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# Effects of titanium surface anodization with CaP incorporation on human osteoblastic response

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## Abstract

In this study we investigated whether anodization with calcium phosphate (CaP) incorporation (Vulcano®) enhances growth factors secretion, osteoblast-specific gene expression, and cell viability, when compared to acid etched surfaces (Porous®) and machined surfaces (Screw®) after 3 and 7 days. Results showed significant cell viability for Porous and Vulcano at day 7, when compared with Screw ($p=0.005$). At the same time point, significant differences regarding runt-related transcription factor 2 (*Runx2*), alkaline phosphatase (*ALP*) and bone sialoprotein (*BSP*) expression were found for all surfaces ($p<0.05$), but with greater fold induction for Porous and Vulcano. The secretion of transforming growth factor β1 (TGF-β1) and bone morphogenetic protein 2 (BMP-2) was not significantly affected by surface treatment in any experimental time ($p>0.05$). Although no significant correlation was found for growth factors secretion and *Runx2* expression, a significant positive correlation between this gene and *ALP/BSP* expression showed that their strong association is independent on the type of surface. The incorporation of CaP affected the biological parameters evaluated similar to surfaces just acid etched. The results presented here support the observations that roughness also may play an important role in determining cell response.

### Keywords

Osteoblast; Implant surface; Gene expression; Growth factors; Osseointegration

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## Introduction

A large number of methods have been used over last decade to change dental implant surface texture and chemistry in a concentrated effort to improve the early bone-to-implant response. Specifically to surface chemistry modifications, alteration of the native TiO2 layer [1, 2] and the incorporation of calcium phosphate (CaP) based bioactive ceramics have received significant attention [3, 4]. This interest is in part, because the biocompatibility of titanium is closely related to the properties of the surface oxide layer [5]. Moreover, CaP is known as a bioactive material that interacts with surrounding bone directly, improving the osteoblast cell responses and further osseointegration [6]. Several studies combining surface anodization to change the oxide layer and CaP incorporation and/or deposition have been reported [1, 2, 7–11]. Despite the extensive physical and chemical characterization of these surfaces described in the literature, *in vitro* biological responses to them are still not clarified. Most of the data available are related to early responses, such as cell attachment [1, 7–12], cell shape [1, 9–12], and cell proliferation [1, 2, 8, 9, 11–13]. Although cell adhesion and proliferation on implant surfaces are prerequisites for the initiation of bone regeneration, the challenge in research on dental implants is the surface ability to guide the differentiation [14].

Osteoblast differentiation is tightly controlled by a range of hormones, cytokines, growth factors and multiple transcription factors [15, 16]. *Runx2* (also known as core-binding factor alpha 1; *Cbfa-1*) is a transcription factor whose deletion has been associated with lack of ossification [17, 18]. At early differentiation stage, *Runx2* plays a major role on directing pluripotent mesenchymall cells to the osteoblast lineage and triggering the expression of many extracellular bone matrix protein genes [18]. It is known that the expression of *Runx2* in osteoblastic cells is under the regulation of bone morphogenetic proteins-2 (BMP-2) and transforming growth factor-β1 (TGF-β1) [15, 16]. BMP-2/TGF-β1 shares a common signaling transduction pathway which converges at the *Runx2* gene to control mesenchymal pre-cursor cell differentiation [19].

We hypothesized that anodization with CaP incorporation can enhance BMP-2 and TGF-β1 secretion, which would upregulate *Runx2* expression, and consequently modulate the gene expression of important osteoblast-related matrix proteins, such as alkaline phosphatase (*ALP*) and bone sialoprotein (*BSP*). To test this hypothesis, we investigated three commercially available surfaces in order to analyze whether the combination of roughness and chemical modification, by anodization with CaP incorporation, positively interferes in the process of osteogenesis *in vitro* when compared to surfaces just physically modified (acid etched) and surfaces without treatment (machined).

