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Effect of porous titanium coating thickness on in vitro osteoblast phenotype expression

ABSTRACT

Aim This study aimed at determining the effect of different thickness of porous titanium (Ti) coating, 0.5, 1.0 and 1.5 mm thick (PC-0.5, PC-1.0 and PC-1.5), on osteoblast phenotype expression.

Materials and methods Dense Ti discs coated with 0.5, 1.0 and 1.5 mm of porous Ti (PC-0.5, PC-1.0 and PC-1.5, respectively) were fabricated by powder metallurgy process with pore size typically between 50 and 400 µm and porosity of 60%. Osteoblastic cells obtained from human alveolar bone were cultured on dense Ti (D-Ti) and PC-Ti discs for periods of up to 17 days.

Results Cultures grown on PC-Ti exhibited higher cell proliferation rate than on D-Ti. By comparing PC-Ti groups, it was observed statistical differences on culture grown only at day 10 (PC-0.5<PC-1.0 and PC-1.5). Cells cultured on PC-1.5 presented the highest gene expression of type I collagen (COL), alkaline phosphatase (ALP), and osteocalcin (OC). The calcium content was significantly greater on PC-1.5 compared to all other groups.

Conclusion These results indicate that PC-Ti favored osteoblastic cell proliferation. In addition, they increased gene expression of osteoblastic markers and higher content of mineralized matrix was observed on the thicker PC-Ti coating (PC-1.5). Therefore, further in vivo evaluations should be done in order to investigate whether this structure should be considered for clinical implant applications.

KEY WORDS Bone; Cell culture; Cell-material interactions; Porous coated; Titanium.

INTRODUCTION

Titanium (Ti) is the most widely used biomaterial for orthopedic and dental implants thanks to its good mechanical properties and high biocompatibility, which allows direct bone-to-implant contact, named osseointegration (1-6). Implants are generally produced in dense forms, which could present limitations such as interfacial instability with host tissues and lack of biological anchorage for tissue ingrowth (7-10). In this context, bone-implant fixation approaches have been investigated and porous biomaterials with three-dimensional structure have been proposed as an alternative to provide biological anchorage through bone ingrowth into the pores (11–13). For such three-dimensional structures, the optimal pore size for bone ingrowth is typically between 50 to 500 µm. The presence of interconnected pores allows the vascular system development, which is required for continuing bone growth (8). A novel powder metallurgy process has recently been developed for the fabrication of Ti foams with unique microstructure. The process make possible the adjustment of the pore size within the range required for bone ingrowth (14,15).

By culturing MC3T3-E1 pre-osteoblastic cells on such porous Ti prepared with three different average pore sizes, St-Pierre et al. observed that cells are capable of growing and differentiating without relevant effect of pore size within the interval studied (15). In addition, we have shown that human alveolar bone-derived cells are able to develop the osteoblast phenotype and to produce mineralized extracellular matrix when grown on porous Ti produced by the same process with pore size ranging from 50 to 400 µm (16).

For clinical applications, implants may either be completely porous or coated with a porous coating.

Because in vitro tissue ingrowth may be restricted to the superficial regions of the porous Ti structure and a fully porous structure may exhibit reduced mechanical properties, porous coated implants are often used in clinical applications (8). Based on this, we hypothesized that not only the percentage of porosity and the pore size, but also the thickness of the porous layer could affect bone cell behavior. As up to now there are no works focused on this topic, we design our study to investigate the effect of three different porous Ti thicknesses on the in vitro development of osteoblast phenotype.

MATERIALS AND METHODS

Production of Ti samples

Porous Ti foams were produced as previously described (14). Ti powder was dry-mixed with a polyethylene binder and a chemical foaming agent (p,p'-oxybis[benzenesulfonyl hydrazide]). This powder mixture was poured into a mold and foamed at 210°C in argon. The resulting material was then debinded at 450°C and presintered at 800°C in argon. After machining, the porous Ti discs of different thicknesses (0.5, 1.0 and 1.5 mm) were washed for 15 min in acetone in an ultrasonic bath (Bandelin Sonorex, Amtrex Technologies Inc., St. Laurent, Quebec, Canada). The discs were then put in contact with dense Ti discs (12 mm diameter) and sintered at 1400°C under vacuum to obtain Ti discs coated with porous Ti of different thicknesses (PC-0.5, PC-1.0 and PC-1.5, respectively). The resulting coating has pore size varying between 50 and 400 µm and porosity of 60%. All samples were autoclaved at 120ºC for 40 min before using in the cell culture experiments.

