



# Ionized Ti Surfaces Increase Cell Adhesion Properties of Mesenchymal Stem Cells

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Titanium (Ti) surface topography of dental implants strongly influences osseointegration. To this view, in the present work we have analyzed the influence of an ionized titanium surface on stem cell adhesion properties. Human Dental Pulp Stem Cells (DPSCs) has been used in order to evaluate the influence of the ionized surface to the osteogenic commitment, extracellular matrix component production and cell adhesion molecules. The morphology of the cells grown onto this surface has been analyzed with SEM and the safety of the surface has been tested by means of karyotype analysis, by AMES test and by hemocompatibility assay. Results confirmed that at 25 days of cultures DPSCs show a substantial expression of some osteoblast specific markers and a strong increase of cell adhesion molecules.

## Keywords:

## 1. INTRODUCTION

The clinical success of implant therapy depends on dental implant osseointegration, which is the direct structural and functional connection between the implant surface and living bone tissues.<sup>1,2</sup> Titanium (Ti) and its alloys are widely used as materials to produce dental implants thanks to their excellent mechanical properties and superior biocompatibility.<sup>3</sup> However, Ti and its alloys are generally considered to be bioinert and not likely to form direct bonding with bone tissue. The adhesion of bone cells on Ti implant depends strictly on implant surface properties, such as roughness, topography, chemistry, charge, and wettability.<sup>4</sup> Studies demonstrate that Ti implants with roughened surfaces have greater contact with bone than Ti implants with smoother surfaces.<sup>5,6</sup> Different methods have been developed in order to create Ti implants with rough surface: surface coating, sand-blasting, and acid-etching are the main approaches to enhance bone adhesion around dental implants. Various bioactive materials, such as hydroxyapatite, are used to coat the surfaces of dental implants in order to form a biologically active bone-like layer. This bioactive layer stimulates bone formation leading to a physicochemical

bond that favors anchorage of implant to host bone. However, over time the coating layer tends to disintegrate in host environment, with low fatigue strength and weak adherence to metallic substrates.<sup>7</sup>

The roughness of Ti surface can also be increased through the sand-blasting of implant with hard ceramic, such as Ti oxide (TiO<sub>2</sub>) particles. Different surface roughness can be produced depending on the size of used ceramic particles: TiO<sub>2</sub> particles with an average size of 25 μm produce a moderately rough surface in the 1–2 μm range on dental implants.<sup>8</sup> Studies report that TiO<sub>2</sub> blasted implants improve osseointegration<sup>9</sup> and have high clinical success rates, up to 10 years after implantation.<sup>10</sup>

Furthermore, chemical treatment seems to be a simple and effective technique to achieve a bioactive Ti surface.<sup>11</sup> Etching dental implants with strong acids, such as HCl or H<sub>2</sub>SO<sub>4</sub>, is a method for producing micro pits on Ti surfaces. This chemical treatment enhances the osteoconductive properties of implants by fibrin attachment and osteoblast adhesion, resulting in bone formation directly on the surface of the implant.<sup>12</sup> However, the effects of acid-etching on the long-term stability of the Ti dental implant are rather limited: the acid-etching technique leads to hydrogen absorption on implant surface which produces fractures in Ti implant. Such cracks compromise the

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good mechanical properties, especially fatigue resistance of the Ti implant.<sup>13</sup> To avoid this drawback, acid-etching is used in combination with sand-blasting: the Ti implant surface is first sand-blasted with large grits, making the surface grossly rough. Then, the implant is acid-etched to form micropits on its surface. The resulting implant surface is both macrotopographically wavy and rough at the microlevel.<sup>14</sup> *In vitro* and *in vivo* studies demonstrated that sand-blasted and acid-etched surfaces show great biomechanical stability, high mechanical resistance, low risk of clinical failures, and high bond between implant and bone.<sup>15</sup>

The integration of dental implant with bone depends on reactions of cells towards the biomaterial: cell attachment, adhesion, and spreading are the first phases of the interactions between the host cells and the implant, that subsequently will lead to bone tissue formation around the implant.<sup>16</sup> These reactions not only depend on topography and roughness of implant surface but also by surface energy, or rather by the material's surface charge density. When a biomaterial is inserted into a living body, it adsorbs proteins and adhesion molecules responsible for cell-implant interactions. In a biological environment, all molecules surrounding an electrically neutral implant, including organic and inorganic ions, proteins, ionic groups, amino acids, and water have equal opportunity to contact and accumulate on implant's surface. On the contrary, a charged implant surface can induce electrical attraction or repulsion between the implant's surface and the surrounding chemical species, depending on their polarity.<sup>17</sup> Therefore, the surface charge regulates the wettability and the orientation of adsorbed molecules, thus influencing osteoblasts and fibroblasts adhesion.<sup>18</sup>

In the light of these considerations, the treatment of sand-blasted and acid-etched Ti implants with inorganic ions is emerging as a new strategy to regulate the surface charge of implants for improving their osseointegration.

The aim of this *in vitro* study was to investigate the influence of ionized surfaces on human mesenchymal stem cells (MSCs) behavior in term of ability to direct the osteogenic commitment and cell adhesion properties. In addition, we analysed the mutagenicity and the hemocompatibility of sand-blasted and acid-etched Ti implants treated or not with inorganic ions.

