# Effect of rhTGF-β1 combined with bone grafts on human periodontal cell differentiation

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**ABSTRACT**

Various techniques and materials have been proposed for the treatment of periodontal defects. In periodontal regeneration, periodontal ligament (PDL) cell differentiation as well as certain growth factors and their delivery system applied are critical. The purpose of this study was to evaluate the *in vitro* effect of rhTGF-β1 combined with two different bone grafts on human PDL cell differentiation. Human PDL cells were treated with TGF-β1 alone or in combination with a calcified freeze dried bone allograft (FDBA) and a porous biphasic calcium phosphate (BC) bone graft. Cell differentiation effect was estimated by measuring alkaline phosphatase (ALPase) activity and osteocalcin (OCN) secretion. Results demonstrated that rhTGF-β1 alone or in combination with FDBA and BC provoked a significant ($p<0.05$) increase in ALPase activity as compared to controls. The findings of this study confirmed the beneficial role of rhTGF-β1 combined with FDBA and BC as carriers in periodontal regeneration.

## INTRODUCTION

Periodontal repair involves the reformation of lost periodontal supporting tissues including alveolar bone. Previous research studies demonstrated that the entire process is regulated by the production and activity of growth factors present at the defect site. Additionally, growth factors may act as natural biological mediators that regulate chemotaxis, proliferation, differentiation and attachment of various cell types including cells derived from the periodontium. Most importantly, some of these cells type express a prominent osteoblast-like phenotype (Anusaksathien and Giannobile, 2002, Rosenkranz and Kazlauskas, 1999). Such cellular events are required for bone regeneration during periodontal therapy (Sodek and McKee, 2000).

Transforming growth factor-beta 1 (TGF-β1) is a biologically active polypeptide protein hormone which belongs to the family of TGF growth factors. They are related to bone morphogenetic proteins (BMPs) but function in a different way (Howell et al., 1996, Lee, 1997).

TGF- β1 is also considered as a chemotactic agent for bone cells, it increases matrix production and influences their proliferation depending on the cell differentiation state, the culture conditions and its concentration used (Cochran and Wozney, 1999). Furthermore, TGF-β1 has been reported as a potent anabolic factor that enhances connective tissue deposition and repair (Javelaud and Mauviel, 2004, Verrecchia and Mauviel, 2002).

However, its effects on different cell types as well as on PDL cells remain controversial; it has been shown that TGF-β1 stimulates collagen synthesis by PDL cells but still remains unclear whether TGF-β1 enhances PDL proliferation and differentiation

(Howell et al., 1996). Furthermore, it has been evident that carrier vectors for TGF-β1 play a crucial role modifying its activity on cells, in a dose and cell type dependent manner (Tatakis et al., 2000).

As only a limited number of studies have studied the *in vitro* effects of TGF-β1 combined with different carriers on human periodontal cells, an effort was made to investigate the potential effect of TGF-β1 combined with different bone grafts in periodontal repair. In this experimental study, TGF-β1 is combined with a freeze dried bone allograft (FDBA) or a porous biphasic calcium phosphate (BC) bone graft and its *in vitro* effects on the differentiation of human periodontal ligament (hPDL) cells are evaluated.

## MATERIAL AND METHODS

### Growth factors and bone grafts

Recombinant human transforming growth factor-beta 1 (rhTGF- β1) was purchased from a commercial supplier (R&D Systems, Abingdon, UK). Its calculated molecular mass was approximately 25 kDa and its purity was >97%. The endotoxin level was determined <1.0 EU per 1μg of the cytokine.

The bone replacement graft materials tested in this study were:

- A human freeze dried bone allograft (FDBA) supplied from the Human Tissue Bank of the National Centre for Scientific Research 'Demokritos' (Athens, Greece). FDBA is prepared from cancellous bone of femur heads from living donors. Tissue collection and process were accomplished according to European (Directive 23/2004) and International standards for radio-sterile freeze-dried human tissue grafts. The Bank was the first to operate in Greece and so far it has delivered over 40 000 tissue allografts in hospitals to be used in reconstructive surgery.
- A commercially available porous biphasic calcium phosphate bone graft (Bone Ceramic-BC 90% porous, 400-700μm) (Straumann Bone Ceramic®, Berne, Switzerland).