Surface analysis

Topography of both D-Ti and the PC-Ti surfaces were examined under scanning electron microscopy (SEM - Jeol JSM 6301F, MA, USA). Analyses of surface roughness were carried out using a contact-scanning instrument designed for 3D measurements (Talyscan 150, Taylor Hobson, Leicester, England). Measurements were made on three samples of each group. Three areas of 5.04 mm x 5.00 mm each were scanned using a Gaussian filter with a cut-off wavelength of 0.8 mm. One hundred and twenty-one profiles were drawn on each sample and analyzed using the software TalyMap (Taylor Hobson). Amplitude roughness parameters (Sa and St), which represent the depth of the grooves, were evaluated. Spatial (Str) and hybrid (Sdr) parameters were used to identify texture strength (uniformity of texture aspect) and to verify the ratio between the surface area (taking the z height into account) and the area of the flat x, y plane, respectively. In addition, 2D parameters, Ra, Rt, and Rz, were also evaluated.

Culture of osteoblastic cells derived from human alveolar bone

Human alveolar bone fragments (explants) were obtained from healthy donors during third molar surgery extraction, using the research protocols approved by the Committee of Ethics in Research of the School of Dentistry of Ribeirao Preto – University of Sao Paulo. Osteoblastic cells were obtained from these explants by enzymatic digestion using type II collagenase (Gibco – Life Technologies, Grand Island, NY, USA) as previously described (17,18). These cells were cultured in minimum essential medium (Gibco), supplemented with 10% fetal bovine serum (Gibco), 50 µg/ml gentamicin (Gibco), 0.3 µg/ml fungizone (Gibco), 10^{-7} M dexamethazone (Sigma, St. Louis, MO, USA), 5 µg/ml ascorbic acid (Gibco), and 7 mM β-glycerophosphate (Sigma). Subconfluent cells in primary culture were harvested after treatment with 1 mM ethylenediamine tetraacetic acid (EDTA) (Gibco) and 0.25% trypsin (Gibco) and subcultured in 24-well culture plates (Falcon, Franklin Lakes, NJ, USA) on D-Ti and CP-Ti at a cell density of $2x10^4$ cells/sample. Cells were subcultured on polystyrene as a control of the culture conditions. During the culture period, cells were incubated at 37ºC in a humidified atmosphere of 5% CO2 and 95% air; the medium was changed every 3 or 4 days.

Culture growth

Culture growth was evaluated by 3-[4,5-dimethylthiazol-2-yl]-2,5- diphenyl tetrazolium bromide (MTT) assay at days 1, 3, 7 and 10 (19). Cells were incubated with 100 µl of MTT (5 mg/ml) in PBS at 37°C for 4 h. The medium was then aspirated from the well and 1 ml of acid isopropanol (0.04 N HCl in isopropanol) was added to each well. The plates were then agitated on a plate shaker for 5 min, and 100 µl of this solution were transferred to a 96-well format using opaque-walled transparent-bottomed plates (Fisher Scientific, Pittsburgh, PA, USA). The optical density was read at 570–650 nm on the plate reader (Quant, Biotek, Winooski, VT, USA) and data were expressed as absorbance. Samples of D-Ti and PC-Ti were incubated in culture medium without cells, assayed and the absorbance was subtracted from the absorbance of experiments carried out with cells to eliminate any background.