## Material and methods

### Surface preparation and analysis

Commercially pure grade IV titanium disks (8.0 × 4.0mm) were manufactured for this research by Conexão Sistema de Próteses (São Paulo, SP, Brazil). The specimens (n= 14 disks/group) underwent three types of surface treatment similar to the commercially

available implants: machined (Screw®), acid etched (Porous®) and anodized (Vulcano®). The machined disks (control) were obtained from cpTi bars in a turning procedure and did not receive any additional treatment. The acid etched titanium discs were obtained by immersion in a mixture of HNO3, HCl and H2SO4, resulting in surfaces with a roughness mean (Ra) of approximately 0.67μm. The anodized samples were prepared using micro-arc oxidation with electrolyte solution containing Ca and P at a high anodic forming voltages and current densities in the galvanostatic mode [20], showing surface roughness mean (Ra) of approximately 0.87μm. The roughness, wettability and morphology of the referred implants surfaces were previously evaluated using a laser profilometer, a contact angle goniometer and a scanning electron microscopy, respectively [21]. Surface composition was investigated by X-ray photoelectron spectroscopy [21].

## Cell culture

Human fetal osteoblastic cells line (hFOB 1.19) from American Type Culture Collection (ATCC, Rockville, MD, USA) was used for this investigation. The cells were routinely cultured in Dulbecco's modified Eagle's medium/Hams F12 (DMEM/F12) (Invitrogen, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (Gibco Life Technologies, Grand Island, NY, USA) and antibiotic/antimycotic (penicillin/streptomycin/amphotericin) (Invitrogen) at 37°C, 5% CO2. The culture media was replaced every third day. When nearly confluent, cells were trypsinized, counted and seeded at $2\times10^4$ cells/well over titanium disks in 24-well culture plates (Corning Inc., NY, USA). After 3, and 7 days of culture, supernatant was collected for growth factors quantification. At the same time points, cell viability was assessed, as well as disks with adherent cells and forming tissue layers were collected for RNA isolation and gene expression analysis.

## Cell viability

Viable cells amount was evaluated using 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT) assay. It is based on the reductive cleavage of MTT (a yellow salt) to formazan (a dark blue compound) by mitochondrial dehydrogenase of living cells. Enzyme activity was determined adding 40μL of a 5mg/ml MTT (M-2128, Sigma-Aldrich, St Louis, MO, USA) to each well and incubating the cells at 37°C for 4h. After incubation period, the resulting formazan crystals were dissolved with 400μL/well of dimethyl sulfoxide (DMSO) (Labsynth, Diadema, SP, Brazil). A 100μL aliquot of this solution was transferred to separated wells of a 96-well ELISA plate (Corning Costar, Corning, NY, USA) and the absorbance was measured at 570 nm through a microplate reader (Instrutherm Espectrofotômetro UV-2000A, São Paulo, SP, Brazil). The absorbance levels of each well were proportional to the amount of coloring. Cell viability tests were performed in quadruplicate for each time point.

## RNA isolation and Real-time RT-PCR analysis

Real-time reverse transcription polymerase chain reaction was used to measure the mRNA levels of *Runx2*, *ALP*, *BSP* in cells adherent to titanium disks, in triplicate. Briefly, disks were removed from the culture plates and rinsed twice with cold phosphate-buffered saline (PBS). Adherent cells on each disk were lysed using Trizol (Invitrogen, Carlsbad, CA) and

lysates were collected by pipetting and centrifugation. Total RNA in the cell lysates was isolated according to the manufacturer's protocol and quantified using UV spectrophotometry. From each total RNA sample, cDNA was generated using $RT^2$ First Strand Kit reverse transcriptase (SABiosciences, Frederick, MD) in a standard 20μl reaction using 500ng of the total RNA. Subsequently, equal volumes of cDNA were used to program real-time PCR reactions specific for mRNAs encoding the osteogenic markers: *Runx2*, *ALP*, and *BSP*. Reactions were performed using primers for the above mentioned genes (SABiosciences) and thermocycling in an ABI 7200 real-time thermocycler (Applied Biosystems, Foster City, CA). The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control and its mRNA abundance was used for normalization of each sample. Relative mRNA abundance was determined by the $2^{-\Delta\Delta Ct}$ method. Results were expressed as fold differences of gene expression relative to the result of the machined surface at 3 days of culture.

### Growth factors production

Human specific ELISA kits were used to measure TGF-β1 (e-Bioscience, San Diego, CA, EUA) and BMP-2 (Peprotech, Rocky Hill, NJ, USA) levels produced by the cells, from the supernatant. The assays were performed according to the manufacturer's directions, in quadruplicate. Intensity measurements were conducted at 450nm for TGF-β1 and 405nm for BMP-2 using a microplate reader (Instrutherm Espectrofotômetro UV-2000A). Sample concentrations were determined by comparing the absorbance value to a known concentration standard curve for each growth factor.