### Human periodontal ligament cells

Human periodontal ligament (hPDL) cells were harvested from four maxillary premolars (Oates et al., 1993, Somerman et al., 1988). Caries-free teeth were extracted for orthodontic reasons from 4 adult patients, 2 males and 2 females (19-23 years of age) with clinically healthy periodontium and without any known medical disorder. The

patients signed an informed consent and the Faculty of Dentistry, University of Athens, approved the experiments of this study.

Under sterile conditions, PDL tissue was mechanically removed by scraping the middle third part of the root surface with a sharp blade No15. PDL cells were grown at $37^0$ C in a humidified atmosphere of 5% $CO_2$, in Eagle's minimal essential medium (MEM) supplemented with L-glutamine (292mg/lt), nonessential amino-acids (10mg/lt), NaHCO2 (2g/lt), penicillin (50mg/lt), streptomycin (50mg/lt) and 10% (v/v) fetal bovine serum (FBS); all media were purchased from Gibco (Paisley, Scotland, U.K.). Cells cultures were also tested periodically and found to be mycoplasma-free. Cells were plated at a density of $10^4$ cells/cm² in MEM containing 10% FBS. After 1 week of growth, cells reached confluence (approximately 7 x $10^4$ cells/cm²). Then the medium was replaced with MEM containing 0.05% FBS in which the cells remained for 2 days. Under these conditions, more than 95% of the cells in culture remain quiescent. Characterization of the cell cultures was also performed by immunohistochemistry for vimentine, osteopontin and bone sialoprotein. All cells used in this study were at 4-6 passages. For each experiment the same passage of cells was used.

Then, cells were grown in the presence or absence of 20mg from each bone allograft separate and maintained quiescent as described above. On day 2 of quiescence new medium was added along with 25ng/ml of rhTGF- β1 and 20mg of FDBA or BC. Supernatant (0.5 ml) was harvested at 3 days and 5 days and stored at -80$^o$C.

**Alkaline phosphatase assay**

Alkaline phosphatase (ALPase) activity was estimated to determine the effect on hPDL cell differentiation. Assay was performed as follows: From each sample, 30 µl of supernatant was selected and pNPP (final concentration 10mmol/ml) in 1300 µl diethalamine buffer (diethanolamine 1 mol/l, $MgCl_2$ 0.5 mmol/l, pH 9.8, 37$^{o}$C) was added as a substrate and incubated at37$^{o}$C for 40min. ALPase activity of the reaction mixture was measured using visual photometer methods at 4065 nm. Each sample was assayed in triplicate and enzyme activity expressed in units, 1 unit being defined as 1 µmol/l para-nitrophenol released per minute (pH 9.8, 37$^{o}$C) (Calcium kit, Sigma and Procedure No 0150, Stanbio Laboratory).

**Osteocalcin detection assay**

Osteocalcin ELISA kits (Takara BioInc., Shiga, Japan) were used to detect OCN levels. The culture medium was collected and measured for OCN levels. These samples were placed in 96-well microtiter plates coated with monoclonal detective antibodies and incubated for 2 h at room temperature. After removing unbound material with washing buffer (50 mM Tris, 200 mM NaCl, and 0.2% Tween 20), horseradish peroxidase conjugated streptavidin was added to bind to the antibodies. Horseradish peroxidase catalyzed the conversion of a chromogenic substrate (tetramethylbenzidine) to a coloured solution, with colour intensity proportional to the amount of protein present in the sample. The absorbance of each well was measured at 450 nm by a plate reader (versa max, Molecular Devices Co., CA, USA).

**Statistical analysis**

All assays were repeated three times to ensure reproducibility. Statistical analysis was performed by a linear regression model with independent variables the day of the measurement, group (treatment) and their interaction. A p-value $< 0.05$ was considered as statistical significant**.** The graph display of the results was performed by box-plot by time and factor.

## RESULTS

During the experiments, the combination of rhTGF- β1 with the bone grafts seemed to be compatible with the cells since their morphology did not change; osteoblastic-like cells with spindle shape and cytoplasmatic extensions could be observed around the grafts.

### Alkaline phosphatase activity

After 3 days of incubation, the presence of rhTGF- β1, FDBA, BC individually or in combination of each bone graft with rhTGF- β1 provoked a statistically significant ($p<0.001$) increase of ALPase activity compared to controls (Figure 1, Table 1 and 2). Furthermore, the enrichment of FDBA and BC with rhTGF- β1 significantly ($p<0.001$) stimulated ALPase activity compared to the effect of FDBA and BC alone. However, the combination of FDBA or BC with rhTGFb1-in did not affect significantly the ALPase activity compared to the effect caused by rhTGF- β1 alone.

