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Surface topography modulates the osteogenesis in human bone marrow cell cultures grown on titanium samples prepared by a combination of mechanical and acid treatments

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Titanium samples of different roughness (R_a) and morphology were prepared using a combination of mechanical (grinding with a SiC paper or blasting with aluminum oxide particles with 65 or 250 μm) and chemical (attack with a sulphuric acid based solution or a hydrofluoric acid based solution) treatments. The biological performance of the prepared surfaces was evaluated using human bone marrow osteoblastic cell cultures. Mechanically treated samples presented different R_a values and surface morphology. The hydrofluoric acid solution was more effective than the sulphuric acid solution in smoothing titanium surface and also in eliminating aluminum contamination resulting from the blasting process. Bone marrow cells seeded on the different titanium samples showed a similar pattern of behavior during cell attachment and spreading. Cells proliferated very well on all the titanium surfaces and cell growth was observed during approximately two to three weeks. The samples treated with the hydrofluoric acid solution presented higher alkaline phosphatase activity. Only the blasted samples treated with the acid solutions allowed seeded bone marrow cells to form a mineralized extracellular matrix. The best biological performance was found in the blasted samples treated with the hydrofluoric acid solution, which could be related to the characteristic microtopography of these samples that presented a homogeneous and smooth roughness.
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1. Introduction

Dental implants are usually made from commercially pure titanium because of its biological acceptance in bone. This material is biocompatible [1–3], has high corrosion resistance, is lightweight and durable, and can be easily prepared in many different shapes and textures without affecting its biocompatibility [4].

Events occurring at the bone–implant interface are of major importance to the osteointegration of the material. The response of cells and tissues at implant interfaces are affected by the surface properties such as the chemical composition, surface energy, topography and roughness of the implant [5, 6]. Relevant work has been performed in order to develop new designs and to optimize surface treatments to produce implants with appropriate characteristics. Surface treatments can be divided in two major approaches: coating the implants with bioactive substances (e.g. calcium phosphate ceramics which present similar composition to the mineral part of the bone) [7–10] and modifying-surface processes [11, 12]. This methodology includes treating the titanium with plasma-sprayed titanium powder or using oxide blasting treatments associated, or not, with chemical treatment.

Bioactive materials used to coat dental implants can create a chemical bonding between implant and bone, while the blasting process followed, or not, by chemical treatment can generate a suitable topography to allow bone–implant mechanical anchor. Bone bonding shear strength of an implant coated with titanium by plasma spray can be 10–20 times higher than the strength

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The studies concerning the *in vitro* biological performance of the titanium surfaces were performed at the Faculdade de Medicina Dentária da Universidade do Porto, Portugal.

measured on surfaces exhibiting lower roughness values [12]. It seems that there is a direct correlation between shear strength and the arithmetic average of roughness (R_a) but Wennerberg *et al.* [11] found better results with respect to bone healing for a moderately increased surface topography compared to a highly increased surface topography.

Commercial implants have a wide variety of morphologies and the ideal microtopography is yet unknown, as it is very difficult to correlate surface properties with clinical results [11, 12]. Moreover, several aspects not only related to topography, material itself or the fabrication process act altering clinical performance. Each patient exhibits a specific answer to a particular implant depending on bone characteristics, patient's health and surgical technique [13]. Systematic studies, performed on animals [14] or using cell culture methodology [15], continually provide important information concerning the relevance of the surface properties and the biological performance of the implants.

This work describes the effect of acid treatments on surface composition, roughness and morphology parameters of titanium sheets submitted to grinding or blasting. For chemical attack, a sulphuric/hydrochloric acid solution or a hydrofluoric acid solution were employed [12]. Both solutions can alter topography and/or surface composition. In addition, the biological performance of the prepared titanium surfaces was evaluated using human bone marrow osteoblastic cell cultures. Cell morphology, cell viability/proliferation, alkaline phosphatase (ALP) activity and ability to form a mineralized extracellular matrix were monitored as indicators of cellular response.

2. Materials and methods

2.1. Preparation of the titanium samples

The titanium samples used in this study were prepared from titanium sheets (ASTM grade 2). A titanium sheet was divided in three pieces that were processed as described below: (1) submitted to grinding with a 600 grit SiC paper (samples G_0), (2) blasted with aluminum oxide particles with 65 μm (samples $B65_0$), and (3) blasted with aluminum oxide particles with 250 μm (samples $B250_0$). After these mechanical treatments, the three titanium pieces were then cut in 8 × 8 mm samples and cleaned using a sequence of acetone, absolute ethanol and distilled water in an ultrasonic cleaner.

Each of the three types of samples were further divided in three groups and treated as follows: (i) maintained as treated before – samples G_0, $B65_0$ and $B250_0$, (ii) submitted to chemical attack with a sulphuric acid based solution (solution S) – samples G_S, $B65_S$ and $B250_S$, and (iii) submitted to chemical attack with a hydrofluoric acid-based solution (solution F) – samples G_F, $B65_F$ and $B250_F$. The chemical attack with solution S or solution F was performed using the conditions shown on Table I. According to the mechanical and chemical treatments described, nine different surfaces were prepared, grouped in three families: G_0, G_S and G_F (G_i family); $B65_0$, $B65_S$ and $B65_F$ ($B65_i$ family); $B250_0$, $B250_S$ and $B250_F$ ($B250_i$ family).

TABLE I Chemical treatment of the titanium samples

Solution	Composition and conditions
S	H_2SO_4 (48%) plus HCL (18%), 30 min
F	First step: HF (4%), 60 s
	Second step: HF (4%) plus H_2O_2 (8%), 15 s

2.2. Surface characterization of the titanium samples

The R_a was determined using a profilometer SLOAN, model Dektak IIA with vertical precision of 5 Å. For each surface condition, three samples were employed and at least 10 measurements were done on each sample.

X-ray photoelectron spectroscopy (XPS) was carried out in an ESCALAB 200 A – VG SCIENTIFIC operating at 15.0 kV, 399 W with the Mg–K_α energy (1253.6 eV). This analysis allows for the identification of residual contamination and for the semi-quantification, with an error in the order of 10%, of the composition of the external layers of the surface.

Scanning electron microscopy (SEM) of the prepared titanium samples was carried out in a ZEISS DSM 940A, operating at 15 kV to characterize surface topography while a LINK energy dispersive X-ray detector (EDS) was used to determine elemental composition of residual particles.

2.3. Cell culture

Human bone marrow, obtained from surgery procedures, was cultured in α-Minimal Essential Medium (α-MEM) containing 10% fetal bovine serum, 50 μg/ml gentamicin and 2.5 μg/ml amphotericin B. Primary cultures were maintained until near confluency and, at this stage, adherent cells were enzymatically released (0.04% trypsin and 0.025% collagenase), counted using a Celltac-NIHON KOHDEN equipment and seeded in 24-well dishes at a density of 2×10^4 cells/cm^2. Incubation was carried out in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C and culture medium was changed twice a week.