## 2. MATERIALS AND METHODS

### 2.1. Biomaterials

Two types of Ti discs (5 mm diameter, 2 mm thickness; Implacil De Bortoli-Dental Product, São Paulo, Brazil) were used in this *in vitro* study:

—*control Ti discs*: Ti discs sand-blasted with microparticles of Ti dioxide (TiO<sub>2</sub>), with an average particle size of 180 μm, and acid-etched with maleic acid;

—*treated Ti discs*: Ti discs sand-blasted and acid-etched as above, then treated with inorganic ions.

All experimental discs were cleaned and sterilized by γ-rays.

### 2.2. Ames Test

The Ames test was performed with the Salmonella Mutagenicity Complete Test Kit (Moltox, Molecular toxicology Inc., Boone, NC, USA) to evaluate the mutagenic potential of the control and treated Ti discs. Nutrient Broth (blank) was used as the extraction vehicle; aluminium oxide ceramic rod (VITA In-Ceram Alumina CA-12, CE 0124, lot 15320) was used as negative control; ICR 191 Acridine (Moltox, 60-101) and Sodium Azide (Moltox, 60-103) were used as positive controls. Extraction conditions were (24 ± 2) h at (37 ± 1) °C. Three replicates were performed for each sample. The bacteria plates were incubated with the different extracts for 48 h at 37 °C, then the number of revertant colonies per plate was counted. Interpretation of results: negative (not mutagenic) if the number of reverted colonies was equivalent to those observed with blank and negative controls; positive (mutagenic) if the number of reverted colonies was equivalent to those observed with positive controls.

### 2.3. Hemolysis Assay

The hemolysis assay was performed following standard practices set forth in ASTM F756 in order to evaluate the blood compatibility of the treated and control Ti discs (test materials). Blood was obtained from three healthy New Zealand rabbits, pooled, then diluted in PBS to a total hemoglobin concentration of 10 ± 1 mg/mL. One mL of diluted rabbit blood was added to 7 mL of the following PBS extracts. For the extraction of the test materials, triplicate 2 gr portions of Ti discs were covered with 10 mL PBS. For the negative control, triplicate 30 cm<sup>2</sup> portions of High Density PolyEthylene (HDPE) were covered with 10 ml of PBS. For the positive control, triplicate 10 mL portions of Sterile Water for Injection (SWFI) were used. Extraction conditions were 50 °C for 72 h for all samples. Each tube was incubated for 3 h at 37 °C with periodic inversions. Following incubation, the tubes were centrifuged for 15 min at 800 g. A 1 mL aliquot of the resulting supernatant from test materials, negative and positive controls was added to 1 mL of Drabkin's reagent (Sigma-Aldrich, St. Louis, MO, USA) and incubated at room temperature for 15 min. The reaction product between hemoglobin and Drabkin's reagent is a cyanoderivative that was quantified by measuring absorbance at 540 nm with a multilabel plate reader (Victor 3 Perkin Elmer, Milano, Italy). The hemolysis index (HI) was then calculated using the mean absorbance value (OD) for each group as follows:

$$\text{HI (\%)} = \frac{\text{OD (test material)} - \text{OD (negative control)}}{\text{OD (positive control)} - \text{OD (negative control)}} \times 100$$

Interpretation of results: nonhemolytic if the HI was 2% or less; hemolytic if HI was higher than 2%.

#### 2.4. Cell Cultures

Human dental pulps were extracted from healthy molar teeth of subjects, who had given written consent. Human dental pulp stem cells (DPSCs) isolation was performed according to our previously published protocol.<sup>19</sup> Briefly, dental pulp was obtained by means of a dentinal excavator or a Gracey curette after mechanical fracturing. The pulp was gently removed and immersed for 1 h at 37 °C in a digestive solution, containing 3 mg/mL type I collagenase and 4 mg/mL dispase in Phosphate Buffer Saline (PBS) (Lonza S.r.l., Milano, Italy) solution. Once digested, the solution was filtered through 70 µm cell strainers (BD Biosciences, Mississauga, Ontario, Canada). The cells were then cultured with Dulbecco's Modified Eagle Medium (DMEM) (Lonza S.r.l.) supplemented with 10% Fetal Bovine Serum (FBS) (Bidachem S.p.A., Milano, Italy) and 100 units/mL penicillin and 100 µg/mL streptomycin to form complete DMEM (cDMEM).

At confluence, the cells were harvested by trypsin treatment. After detachment from culture plates, cells were seeded onto the control and treated Ti discs at a density of  $5 \times 10^3$  cells/disc in a 96-well plate. The cells were cultured in cDMEM at 37 °C with 5% CO<sub>2</sub> up to 25 days, and the medium was changed twice a week.

