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Biological response of human bone marrow stromal cells to sandblasted titanium nitride-coated implant surfaces

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Abstract Titanium nitride (TiN) coating has been proposed as an adjunctive surface treatment aimed to increase the physico-mechanical and aesthetic properties of dental implants. In this study we investigated the biological response of primary human bone marrow stromal cells (BMSC) to TiN-coated sandblasted (TiN-SB) compared to uncoated sandblasted (SB) surfaces. SB and TiN-SB disks were qualitatively and quantitatively analyzed by atomic force microscopy. BMSC were obtained from healthy donors and their adhesion and proliferation on the titanium disks were evaluated by scanning electron microscopy and viability assay. The osteoblastic differentiation, in terms of alkaline phosphatase activity, osteocalcin synthesis, and extracellular mineralization, was assessed by specific immunoenzymatic or spectrophotometric assays. No difference ($P > 0.05$) between TiN-SB and SB disks was found in terms of any of the investigated parameters. TiN-coating showed to maintain the topographical characteristics of sandblasted titanium surfaces and their biological affinity toward bone precursors.

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1 Introduction

Titanium and its alloys have been widely used and have proved their safety and efficacy as dental implant materials, as demonstrated by the long-term success rate of implant-supported rehabilitations [1–4]. They show an excellent biocompatibility and are highly tolerated by the organism. Osseointegration of dental implants results from the interaction between the titanium surface and the cellular and matrix constituents of the surrounding bone tissue. Topographic and chemical surface modifications have been shown to significantly affect osseointegration [5–7]. In particular, micro-rough surfaces, obtainable by several techniques, such as sandblasting, titanium plasma-spraying, acid etching, etc., have been demonstrated to variably improve and accelerate the osseointegration process. Indeed, these modified surfaces are able to increase the implant affinity for bone cells [8–11] and lead to higher values of bone-to-implant contact rate and retention into the bone [12–14].

However, titanium implants, especially in the commercially pure form, have a low strength and can undergo to physical abrasion in the oral environment, e.g., due to oral prophylaxis procedures [15–17]. Furthermore, the titanium gray color can raise aesthetic problems when it is not adequately masked by soft tissue at level of the gingival area.

Titanium nitride (TiN) is a ceramic material commonly used to cover a number of metal tools, including surgical instruments, in order to improve their surface properties and aesthetic appeal (thanks to its characteristic gold-like color). TiN-coating has been recently introduced also in the dental implant field with the aim of increasing surface resistance to abrasion and corrosion [18, 19], and of providing a better camouflage under the gingival tissue.

Furthermore, TiN-coated surfaces have been shown to reduce bacterial colonization compared with other implant surfaces [20, 21], with possible implications for the maintaining of peri-implant mucosa health. However, whether this treatment could affect the osseointegration and the behavior of bone cells has not been extensively investigated. Bone marrow stromal cells (BMSC) contain multipotent mesenchymal stem cells able to self-renew and to differentiate into precursors of several tissues, including osteoprogenitor cells. They are involved in the normal remodeling and reparative mechanisms of bone, and play a central role in the osseointegration process.

The purpose of the present study was to investigate if an additional treatment of TiN-coating of conventional sandblasted titanium implant surfaces could affect the biological response of human bone marrow stromal cells in terms of cell adhesion, proliferation, and differentiation.

2 Materials and methods

2.1 Products and reagents

All cell culture biologics were purchased from Gibco BRL (Grand Island, NY, USA), and all chemicals were from Sigma Chemical Co. (St. Louis, MO, USA) when not otherwise specified.

2.2 Specimen preparation

Two different titanium implant surfaces were analyzed: sandblasted (SB) and TiN-coated sandblasted (TiN-SB). All specimens were prepared by a commercial firm (P.H.I. S.r.l., San Vittore Olona, Milano, Italy) in form of 15-mm wide and 1-mm thick disks of Ti_6–Al_4–V. The disks were sandblasted and then cleaned of surface organic contaminants by ultrasonic agitation in a series of detergent solutions, acetone, ethanol, and deionized water. The TiN-coating was obtained by a process of physical vapor deposition (PVD). All disks were singularly packed and finally sterilized in a steam autoclave. For cell culture assays, titanium disks were put on the bottom of 24-well plates. The polystyrene surface of the multiwell plates was used as control.

