In Vitro Behavior of Primary Human Osteoblasts Onto Microrough Titanium Surface

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Objective: *The aim of this study was to evaluate* in vitro *the behavior and the biocompatibility of primary human osteoblasts (HOs) grown onto different implant surface.*

Methods and Materials: *HOs were cultured onto sandblasted/acid-etched (control group) and sandblasted/acid-etched followed by coating with inorganic ions (test group) experimental titanium discs. At established times, SEM analysis, LDH assay, MTT assay, and enzyme-linked immunosorbent assay for type 1 collagen, interleukin (IL)-6, and PGE_2 secretion were performed.*

Results: *Both surfaces promote HOs adhesion and proliferation. After 21 days, cells on test surfaces are well spread, flattened, and attached by cellular extensions, whereas cells on control discs appear mainly elongated. Lower LDH levels and higher values of MTT assay are recorded for cells on test respect to control surfaces at each experimental time. Type 1 collagen release increases until 14 days, significantly decreasing at day 21 in cells grown on both surfaces. IL-6 and PGE_2 secretion shows a peak in control group samples at day 7, whereas their levels do not significantly modify in both groups at days 14 and 21.*

Conclusion: *Results indicate that the test group surface is more biocompatible, well tolerated, and suitable for supporting osteoblasts growth and proliferation. (Implant Dent 2015;24:377–383)*

Key Words: cell adhesion, cell-material interactions, surface modification, in vitro

From early Brånemark's studies, the use of osseointegrated dental implants has become increasingly widespread, and implant dentistry has made great scientific achievements in the past decade. Clinical success of osseointegrated implants is based on a number of factors, all of which have as common denominator the achievement of osseointegration, that is, the intimate contact between implant surface and bone tissue. This phenomenon is closely influenced by chemistry and surface topography.[1] In recent years, scientific research on dental implants has been primarily focused on the development of new surface modifications or special coatings that were able to improve the biological characteristics of titanium.[2] Topography of titanium surfaces is considered one of the most important factors for the success of dental implants.[2,3] Indeed, the degree of surface roughness influences the behavior of mesenchymal precursors of bone cells, modulating proliferation, and differentiation towards the osteoblastic phenotype.[4,5] Moreover, it is very important that implant surface allows formation of a stable clot inducing proliferation, differentiation, and migration of osteoblastic cells from mesenchymal precursors elements, followed by regeneration of an adequately vascularized bone tissue that is able to reshape, improve the healing and mechanical stability of implant fixture, avoiding resorptive phenomena or any adverse reaction.[6–8] In fact, osteoblastic cells dislike extremely rough surfaces, but require intermediate values of roughness allowing them to organize their cytoskeleton and to adhere with focal contacts, reducing overall adhesive strength.[9,10] For this purpose, various methods such as sandblasting, sandblasting and acid-etching, titanium plasma spray, laser sintering of

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titanium microspheres have been proposed.[2,11–13] *In vitro* and *in vivo* studies demonstrated that, currently, sandblasted and acid-etched surfaces showing greater biomechanical stability, high bone-to-implant contact percentages, reduced release of inflammatory cytokines, high mechanical resistance, a lower risk of clinical failures[2] seem to offer the best compromise. However, using current procedures for titanium surface modification osseointegration is still not complete as, even for successful implants, the average bone-to-implant contact area is 70% to 80%.[14] In recent years, new innovative implant surface treatments have been proposed, to improve surface quality of titanium dental implants, to obtain a higher rate of bone-to-implant contact, and to reduce healing times.[15–21] By acting on the titanium surface charge, it could be possible to enhance osteogenic cells adhesion and early phases of mineralized matrix deposition at the bone-implant interface.

The aim of this study was to evaluate *in vitro* the behavior and the biocompatibility of human primary osteoblasts grown onto an implant surface obtained by sandblasting and acid-etching and then treated by coating with inorganic ions.