#### 2.5. MTT Assay

To determine the proliferation rate of cells grown on control and treated Ti discs, the MTT-based (methyl thiazolyl-tetrazolium) cytotoxicity assay was performed according to the method of Denizot and Lang with minor modifications.<sup>20</sup> The test is based on mitochondria viability, i.e., only functional mitochondria can oxidize an MTT solution, giving a typical blue-violet end product. After harvesting the culture medium, the cells were incubated for 3 h at 37 °C in 1 mL of 0.5 mg/mL MTT solution prepared in PBS solution. After removal of the MTT solution by pipette, 0.5 mL of 10% dimethyl sulfoxide in isopropanol (iDMSO) was added for 30 min at 37 °C. For each sample, absorbance values at 570 nm were recorded in duplicate on 200 µL aliquots deposited in 96-well plates using a multilabel plate reader (Victor 3 Perkin Elmer). All samples were examined after 15 and 25 days of culture.

#### 2.6. Scanning Electron Microscopy (SEM)

For SEM imaging, DPSCs grown on control and treated Ti discs for 15 and 25 days were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 1 h, then progressively dehydrated in ethanol. Control and treated Ti discs without cells were also examined. The SEM analysis was carried out at the Interdepartmental Service Center C.U.G.A.S. (University of Padova, Italy).

#### 2.7. RNA Extraction and First-Strand cDNA Synthesis

Total RNA was extracted with RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany), including DNase digestion with the RNase-Free DNase Set (Qiagen), from DPSCs seeded on the control and treated Ti discs after 15 and 25 days of culture. The RNA quality and concentration of the samples was measured using the NanoDrop™ ND-1000 (Thermo Scientific). For the first-strand cDNA synthesis, 200 ng of total RNA of each sample was reverse transcribed with M-MLV Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA), following the manufacturer's protocol.

#### 2.8. Real-Time PCR

Human primers were selected for each target gene with Primer 3 software (Table I). Real-time PCRs were carried out using the designed primers at a concentration of 300 nM and FastStart SYBR Green Master (Roche Diagnostics, Mannheim, Germany) on a Rotor-Gene 3000 (Corbett Research, Sydney, Australia). Thermal cycling conditions were as follows: 15 min denaturation at 95 °C; followed by 40 cycles of denaturation for 15 sec at 95 °C; annealing for 30 sec at 60 °C; and elongation for 20 sec at 72 °C. Differences in gene expression were evaluated by the  $2^{-\Delta\Delta Ct}$  method,<sup>21</sup> using DPSCs cultured onto control Ti discs for 15 days as control condition. Values were normalized to the expression of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) internal reference, whose abundance did not change under our experimental conditions.

#### 2.9. Karyotype Analysis

After 25 days of culture on treated Ti discs, cells were exposed to colchicine (Sigma-Aldrich) for 6 h, washed

**Table I.** Results of the Ames test.

Sample	STDisc™ TA1535		STDisc™ TA1537	
	Rev/plate <sup>a</sup>	Result	Rev/plate <sup>a</sup>	Result
Blank	4 ± 3	Not mutagenic	5 ± 3	Not mutagenic
NC <sup>b</sup>	3 ± 2	Not mutagenic	4 ± 2	Not mutagenic
PC1 <sup>c</sup>	922 ± 76	Mutagenic	928 ± 76	Mutagenic
PC2 <sup>d</sup>	847 ± 50	Mutagenic	851 ± 50	Mutagenic
TS1 <sup>e</sup>	4 ± 2	Not mutagenic	4 ± 2	Not mutagenic
TS2 <sup>f</sup>	3 ± 2	Not mutagenic	3 ± 2	Not mutagenic
Sample	STDisc™ TA98		STDisc™ TA100	
	Rev/plate <sup>a</sup>	Result	Rev/plate <sup>a</sup>	Result
Blank	5 ± 3	Not mutagenic	3 ± 3	Not mutagenic
NC <sup>b</sup>	2 ± 2	Not mutagenic	4 ± 2	Not mutagenic
PC1 <sup>c</sup>	921 ± 76	Mutagenic	929 ± 76	Mutagenic
PC2 <sup>d</sup>	844 ± 50	Mutagenic	849 ± 50	Mutagenic
TS1 <sup>e</sup>	4 ± 2	Not mutagenic	3 ± 2	Not mutagenic
TS2 <sup>f</sup>	3 ± 2	Not mutagenic	4 ± 2	Not mutagenic

Notes: Number of revertants/plate: Mean of three independent experiments ± SD; <sup>b</sup>NC, negative control: Aluminium oxide ceramic rod; <sup>c</sup>PC1, positive control 1: ICR 191 Acridine; <sup>d</sup>PC2, positive control 2: Sodium Azide; <sup>e</sup>TS1, tested sample 1: Control Ti disc; <sup>f</sup>TS2, tested sample 2: Treated Ti disc.

in PBS, dissociated with trypsin (Lonza S.r.l), and centrifuged at 300 g for 5 min. The pellet was carefully resuspended and incubated in 1% sodium citrate for 15 min at 37 °C, then fixed and spread onto -20 °C cold glass slides. Metaphases of cells were Q-banded and karyotyped in accordance with the International System for Human Cytogenetic Nomenclature recommendations. Twenty-five metaphases were analyzed for three expansions.