Cells were cultured in control conditions (standard plastic tissue culture plates) and on the surface of the nine prepared titanium samples for 28 days and in the presence of ascorbic acid (50 μg/ml), β-glycerophosphate (βGP, 10 mM) and dexamethasone (10 nM). Before being seeded, titanium samples were washed with ethanol and distilled water in an ultrasonic cleaner and sterilized by autoclaving.

Cell cultures were characterized throughout the culture time at defined time intervals. Cell morphology was observed by SEM in the initial stage of the incubation (4, 7 and 24 h) in order to assess the cell adhesion process to the material surface and also at 3, 7, and 21 days. Cell viability/proliferation and ALP activity were evaluated at 3, 7, 14, 21 and 28 days.

2.3.1. Cell/viability proliferation and ALP activity

MTT assay (reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to a purple Formazan

product) was used to estimate cell viability/proliferation [16]. Cells were incubated with 0.5 mg/ml of MTT in the last 4 h of the culture period tested; the medium was then decanted, Formazan salts were dissolved with dimethylsulfoxide and the absorbance was determined at 600 nm in an ELISA reader.

ALP activity was determined in cell lysates (obtained by treatment of the cultures with 0.1% triton) and assayed by the hydrolysis of p-nitrophenyl phosphate in alkaline buffer solution, pH 10.3, and colorimetric determination of the product (*p*-nitrophenol) at $\lambda = 405$ nm (hydrolysis was carried out for 30 min at 37 °C). Results are expressed in nanomoles of p-nitrophenol produced per min per cm^2 ($nmol/min \cdot cm^2$).

2.3.2. Scanning electron microscopy
SEM observation was performed on the seeded titanium surfaces and control cultures. Samples were fixed with 1.5% glutaraldehyde in 0.14 M sodium cacodylate (pH 7.3), then dehydrated in graded alcohols, critical-point dried, sputter-coated with gold and analyzed in a JEOL JSM 6301F scanning electron microscope equipped with a X-ray EDS microanalysis capability, Voyager XRMA System, Noran Instruments.

2.4. Statistical analysis
For cell culture studies, data are presented from one of two replicate experiments, both of which yielded comparable results. For any given experiment, each data point represents the mean $\pm$ standard error of three replicates. Statistical analysis was done by one-way analysis of variance (ANOVA). The statistical differences between the different groups were determined by the Bonferroni method. P values ≤ 0.05 were considered significant.

Analysis of variance was also used for the comparison of the surface properties of the prepared titanium samples.

3. Results
3.1. Material surface characterization
Table II shows the R_a parameter (media and standard deviation) for the nine prepared titanium surfaces. The mechanical treatments produced surfaces with significantly different roughness; the highest and the lowest R_a values were observed respectively in $B250_0$ and G_0 samples. Treatment of the samples submitted to the grinding or the blasting processes with the chemical solutions resulted in a reduction of the roughness, although differences were not significant.

TABLE II Roughness of the titanium samples

Samples	Mechanical treated	After solution S	After solution F
G_i	0.47 ± 0.17	0.31 ± 0.09	0.38 ± 0.15
$B65_i$	1.00 ± 0.18*	0.88 ± 0.18*	0.80 ± 0.14*
$B250_i$	3.59 ± 0.73*	3.31 ± 0.77*	3.09 ± 0.70*

*Significantly different from the G_i family samples.

SEM micrographs of the different surfaces (Fig. 1) show that G_0 samples presented a smooth surface with some defects of decreased size and $B65_0$ and $B250_0$ samples an irregular morphology with many cracks and pits among flatter-appearing areas of various sizes, characteristics that were more pronounced in $B250_0$ samples. Treatment of the blasted samples with the sulphuric acid solution did not result in major morphological changes, although some decrease on the depressions and indentations and also some smoothing of the edges was apparent (samples $B65_S$ and $B250_S$); in the G_0 samples, some defects were created by this treatment (samples G_S). By contrast, treatment of G_0, $B65_0$ and $B250_0$ with the hydrofluoric acid solution resulted in surfaces with a completely different morphology. The effect was more pronounced in the blasted samples, and $B65_F$ and $B250_F$ surfaces exhibited a homogeneous morphology consisting in uniform craters.

Blasted samples ($B65_0$ and $B250_0$) and blasted samples treated with the sulphuric acid solution ($B65_S$ and $B250_S$) presented aluminum-rich particles. Those particles with 20 µm or less were incrusted in the titanium surface as a consequence of the blasting process and were easily identified using backscattered image as aluminum-rich particles (with low atomic number) appeared darker than the titanium matrix (with high atomic number), as shown in Fig. 2 for $B250_i$ family samples. It seems that solution S promotes some fragmentation of alumina particles, while solution F removes all aluminum contamination as evident by the observation of $B65_F$ and $B250_F$ samples.

XPS spectra for G_i samples show the presence of nitrogen, carbon and oxygen peaks, as might be expected as those elements are commonly adsorbed on titanium surface. A layer of titanium oxide, TiO_2, was present on the surface of all the prepared samples and its stoichiometry could be confirmed through the identification of an oxygen peak with binding energy of 531 eV.

Fig. 3 shows the XPS spectra for $B65_0$, $B65_S$ and $B65_F$ samples; similar spectra were observed for $B250_i$ family samples. The results of XPS quantification are shown on Table III. Sample $B65_0$ shows the presence of an aluminum peak with low intensity corresponding to 10.5%; the oxygen around 532 eV can be associated to aluminum oxide while the oxygen peak around 531 eV is related to TiO_2. XPS analysis shows that treatment with solution S did not remove aluminum contamination occurred during the blasting process, although the oxygen peak related to the aluminum oxide was drastically reduced (samples $B65_S$ and $B250_S$). However, $B65_F$ and $B250_F$ samples did not present any aluminum contamination (Fig. 3 and Table III).

3.2. Biological performance of the titanium surfaces
Human bone marrow cells (first subculture) were cultured in the presence of ascorbic acid, βGP and dexamethasone, experimental conditions described to favor the expression of the osteoblast phenotype in