Materials and Methods

Samples Preparation

For this *in vitro* study, a total of 40 (5 mm diameter and 2 mm thick) experimental titanium discs (Implacil De Bortoli-Dental Product, São Paulo, Brazil) were used. Discs were divided into 2 experimental groups according to the type of treatment: 20 experimental titanium discs sandblasted and acid-etched (control group) and 20 experimental titanium discs sandblasted and acid-etched as above and then treated by coating with inorganic ions (test group). In brief, all titanium discs after machining were cleaned by ultrasonic treatment in an enzymatic detergent solution, then rinsed with sterile purified water (cell-culture grade), and ultrasonically treated again for 5 minutes in absolute alcohol. Then, surface was blasted with microparticles of titanium oxide (TiO_2), with an average particle size of approximately 180 μm, cleaned in ultrasound with an enzymatic detergent for removing residues from blasting, and then etched with glacial acetic acid. After this treatment, test group discs were immersed in a CaP solution, according to Ibasco et al,[22] to allow deposition of calcium and phosphorus ions on the titanium surface.

The discs to be used for in vitro experiments were transferred aseptically to sterile 48-well cell-culture trays and submerged in acetone, rinsed with sterile deionized water and then with absolute alcohol, and dried at 80°C under aseptic conditions.

Atomic Force Microscope Analysis of Titanium Discs Surface

Topographic analysis of titanium discs surface on a nanoscale level was performed by an atomic force microscopy (AFM; Multimode 8; Bruker, Santa Barbara, CA). Surfaces were imaged with a scan rate from 0.1 to 1.95 Hz. The examination was performed by using a silica tip. AFM surface images were acquired in a tapping mode in area up to 5 × 5 μm. All images were processed by Nanoscope Analysis software. Nanoroughness evaluation was performed on the same instrument in Peak Force QNM operation mode. All images were processed by Nanoscope Analysis software using the function roughness.

Primary Human Osteoblasts Isolation and Culture

Primary human osteoblasts (HOs) were obtained from fragments of femoral head of patients undergoing total hip prosthetic replacement surgeries for osteoarthritis (age range, 75–85 years). Each subject gave informed written consent for participating in this study as a donor of femoral head in accordance with the local ethics committee, in compliance with Italian legislation, and with the code of Ethical Principles for Medical Research involving Human Subjects of the World Medical Association (Declaration of Helsinki). Soft tissues from the outer surfaces of the femoral head were removed by scraping with a sterile scalpel blade. Bone chips were withdrawn from the medullar part, minced into 1 to 2 mm width fragments, and washed in phosphate-buffered saline (EuroClone S.p.A, Milan, Italy). Bone fragments were then transferred to Petri dish and cultured in a Dulbecco's Modified Eagle's Medium (DMEM; EuroClone S.p.A) supplemented with 10% Fetal bovine serum (FBS; EuroClone S.p.A), 1% penicillin/streptomycin (EuroClone S.p.A) at 37°C in a humidified atmosphere and 5% (vol/vol) CO_2. Within 7 to 10 days of culture, cells were observed migrating out of the bone fragments. Culture medium was changed every 4 days, and cells became confluent within 4 to 5 weeks. After reaching confluence, bone fragments were removed with sterile forceps, and cells were washed, trypsinized (EuroClone S.p.A), and transferred to culture flasks.

Osteoblastic phenotype was assessed through alkaline phosphatase assay and Western blotting analysis for osteocalcin expression as described below.

When 58% of the cells became alkaline phosphatase (ALP) positive and were seeded and cultured on titanium surfaces in the presence of DMEM (EuroClone S.p.A) supplemented with 10% of FBS (EuroClone S.p.A), 1% penicillin/streptomycin (EuroClone S.p.A) at 37°C in a humidified atmosphere, and 5% (vol/vol) CO_2 for a total of 21 days. The culture medium was replaced every 3 to 4 days. At established experimental times (7, 14, and 21 days), cells were yielded and processed. Because osteoblastic phenotype was maintained through five passages, HOs after 3 to 4 passages were used.