RNA extraction and quantitative real-time reverse transcriptase-polymerase chain reaction (Real-Time PCR)

Gene expression of type I collagen (COL), alkaline phosphatase (ALP), osteocalcin (OC), and osteopontin (OPN) were evaluated by real-time PCR at day 7. Gene-specific primers were designed with Primer

Express 2.0 (Applied Biosystems, Foster City, CA, USA) and are presented in Table 1.
The total RNA from cells was extracted using the Promega RNA extraction kit (Promega, Madison, WI, USA), according to the manufacturer instructions. The concentration of RNA was determined by optical density at a wavelength of 260 nm, using the Biomate 3 spectrophotometer (Thermospectronic, Rochester, NY, USA). Complementary DNA (cDNA) was synthesized using 2 µg of RNA through a reverse transcription reaction (M–MLV reverse transcriptase, Promega). Real-time PCR was performed in an ABI Prism 7000 Sequence Detection System using the SybrGreen system (Applied Biosystems, Warrington, UK). SybrGreen PCR MasterMix (Applied Biosystems), specific primers and 2.5 ng cDNA were used in each reaction. The standard PCR conditions were 95°C (10 min) and 40 cycles of 94°C (1 min), 56°C (1 min) and 72°C (2 min), followed by the standard denaturation curve. For mRNA analysis, the relative level of gene expression was calculated in reference to β-glucuronidase (β–GUS) expression and normalized by the gene expression of cells subcultured on D-Ti using the cycle threshold (Ct) method (20).

Extracellular matrix mineralization

Matrix mineralization was detected at 17 days by Alizarin Red S (Sigma), which stains areas rich in calcium. Attached cells were fixed in 10% formalin for 2 h at room temperature. After fixation, the specimens were dehydrated through a graded series of alcohol and stained with 2% Alizarin Red S (Sigma), pH 4.2, for 10 min. The calcium content was evaluated using a colorimetric method as previously described (21). Briefly, 280 µl of 10% acetic acid was added to each well containing Ti samples stained with Alizarin Red S, and the plate was incubated at room temperature for 30 min under shaking. This solution was transferred to a microcentrifuge tube and after vortexing for 1 min, the slurry was overlaid with 100 µl of mineral oil (Sigma), heated to exactly 85ºC for 10 min, and transferred to ice for 5 min. The slurry was then centrifuged at 20,000 g for 15 min and 150 µl of supernatant was transferred to a new microcentrifuge tube. Then, 60 µl of 10% ammonium hydroxide was added to neutralize the acid and this solution containing 210 µl was read at 405 nm in 96-well format using opaque-walled transparent-bottomed plates (Fisher Scientific) on the plate reader Quant (Biotek). Data were expressed as absorbance. Samples of D-Ti and PC-Ti were incubated in culture medium without cells, assayed and the absorbance was subtracted from the absorbance of experiments carried out with cells to eliminate any background.

Target*	Primer Sense Sequence Primer Anti-Sense Sequence	TM (°C)	bp
COL	AATCACCTGCGTACAGAACGG CAGATCACGTCATCGCACAAC	84	114
ALP	CGAGCTGAACAGGAACAACGT CCACCAGCAAGAAGAAGCCTT	84	104
OC	CAAAGGTGCAGCCTTTGTGTC TCACAGTCCGGATTGAGCTCA	85	150
OPN	AGACACATATGATGGCCGAGG GGCCTTGTATGCACCATTCAA	79	154
,-GUS	CGTCCCACCTAGAATCTGCTG ACATACGGAGCCCCCTTGTC	82	130

**COL, type I collagen; ALP, alkaline phosphatase; OC, osteocalcin; OPN, osteopontin; β–GUS, β–glucuronidase*

Table 1 *Primer Sequences, Melting Temperature (TM), and Product Size (bp) for Real-Time PCR Reactions*

Statistical analysis

Quantitative data from cell culture experiments presented in this work are the representative results of three separate experiments using three sets of cell cultures established from three different donors. Culture growth and extracellular matrix mineralization were evaluated in quadruplicates (n=4) and gene expression, in triplicates (n=3). Comparisons were done using analysis of variance (ANOVA) followed by Duncan's multiple range test or Kruskal-Wallis followed by Fischer test based on rank, where appropriate (level of significance: 5%).