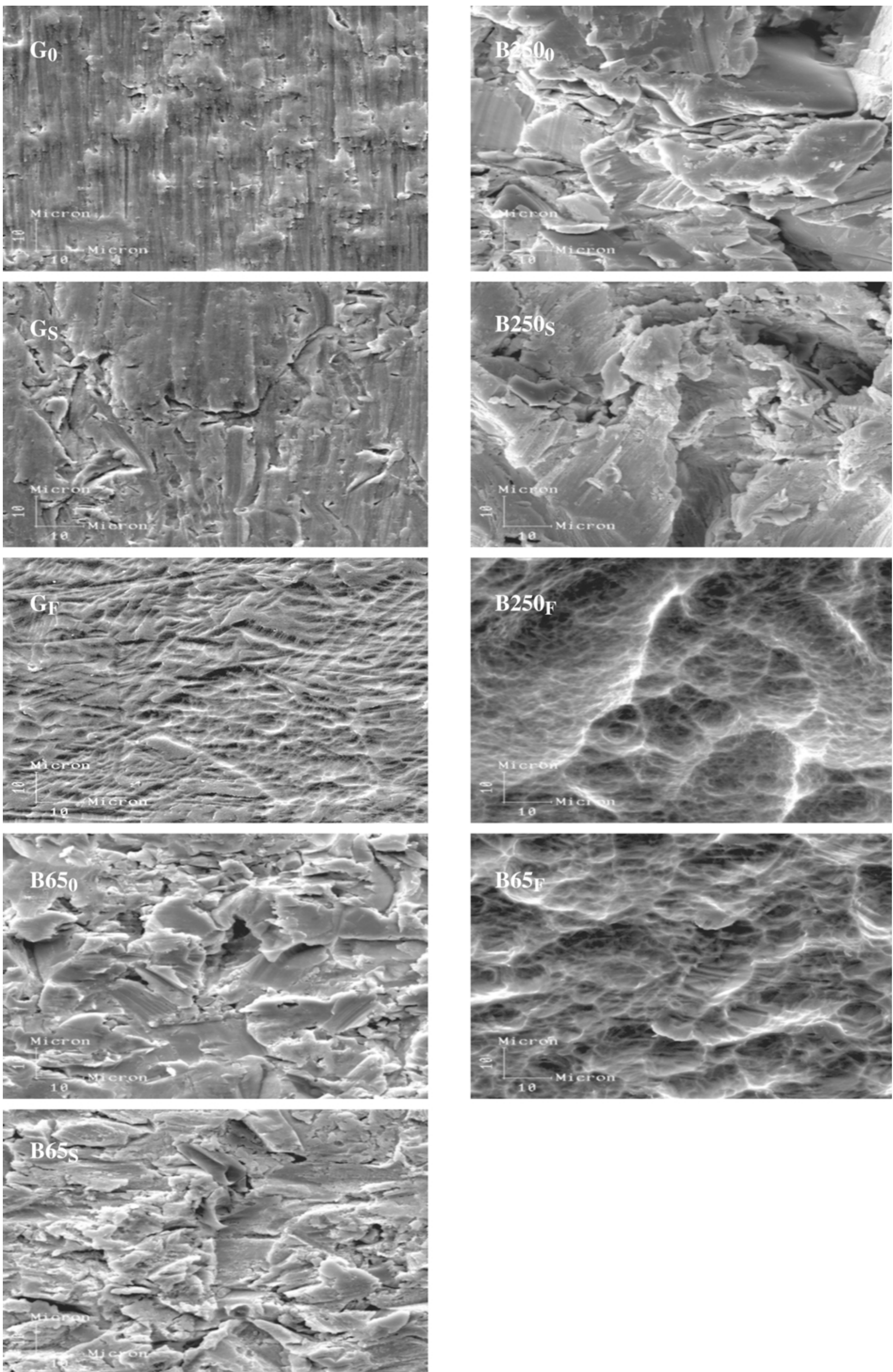


Figure 1 SEM micrographs (secondary image, × 1000) of the prepared titanium samples.

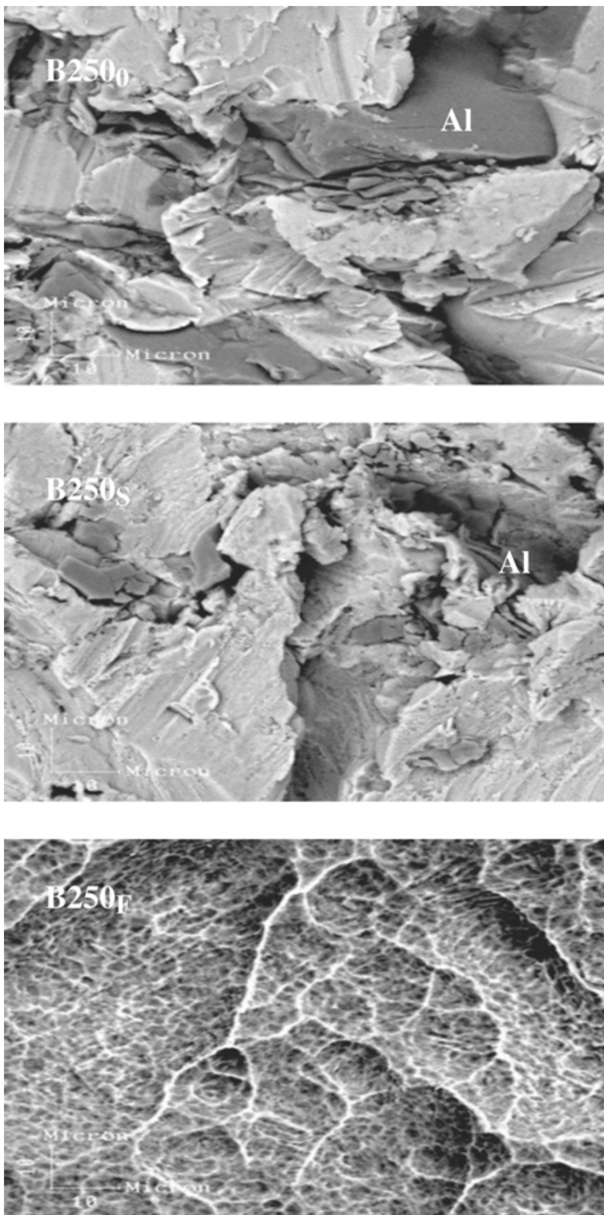


Figure 2 SEM micrographs of B250$_i$ family samples (backscattered image, × 1000).

several bone cell systems [17–21]. Cells were grown for 28 days in control conditions (absence of material, standard plastic culture plates) and on the prepared titanium surfaces. Cultures were evaluated concerning cell morphology, cell viability/proliferation, ALP activity and ability to form calcium phosphate deposits.

3.2.1. Control cultures

Cultures grown on the standard plastic culture plates proliferated during the two first weeks; maximum values for the MTT assay were observed around day 14, decreasing after that. ALP activity increased especially during the second week, attained a stationary phase during the third week and decreased afterwards. SEM observation of 21-day cultures showed the presence of abundant mineral deposits that contained Ca and P as shown by X-ray microanalysis. The behavior of control cultures is presented in Fig. 4.

3.2.2. Seeded titanium samples

Cell adhesion to the material surface. Bone marrow cells began to adhere and spread on the material surface within minutes after being seeded. Observation of the cultures at time intervals during the first 24 h showed that expansion of the cytoplasm began to occur after 30 min and at 4 h, cells presented a typical morphology of a central spherical body with the cytoplasm extending away from the central area in all directions and adhering to the adjacent titanium surface. As this process went on, the cells appeared to flatten and spread and, after 24 h, they were completely spread, presenting an extended morphology (similar to that observed at three days of culture). This pattern of behavior was similar in all the samples and is exemplified in Fig. 5 for G_i and B250$_i$ family samples. In G_0 and G_S samples, cells appeared to be oriented by the shape of the surface but in the other surfaces they were randomly distributed. On the blasted materials, most of the cells remained on the surface with filopodial-like extensions adapted to the irregular rough material but some of them took on the morphology of the underlying substrate and intimately conform to the interstices of the irregular surface (Fig. 5).