Evaluation of the Osteoblastic Phenotype and ALP Assay

Osteoblastic phenotype was assessed by means of Sigma 86-R staining kit (Sigma-Aldrich, St. Louis, MO). Samples were observed under a Leica DM 4000 light microscopy equipped with a Leica DFC 320 camera (Leica Cambridge Ltd., ambridge, United Kingdom).

Percentage of ALP-positive cells was obtained by direct visual counting of ten fields per sample chosen

through systematic and uniformly random sampling of the fields of vision through a predefined XY step, by identifying the intensity of the staining and of the granules' size, according to the manufacturer's instruction. QWin Plus 3.5 assessments were logged into Microsoft Excel and processed for SDs and histograms.

Western Blotting Analysis of Osteocalcin Expression

Total eukaryotic cell lysates (20 μg) were electrophoresed on a 4% to 20% sodium dodecyl sulphate–polyacrylamide gel and transferred to nitrocellulose membrane. Nitrocellulose membranes, blocked in 5% nonfat milk, 10 mM Tris-HCl pH 7.5, 100 mM NaCl, 0.1% Tween-20, were probed with rabbit anti-osteocalcin primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA; catalog number: sc-30044, antibody dilution 1:200) and mouse anti β-tubulin primary antibody (Sigma-Aldrich, Milan, Italy; catalog number T4026, antibody dilution 1:1000). After that nitrocellulose membranes were incubated in the presence of specific horseradish peroxidase–conjugated IgG (goat anti-rabbit, Calbiochem, Merck KGaA, Darmstadt, Germany, catalog number 401393, antibody dilution 1:20,000). Immunoreactive bands were detected by Lite Ablot Extend system (Euroclone, Milan, Italy) and analyzed by densitometry. Densitometric values, expressed as integrated optical intensity, were estimated in a ChemiDoc XRS system by the QuantiOne 1-D analysis software (Bio-Rad, Richmond, CA). Values obtained were normalized based on densitometric values of internal β-tubulin. Statistical analysis was performed using the analysis of variance. Results were expressed as mean ± SD; values of $P < 0.05$ were considered statistically significant.

SEM Analysis

SEM analysis was conducted onto control and test group titanium discs before cell seeding, for surface microstructure observation. Moreover, at the established time points, HOs cultured on titanium discs were fixed in 2% glutaraldehyde in 0.1 M phosphate buffer for 30 minutes at 4°C, then postfixed in OsO_4 (osmium tetraoxide), dehydrated in an increasing ethanol series, and finally dried in HSMDA (hexa-methyl-disilazane). SEM analysis was performed at the InterDept.al Service Center C.U.G. A.S. (University of Padova, Italy) using a Quanta 200 (FEI company, Hillsboro, OR) instrument equipped with an energy dispersive x-ray (EDS; EDAX Inc., Mahwah, NJ) detector. Samples were analyzed directly after placing on a vitreous carbon planchet, after being metalized with gold in a sputtering. Backscattered electron images were collected at 25 kV. Representative EDS spectra were collected in potentially interesting sites to obtain a qualitative indication of the elemental composition of the surface and particles identified in the samples.

MTT Assay

The metabolic activity of the HOs was evaluated after 7, 14, and 21 days of culture on titanium test and control discs through the MTT (3-[4,5-dimethyl-thiazol-2-yl-]-2,5-diphenyl tetrazolium bromide) assay (Sigma-Aldrich, Saint Louis, MO), based on the capability of viable cells to reduce MTT into a colored formazan product. At the established time points, the medium was replaced by a new one containing 0.5 mg/mL MTT and probed with cells for 5 hours at 37°C. The plate was incubated in dimethyl sulfoxide solution for 30 minutes at 37°C to solubilize salts and then read at 570 nm. Optical density was measured by means of spectrophotometric reading performed at 570 nm with a spectrophotometer Anthos 2010 96 (Anthos Labtec Instruments, Salzburg, Austria). Values obtained in the absence of cells were considered as background.