2.3 Surface topographic characterization

Qualitative and quantitative measurements of implant surfaces were made by atomic force microscopy (AFM). AFM analysis was carried out using an Easy Scan 2 microscope (Schaefer, Rovigo, Italy). Scanning was performed in contact mode on 50 × 50 μm areas, with a resolution of 512 × 512 pixel/line, with a photodetector direction of 90° and a sampling rate of 0.4 Hz. All images were obtained at ambient conditions. Mean roughness (Sa), root mean square roughness (Sq) and ten-point average roughness (Sz) were calculated as typical surface texture parameters [22]. All measurements were performed on six different points, randomly distributed over the surface, with at least one scan effected close to the center and one close to the edge of each specimen.

2.4 Preparation of a collection of human bone marrow stromal cells

Ten ml samples of human bone marrow were harvested from three healthy donors (two woman and one man, aged 25–42 years). Informed consent was provided according to the Declaration of Helsinki. Informed consent and research protocol were institutionally approved. BMSC cultures were initiated as previously described [23]. Briefly, heparinized bone marrow was diluted 1:5 with complete culture medium consisting of OptiMEM containing 10% (v/v) fetal calf serum (FCS), 100 units/ml penicillin, 100 μg/ml streptomycin and 50 μg/ml sodium ascorbate, and incubated at 37°C in a 5% CO_2 humidified atmosphere. Although present in a percent extremely low with respect to the total of mononuclear cells originally present in the bone marrow, BMSC can be obtained on the basis of their ability to adhere on polystyrene plates, while the cells of the haemopoietic lineage remain in suspension and can be easily removed. After 48 h, the medium containing all non-adherent cellular elements was centrifuged 10 min at 800 × *g* in order to remove the haematopoietic cells and added again to the dish. In 3–4 days, several foci of adherent spindle-like cells appeared and reached the sub-confluence in 2 weeks. The medium was refreshed every 3 days, each time leaving one half of the conditioned medium. The cells harvested from each donor were kept separately and not pooled with other preparations. Cultures between the second and fourth passage were used in our experiments.

2.5 Cell adhesion and proliferation

BMSC were seeded on implant surfaces and control wells at a density of 15,000 cells/cm^2 in complete culture medium. Cell adhesion to implant surfaces at 6 h from plating and cell proliferation at 3 days were assessed by MTT vitality assay. The key component of this assay is 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Mitochondrial dehydrogenases of living cells reduce the tetrazolium ring, yielding a blue formazan product, which can be measured spectrophotometrically. Cells were washed with phosphate buffered saline (PBS) and incubated with 0.5 mg/ml MTT solution for 4 h at 37°C. At the end of this time, the liquid was aspirated and the insoluble formazan produced was dissolved in

isopropanol–HCl 0.1 M. The optical density was measured at 570 nm, subtracting background absorbance determined at 690 nm.

Cell adhesion and morphology were also evaluated by scanning electron microscopy (SEM). Cells were plated on titanium surfaces as above mentioned. After 6 h cells were rinsed three times with PBS and fixed for 30 min with 2.5% glutaraldehyde. The fixed cell layers were washed in PBS and dehydrated by graded ethanol solutions and critical point drying. Samples were mounted on stubs, coated with Au/Pd alloy and examined by SEM (Philips SEM XL20).

2.6 Osteoblastic differentiation

The effects on cell differentiation was evaluated analyzing the expression of specific markers of the osteoblastic phenotype, namely alkaline phosphatase activity, osteocalcin production and the mineralization of the extracellular matrix.