Cell viability/proliferation and ALP activity. Bone marrow cells proliferated very well on all the titanium surfaces and cell growth was significantly higher than that observed in control cultures. Cell growth was observed during approximately three weeks, decreasing after that (Figs 6 and 7).

A comparison of results concerning the samples submitted to the three mechanical treatments – G_0, B65$_0$ and B250$_0$, showed that the blasted samples presented increased cell growth during the third week as compared with G_0 samples, especially B65$_0$ samples (around 30% at day 21, Fig. 6). However, ALP activity was significantly higher in G_0 samples (Fig. 6).

Treatment of G_0 samples with the acid solutions resulted in an evident increase in cell proliferation during the third week (Fig. 7(A)). However, treatment of B65$_0$ surfaces resulted in a small decrease in cell growth rate, especially in the Samples treated with the hydrofluoric acid solution (Fig. 7(B)). The chemical treatment of B250$_0$ samples did not affect cell proliferation (Fig. 7(C)).

Determination of ALP activity in the seeded titanium samples showed that the levels of the enzyme were higher in the G_i family samples (Figs 6 and 7). Treatment of G_0, B65$_0$ and B250$_0$ with the sulphuric acid solution did not affect significantly ALP activity, whereas an evident increase on the levels of the enzyme was observed in the samples treated with the hydrofluoric acid solution (Fig. 7(A)–(C)). As compared with the cultures performed in the standard plastic culture plates, cultures grown on the titanium surfaces presented a significantly lower ALP activity.

Formation of calcium phosphate deposits. Fig. 8 shows the SEM appearance of 21-day seeded titanium samples. G_i family samples presented the surface covered by cell layers with a homogeneous appearance but no clear evidence of the presence of calcium phosphate deposits was found. B65$_0$ and B250$_0$ also presented most of the surface covered by cells with numerous extending processes that adapted firmly to the irregular surface,

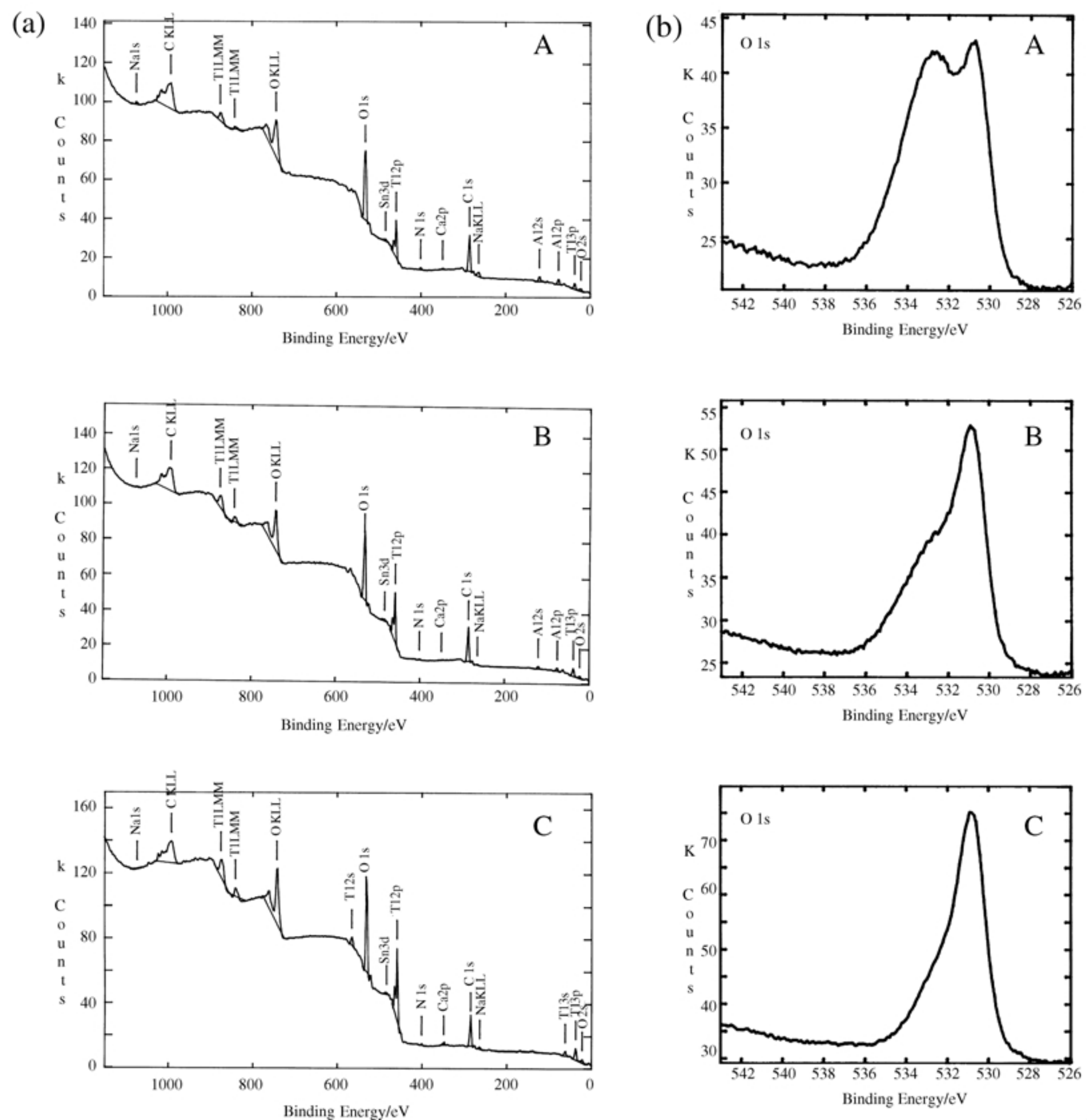


Figure 3 XPS spectra of $B65_0$ (A), $B65_S$ (B) and $B65_F$ (C) samples; (a) general view, (b) detail near oxygen peak.

however, mineral deposition did not seem to occur. By contrast, blasted samples submitted to the acid treatments presented clear evidence of the formation of calcium phosphate deposits. $B65_S$ showed a low incidence of the mineral deposits as compared to $B250_S$ samples. However, $B65_F$ and $B250_F$ showed a similar appearance with abundant extracellular matrix and numerous globular mineral deposits.

4. Discussion

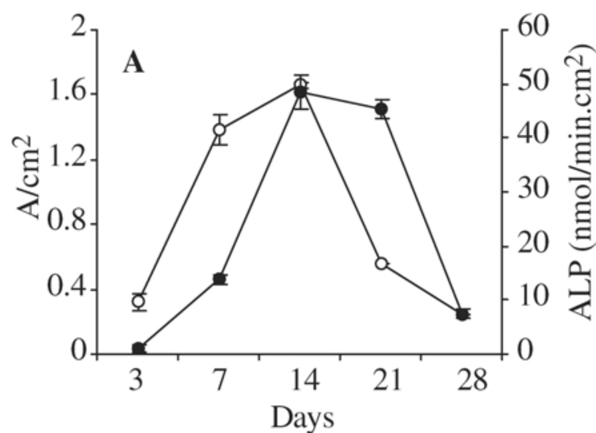
Bone formation on the implant surface requires the recruitment of osteoprogenitor cells and their proliferation and differentiation into functional osteoblasts that are able to produce a mineralized extracellular collagenous matrix at the interface. The surface properties of an implant plays a critical role in this process as bone cells can recognize and respond to substratum structures both *in vivo* and in *in vitro* conditions [22–24]. Methods of implant surface preparation can significantly affect the resultant properties of the surface and, subsequently, the biological response of the osteoblastic cells including cell attachment, cell growth and functional activity, as described in a number of studies [25–32].