### 2.10. Statistical Analysis

One-way analysis of variance (ANOVA) was used for data analyses. A repeated-measures ANOVA with a post-hoc analysis using Bonferroni's correction for multiple comparisons was performed, and *t*-tests were used to determine significant differences ( $p < 0.05$ ). Repeatability was calculated as the standard deviation of the difference between measurements. All testing was performed using SPSS 16.0 software (SPSS Inc., Chicago, Illinois, USA) (licensed by the University of Padova).

## 3. RESULTS

### 3.1. Evaluation of the Mutagenicity of Ti Discs

The Ames test was performed in order to assess the mutagenic potential of the treated and control Ti discs. Four different histidine dependent mutant strains (TA1535, TA1537, TA98 and TA100) of *Salmonella typhimurium* were used. As reported in Table I, no mutagenic activity has been revealed for the two tested materials.

### 3.2. Evaluation of the Hemocompatibility of Ti Discs

The hemolysis assay was performed in order to evaluate the blood compatibility of the Ti discs, which are intended for blood contacting applications. The HI of the control and treated Ti discs was less than 2%, indicating the absence of any hemolytic activity of the two tested materials (Table II).

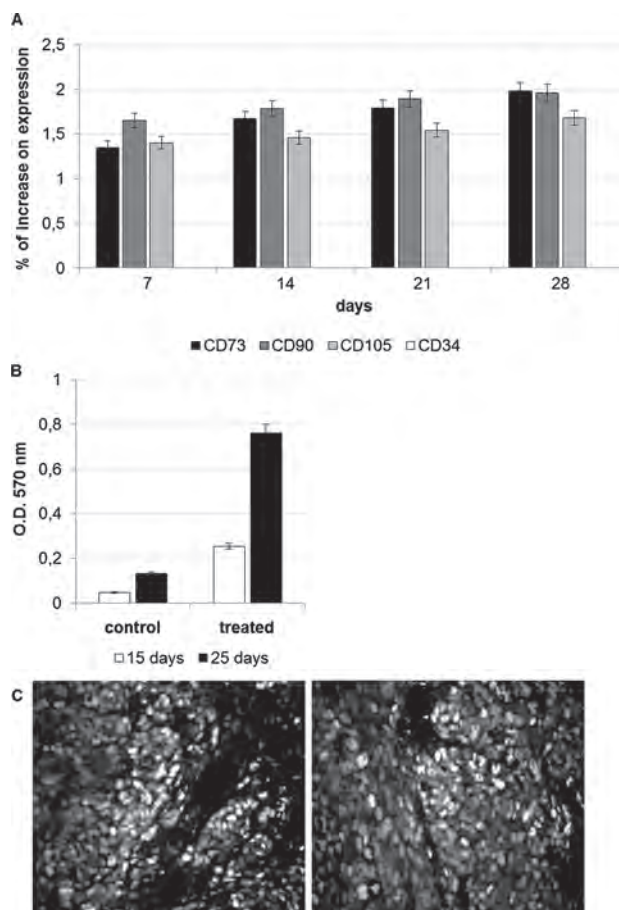
### 3.3. Biocompatibility of Ti Discs

Real-time PCR was performed on DPSCs cultures in absence of differentiative factors (Fig. 1(A)) in order to evaluate their mesenchymal stem lineage. The gene expression detected by means of the molecular markers

**Table II.** Results of the hemolysis assay.

Sample	OD <sup>a</sup>	HI <sup>b</sup> (%)	Result
PC1 <sup>c</sup>	0.8762 ± 0.012	100	Hemolytic
NC <sup>d</sup>	0.0143 ± 0.002	0	Nonhemolytic
TS1 <sup>e</sup>	0.0147 ± 0.002	0,046	Nonhemolytic
TS2 <sup>f</sup>	0.0153 ± 0.002	0,116	Nonhemolytic

Notes: <sup>a</sup>OD, absorbance value at 540 nm: Mean of three independent experiments ± SD; <sup>b</sup>HI, hemolysis index; <sup>c</sup>PC, positive control: Sterile Water for Injection (SWFI); <sup>d</sup>NC, negative control: High Density PolyEthylene (HDPE); <sup>e</sup>TS1, tested sample 1: Control Ti disc; <sup>f</sup>TS2, tested sample 2: Treated Ti disc.