L-lactate dehydrogenase Assay

LDH released into the medium was quantified *in vitro* using kit-LDH (Sigma-Aldrich, St. Louis, MO), following the directions of the manufacturer, after 7, 14, and 21 days of culture on titanium discs. At each experimental time, LDH released was

Table 1. AFM Analysis of Titanium Discs Surface

	Rq (nm)	Ra (nm)	Image Rmax (nm)	Frequency (x, y/μm^{-1})	Equivalent RMS (nm)	Sdr	Sdq
Test	62.2	45.0	867	24.8, 0.20	138	23.0%	35.9°
Control	67.2	54.4	343	1.00, 126	62.3	43.5%	47.4°

Values are referred to a significant area for each sample groups.
Rq indicates roughness least square value; Ra, average of the absolute values of the surface high deviations; Image Rmax, average feature height; Sdq, root-mean-square of the surface; Sdr, developed interfacial area ratio.

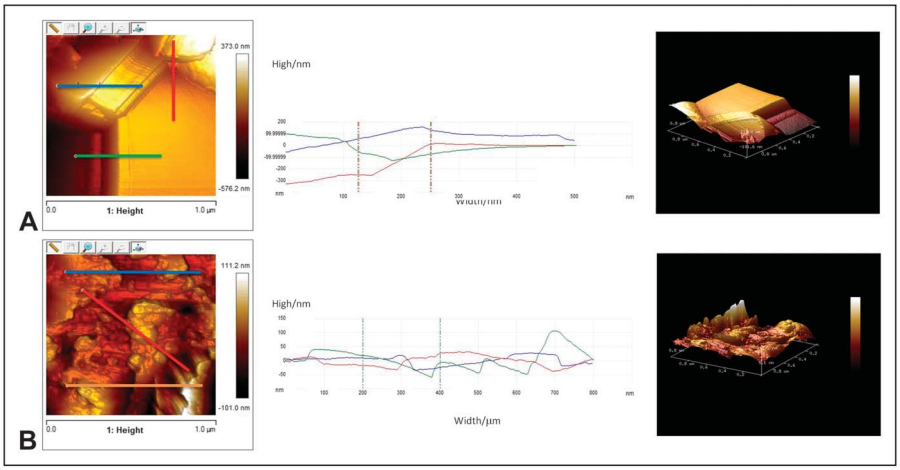


Fig. 1. AFM analysis of control group (**A**) and test group (**B**) titanium discs surface at the scan size of 1 × 1 μm. Figures on the left represent 2D images, figures on the right 3D reconstruction, central plots indicate roughness in terms of peak high along the highlighted 3 sections.

measured in different wells and normalized to the relative number of viable cells (OD 490 nm/10^4 viable cells), as previously determined with the MTT assay.

Enzyme-Linked Immunosorbent Assay Test of Collagen Type I, Interleukin 6, and PGE_2 Secretion

Collagen type I, interleukin (IL)-6, and PGE_2 secretion in the culture medium at the different experimental times for both samples was detected by following the instructions provided by the manufacturer. The amount of collagen type I was assessed by enzyme-linked immunosorbent assay (ELISA) Human Collagen type I, (Cosmo Bio Co. Ltd., Tokyo, Japan); EIA kit (Enzo Life Sciences, Farmingdale, NY) was used to determinate IL-6 and PGE_2 concentrations. The absorption values were obtained by spectrophotometric reading at 405 and 450 nm respectively by means of plates for reading in the spectrophotometer 96 Anthos 2010 (Anthos Labtec Instruments, Salzburg, Austria). Secretion levels of collagen type I, IL-6, and PGE_2 were measured in different wells and normalized for relative number of viable cells (viable cells μg/mL/10^4 for collagen type I and pg/mL/10^4 viable cells for IL-6 and PGE_2) as previously determined by MTT assay.