RESULTS

The SEM micrographs of the D-Ti and the PC-Ti surfaces are presented in Fig. 1. The dense Ti surface was not perfectly smooth and cutting lines could be observed (Fig. 1A). The open-pores and the spherical nature of the powder used to produce the foam were clearly noticed on the PC-Ti surface (Fig. 1B). All PC-Ti have similar pore sizes and the only difference was the thickness of the porous Ti coating.
Computer-generated image of D-Ti and CP-Ti surfaces confirmed the characteristics observed on SEM images (Fig. 2A-D). Profiles of D-Ti (Fig. 2E) showed a periodic morphology at lower scale than PC surfaces (Fig. 2F-H). The autocorrelation of Ti surfaces presented a non-periodic component meaning a homogeneous pattern (Fig. 2I-L). All roughness parameters were greater on PC-Ti surfaces compared to D-Ti samples (Table 2). Besides, it was not possible to detect significant differences in roughness among PC-Ti surfaces (Table 2). These surfaces were quite homogeneous (Fig. 2F-H), as indicated by the low dispersion of Rz values (Table 2). However, the amplitude roughness values of PC-Ti surfaces were significantly higher

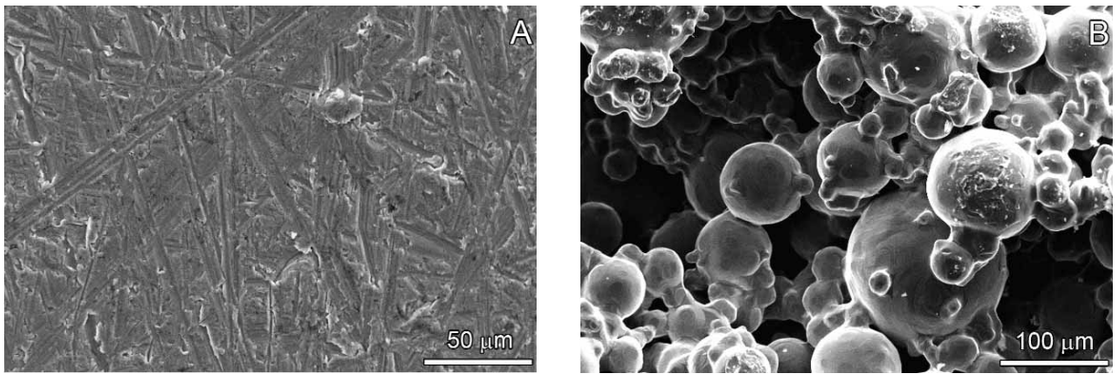

Fig. 1 *High resolution scanning electron micrographs of the dense Ti (D-Ti) and the porous-coated Ti (PC-Ti). The D-Ti surface (A) showed a slight roughness with some cutting lines. The PC-Ti structure (B) exhibited a unique topography characterized by a network of spherical particles.*