2.7 Alkaline phosphatase specific activity

The alkaline phosphatase (AP) specific activity of BMSC grown on the titanium surfaces was evaluated after 7 and 14 days of culture. Once removed the medium, the wells were rinsed with 20 mM Tris–HCl–0.5 M NaCl, pH 7.4 (TBS) and the cells lysed with a specific buffer (20 mM Tris/HCl, pH 7.4, 0.5 mM NaCl, 0.25%Triton X-100, 0.5 mM PMSF, 0.5 mM DTT). After 30 min in ice, the cell lysates were centrifuged at 13,000*g* for 5 min, and the supernatants assayed for AP activity. Protein concentration was determined according to the method of Bradford. AP activity was determined by measuring the release of *para*-nitrophenol (PNP) from disodium *para*-nitrophenyl phosphate disodium *para*-nitrophenyl phosphate (PNPP). The reaction mixture contained 10 mM PNPP, 0.5 mM $MgCl_2$, diethanolamine phosphate buffer pH 10.5, and 10–30 μg of cell lysate in a final volume of 0.5 ml. After 30 min at 37°C, the reaction was stopped by adding 0.5 ml of 0.5 M NaOH. PNP levels were measured spectrophotometrically at 405 nm. The AP activity was normalized to the protein content and expressed as units/mg protein, where one unit was defined as the amount of enzyme that hydrolyzes 1 nmol of PNPP/min under the specified conditions.

2.8 Osteocalcin synthesis

To evaluate osteocalcin synthesis confluent cultures grown on the different surfaces for 2 weeks were incubated in FCS-free Opti-MEM in presence of 0.1% bovine serum albumin and 100 nM 1,25-dhydroxycolecalciferol for 48 h. The levels of polypeptide secreted in the medium were measured by means of an immunoenzymatic assay (Biosource International, Camarillo, CA, USA) that utilizes monoclonal highly specific antibodies and a peroxidase as conjugated enzyme. The amount of osteocalcin was calculated in ng/ml and then normalized to the protein content.

2.9 Extracellular matrix mineralization

The ability of titanium surfaces to promote the extracellular matrix mineralization was tested by quantification of the calcium levels. BMSC confluent cultures were incubated for 20 days with an osteogenic medium composed of 100 nM dexamethasone and 10 mM β-glycerophosphate. The calcium levels were measured colorimetrically using arsenazo III reagent: cells were decalcified with 0.6 N HCl for 24 h and the calcium released in the supernatant was determined at 575 nm using a plate reader and calculated according to a standard solution.

2.10 Statistical analysis

All the experiments were performed in triplicate on at least two different cell preparations. Data are expressed as the mean ± standard deviation (SD) of relative units (percentage of control). The means of each experimental group were compared by unpaired Student's *t*-test, with the value of significance set at $P < 0.05$.

3 Results

3.1 Surface roughness and topographical characterization

The AFM images of SB and TiN-SB surfaces are shown in Fig. 1a. The two samples appear similar, with a uniformly rough and isotropic surface. Sa, Sq and Sz values of the experimental surfaces are reported in Fig. 1b. No difference ($P > 0.05$) was detected between SB versus TiN-SB surfaces.

3.2 Cell adhesion and proliferation

Cell adhesion on implant surfaces was evaluated 6 h after plating. Both SB and TiN-SB surfaces showed a good affinity for BMSC, resulting in comparable cell morphology (Fig. 2a) and adhesion values (Fig. 2b). In both SEM images (Fig. 2a) BMSC appeared in phase of early adhesion and spreading with many evident filopodia.

On a parallel set of samples, BMSC were cultured for 72 h. At the end of the incubation, no difference in terms cell number between the two surface types was detected (Fig. 2c).