At the time-points studied there are no differences of ALPase activity among the grafts as it is shown in Tables 1 and 2

The induced cell response to the agents examined in this study after 5 days of cell culture, is depicted in Table 2. ALPase activity was statistically ($p<0.001$) promoted by the presence of rhTGF- β1, FDBA and BC alone as well as by their combination, compared to controls. The combination of FDBA or BC with rhTGF- β1 does not effect significantly of ALPase activity compared to rhTGF- β1 alone. Furthermore, the combination of FDBA or BC with rhTGF- β1 significantly ($p<0.001$) stimulated ALPase activity compared to FDBA or BC alone.

The comparison of the results in ALPase activity between 3 and 5 days did not reveal any significant difference (Table 2). The presence of BC alone provoked a further increase of ALPase activity on the 5$^{th}$ day (Tables 1 and 2).

**Osteocalcin secretion**

All cell cultures produced similar no comparable small amounts of OC (data no shown).

## DISCUSSION

Periodontal regeneration is the end goal of periodontal therapy. Several procedures for periodontal regeneration have been proposed so far as an essential part of periodontal treatment. Certain procedures include different combinations of growth factors with bone replacement graft materials that may play the important role of carrier molecules for bioactive components. Mechanisms for the delivery of growth factors may include extracellular matrix analogs (Roy et al., 1993), gels (de Obarrio et al., 2000, Weiser et al., 1999), different types of bone grafts (Boyan et al., 1999, Salkeld et al., 2001) and gene delivery systems (Giannobile et al., 2001). Growth factor delivery systems play a prominent role in directing a polypeptidic growth factor to reach the desired defect site within the requisite time and they can further control the release time course to the defect. Thus, it is evident that interaction between growth factors and bone graft materials is of great importance to the regulation of polypeptidic growth factor delivery to the healing site.

TGF-β1 exerts its effects on cell proliferation, differentiation and migration partly due to its ability to modulate the deposition of extracellular matrix components (Verrecchia and Mauviel, 2002). TGF-β1 is expressed during the development of the alveolar bone, PDL and cementum. Also, it is expressed through all stages of tooth formation in the osteoblasts, PDL cells and cementoblasts close to the apical portion of root (Gao et al., 1998). Research results have indicated that TGF-β1 is released from human cultured gingival epithelial sheets in significant amounts suggesting that TGF-β1 may promote wound healing and periodontal tissue regeneration (Momose et al., 2002).

Research data indicates that the effect of TGF- β on cell activities is rather ambiguous depending on the experiment conditions, the type of the cellular population as well as the presence of other growth factors and different carriers (Dereka et al., 2006).

Previous studies have demonstrated a positive effect of rhTGF-β1 on the proliferation of hPDL cells and human gingival fibroblast (GFs) (Marcopoulou et al., 2003). TGF-β1 alone or in combination with PDGF-BB and IGF-I had similar effects on the rate of proliferation and adhesion of hPDL cells (Sant'Ana et al., 2007). The adhesion of hPDL cells was significantly upregulated by the combination of TGF-β1 with enamel matrix derivatives (EMDs), comparatively to EMD alone and other treatments. ALPase and total protein synthesis was significantly increased after EMDs treatment while the addition of TGF-β1 did not ameliorate the behavior of hPDL cell (Rodriguez et al 2007).

Our results demonstrate that TGF-β1 induced a significant increase in ALPase activity compared to control group. Both control and test group produced similar no comparable small amounts of OC after 3 and 5 days of culture. Since the ALPase activity is often used as an early marker of osteoblastic differentiation and OC secretion as a late osteoblastic marker (Mimori et al., 2007, Somerman et al., 1988), the above results are justified. Also, our findings confirm a previous in vitro study where the addition of TGF-β1 (0,0050- 0,1000mg/L) in PDL cells increased significantly the ALPase activity while it did not influence the synthesis of OC (Si and Liu, 2001).

Takeuchi et al. studied the effect of the addition of TGF-β1 on human gingival fibroblast and hPDL cells at 24, 48 and 72 hrs. They found that the expression of connective tissue growth factor, mRNA and protein were increased significantly in a dose- and time-dependent manner (Takeuchi et al., 2009). However, Howell et al. found

that TGF-β1 gave opposed results when studied on different cell types (Howell et al., 1996).