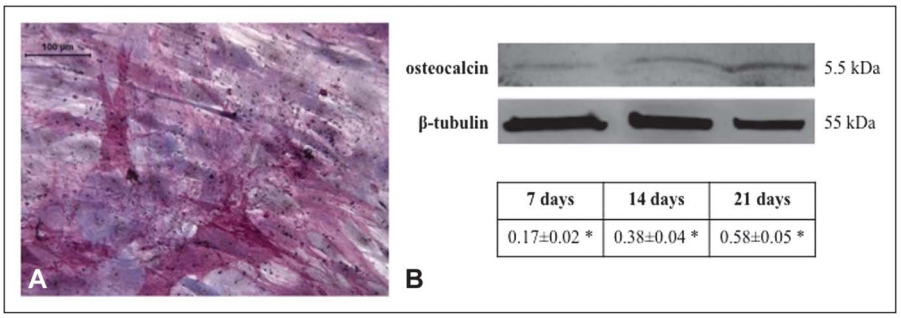
Statistical Analysis

Statistical analysis was performed using SPSS software GraphPad Prism 5 and evaluation with *t* test. The results were expressed as means ± SD significant. We considered significant *P* values <0.05.

Results

AFM Analysis of Titanium Discs Surface

The values obtained through AFM analysis are reported in Table 1. Although data are characterized by a high error, all the roughness indexes do differentiate test group discs surface from control group. Nevertheless, the roughness least square value (Rq) and the average of the absolute values of the surface high deviations (Ra) are more concerned with a best fit of all height points than with the spatial frequency of features. In terms of Rq and Ra, control group discs surface seems to manifest a higher roughness than test group sample because Rq and Ra do not reveal smaller surface features. Instead, it is worth considering frequency profile of peaks and valleys. The frequency can be highlighted by counting, in the central plot of



7 days	14 days	21 days
0.17±0.02 *	0.38±0.04 *	0.58±0.05 *

Fig. 2. **A**, Evaluation of the osteoblastic phenotype through ALP assay. **B**, Western blotting analysis for osteocalcin secretion. Values obtained were normalized based on densitometric values of internal β-tubulin. *$P < 0.05$.