TABLE III Surface composition obtained from XPS spectra (at %) for $B65_i$ and $B250_i$ family materials

	C	Al	Ti	O	N
$B65_0$	44.9	10.5	6.1	36.0	1.7
$B65_S$	44.6	9.0	9.3	36.2	0.9
$B65_F$	41.1	—	14.5	43.1	1.3
$B250_0$	45.9	7.6	7.7	38.0	0.8
$B250_S$	41.1	7.0	11.2	39.3	1.3
$B250_F$	42.2	—	15.0	42.7	—

In this work, a combination of mechanical and chemical treatments were used to produce titanium surfaces of different roughness and morphology. Biological performance of the prepared samples was evaluated using osteoblastic cell cultures obtained from human bone marrow, the biological compartment with which the bone substitution material is confronted *in vivo*.

Bone marrow cells were cultured in the presence of ascorbic acid, necessary for the formation and maturation of the collagen [33], βGP, a potential source of phosphate ions for the mineralization process [34, 35], and dexamethasone, a compound that induces the proliferation and/or the differentiation of osteoblastic cells [33, 36]. These experimental conditions allow the



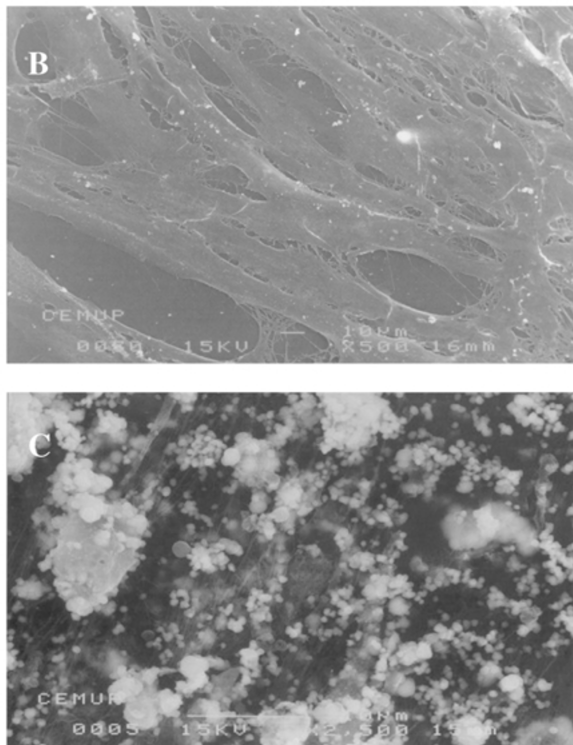


Figure 4 Cell proliferation and differentiation behavior of human bone marrow cell cultures grown in control conditions. Cell viability/proliferation (○) and ALP activity (●) (A); SEM appearance of 14-day (B) and 21-day (C) cultures.

complete expression of the osteoblast phenotype, that is, the formation of a collagenous mineralized matrix in cultures maintained for three to four weeks, as described in previous studies [37, 38], and also as it is shown in this work (Fig. 4). These cultures have been used as a useful tool to study the interactions of osteoblastic cells with biomaterials [39, 40] and their degradation products [20, 41–43].

Results showed that samples submitted to the three mechanical treatments (G_0, $B65_0$ and $B250_0$) presented different roughness and morphology. G_0 samples showed a smooth appearance and the blasted samples a very irregular morphology. In each material family, both chemical treatments reduced R_a, although the differences observed were not significant (Table II). The hydrofluoric acid solution was more effective than the sulphuric acid solution in smoothing titanium surface, especially for samples previously blasted with alumina and $B65_F$ and $B250_F$ samples presented a homogenous topography. Aluminum-rich particles were identified on samples just blasted ($B65_0$ and $B250_0$) or blasted and treated with the sulphuric acid solution ($B65_S$ and $B250_S$) (Fig. 2 and Table III). Treatment with the hydrofluoric acid solution seems to be an efficient way to dissolve aluminum oxide as in $B65_F$ and $B250_F$ samples aluminum content dropped to zero and the oxygen peak related to the aluminum oxide disappeared (Table III and Fig. 2). XPS results were in agreement with the SEM observation of the samples. As expected, all the samples presented a layer of titanium oxide on their surface.

The surface topography of the substratum is believed to be an important factor in controlling the shape, orientation and adhesion of cells and this subject has been extensively studied and reviewed [23, 29, 30, 32, 44, 45]. Observation of the seeded titanium samples by SEM during the first hours of incubation showed that surface topography did not appear to inhibit cellular spreading following initial attachment to the material. In addition, a similar pattern of behavior was observed and, after 24 h, cells have flattened on the surfaces and were completely spread. Morphological changes occurring during the attachment and spreading of the cells correspond to the reorganization of the cytoskeleton, a structure that plays an important role in the control of the cell shape and behavior [46]. Observation of the seeded material samples showed that the cell layer organization was influenced by the topography of the underlying substrata, results that are in agreement with other studies [24, 26, 29, 30, 44, 45]. On G_0 and G_S samples, cells seem to be oriented according to the shape of the material and for later culture times they were organized in a parallel order, covering the all surface as they did on the plastic culture dish (Fig. 4b). On the other surfaces, cells were randomly distributed and appeared to successfully adapt to the irregular rough surfaces.

Results concerning the cell proliferation evaluated by the MTT assay showed that cell growth was observed until later on the blasted samples, as compared to the G_0 samples (Fig. 6). These results suggest that on the rougher surfaces cell growth was enhanced, observation that is in agreement with other studies that suggest that surface roughness directly affects cell proliferation [47, 48]. However, it is also possible that the greater surface area of the blasted samples allowed for a longer period of cell growth. By contrast, ALP activity was higher on G_0 samples than on $B65_0$ and $B250_0$ surfaces (Fig. 6), suggesting a lower expression of this marker. This observation may be related with the increased proliferation rate observed in the blasted samples, as a reciprocal relationship between proliferation and differentiation has been described in the development of the osteoblast phenotype [49].

