantly lower compared with allograft groups.

## Conclusion

- i. Higher osteoblast proliferation in the control group compared

with other groups. In the early period the proliferation of osteoblast cells that don't have contact with graft material and growth factors was higher.

- ii. PDGF increased the proliferation of osteoblasts in the first 24h. After 24h due to the absence of carrier material it is subjected to degradation without any contribution to proliferation.
- iii. Tricalcium phosphate graft material increased proliferation in the early period. However, it played a negative role in proliferation due to phosphate release after 24hours.
- iv. rhPDGF-AA in combination with allograft was more effective and successful in osteoblast proliferation compared with other materials. As a result, carrier material should be used with rhPDGF-AA for optimal effectiveness.

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## Conflicts of interests

Authors declare that they have no conflict of interests.

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