### Statistical Analysis

Descriptive statistics were calculated using SigmaPlot 11.0 Software (Systat Software Inc., Chicago, IL, USA). Data were submitted to normality test (Shapiro-Wilk) and equal variance test (Levene). Kruskall-Wallis was applied to compare cell viability at 3 days. TGF-β1 and BMP-2 quantification, as well as cell viability at 7 days, were compared by one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test when necessary. For the gene expression analysis, Student's *t*-test was performed for comparison of mRNA levels when compared with machined surface at 3 days [22]. All variables considered as possibly associated to *Runx2* expression, such as TGF-β1, BMP-2, *ALP*, and *BSP*, were analyzed individually in relation to the transcriptional factor by Pearson's Correlation. For those with significant association ($p<0.05$), Simple Linear Regression was run. Multiple Linear Regression was also run with *Runx2* as the dependent variable, considering TGF-β1 and BMP-2 might influence its expression. For all statistical analysis significance level was set at $p<0.05$.

## Results

### Cell viability

Results concerning cell viability showed greater absorbance levels for Porous and Vulcano than for Screw in both time periods (Fig. 1). Even though, no significant difference among the groups was found at day 3 ($p=0.540$, Fig. 1). At 7 days, there was a significant increase on cell viability for Porous and Vulcano ($p=0.005$, Fig. 1), compared with control group.

However, between Porous and Vulcano there was no statistical difference at 7 days ($p=0.995$).

### Real-time RT-PCR analysis

The surface-specific gene regulation was observed for the three genes evaluated (Fig. 2). One general observation was that differences among the surfaces at day 3 were often of lower magnitude than significant differences observed at 7 days. At 3 days, no statistical differences were found in gene expression among the groups when compared to machined day 3 (control). However, at day 7, all the surfaces presented a marked increase ($p<0.05$) on the mRNA levels for the three genes studied, when compared with Screw 3 days. At 7 days, *Runx2* relative mRNAs levels (Fig. 2a) were upregulated in hFOB on Porous and Vulcano surfaces (12.5- and 8.6-fold, respectively), when compared to expression for Screw surface (5.8-fold). At the same time point, the relative expression levels of *ALP* (Fig. 2b) were 8.1-fold greater for Vulcano, 7.6-fold for Porous, and 4.8-fold for Screw. *BSP*-relative mRNA expression (Fig. 2c) was similarly for both Screw and Vulcano at day 7 (6.2- and 6.9-fold) and increased on Porous group (13.4-fold upregulated).

### Growth factors production

For both TGF-β1 and BMP-2 secretion, no statistical difference was observed among the three surfaces at both experimental times (Fig. 3). In general, growth factors levels were higher at 7 days than at 3 days for all groups, except for TGF-β1 secretion at Screw surface, which decreased its levels after 7 days.

### Association between growth factors secretion vs. Runx2 expression and Runx2 expression vs. osteoblast-related matrix proteins expression

The influence each surface treatment had on the association of growth factors secretion (BMP-2 and TGF-β1) with *Runx2* expression, as well as on the association of *Runx2* and osteoblast-related matrix proteins (*ALP and BSP*) expression are represented at Table 1. There was no significant correlation between the secretion of growth factors and expression of *Runx2* for any type of surface studied. Multiple Linear Regression confirmed the null hypothesis that *Runx2* expression is not dependent upon BMP-2 and TGF-β1 secretion ($p>0.05$) at any group evaluated (data not shown). Differently, irrespective of the surface, we found significant positive correlation between *Runx2* and *ALP/BSP e*xpression, suggesting that these proteins may be dependent on *Runx2* expression. It was confirmed by significant linear determination coefficient ($r^2$) obtained on Simple Linear Regression ($p<0.05$) (see Fig. 4).

## Discussion

Many *in vitro* studies support the hypothesis that surface topography modulates cell response [12, 14, 23–26]. Implant industry continues to manufacture surfaces with no additional treatment or just physically modified or even combining topography and composition modification. The incorporation of CaP on implant surfaces through the anodization technique results in roughness and chemical modification, and is supposed to render a faster osteoblast cell response and osseointegration. The goal of this study was to

evaluate whether anodization with CaP incorporation can enhance growth factors secretion, modulating gene expression, and consequently controlling osteoblast differentiation, compared to surfaces just physically modified and without modification.