G_S and G_F surfaces allowed for a longer period of cell growth as compared to G_0 samples (Fig. 7(A)). This is most probably due to surface morphology modifications and/or increase surface area induced by the acid treatments as both parameters influence cell growth. Chemical treatment of the blasted samples did not result in relevant effects in the cell proliferation (Fig. 7(B),(C)). Concerning ALP activity, it is interesting to note that, for each material family, the samples treated with the hydrofluoric acid solution presented higher ALP activity

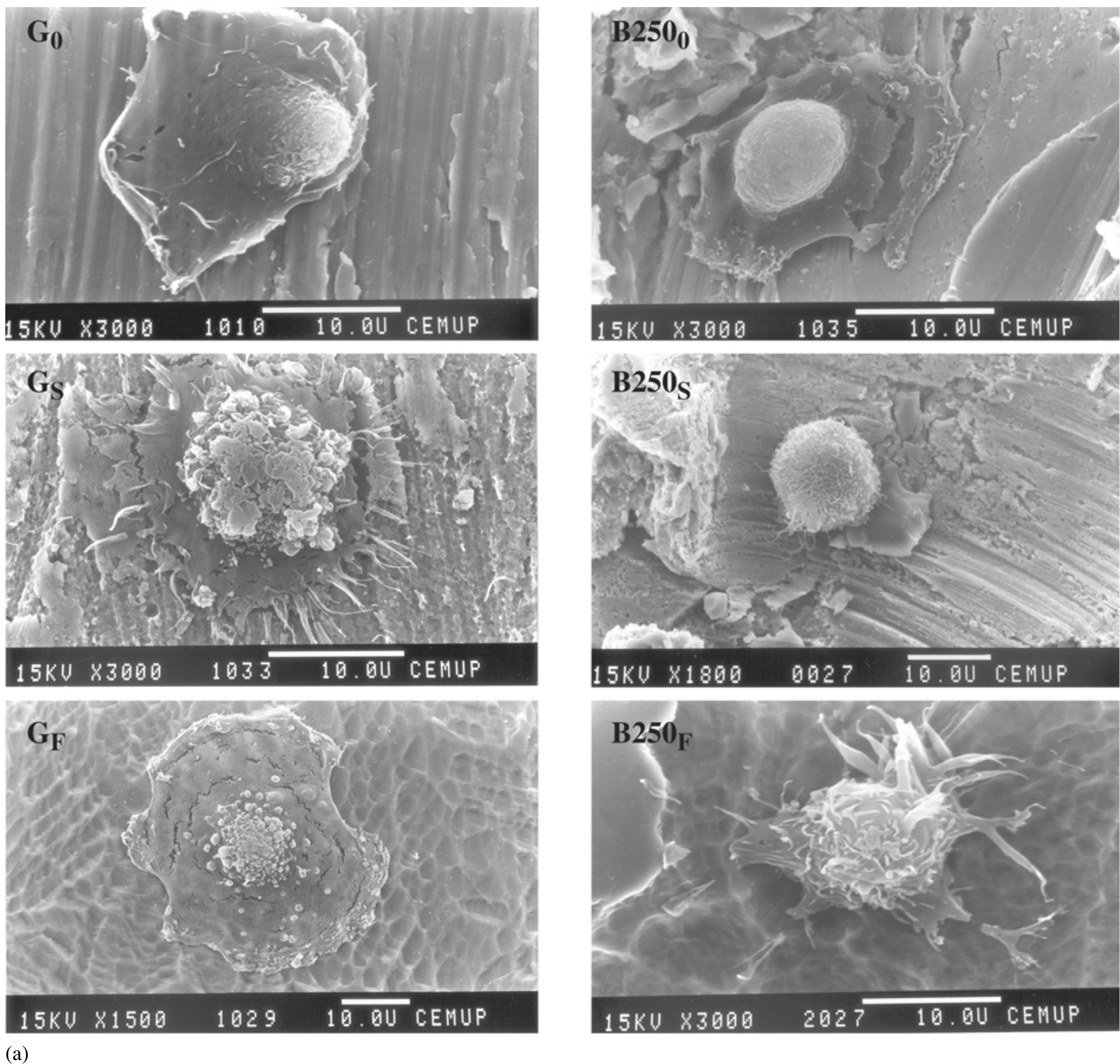
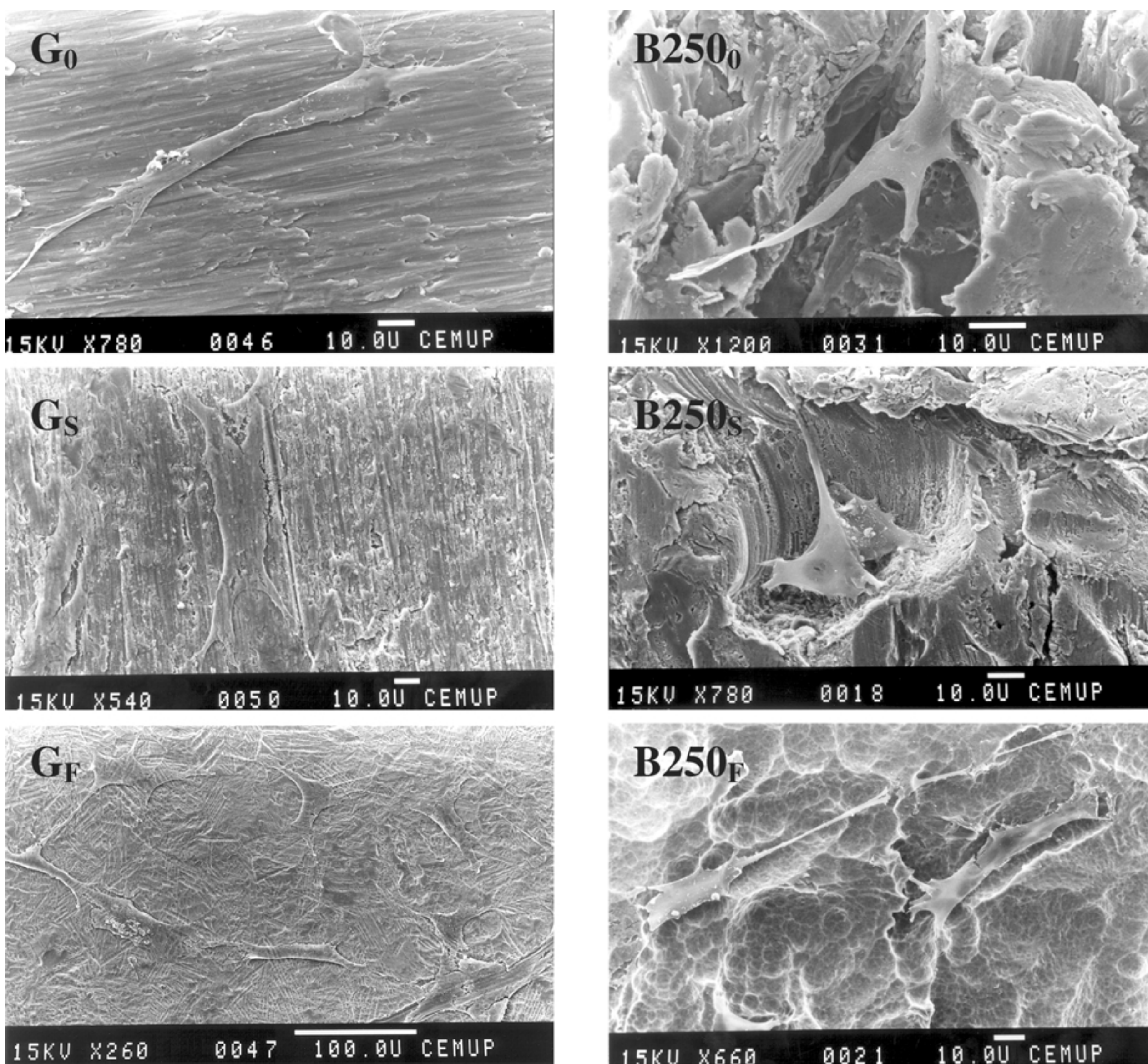


Figure 5 SEM morphology of human bone marrow cells cultured on the G_i and $B250_i$ family samples after 4 h (a) and 24 h (b).