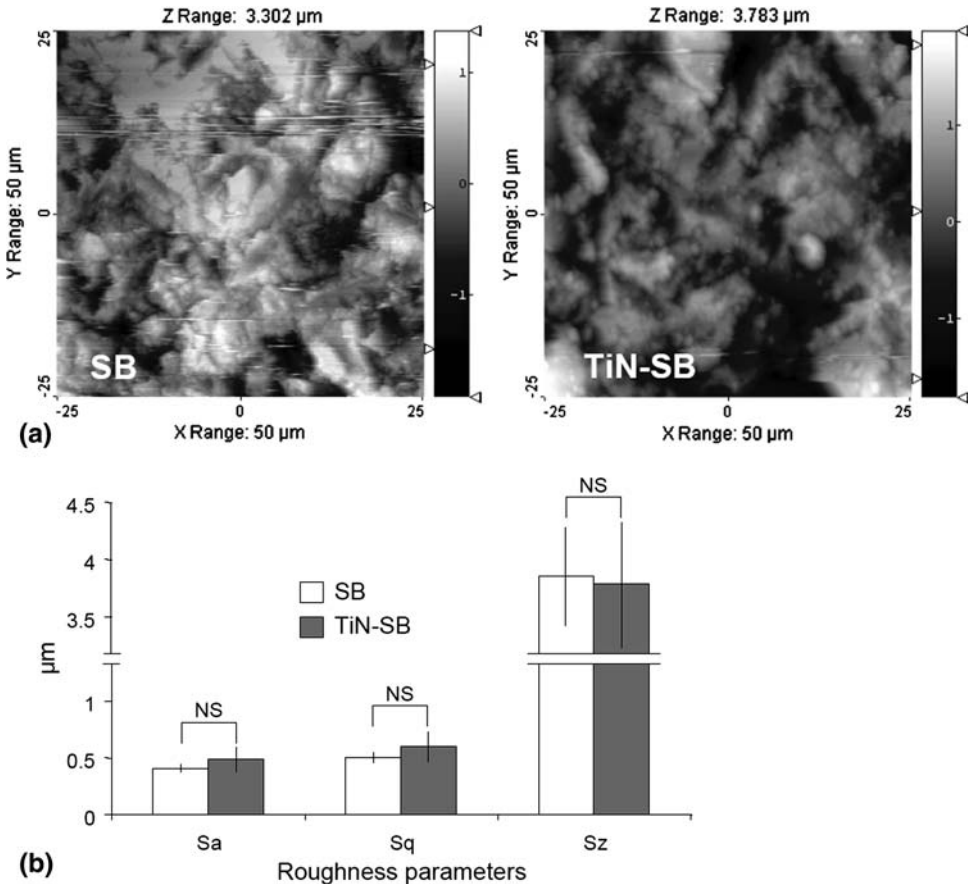

Fig. 1 Qualitative and quantitative characterization of implant surfaces. (**a**) Atomic force microscopy images of SB and TiN-SB implant surfaces. (**b**) Roughness values measured on SB and TiN-SB surfaces. Data are expressed as mean ± standard deviation (SD). NS = not significant ($P > 0.05$); Sa = mean roughness; Sq = root mean square roughness; Sz = ten-point average roughness