The results showed that the process of osteogenesis *in vitro* is influenced by cell-surface interaction. However, the proposed hypothesis was rejected. The higher cell viability for Porous and Vulcano at both time points indicates that rough and hydrophilic surfaces have a greater affinity with osteoblasts and hence, are more biocompatible than smooth ones. These observation is consistent with previous investigations [1, 2, 12, 13]. Cell viability over time suggests that there was a marked increase on cell proliferation rate between 3 and 7 days for Porous and Vulcano, and that their similar viability at 7 days might be due to confluence and stop on proliferation to start differentiation. Probably if a 5-day time point was evaluated, differences between these two surface treatments would be more pronounced. Although simultaneous and enhanced cell proliferation and differentiation would provide an ideal situation for bone growth and repair, the development of the osteoblast phenotype requires a regulated interrelation between proliferation and differentiation with transcriptionally restricted transitions that mark the end point of proliferation and the onset of differentiation [23].

The extent of osteoblastic differentiation as demonstrated by osteoblast-specific gene expression was greater for cells adherent to rough surfaces, which is in agreement with others [24–26]. *Runx2* is a transcription factor essential for osteoblast differentiation being strongly detected in preosteoblasts, immature osteoblasts, and early mature osteoblasts [18]. The significant increase in *Runx2* expression after 7 days suggests the beginning of mature phenotype determination for hFOB 1.19 osteoblasts. This result is in contrast with those obtained by Setzer et al. [26], who observed a statistically up-regulation of *Runx2* at day 3 for hFOB 1.19 cultured on rough surfaces. In our study, after 3 days of culture, Vulcano presented high levels of *Runx2* expression compared with other groups, indicating this surface might have a positive effect on early osteoblast differentiation. Mendonça et al. [25] only demonstrated significant increase on *Runx2* levels after 14 days for acid etched and grit-blasted surfaces. These may be due to the cell culture model used by these authors, who performed the experiment with human mesenchymal stem cells (hMSCs). hMSCs requires much more steps until differentiation into mature osteoblasts than an osteoprogenitor cell line does, like the one used in this work.

Besides the commitment of pluripotent mesenchymal cells to osteoblast lineage, Runx2 has been related with the modulation of important bone matrix protein gene including type I collagen (Coll), alkaline phosphatase (ALP), osteocalcin (OCN), osteopontin (OPN), and bone sialoprotein (BSP) [18, 24, 25]. In this study, it was observed a significant increase on expression of ALP and BSP at the same time point Runx2 presented elevated levels (7 days). This strong association was confirmed by significant positive correlation among these genes. Thus, we speculate that greater Runx2 levels favor its binding to ALP and BSP promoter regions resulting in greater expression of these genes. However, some studies indicate that BSP expression can be inhibited by the increase on Runx2 levels, depending on which cofactor is recruited [27, 28]. Considering ALP and BSP are markers of early [29]

and late [28] stages of differentiation, respectively, their significant high levels at day 7 reinforces cells are differentiating into mature osteoblasts, especially on rough surfaces.

Regarding TGF-β superfamily and its influence on gene expression, studies using pluripotent mesenchymal precursors cells (C2C12) [15] and osteoblast progenitor cell line ROB-C 26 (C26) [16] treated with BMP-2 and TGF-β1 have demonstrated that *Runx2* expression is under their regulation. However, in the present study both BMP-2 and TGF-β1 did not have a similar pattern of release among the groups which could be positively or negatively correlated with *Runx2* expression at each time point. These contradictory results might be due to the fact that, differently from the previous studies, we did not supplement culture media with growth factors, which might be less expressive in modulating *Runx2*. We expected that BMP-2/TGF-β1 secreted by the own cells would be able to affect significantly *Runx2* expression through autocrine and paracrine stimulation, what did not happened. Moreover, there was no significant increase on BMP/TGF superfamily secretion on rough or smooth surface. In contrast, *in vitro* [30] and *in vivo* [31] studies found greater *TGF-β1* synthesis on rougher surfaces than on smoother ones. Substrates containing CaP coatings are expected to render a faster osteoblast cell responses and further osseointegration, when compared to those without CaP coatings [4]. In the present study, roughened surfaces, apart surface treatment, showed increased cell viability/proliferation and differentiation in comparison to smooth surfaces. These results are supported by Le Guehennec et al. [32] who concluded in his surface treatments review that surface roughness enhances osseointegration, but the exact role of the composition and topography in early events of osteogenesis is still poorly understood. Therefore, it is important that commercially available surfaces combining physical and chemical modification continue to be evaluated to warrant their clinical use. In this way, further studies at long-term periods of culture should be performed, in order to investigate other transcription factors, osteoblast-related genes expression and secretion, and cytokines involved with the process of bone repair. Thus, it would be possible to figure out the effect of CaP incorporation on biological responses.