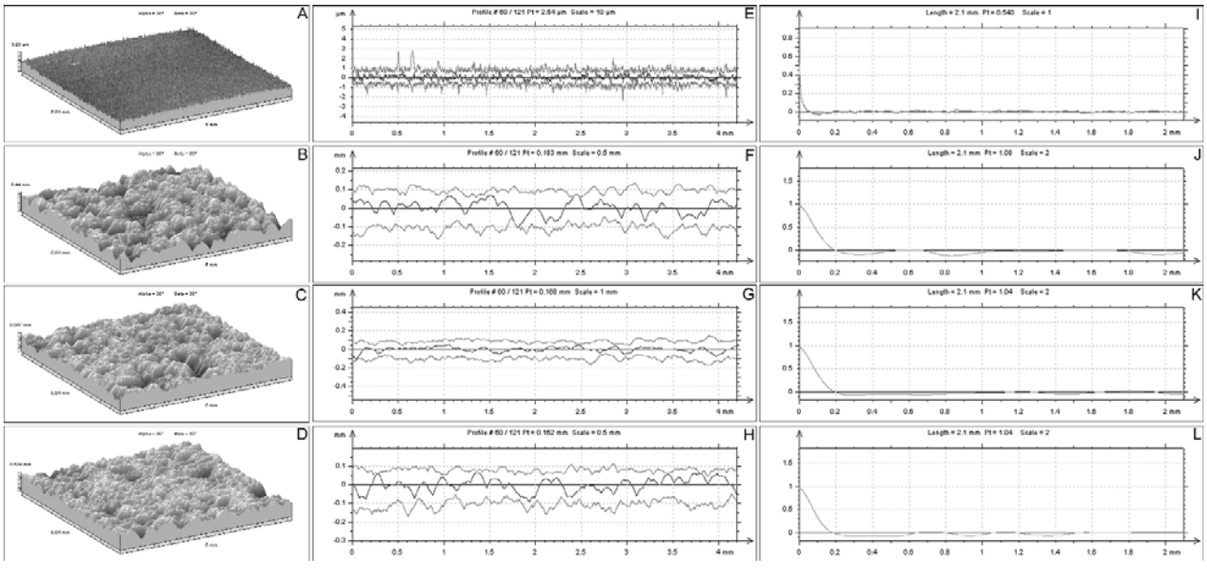

Fig. 2 *Computer generated surface images, original roughness profiles and autocorrelation function profiles for dense Ti (D-Ti) and porous-coated Ti (PC-Ti) surfaces. D-Ti (A, E and I), scale profiles (μm); Porous-coated 0.5 mm (PC-0.5 - B, F and J), scale profiles (mm); Porous-coated 1.0 mm (PC-1.0 - C, G and K), scale profiles (mm); Porous-coated 1.5 mm (PC-1.5 - D, H and L), scale profiles (mm).*

than D-Ti surface (Table 2). PC-Ti surfaces also exhibited the highest isotropy as Str value tends toward 1, meaning a spatially isotropic texture, i.e. uniform texture in all directions with no defined lay (Fig. 2B-D, Table 2). In contrast, D-Ti samples presented a surface with a dominating pattern (Fig. 2A) and a smaller Str value (Table 2), indicating an increasingly strong directional structure. We considered PC-0.5, PC-1.0 and PC-1.5 as rough isotropic surfaces and D-Ti as a smooth anisotropic surface.

Culture growth analyses indicated that cell number increased from day 1 to day 10, irrespective of the substrate surface ($p<0.0001$) and there were significantly more cells ($p<0.0001$) in cultures grown on PC-Ti surfaces at days 3, 7 and 10 than on D-Ti. By comparing PC-Ti groups, it was noticed statistical differences on culture growth only at 10 days (PC-0.5 and PC-1.0<PC-1.5) (Fig. 3). Osteoblast phenotype was confirmed at the transcriptional level by mRNA expression of the bone markers in cultures grown on D-Ti and PC-Ti surfaces at 7 days. Overall, cells cultured on PC-1.5 presented the highest ($p<0.05$) expression of COL, ALP, and OC (Fig. 4). The calcium content measured by the extraction of Alizarin Red S from mineralized matrix was significantly greater ($p<0.05$) on PC-1.5 compared to all other groups (Fig. 5).

DISCUSSION

There is a general agreement that implant surface properties affect bone cell responses and ultimately the

Ti surfaces	Ra* (µm)	Rt* (µm)	Rz* (µm)	Sa* (µm)	St*	SsK*	Str*	Sdr* (%)
D-Ti	0.15±0.03	1.70±0.36	1.29±0.26	0.17±0.04	5.31±0.49	-0.01±0.031	0.00±0.00	0.14±0.04
PC-0.5	22.00±0.72	135.00±5.57	105.67±4.04	36.00±0.70	313.67±10.69	-0.36±0.07	0.78±0.09	43.17±0.75
PC-1.0	21.63±0.57	138.00±6.08	106.33±4.04	35.30±1.20	315.67±14.36	-0.33±0.05	0.81±0.08	42.57±0.67
PC-1.5	20.87±0.40	132.00± 2.65	102.67±1.53	34.73±1.72	328.33±46.001	-0.41±0.09	0.82±0.08	43.10±0.36

Ra, arithmetic mean deviation of the roughness profile; Rt, total height of roughness profile; Rz, maximum height of roughness profile; Sa, arithmetic mean deviation of the surface; St, total height of the surface; Ssk, skewness of the topography height distribution; Str, texture aspect ratio of the surface; Sdr, developed interfacial area ratio.
Parameters values presented as mean ± standard deviation.
**Statistically significant (p<0.05) – PC-1.5, PC-1.0 and PC-0.5>D-Ti*