(Fig. 7(A)–(C)), an important osteoblastic differentiation marker [50].

Biological performance of the titanium samples was also evaluated concerning their ability to allow for the formation of a mineralized extracellular matrix, the last event of the osteoblastic differentiation [50] and essential to the osteointegration of the implant, that is, the bone formation at the bone/material interface. Major behavior patterns were observed. G_i family samples did not show the formation of mineral deposits and the same behavior was found on $B65_0$ and $B250_0$ surfaces. Only the blasted samples treated with the acid solutions allowed seeded bone marrow cells to form mineral deposits, especially those treated with the hydrofluoric acid solution. These results showed that the blasted samples treated with the acid solutions presented a better biological performance concerning the ability to form a mineralized matrix than the G_i samples submitted to the same chemical treatments. Samples submitted to the grinding (G_i samples) or the blasting processes ($B65_i$ and $B250_i$ samples) presented different surface roughness and several studies suggest that increasing surface roughness enhances *in vitro* osteoblastic differentiation [47, 48] and also the *in vivo* production of certain cytokines and growth factors by host osteoblastic cells, both of which may increase bone formation [51]. A review by Kieswetter *et al.* [52] highlighted evidence that osteoblasts tend to exhibit a more mature phenotype when grown on rougher surfaces. Another interesting observation is that $B65_S$ samples presented low ability to induce the formation of mineral deposits as compared to $B250_S$ samples, which also may be related to differences in surface roughness ($B250_S$ has higher R_a value). However, $B65_F$ and $B250_F$ samples, despite of the differences in R_a, presented a similar behavior with the formation of an exuberant mineralized extracellular matrix. Also, $B65_0$ and $B250_0$, with similar roughness to, respectively, $B65_F$ and $B250_F$ did not show evidence for the presence of mineral deposits. These results suggest that, in a way similar to that observed in other studies, other surface properties such as surface texture, a combination of topography and roughness, appear to be



(b)

Figure 5 (Continued).

an important variable in the biological performance of the materials [52, 53]. The lack of mineralization observed on the seeded G_i family samples and the blasted surfaces ($B65_0$ and $B250_0$) may be related to surface characteristics that did not allow for the formation of an extracellular matrix in quantity and/or quality to support the mineralization process [49].

Aluminum contamination presented on the surface of $B65_0$, $B65_S$ and $B250_0$ and $B250_S$ did not appear to significantly affect cell behavior or, at least, be a factor as important as the surface topography. This observation is suggested by the similar results concerning cell response observed in $B250_S$ and $B250_F$.

Taking the results together, the best biological performance was found in the blasted samples treated with the hydrofluoric acid solution (that presented an increase in ALP activity and in the ability to form mineralized deposits). This behavior could be correlated to the characteristic microtopography of these samples as the hydrofluoric acid-based solution produced a homogeneous and smooth roughness. These samples may provide a more appropriate surface for the reactions that occur as the material surface is conditioned by the culture medium. The initial interaction results in the adsorption of biologically active molecules to the material surface, including cell-attachment proteins that play a critical role in the adhesion and spreading of bone cells and, also, cell growth and function [54, 55]. The surface properties would also influence the type, amount and conformation of the adsorbed proteins and may further influence the secretion of the extracellular matrix proteins [56]. Also, differences in the oxide layer formed on the various surfaces will change the surface energy of the material and affect cell behavior and most probably will cause different serum components to be adsorbed to the material, as suggested previously [57, 58]. These are important aspects because there is a general agreement that growth enhancing surface properties of the material are a prerequisite for sufficient long-term performance of an implant.

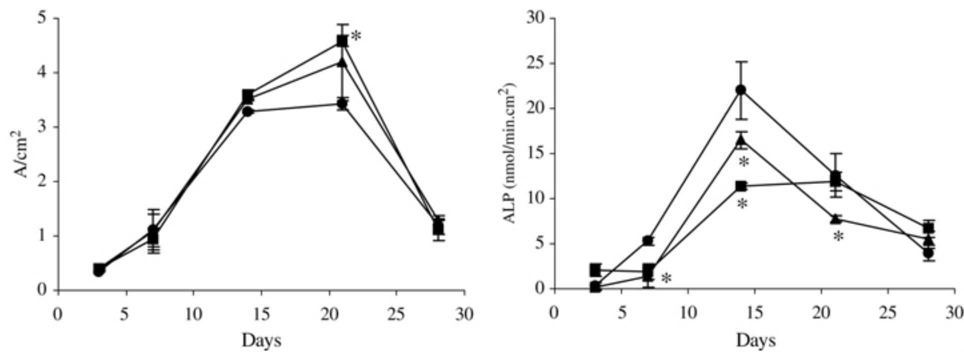


Figure 6 Cell viability/proliferation (A/cm^2) and ALP activity ($nmol/min\ cm^2$) of human bone marrow cell cultures grown on the mechanical treated titanium surfaces for 28 days. G_0 (●), $B65_0$ (■) and $B250_0$ (▲). *Significantly different from G_0 samples.