## Conclusions

Within the methodology of this study, it can be concluded that the anodization with CaP incorporation modulated positively cell viability and osteoblast-related gene expression (especially ALP). No significant correlation was found between greater secretion of BMP-2/TGF-β1 with a higher expression of *Runx2* for neither group. Even though, irrespective of the surface treatment, *ALP/BSP* expression was highly dependent on *Runx2* expression. The results presented here support the observations that roughness may play a more important role in determining cell response than surface composition does.

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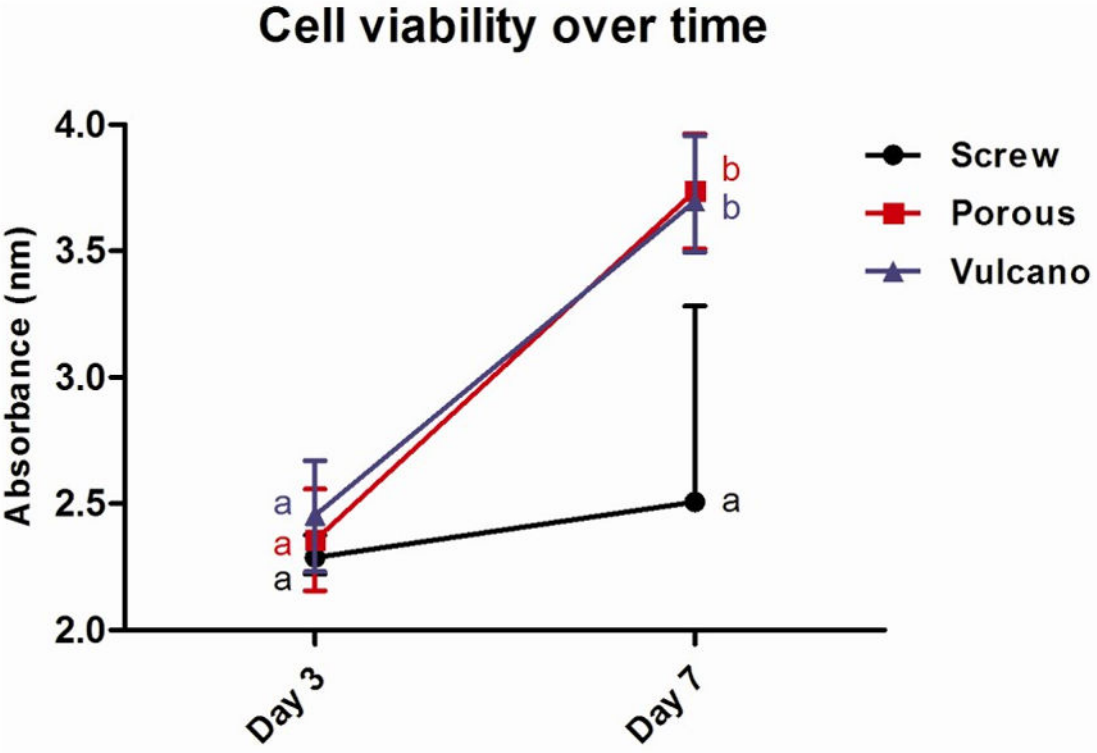


**Fig. 1.**

MTT assay of hFOB cells on Screw (machined), Porous (acid etched), and Vulcano (anodized and CaP incorporation) surfaces after 3 and 7 days of culture. The same letters indicate non-significant differences among groups at each time point.