In addition, there are other studies indicating that TGF-b1 stimulated PDL regeneration and repair, by increasing SPARC (secreted protein acidic and rich in cytokine / osteonectin), DNA and fibronectin levels in PDL cells (Fujita et al., 2004), but suppressed the osteoblast-like phenotype of human PDL cells (Brady et al., 1998, Fujita et al., 2004).

Concerning TGF-β1 participation to matrix maintenance, it is indicated that stimulates type I collagen synthesis (Brady et al., 1998) depending on the experiment conditions, the cell line and the stage of the cell development (Javelaud and Mauviel, 2004).

Furthermore, in another *in vitro* study it was found that TGF-β1 and BMP-2 were found to exert opposite effects on osteoblast differentiation and murine cell lines maturation. TGF-β1 was unable to induce ALPase activity and OC secretion. It also inhibited the positive activity of BMP-2 (Spinella-Jaegle et al., 2001). On the other hand, in an animal model, TGF- β1 production by rat osteoblasts was enhanced in the presence of PepGen P-15 (Trasatti et al., 2004). During dog alveolar bone defect repair, the expression of TGF-β1 induced peripheral blood stem cells, in combination with tissue engineered bone, to differentiate and proliferate into osteoblasts. The locally applied TGF-β1 subperiostally in rabbits improves the mineral density of distraction gap (Ozkan et al., 2007).

In this study we have tested as well the effect of FDBA and BC alone or combined with TGF-β1, on the differentiation of hPDL cells. The grafts increased the ALPase

activity but their combination with TGF-β1 did not further enhance the ALPase activity compared to TGF-β1 alone. The presence of BC, used alone, gave rise to a further increase of ALPase activity the 5$^{th}$ day of incubation comparatively to the 3$^{rd}$. All cell cultures produced similar small amounts of OC. The results of this study imply that TGF-β1 combined with FDBA and BC stimulates the early differentiation of hPDL cells. FDBA and BC could, therefore, serve as carriers for TGF-β1.

A previous *in vitro* study demonstrated that FDBA increased the activity of ALPase on hPDL cells, alone or in combination with rhBMP-2. The highest value of ALPase activity, indicating cells differentiation, was noticed between the 3$^{rd}$ and 5$^{th}$ day (Markopoulou et al., 2003). In addition, the combination of FDBA with PDGF-BB showed significant proliferative effect on hPDL cells (Papadopoulos et al., 2003).

From *in vivo* studies it is evident that TGF-β1 effect on periodontal regeneration when used with different carriers is ambiguous. In an animal study, Tatakis et al. implanted TGF-β1 in supra- alveolar periodontal defects in beagle dogs with a calcium carbonate carrier. After 4 weeks they have noticed that there were sites of significant bone and cementum regeneration but elsewhere this phenomenon was minimal. They concluded that TGF-β1 activity is dose, defect type and carrier dependent (Tatakis et al., 2000).

When grafts used as carriers of different growth factors they seem to play an important role. When calcium carbonate was used as carrier in the implantation of TGF-β1 in beagle dogs' defects, periodontal regeneration was accelerated (Koo et al., 2007). In another case a similar graft, tricalcium phosphate combined with PDGF-BB promoted periodontal regeneration in human intraosseous periodontal defects (Ridgway et al.,

2008). In this experimental study, it became evident that the combination of TGF-β1 with bone grafts did not altered the positive activity of TGF-β1 alone. Finally, in previous animal studies it was shown that TGF-β1 had significant effect on periodontal regeneration in combination with other carriers like collagen sponge (Shigeno et al., 2002), hydrogel scaffold (Srouji et al., 2005) and chitozan/collagen scaffold (Zhang et al., 2006).

In addition, it has been hypothesized that the short half-life of TGF-β1 in aqueous solutions has led to a decreased bioactivity. Thus, a controlled presentation of TGF-β1 would maintain its bioactivity, improving its regeneration potential. It becomes evident that carrier molecules of TGF-β1 are critical for its controlled presentation to the healing site. In this way, TGF-β1 maintains its bioactivity and its potential for *in situ* periodontal regeneration is enhanced. Controlled release of TGF-β1 allows also chemotactic agents to recruit host infiltration and enhance regeneration.

Our study clearly demonstrated that the combination of FDBA or BC with TGF-β1 did not increase the activity of APLase significantly comparatively to that of TGF-β1 alone. Further studies are required to evaluate bone grafts as potential TGF-β1 carriers.