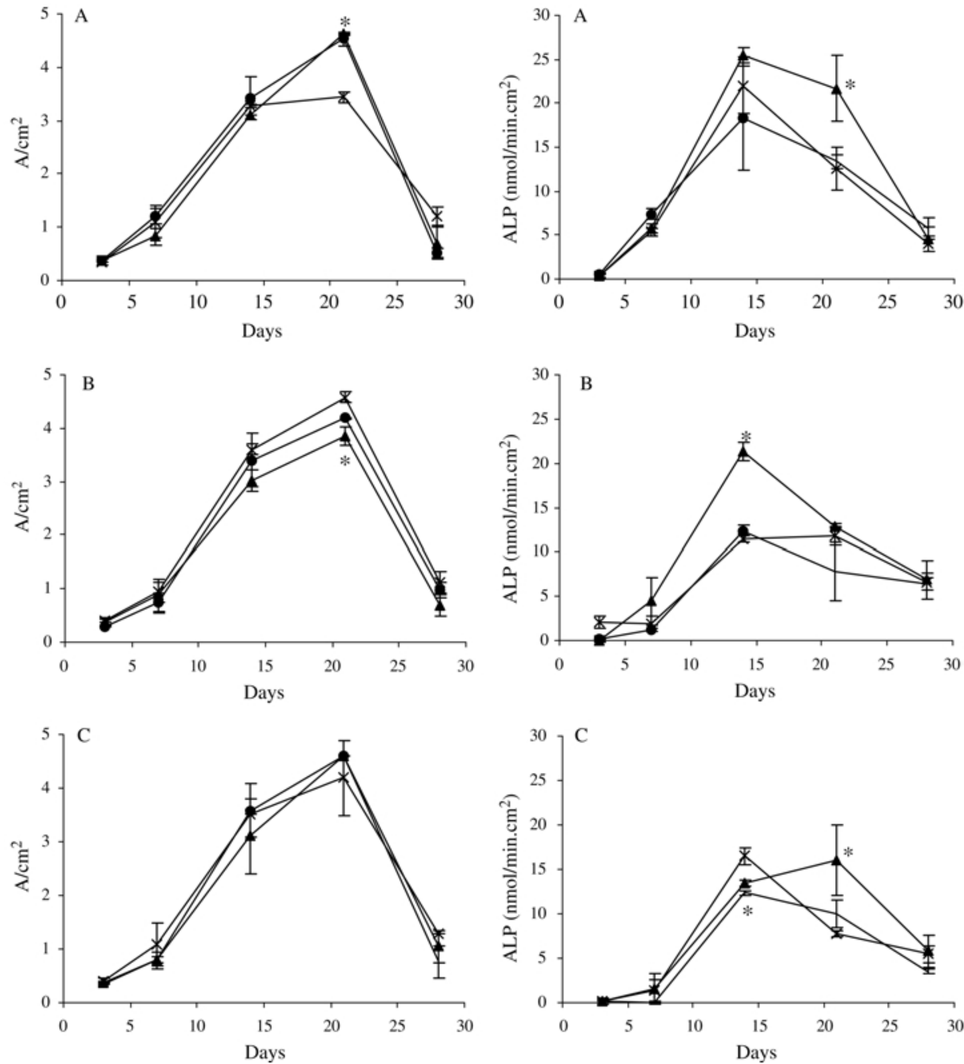


Figure 7 Cell viability/proliferation (A/cm^2) and ALP activity ($nmol/min.cm^2$) of human bone marrow cell cultures grown on titanium surfaces for 28 days. G_i family (A), $B65_i$ family (B) and $B250_i$ family (C). G_0, $B65_0$, $B250_0$ (×); G_S, $B65_S$, $B250_S$ (•);G_F, $B65_F$, $B250_F$ (▲). *Significantly different from the non-chemical treated samples, respectively, G_0 (A), $B65_0$ (B) and $B250_0$ (C).

5. Conclusion

Titanium samples of different roughness and morphology were prepared using a combination of mechanical and acid treatments. Both chemical solutions act reducing the arithmetic average roughness (R_a) of the mechanical treated samples, with hydrofluoric acid solution producing the most homogeneous and clean surface.

Human bone marrow cells appear to be very sensitive to variations in surface topography resulting in differences in the biological performance of the prepared titanium samples. Results suggest that surface roughness

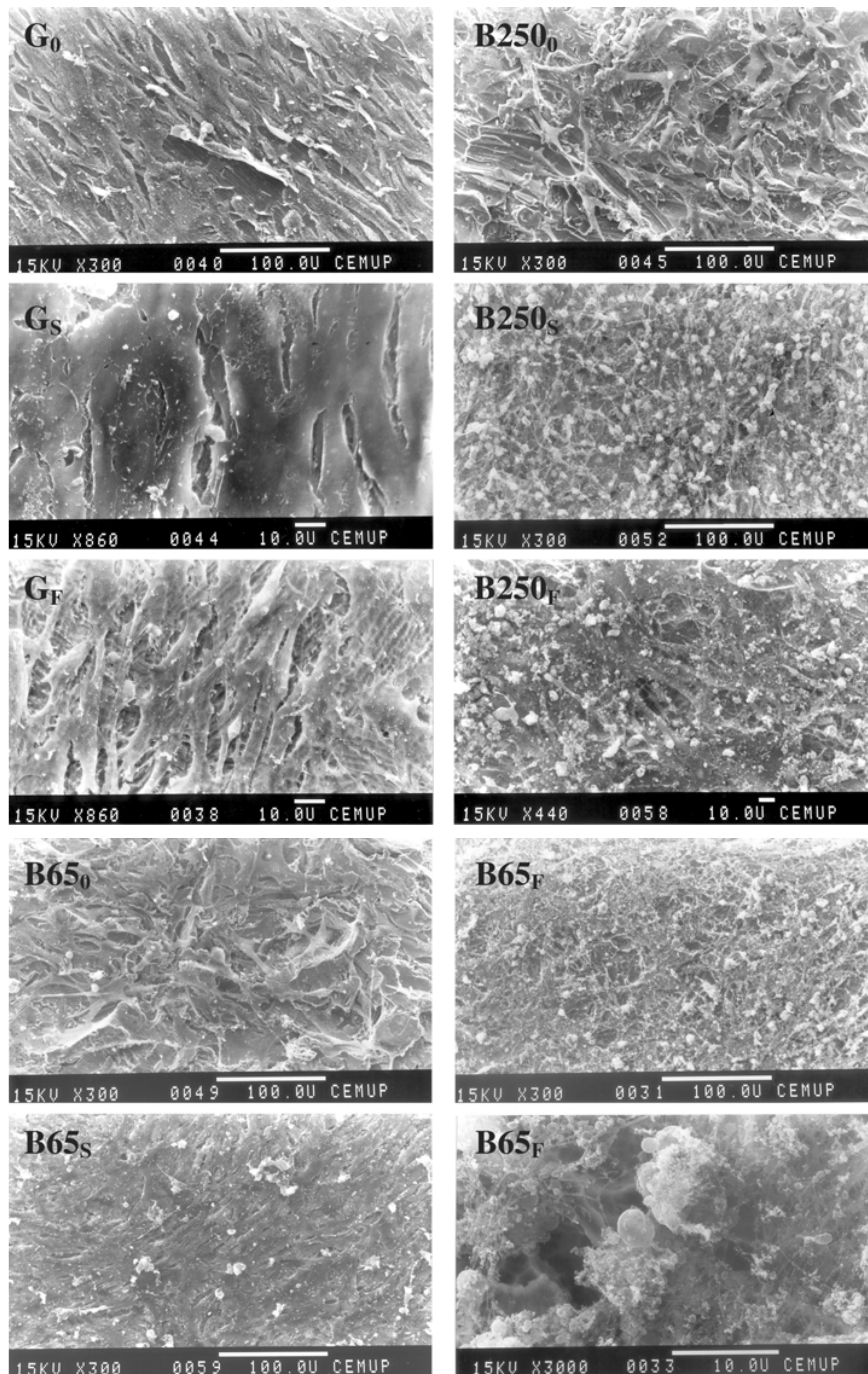


Figure 8 SEM appearance of 21-day seeded titanium samples. A higher magnification of the seeded $B65_F$ sample showed an abundant extracellular matrix that is heavily mineralized.

and morphology may modulate osteoblast differentiation, namely the formation of an extracellular mineralized matrix, an essential step for the bone apposition on the material surface and the long-term performance of the implant.

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