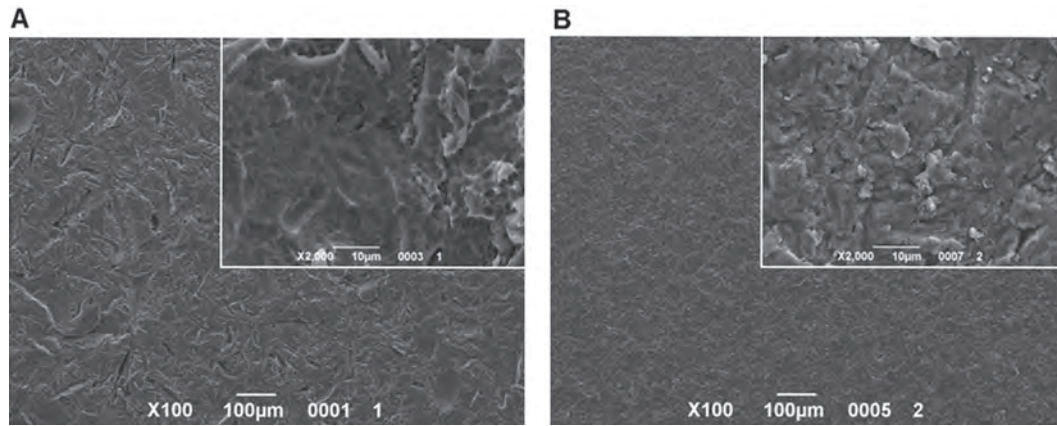


**Fig. 1.** (A) Real-time PCR confirms the expression for mesenchymal stem cells markers such as CD73, CD90, and CD105, whether no expression for CD34 (for hematopoietic phenotype) has been revealed. (B) MTT assay of DPSCs cultured on the control and treated Ti discs. DPSCs proliferation rate is higher on the treated discs respect to the control discs both at 15 and 25 days after seeding. (C) Immunofluorescence for Proliferating Cell Nuclear Antigen (PCNA) indicate that DPSCs seeded onto control (left) and treated (right) Ti discs are able to proliferate. Magnification 40×.

selected can provide information on the mesenchymal stem cell phenotype. As shown in Figure 1(A) a well-defined expression for CD73, CD90 and CD105 is reported, whether no expression for CD34 has been revealed.

In order to evaluate the biocompatibility of the control and treated Ti discs, DPSCs were seeded and cultivated onto these surfaces up to 25 days. The results of MTT assay show that the cells were able to adhere and grow onto Ti discs. However, the cell proliferation rate was higher on treated discs with respect to the control group both after 15 and 25 days of culture (Fig. 1(B)).

The ability to growth and to proliferate of DPSCs has been also detected by the immunofluorescence for Proliferating Cell Nuclear Antigen (PCNA) (Fig. 1(C)). PCNA is indeed is a DNA clamp that acts as a processivity factor for DNA polymerase  $\delta$  in eukaryotic cells and is essential for replication. PCNA is a homotrimer and achieves its processivity by encircling the DNA,



**Fig. 2.** SEM analysis of the control (A) and treated (B) disc surfaces at 100× and 2000× (inset) magnification before cells seeding.

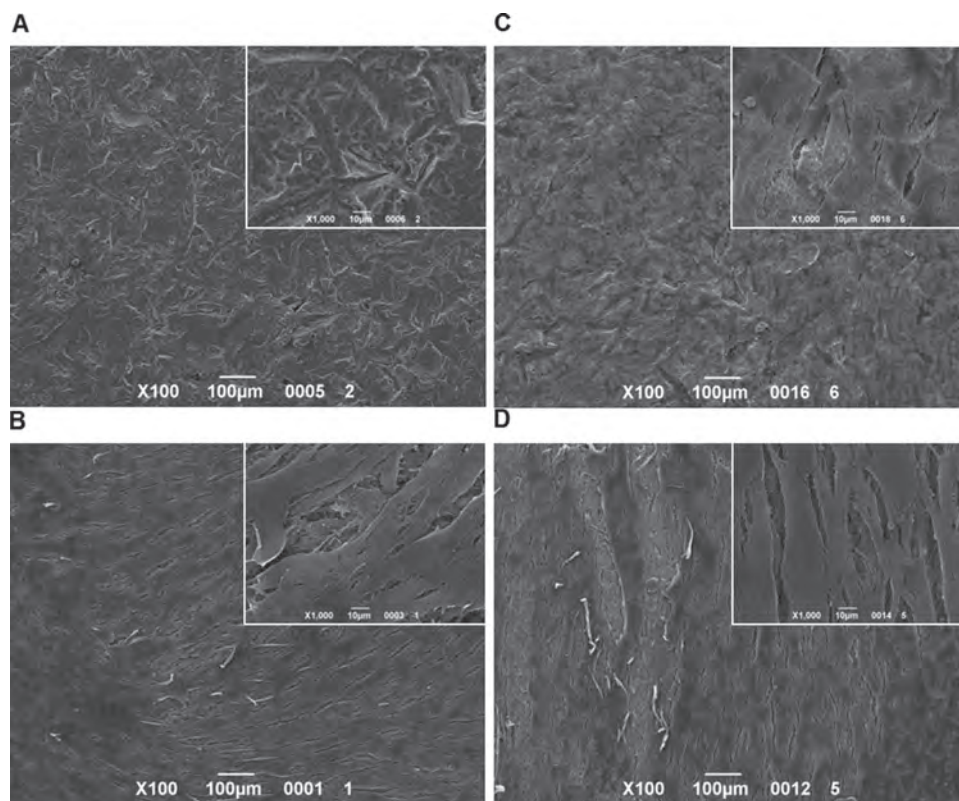
where it acts as a scaffold to recruit proteins involved in DNA replication, DNA repair, chromatin remodeling and epigenetics.