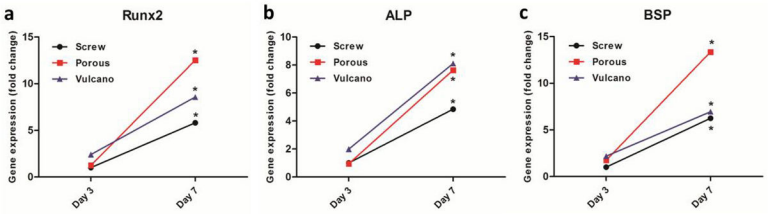


**Fig. 2.**

Adherent hFOB of bone-specific mRNA expression. Total RNA was isolated from cells at 3, and 7 days of culture on machined (Screw), acid etched (Porous), and anodized with CaP (Vulcano) titanium disks. Expression levels of (a) *Runx2*, (b) *ALP*, and Ct (c) *BSP* are compared for all surfaces. The results are shown as fold change ($2^{-\Delta\Delta}$ method, the mRNA expression relative to *GAPDH* was determined and the fold changes were calculated using the values of machined day 3 as a calibrator). *Statistically significant difference when compared with machined day 3 ($p<0.05$).

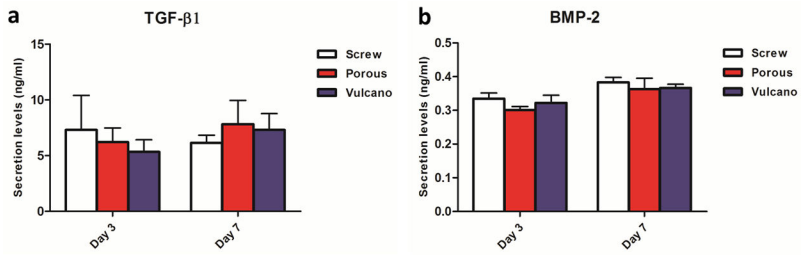


**Fig. 3.**

TGF-β1 (a) and BMP-2 (b) production by hFOB cells cultured on machined (Screw), acid etched (Porous) and anodized with CaP (Vulcano) surfaces after 3, and 7 days. At harvest, the media were collected, and growth factors content measured by ELISA. Values are expressed as mean ±SD.

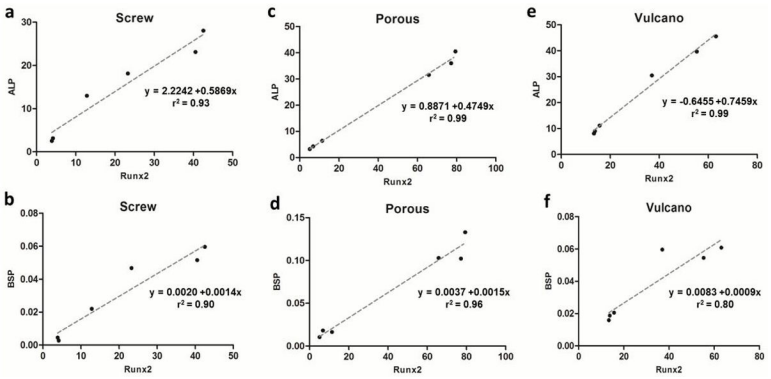


**Fig. 4.**

Relationship of *Runx2* expression to *ALP* and to *BSP* expression for each surface treatment. Pearson correlation analysis showed a significant positive correlation of *Runx2* versus *ALP/BSP* expression in all groups (Screw, machined; Porous, acid etched; Vulcano, anodized and CaP incorporation) (Pearson $r > 0.90$; $p < 0.05$). Line represents linear regression of data ($y = ax+b$; $r^2 > 0.80$; $p<0.05$).

**Table 1**

Pearson correlation (r) analysis among *Runx2* expression and variables of growth factors secretion and osteoblast-related matrix proteins expression.

| | Screw | | Porous | | Vulcano | |
|---|---|---|---|---|---|---|
| **Variables** | **r** | **p-value** | **r** | **p-value** | **r** | **p-value** |
| TGF-β1 | −0.2223 | 0.6720 | 0.4738 | 0.3424 | 0.7987 | 0.0567 |
| BMP-2 | 0.4459 | 0.3754 | 0.7255 | 0.1026 | 0.2957 | 0.5694 |
| *ALP* | 0.9713 | 0.0012* | 0.9979 | <0.0001* | 0.9943 | <0.0001* |
| *BSP* | 0.9601 | 0.0023* | 0.9856 | 0.0003* | 0.9181 | 0.0098* |

* statistically significant