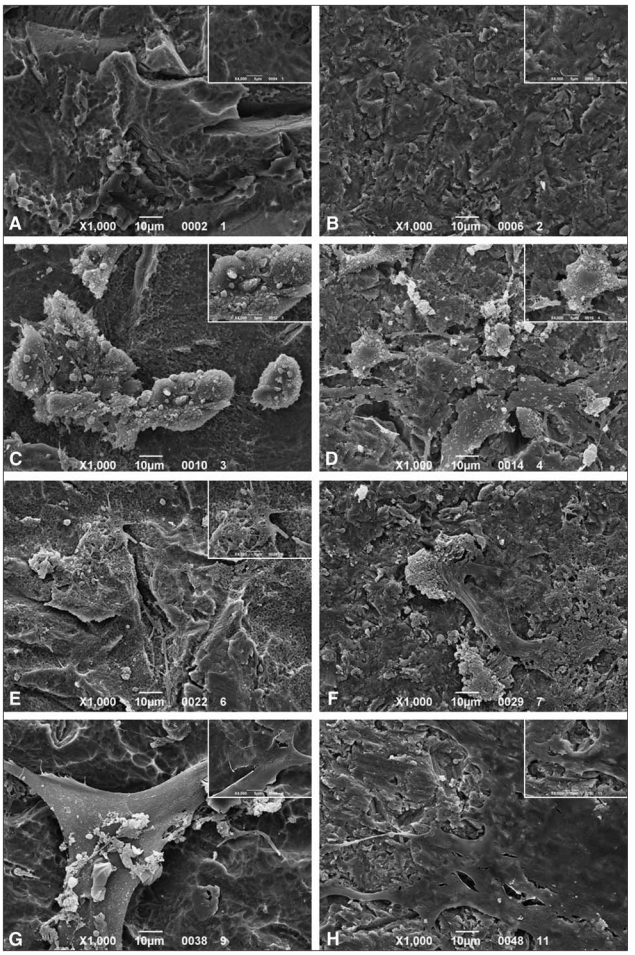


Fig. 3. SEM analysis of the control group and test group titanium discs surface in different experimental conditions at 1000× and 4000× (insets) magnification. **A**, Control group before HOs culture. **B**, Test group before HOs culture. **C**, Control group after 7 days of HOs culture. **D**, Test group after 7 days of HOs culture. **E**, Control group after 14 days of HOs culture. **F**, Test Group after 14 days of HOs culture. **G**, Control group after 21 days of HOs culture. **H**, Test group after 21 days of HOs culture.

Figure 1, the number of peaks obtained from each section evidenced in the images on the left. By considering frequency, test appears to be characterized by slightly lower peaks (Image Rmax) but distributed at narrower (higher frequency) intervals in the samples.

The root-mean-square of the surface (Sdq) and the developed interfacial area ratio (Sdr) confirm the previous hypothesis. As a matter of fact, Sdr is expected to give the surface enlargement induced by the different pretreatments. This means that, on average, both surfaces are highly rough (being Sdr >1%) but test group disc surface is characterized by a topography consisting of low peaks that cover the whole surface, whereas the control group disc surface is characterized by steeper and fewer peaks (check Sdq). On increasing the scan size of test sample (data not shown) from 1×1 μm to 5×5 μm, roughness indexes are confirmed.

Primary HOs Sampling and Culture

Before seeding cells on experimental discs, osteoblastic phenotype was verified by means of the ALP assay and confirmed when 58% of the cell population showed positive ALP, a specific marker of osteoblast line (Fig. 2, A).

The expression of osteocalcin, biochemical marker of the bone formation process, analyzed by Western blot analyses, significantly increases from day 7 up to day 21 of culture (Fig. 2, B).

SEM Analysis

SEM analysis of titanium discs, performed before cells seeding to assess the topography of the test and control group surfaces, shows a less rough topography in discs treated with inorganic ions, with respect to control ones, which disclose high peaks and deep microcavities (Fig. 3, A and B).

No significant difference in terms of cell adhesion and spreading on the 2 surfaces examined is evidenced after 7 and 14 days of culture (Fig. 3, C–F), whereas a modest increase of osteoblastic expansion is recognized at day 21 when the cells appear to be well spread, flattened, and attached to the substrates by cellular extensions, only on titanium discs belonging to the test group. Thus, SEM images indicate that the treatment with inorganic ions of titanium surfaces promotes osteoblasts adhesion and proliferation. On the contrary, cells cultured on control group discs appears to be mainly elongated (Fig. 3, G and H).

MTT Assay

MTT assay shows that the metabolic activity is higher in cells grown on test group surfaces with respect to control group surfaces at all experimental times, even if differences are not statistically significant (Fig. 4, A).

L-Lactate Dehydrogenase (LDH Assay)

LDH released into the medium/OD. MTT shows lower levels of LDH in cell cultured at all experimental times on test group surfaces with respect to control group surfaces (Fig. 4, B). Differences between groups are statistically significant at 7 and 21 days of culture ($P < 0.05$).

Collagen Type I, IL-6, and PGE_2 Release Assay

ELISA assay evidences that collagen type I release is higher in cells grown on test group surfaces compared with control group surfaces at 7 and 14 days of culture, whereas at 21 days, it is higher in cells grown on control group surfaces. Moreover, in both groups, the release increases until 14 days decreasing up to basal level at day 21 (Fig. 5, A).

After 7 and 21 days of culture, IL-6 secretion is higher in cells grown on control group surfaces with respect to those grown on test surfaces, whereas at 14 days, the levels are lower on control group surfaces compared with test surfaces (Fig. 5, B).