**3.4. Morphological Analyses**

SEM analyses were performed before seeding cells in order to examine the surface topography of the control and treated Ti discs. The surface of the control discs is characterized by high peaks and deep microcavities (Fig. 2(A)); on the contrary, the treated discs surface shows a less

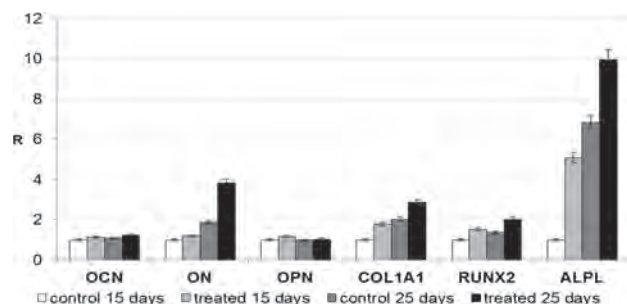
roughness and a more homogeneity after the treatment with inorganic ions (Fig. 2(B)).

SEM analyses of treated Ti surfaces seeded with DPSCs for 15 days showed a better adhesion and proliferation of the cells (Fig. 3(B)), if compared to the control discs (Fig. 3(A)). No significant difference in terms of cell adhesion and spreading on the two examined surfaces is evidenced after 25 days of culture (Figs. 3(C) and (D)). Indeed, in both cases DPSCs seem to be well spread and attached to the substrates.



**Fig. 3.** SEM analysis of the control and treated disc surfaces at 100× and 1000× (inset) magnification after cells seeding. ((A), (B)) DPSCs grown on the control Ti discs at 15 and 25 days of culture, respectively. ((C), (D)) DPSCs grown on the treated Ti discs at 15 and 25 days of culture, respectively.

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**Fig. 4.** Gene expression profile of the osteoblast markers OCN, ON, OPN, COL1A1, RUNX2, and ALPL in DPSCs cultured on the control and treated Ti discs for 15 and 25 days. The results are reported as ratios (*R*) with respect to the mRNA expression of DPSCs seeded 15 days on the control discs.

### 3.5. Expression of Osteoblast Markers

The gene expression level of some osteoblast markers was analyzed at day 15 and 25 by means of real-time PCR in order to verify the osteoinductive properties of the Ti discs used in the present study. The expression of selected genes (ALPL, COL1A1, OCN, ON, OPN, RUNX2) was evaluated in relation to the expression of a reference gene (GAPDH). Cells seeded on control Ti discs for 15 days were used as control for data normalization. As shown in Figure 4, the expression of ON, COL1A1 and ALPL is higher in cells cultured onto treated discs compared with the control ones. The expression of these markers reaches the maximum value at 25 days of culture.

In order to detect the possible influence of ionized surfaces on cell adhesion and extracellular matrix production we performed PCR real time in DPSCs cultured on

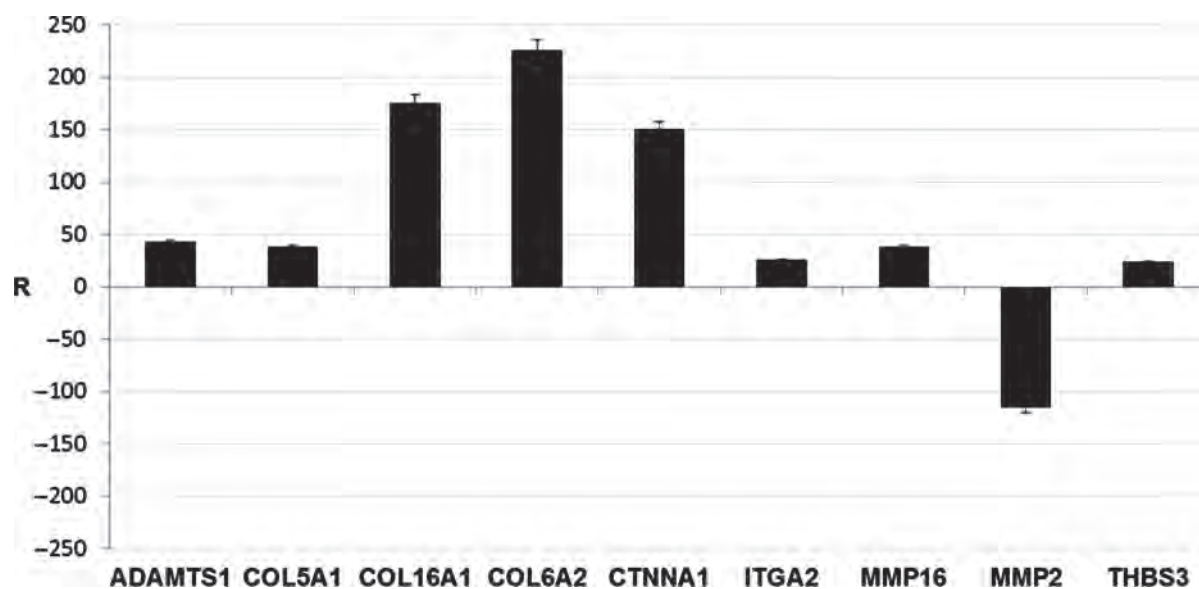
treated Ti discs for 25 days compared to DPSCs seeded 25 days on the control discs. In particular we evaluated the expression of gene related to cell adhesion, such as Integrin alpha 2 (ITGA2), Thrombospondin 3 (THBS3), and Catenin (cadherin-associated protein) alpha 1 (CTNNA1); gene related to ECM components, such as Collagen type V alpha 1 (COL5A1), Collagen type VI alpha 2 (COL6A2), and Collagen type XVI alpha 1 (COL16A1); gene related to ECM component remodeling, such as Matrix metalloproteinase 16 (membrane-inserted) (MMP16), Matrix metalloproteinase 2 (MMP2), and ADAM metalloproteinase with thrombospondin type 1 motif 1 (ADAMTS1). As shown in Figure 5, the presence of ions induces on treated Ti discs an important increase in collagen fibers.