## Conflict of interest

The authors declare that there are no conflicts of interest of this study. The authors alone are responsible for the content and writing of the paper.

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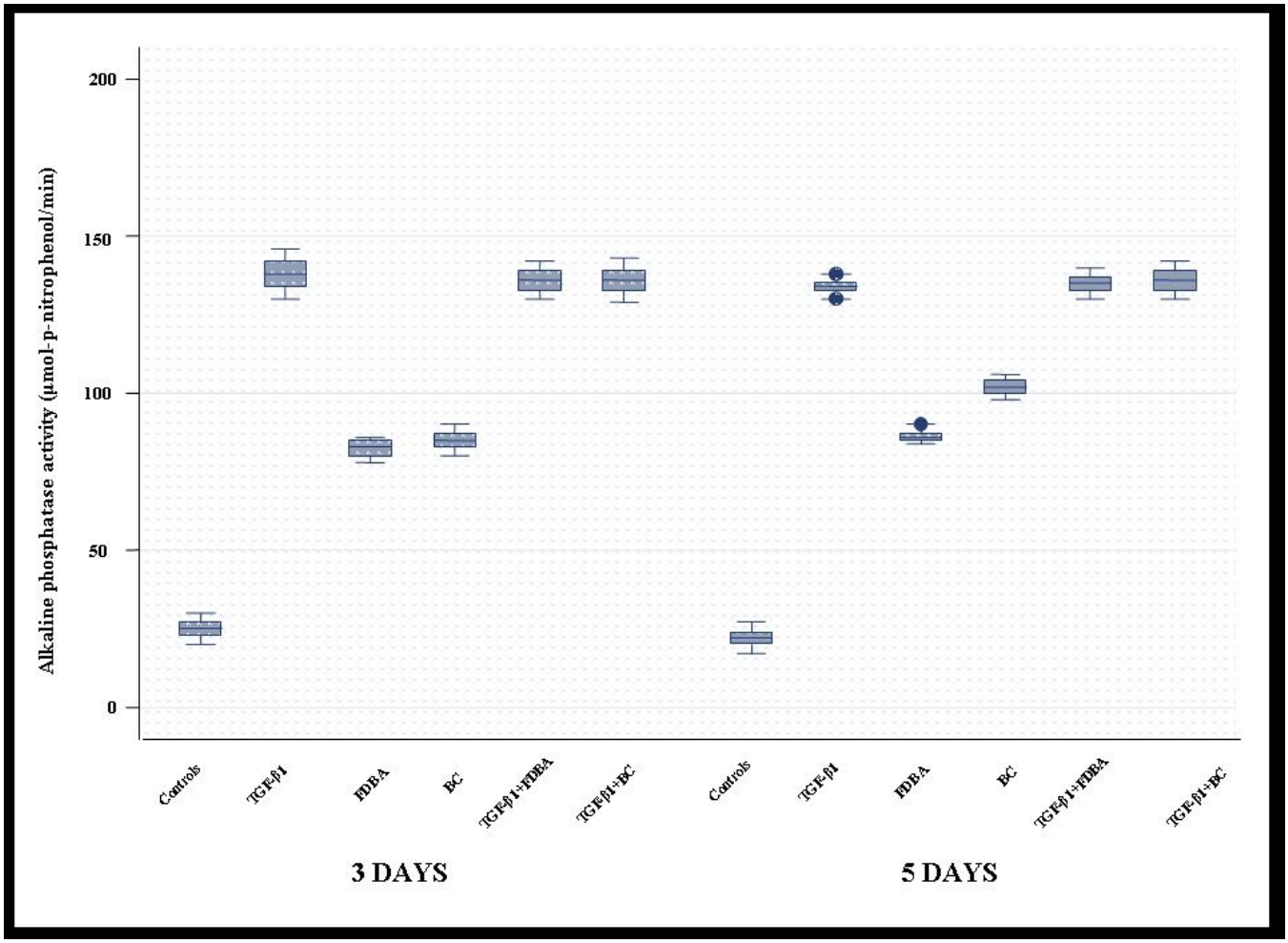


**Figure 1.** Box plot for the colorimetric assay for the determination of alkaline phosphatase activity (µmol-p-nitrophenol/min) at 3 and 5 days.