PGE_2 release is increased significantly at day 7 in cells grown on control group surfaces than to test surfaces, whereas at 14 and 21 days, evidences similarly reduced levels for both

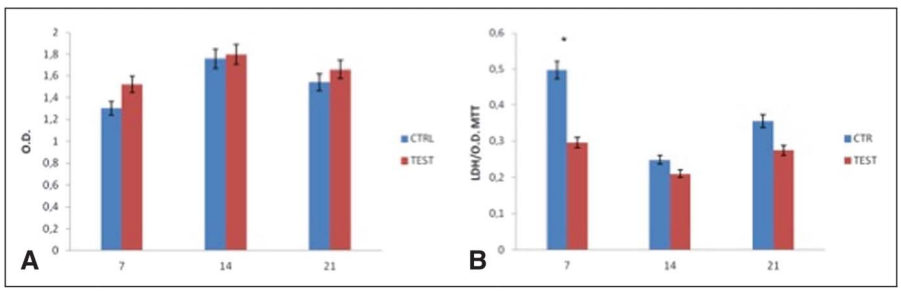


Fig. 4. A, Proliferation assay of HOs cultured on control and test group experimental discs. HOs viability was measured by MTT assay at 7, 14, and 21 days and reported as number of viable cells. The results are the mean of 2 samples from 3 different experiments ± SD; **B**, Cytotoxicity assay of HOs cultured on control and test group experimental discs. LDH leakage was measured at 7, 14, and 21 days and reported as OD 490 nm/10^4 viable cells. The results are the mean of 2 samples from 3 different experiments ± SD. *$P < 0.05$.

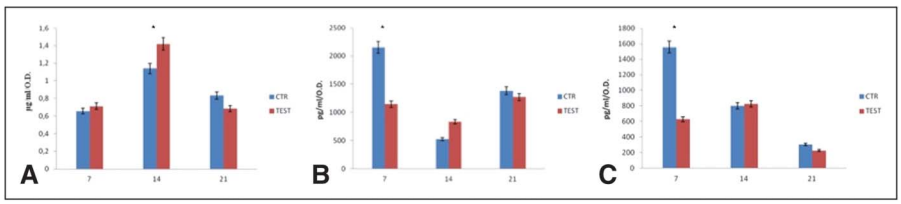


Fig. 5. ELISA for type I collagen secretion (**A**), IL-6 (**B**), and PGE_2 (**C**) of HOs cultured on control and test group experimental discs. Secretion levels were measured at 7, 14, and 21 days and reported, respectively, μg/mL/10^4 for type I collagen and pg/mL/10^4 viable cells for IL-6 and PGE_2. The results are the mean of 2 samples from 3 different experiments ± SD. *$P < 0.05$.

surfaces are evidenced. In control group, PGE_2 release decreases throughout the experimental period, whereas in test group, it shows a peak of expression after 14 days with respect to 7 and 21 days of culture (Fig. 5, C).

Discussion

In recent years, new treatment techniques of titanium surfaces were proposed and tested to improve the clinical performance and, consequently, to reduce the percentage of clinical failures.[2] The efforts of scientific research were especially focused to improve the quantity and quality of bone tissue healing around implant surfaces and to increase the contact area between the implant surface and bone tissue.[6–8,13] It was demonstrated that osteoblasts preferred rougher surfaces on which they adhered through adhesion plaques.[9,10] All methods regarding surface treatments, introduced in recent years, led to specific microstructured surfaces with a higher performance, due to a greater contact area to the bone, ability to absorb biological molecules, promote faster healing, increasing the cellular response and long-term clinical successes. Sandblasted and acid-etched surfaces have shown good biomechanical and biological results. Indeed, sandblasting performed with particles of alumina (Al_2O_3) or TiO, having diameters in order of micrometers, allowed an increased biomechanical connection. Inorganic or organic acid-etching allowed to smoothen the peaks created by the formation of microcavities and helped to promote proteins adhesion, considered essential in early stages of bone healing.[2,23–26] The objective of a micrometric coating is to render a sandblasted and acid-etched surface more hydrophilic, reducing unavoidable contaminations by carbon particles from the atmosphere to a minimum and, by increasing surface energy, to enhance osteogenic cells adhesion and cell activity.