### 3.6. Cytogenetic Analysis

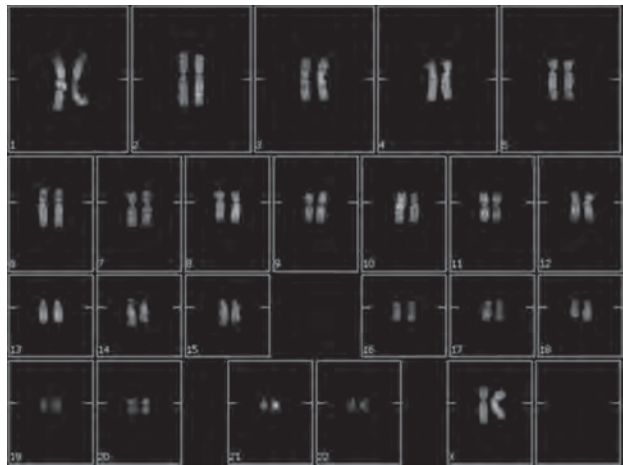
The chromosomal stability of DPSCs seeded on the treated Ti discs was analyzed by means of karyotyping. As reported in Figure 6, no chromosomal alterations are present in DPSCs seeded onto these surfaces for 25 days.

## 4. DISCUSSION

Ti and its alloys are the most commonly used biomaterials in dental implantology. The osseointegration of dental implants strictly depends on their surface properties, such as topography, roughness, and wettability.<sup>4</sup> However, the attachment, adhesion, and spreading of cells are also influenced by the material's surface charge density.<sup>17</sup> In this work, we have compared two sand-blasted and acid-etched Ti discs treated or not with inorganic ions. Preliminary



**Fig. 5.** Gene expression profile of the adhesion and extracellular proteins in DPSCs cultured on treated Ti discs for 25 days. The results are reported as ratios (*R*) with respect to the mRNA expression of DPSCs seeded on control discs for 25 days. ADAM metalloproteinase with thrombospondin type 1 motif, 1 (ADAMTS1); Collagen type V alpha 1 (COL5A1); Collagen type XVI alpha 1 (COL16A1); Collagen type VI alpha 2 (COL6A2); Catenin (cadherin-associated protein) alpha 1 (CTNNA1); Integrin alpha 2 (ITGA2); Matrix metalloproteinase 16 (membrane-inserted) (MMP16); Matrix metalloproteinase 2 (MMP2); Thrombospondin 3 (THBS3).



**Fig. 6.** Karyotype analysis of DPSCs seeded on the treated Ti discs for 25 days. No chromosomal alterations are present.

analyses were performed to test the biocompatibility of the chemical modifications of the surfaces materials by means the test for mutagenicity. The identification of substances capable of inducing mutations has become an important procedure in safety assessment. Indeed, there is considerable evidence that gene mutations are involved in cancer formation in humans. The mutagenic potential of the treated and control Ti discs was examined with the Ames test.<sup>22</sup> The low number of histidine revertant colonies indicates that treated and control Ti discs lacked mutagenic activity at the conditions tested. At this point, we performed the hemolysis assay which is considered to be a very simple and reliable test for estimating blood compatibility of materials. Our attention has been then focuses on the studies of the osteointegrative properties of the surfaces in order to obtain preclinical informations for a possible next step of vivo applications. In the process of bone healing and implant osseointegration, MSCs are the key repair cells, and their cellular response is important because successful osseointegration of implants depends on the adhesion of MSCs onto the implant surface.<sup>23</sup> In this work, human MSCs isolated from dental pulp (DPSCs) were used to evaluate the biocompatibility of the control and treated Ti discs. The results of the MTT assay indicate that DPSCs are able to attach and grow on both the disc types. Nevertheless, cell proliferation rate is higher onto the treated discs compared to the control samples, especially in long-term cultures. It seems that inorganic ions treatment of sand-blasted and acid-etched Ti surfaces positively affects cell proliferation. A possible explanation is that the inorganic ions treatment introduces charges onto the Ti surface. It has been shown that a charged surface may be more hydrophilic than an electrically neutral surface, thus favoring cells attachment, adhesion and proliferation.<sup>18</sup>

The morphology of the cells grown on Ti discs was further observed with SEM. In long-term cultures, DPSCs

appear attached to both the substrates by cellular extensions and they are well spread. However, the morphology of the cells seems quite different: onto the control discs, DPSCs appear mainly elongated (resembling fibroblast-like cells); instead, when grown on treated discs, they are more flat (similar to osteoblastic cells). In order to better understand the different behavior of the cells seeded onto the two substrates, we performed SEM analyses of the Ti discs without the cells. The surface of the control discs is characterized by high peaks and deep microcavities; whereas, the treated discs surface shows a less roughness and a more homogeneity.