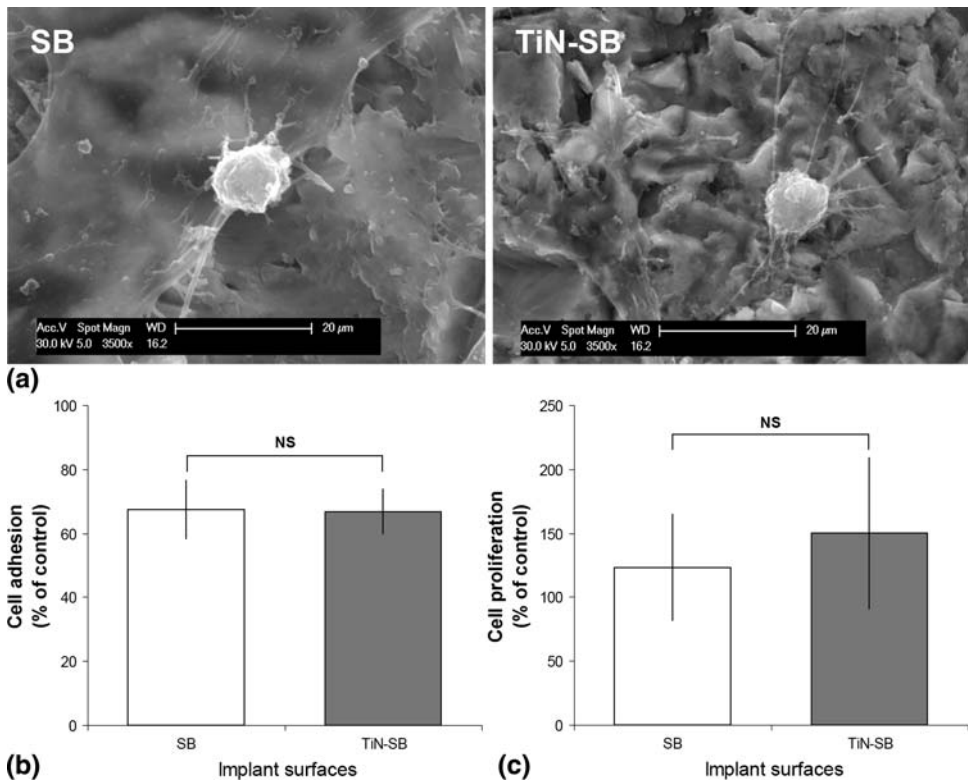

Fig. 2 Cell adhesion and proliferation on implant surfaces. (**a**) Scanning Electron Microscopy images of BMSC adhering on SB and TiN-SB implant surfaces. (**b**) Adhesion values of BMSC at 6 h from seeding on SB and TiN-SB samples. (**c**) Proliferation of BMSC at 72 h from seeding on SB and TiN-SB implant surfaces. In both cases, the presence of viable cells was spectrophotometrically assessed by MTT assay. Results were obtained from three independent experiments. Data are expressed as percentage of those obtained on cells cultured on polystyrene surface (control) ± standard deviation (SD). NS = not significant ($P > 0.05$)

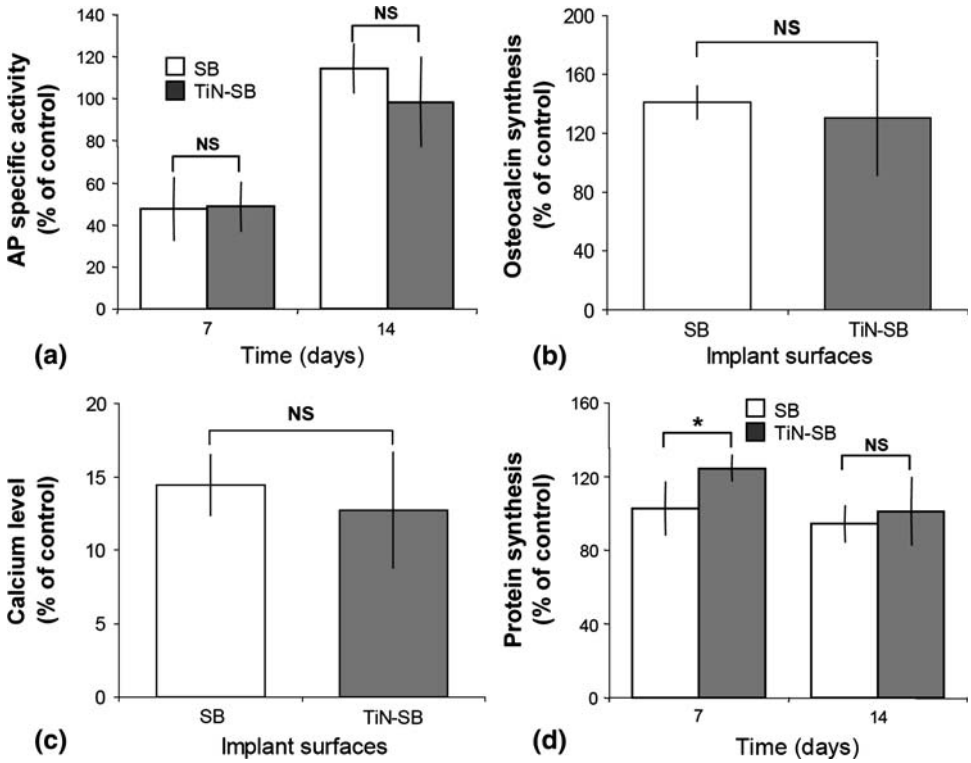

Fig. 3 Cell differentiation in response to implant surfaces. (**a**) Alkaline phosphatase specific activity of bone marrow stromal cells (BMSC) cultured for 7 and 14 days on SB and TiN-SB implant surfaces. (**b**) Osteocalcin synthesis by BMSC at 14 days from seeding on SB and TiN-SB implant surfaces. (**c**) Extracellular matrix mineralization expressed as calcium level produced by BMSC cultured for 20 days on the implant surfaces. (**d**) Overall protein synthesis by BMSC at 7 and 14 days from seeding on SB and TiN-SB implant surfaces. Results were obtained from three independent experiments. Data are expressed as percentage of those obtained on cells cultured on polystyrene surface (control) ± standard deviation (SD). $*P < 0.05$; NS,not significant ($P > 0.05$)