In this study, primary HOs cultured on test group experimental discs showed a high rate of metabolic activity and cell viability. In fact, MTT assay confirmed a greater metabolic activity of the cells grown on test group surfaces when compared with cells grown on control group surfaces.

LDH assay was used to analyze the activity of the enzyme released by osteoblasts cultured on both surfaces, and therefore, to assess the degree of biocompatibility. Thus, a survey of its levels is an indirect measure of the toxicity of a material or surface. Levels of LDH released by cells cultured on test group surfaces appeared lower than those produced by cells on control group surfaces even if the statistical significance is appreciable only after 7 days of culture. This allows to hypothesize that at an early stage of culture, the test group surfaces seemed to be more biocompatible than that of the control group. This result is also supported by the work of Ibasco et al,[22] which evidenced that calcium and phosphorus ions deposition could enhance osteoblast survival and adhesion on the implant surface.

Collagen type I secretion was higher in cells grown on test group surfaces compared with those grown on control group at days 7 and 14, whereas from day 21, there was a decrease. This could be explained by the fact that cells grown on test group surfaces showed the peak of their metabolic activity around day 14 of culture and expressed a more evident osteoblastic phenotype.

It is known that, under antigenic stimulation, macrophages and osteoblasts are induced to release cytokines, chemokines, and other soluble factors responsible for the implementation of major tissue responses. These factors, acting in harmony through autocrine and paracrine effects, are primarily responsible of differentiation, proliferation, and activation of osteoclastic cells, elements belonging to the monocyte-macrophage line, whose function is to implement resorption of bone tissue. It was hypothesized that the presence of molecules on the surfaces could induce macrophages to release factors such as IL-1 and tumor necrosis factor alpha (TNFα), which subsequently stimulate osteoblasts to secrete IL-6 and PGE_2.[27] The high level of IL-1 and TNFα released in vitro at day 7 could resemble the inflammatory response that arises from the first interaction between an implant surface or, in general, a biomaterial with the host tissue.[28,29] However, IL-6 and PGE_2 are usually described as stimulators for osteoclast activity.[30] In particular, the latter could be responsible in vivo for the recruitment and for the osteoclastic cell activation, which is responsible of bone tissue resorption.[3,27] These same factors have an effect on macrophages, which sustain the inflammatory response through release of additional amounts of TNFα and IL-1β; thus, there is an increase of proliferation and differentiation of osteoclasts from hematopoietic mesenchymal precursors, arrived on site after the maximum vasodilation that is characteristic of the primary phases of inflammatory response.[3]

TNFα and IL-6 are also able to counteract the formation of new bone tissue in an indirect manner by acting on osteoblasts, in which activities will be blocked to facilitate RANKL production and secretion; this ligand promotes further development, activity, and survival of osteoclast by binding to RANK receptors expressed on osteoclast cell membranes.[3] In this study, significant differences in the *in vitro* IL-6 and PGE_2 release between test and control group surfaces were recorded after only 7 days, confirming the fact that the test group surfaces were well tolerated by osteoblasts. Moreover, differences in surface roughness and chemical composition did not seem to influence significantly IL-6 and PGE_2 release by the osteoblasts. Thus, the relatively low levels of release of both cytokines suggested that they could not be sufficient to recruit osteoclast precursors and support osteoclastic activity.

Conclusion

In conclusion, the metabolic activity of HOs cultured on a surface sandblasted and acid etched followed by a treatment with inorganic ions was higher after 7, 14, and 21 days *in vitro*. Moreover, this surface was found to be more biocompatible and well tolerated by HOs as shown by the lower LDH levels expressed and by the reduced secretion of inflammatory cytokines as PGE_2 and IL-6. This surface seemed to

be suitable for supporting osteoblasts growth and differentiation.

Disclosure

The authors claim to have no financial interest, either directly or indirectly, in the products or information listed in the article.

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