Table 2 *Roughness parameters measured on dense (D-Ti) and porous-coated (PC) Ti surfaces (PC-0.5, PC-1.0 and PC-1.5).*

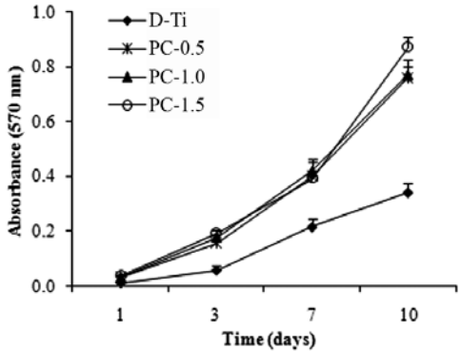

Fig. 3 *Growth curves (absorbance) of osteoblastic cells subcultured on dense Ti (D-Ti) and porous-coated Ti (PC-Ti) at days 1, 3, 7, and 10. It was noticed significantly more cells (p<0.0001) in cultures grown on PC-Ti surfaces at days 3, 7 and 10 than on D-Ti. Among PC-Ti groups, it was observed statistical differences on cultures only at 10 days (PC-0.5 and PC-1.0<PC-1.5). Data are reported as mean ± standard deviation (n=4).*

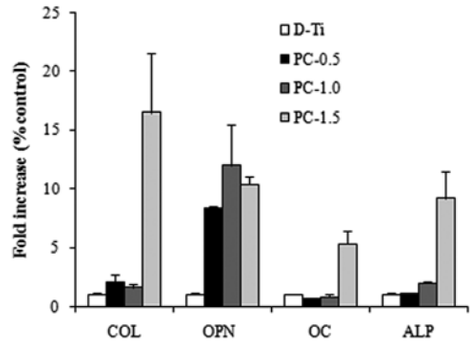

Fig. 4 *Gene expression of type I collagen (COL), alkaline phosphatase (ALP), osteocalcin (OC), and osteopontin (OPN) in osteblastic cells subcultured on dense Ti (D-Ti) and porous-coated Ti (PC-Ti) for 7 days. Cells subcultured on porous-coated 1.5 mm (PC-1.5) surface presented the highest (p<0.05) expression of all evaluated genes compared to the other Ti surfaces. Data are reported as mean ± standard deviation (n=3).*

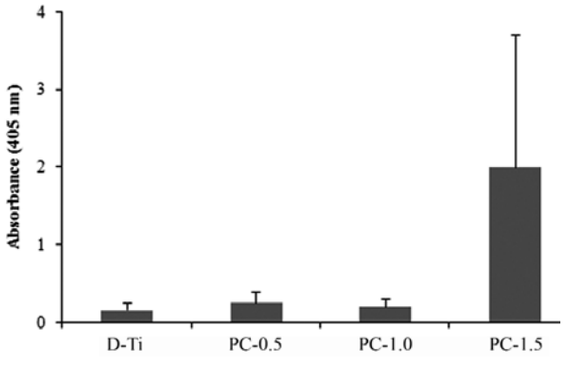

Fig. 5 *Calcium content extracted from mineralized extracellular matrix of osteoblastic cultures grown on dense Ti (D-Ti) and porous-coated Ti (PC-Ti) for 17 days. The amount of calcium was significantly greater (p<0.05) on porous-coated 1.5 mm (PC-1.5) surface compared to all other Ti surfaces. Data are reported as mean ± standard deviation (n=4).*

process of osseointegration. Although Ti exhibits a superb ratio of clinical success as implant biomaterial, a relevant number of studies has been focused on surface modifications to enhance and/or accelerate the bone formation in direct contact with Ti implants. The present study was designed to investigate the osteoblast phenotype development of human alveolar bone-derived cells cultured on dense Ti and Ti coated with three different thickness of porous coating (0.5, 1.0, and 1.5 mm). Indeed, confirming our hypothesis, the results indicate that the thickness of porous Ti coating has effect on osteoblast cell behavior. In addition, it was observed that the expression of osteoblast phenotype and the production of mineralized extracellular matrix were higher on the thicker porous coating evaluated (PC-1.5).