| | Mean change | 95% C.I. | p-value |
|---|---|---|---|
| **3 DAYS** | | | |
| *TGF-β1 vs. Controls* | 113.00 | (107.72, 118.28) | **<0.001** |
| *FDBA vs. Controls* | 57.40 | (52.12, 62.68) | **<0.001** |
| *BC vs. Controls* | 60.00 | (54.72, 65.28) | **<0.001** |
| *TGF-β1+ FDBA vs. Controls* | 111.00 | (105.72, 116.28) | **<0.001** |
| *TGF-β1+ BC vs. Controls* | 111.00 | (105.72, 116.28) | **<0.001** |
| *FDBA vs. TGF-β1* | -55.60 | (-60.88, -50.32) | **<0.001** |
| *BC vs. TGF-β1* | -53.00 | (-58.28, -47.72) | **<0.001** |
| *TGF-β1+ FDBA vs. TGF-β1* | -2.00 | (-7.28, 3.28) | 0.450 |
| *TGF-β1+ BC vs. TGF-β1* | -2.00 | (-7.28, 3.28) | 0.450 |
| *BC vs. FDBA* | 2.60 | (-2.68, 7.88) | 0.327 |
| *TGF-β1+ FDBA vs. FDBA* | 53.60 | (48.32, 58.88) | **<0.001** |
| *TGF-β1+ BC vs. FDBA* | 53.60 | (48.32, 58.88) | **<0.001** |
| *TGF-β1+ FDBA vs. BC* | 51.00 | (45.72, 56.28) | **<0.001** |
| *TGF-β1+ BC vs. BC* | 51.00 | (45.72, 56.28) | **<0.001** |
| *TGF-β1+ BC vs.TGF-β1 + FDBA* | 0.00 | (-5.28, 5.28) | >0.999 |
| **5 DAYS** | | | |
| *TGF-β1 vs. Controls* | 112.00 | (106.72, 117.28) | **<0.001** |
| *FDBA vs. Controls* | 64.40 | (59.12, 69.68) | **<0.001** |
| *BC vs. Controls* | 80.00 | (74.72, 85.28) | **<0.001** |
| *TGF-β1 + FDBA vs. Controls* | 113.00 | (107.72, 118.28) | **<0.001** |
| *TGF-β1 + BC vs. Controls* | 114.00 | (108.72, 119.28) | **<0.001** |
| *FDBA vs. TGF-β1* | -47.60 | (-52.88, -42.32) | **<0.001** |
| *BC vs. TGF-β1* | -32.00 | (-37.28, -26.72) | **<0.001** |
| *TGF-β1 + FDBA vs. TGF-β1* | 1.00 | (-4.28, 6.28) | 0.705 |
| *TGF-β1 + BC vs. TGF-β1* | 2.00 | (-3.28, 7.28) | 0.450 |
| *BΨ vs. FDBA* | 15.60 | (10.32, 20.88) | **<0.001** |
| *TGF-β1 + FDBA vs. FDBA* | 48.60 | (43.32, 53.88) | **<0.001** |
| *TGF-β1 + BC vs. FDBA* | 49.60 | (44.32, 54.88) | **<0.001** |
| *TGF-β1 + FDBA vs. BC* | 33.00 | (27.72, 38.28) | **<0.001** |
| *TGF-β1 + BC vs. BC* | 34.00 | (28.72, 39.28) | **<0.001** |
| *TGF-β1 + BC vs .TGF-β1+FDBA* | 1.00 | (-4.28, 6.28) | 0.705 |

**Table 1.** Different comparisons of relative changes in alkaline phosphatase activity (μmol-p-nitrophenol/min) at 3 and 5 days.

| | 5 vs 3 DAYS | | |
|---|---|---|---|
| | **Mean change** | **95% C.I.** | **p-value** |
| ***Controls*** | -3.00 | (-8.28, 2.28) | 0.259 |
| ***TGF-β1*** | -4.00 | (-9.28, 1.28) | 0.134 |
| ***FDBA*** | 4.00 | (-1.28, 9.28) | 0.134 |
| ***BC*** | 17.00 | (11.72, 22.28) | **<0.001** |
| ***TGF-β1 + FDBA*** | -1.00 | (-6.28, 4.28) | 0.705 |
| ***TGF-β1 + BC*** | 0.00 | (-5.28, 5.28) | >0.999 |

**Table 2.** Differences in relative changes in alkaline phosphatase activity (µmol-p-nitrophenol/min) between 3 and 5 days.