In order to evaluate the osteoinductivity of Ti discs and determine the effect of inorganic ions treatment on osteoblast differentiation of DPSCs, the expression of osteogenic specific markers was evaluated with real-time PCR. Collagens (mainly type I, COL1A1) represent 90% of the total bone protein content.<sup>24</sup> When DPSCs are cultured on the treated Ti discs, the gene expression of COL1A1 is found to be significantly up-regulated compared with cells grown on the control surface. This increase is more evident after 25 days of culture. Such a result is very interesting since COL1A1 synthesis is known to be a prerequisite for extracellular matrix (ECM) formation and mineralization in bone.<sup>25</sup>

Osteocalcin (OCN), a specific osteoblast protein, is the most abundant non-collagenous protein found in bone ECM after collagens.<sup>26,27</sup> It is thought that OCN is implicated in bone mineralization and calcium ion homeostasis.<sup>28</sup> Osteopontin (OPN) is another non-collagenous protein found in bone. It is an important factor in bone remodeling,<sup>29</sup> and different studies have shown that it plays a role in anchoring osteoclasts to the mineral matrix of bones.<sup>30</sup> In this work, the expression levels of both OCN and OPN do not show statistical differences on the treat and control Ti discs both at 15 and 25 days of culture. This result could be explained considering that OCN and OPN expression in bone is strongly dependent by runt-related transcription factor 2 (RUNX2).<sup>31</sup> RUNX2 is an osteoblast-specific transcription factor that binds the promoter of both OCN and OPN, up-regulating their transcription.<sup>32</sup> It is possible that a change in OCN and OPN gene expression occurs after day 25, which is the latest time point evaluated here. This hypothesis may be supported by the observation that, on the other hand, the expression of RUNX2 increases over time, reaching the maximum value at 25 days. RUNX2, indeed, is the earliest osteoblastic marker essential for differentiation of osteoblasts and formation of bone.<sup>33</sup>

Although no significant changes are found in the expression of OCN and OPN, other markers associated with the osteogenic differentiation are up-regulated. For example, the gene expression of osteonectin (ON) and alkaline phosphatase (ALPL) is strongly increased by the inorganic ions treatment of the Ti surfaces, with the highest value

recorded after 25 days of culture. ON and ALPL are other classical non-collagenous proteins, both playing a role in bone ECM formation and mineralization. In particular, ON is a glycoprotein that binds calcium.<sup>34</sup> It is secreted by osteoblasts during bone formation, initiating mineralization and promoting mineral crystal deposition. ON also shows affinity for collagen in addition to bone mineral calcium. ALPL, instead, is a hydrolase enzyme responsible for removing phosphate groups from many types of molecules, and its activity is known to be an early marker of bone formation.<sup>35</sup> The elevated ON and ALPL expression observed on treated Ti discs supports the success of the osteoblastic differentiation of DPSCs and may be an indication of the osteoinductive properties of the scaffolds used. The up-regulation of some osteogenic genes on treated Ti surfaces is a promising indication that inorganic ions treatment is favorable for more rapid osteogenic induction of DPSCs in comparison to the control surfaces. In this context in order to evaluate the possible *in vivo* application of ionized based medical devices we tested the influence of these surfaces on synthesis of extracellular matrix components and adhesion molecules. Our results confirm a strong increase on type I and type VI collagen production that are fundamental for the reconstruction of the supporting matrix around the medical devices that need to be tissue integrated (i.e., osteointegration of dental implant). This environment exerts a positive effect on integrin expression, responsible for the interaction cell-extracellular matrix (ECM) and on negative effect on MMP involved on tissue remodeling, digesting collagen fibers. These important results confirm that these surfaces, increasing the production of adhesion molecules, could be easily *in vivo* colonized by resident cells, aiding the tissue integration of their related medical devices. In order to evaluate the genomic stability of DPSCs during this long-term cultures on treated Ti discs, we performed karyotyping. This method consisted in the analysis of metaphases of cells for testing the presence of chromosome alterations following their proliferation and differentiation onto the treated discs. No chromosomal alterations were found in the karyotype of DPSCs seeded on treated Ti discs for 25 days. This confirms that the cells are able to maintain their genomic stability, an extremely important fact when considering possible clinical use.<sup>36</sup> In the end, assuming that *in vivo* the surfaces will be in contact with blood, we tested its hemocompatibility. The test relies on the measurement of free hemoglobin released into the plasma when blood cells are damaged. Generally, the smaller the HI, the better the blood compatibility of the biomaterial. The materials extracts tested in this study induced less than 2% of contacting erythrocytes to hemolysis over 3 h of contact with blood. These results indicate that the treated and control Ti discs have no hemolytic effects and meet the requirements for clinical application.

Taken together, our results indicate that both treated and control Ti discs are not mutagenic, do not cause hemolysis, are biocompatible when seeded with human DPSCs and able to induce the osteogenic commitment of DPSCs seeded on ionized surfaces. To note, the greater expression of extracellular matrix component especially for collagen type VI, able to influence cell adhesion molecules and then a more rapid colonization of the materials by the resident cells.

### Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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