One possible explanation for these findings is the larger surface available for bone apposition when the thickness of the coating increases.

Porous Ti coating with pore size varying between 50 and 400 µm was used in this study, fitting the optimal pore size range for bone ingrowth (7, 8, 22, 23). For all samples, topography was analyzed using amplitude and frequency parameters as well as surface topography parameters previously used to characterized implants intended for orthopedic and dental applications (24, 25). The average roughness values were

higher on PC-Ti than on D-Ti (Sa was 200-fold greater on PC-Ti surfaces). Regarding the topography height distribution, the skewness value of a Gaussian surface exhibiting a symmetrical shape is zero. For an asymmetric distribution of surface heights, the skewness may be negative or positive. All surfaces evaluated in this study presented negative values, indicating that the distribution has a longer tail on the lower side of the mean plane, which means more valleys than peaks. Additionally, PC-Ti surfaces exhibited the highest isotropy as Str values tend towards 1, meaning a uniform texture in all directions with no defined lay. In contrast, D-Ti samples presented a surface with a dominating pattern and the smaller Str value indicated a strong directional structure. As chemical composition and most of the surface parameters were similar among PC-Ti surfaces, it is possible to suggest that the differences in cell responses to these surfaces are essentially related to the thickness of the porous Ti coating.

The development of osteoblast phenotype is skeletal site-specific, and is most likely related to different embryological origins and functions of each site (26). Thus, cell source should be taken into account in studies of the interaction between cells and biomaterials. Since we are considering dental implant applications, we decided to carry out this study by using osteoblastic cells derived from human alveolar bone, which is one of the most active bone in the human body (27). It has been shown that the architecture of the porous biomaterials may provide a favorable environment for cell growth. Cultures of osteblastic cells derived from human alveolar bone presented a higher proliferation rate on porous Ti compared to dense Ti (16). Also, newborn mouse calvaria-derived MC3T3-E1 pre-osteoblastic cultures and MG63 cells grown on porous Ti exhibited more cells than on dense Ti surface or treated Ti, respectively (15, 28). Corroborating these findings, in the present study we demonstrated that cell proliferation evaluated by the reduction of MTT by the mitochondrial dehydrogenase of viable cells was significantly increased in cultures grown on PC-Ti surfaces. In addition to the three-dimensional structure that mimics the architecture of trabecular bone and supports tissue ingrowth, the positive effect of porous biomaterials on proliferation could also be attributed to the larger surface area available for cell apposition (16, 29-31). By comparing PC-Ti groups, it was only noticed statistical difference on cultures growth at 10 days (PC-0.5 and PC-1.0<PC-1.5). A possible reason is the low penetration of the cells in the structure of the foams as previously demonstrated using similar in vitro tests where cell growth was restricted to the superficial regions of the porous surface (16).

Significant differences between dense Ti and porous Ti were also observed regarding the osteoblast differentiation (15, 16). Our previous study showed a delay in the osteoblast phenotype development in cultures grown on 2 mm thick porous Ti discs (16). The present results demonstrated that the gene expression of COL, ALP, and OC, key markers of the osteoblast phenotype, and the production of mineralized extracellular matrix were higher in cultures grown on the thicker porous Ti coating (PC-1.5) compared to other porous Ti coatings (PC-0.5 and PC-1.0) and dense Ti surfaces. Taken together, the results of our previous study on 2 mm thick porous Ti discs and the present results with 0.5, 1.0, and 1.5 mm thick porous coatings suggest that the thickness of 1.5 mm allowed a better in vitro osteoblastic cell response in terms of proliferation, osteoblast phenotype expression and extracellular matrix mineralization.

In conclusion, this study confirmed that PC-Ti surfaces produced by a novel powder metallurgy process, allowed human alveolar bone-derived cells to adhere, proliferate, differentiate and produce mineralized extracellular matrix. Additionally, we present new data indicating that the thickness of the porous Ti coating exerts a relevant influence on these events. Finally, porous coating Ti with 1.5 mm in thickness seems to favor osteoblast growth, differentiation, and extracellular matrix mineralization. In this way, further in vivo studies should be carried out to evaluate whether this structure is the more suitable for clinical implant applications.

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