3.3 Osteoblastic differentiation

The expression of the early and late osteoblastic markers was not affected by surface type. Indeed, both SB and TiN-SB specimens showed comparable values ($P > 0.05$) in terms of alkaline phosphatase activity at both 7 and 14 days from plating (Fig. 3a), osteocalcin synthesis at 14 days (Fig. 3b) and extracellular matrix mineralization at 20 days (Fig. 3c). The overall protein synthesis appeared to be significantly increased ($P < 0.05$) by TiN-SB surfaces after 7 days compared to SB ones, however, no difference between the two experimental groups was found after 14 days (Fig. 3d).

4 Discussion

In the present study the biological response of human BMSC to TiN-coated/uncoated implant surfaces was analyzed. TiN-coated surfaces showed comparable topographical characteristics and biological performance with respect to the uncoated ones in terms of cell adhesion, proliferation and differentiation.

The influence of the surface characteristics of Ti implants on biomolecules deposition and cell behavior/morphology has been widely reported but there are still open questions about their interaction with different tissue cells in a biological environment. The limits of all in vitro studies, including the present, are related to the impossibility of reproducing all the complex biological events that normally happen in vivo, and that only in their initial phase consist in a direct contact with the substratum. However, the in vitro experimental approach allows to investigate in detail the interaction between bone cells and implant surface. This phase of the osteointegration process is crucial, being the contact of the osteoprogenitor cells with the implant surfaces the basis for all the following events, including the deposition of an organized extracellular matrix and its mineralization.

Among the many variables which need to be controlled in conducting in vitro experiments, the selection of a suitable cell system is crucial. In fact, the results obtained on animal-derived or transformed cells cannot be extrapolated to normal human beings. In our study, the biological response of BMSC to implant surfaces was investigated using primary human cultures. Primary cells from normal tissues represent, in our opinion, the ideal cellular model for pre-clinical evaluations. However, a number of factors, as the donor-to-donor variability, age, gender, etc., must be taken into account. In our study, in order to enhance data reliability, three different BMSC populations were prepared.

Sandblasting represents a recognized method of increasing surface roughness and enlarging the surface area of titanium implants. With this subtractive process, the implant surface is blasted by ceramic particles (usually

alumina) projected on it at high velocity through a nozzle by means of compressed air. The result is a moderately rough surface, in the 1–2 μm range, with an isotropic topographical pattern, i.e., without a visible direction in its roughness [24].

The description of the surface texture of a material is conventionally done using specific parameters, such as Sa, Sq and Sz. Sa is the arithmetic average of the absolute values of the surface height deviations measured from a reference plane. It is the most diffused parameter for measuring surface texture, however, it only quantifies the "absolute" magnitude of the surface heights and is insensitive to the spatial distribution of the surface heights. Sa is also insensitive to the "polarity" of the surface texture in that a deep valley or a high peak will result in the same Sa value. Sq, namely the root mean square roughness, is the statistical measure of the magnitude of the height distribution and correlates well with Sa. Also in this case, a series of high peaks or a series of deep valleys of equal magnitude will produce the same Sq value. Sz, namely the ten-point average roughness, is found from the difference between the average maximum peak height of the ten highest peaks and the average maximum valley depth of the ten lowest valleys found over the complete 3D image. Sz may be used to characterize the extreme features of a surface, being a nominal measure of the "peak-to-valley" range of the surface.

Nowadays, there is consensus on the clinical advantages of implanting moderately rough surfaced implants (Sa 1–2 μm), rather than highly rough surfaces (Sa 3–10 μm) that, if exposed to the oral environment, could enhance bacterial plaque formation and the occurrence of a perimplantitis process [24–26].

As shown by our measurements, the investigated SB surfaces showed Sa values of 0.4–0.5 μm, that are higher than a smooth surface (Sa 0.1–0.2 μm), but lower than a conventional SB one (Sa 1–2 μm).

TiN-coating was applied as an adjunctive treatment to SB surfaces. Different nitriding processes exist, but the physical vapor deposition represents one of the most efficient and widely used technique for TiN-coating in the biomedical field. With this process, the titanium is vaporized in a nitrogen atmosphere by means of a cathodic arc, and the evaporating material and the reactive gas become highly ionized. The result is the formation of a thin layer of titanium nitride which is tightly connected to the substratum by an intermixed zone which is a peculiarity of the cathodic arc technique [27].

An interesting finding of our investigation is that the TiN-coating process did not modify the topographic characteristics of SB surfaces. The roughness values of TiN-SB disks were only slightly and not significantly higher compared to the SB ones, and the AFM images also confirm that their surface topography was similar. This finding was in line with another study [28] in which TiN-SB surfaces showed comparable surface roughness with respect to the uncoated ones.

Cell adhesion to the substratum represents the initial interaction between cells and biomaterials and is considerably influenced by their surface chemical and topographical characteristics. Only few data are available about the influence of TiN-coated surfaces on cell adhesion. In particular, this treatment appears to favor cellular attachment of human gingival fibroblasts [29] and moreover to reduce bacterial adhesion [20, 21], with possible clinical implications in limiting inflammatory signs at level of the transmucosal portion of the implants. On the other hand, an implant surface treatment should not negatively affect the adhesion of bone cells precursors at a deeper level, that is the bone–implant interface. Only some studies have investigated the response of BMSC to TiN-coated surfaces. Clem et al. [30] showed comparable cell adhesion and spreading of human BMSC to TiN-coated (obtained by microwave plasma chemical vapor nitriding) and uncoated substrates, together with a similar vitronectin deposition. In another study [31], that investigated the effect of DC magnetron sputtered TiN surfaces on human BMSC, cells showed an enhanced adhesion and proliferation onto TiN-coated surfaces with respect to the uncoated ones. In our study, TiN-coated and uncoated surfaces showed comparable results in terms of cell morphology and adhesion values at 6 h from plating. In both surface types, BMSC adhered with high affinity onto the titanium samples exhibiting numerous pseudopodia anchored to the substratum. In addition, also the growth rate at three days appeared similar in both groups of specimens.

The only research available in literature which dealt with osteogenic markers in response to TiN surfaces was performed on mature osteoblasts [32]. In that study cells were seeded onto titanium surfaces which were TiN-coated by different techniques including plasma diffusion treatment and plasma-assisted chemical vapor deposition in addition to plasma diffusion treatment. The osteoblasts showed a reduction in adhesion and proliferation with respect to uncoated surfaces, while no difference in terms of osteogenic markers (alkaline phosphatase activity and osteocalcin synthesis) was detected.

The differentiation of BMSC toward the osteoblastic phenotype is a complex process which follows a precise temporal sequence involving several phases. The alkaline phosphatase, a membrane-bound enzyme contained into matrix vesicles, is involved in the hydroxyapatite crystal deposition, and is commonly considered an early marker of osteogenic differentiation. Osteocalcin is a specific late marker of osteogenic differentiation, representing the major non-collagenic protein of the bone matrix, and is

expressed exclusively by cells of osteoblastic lineage. Osteocalcin plays a central role in bone mineralization and calcium ion homeostasis [33–35]. A recent fascinating study has demonstrated that this molecule can be actually considered a hormone by which skeleton exerts a feedback endocrine regulation of energy metabolism [36]. The mineralization of the extracellular matrix, namely the precipitation of hydroxyapatite crystals upon the extracellular collagenic network, proceeds in parallel with the increasing of osteocalcin levels, representing the final step of osteogenic differentiation.

The evaluation of AP activity, osteocalcin and extracellular calcium levels allowed us to follow BMSC through the phases of the differentiation process. In our experiments, AP specific activity, as well as the synthesis of osteocalcin and the deposition of calcium into the extracellular matrix, were found comparable between the TiN-coated and uncoated samples. Thus, TiN-coating did not show to hamper BMSC differentiation in terms of any of the investigated markers with respect to the uncoated titanium surfaces.

Although it is difficult to compare the above mentioned studies among them, due to the existing remarkable difference in terms of many variables, such as the coating technique applied, the surface topography of the specimens and the cellular model used, our results appear in line with the available literature data.

5 Conclusion

Human BMSC showed a similar biological response to TiN-coated titanium surfaces compared to the uncoated ones. In the limits of the present study, TiN-SB implant surfaces can be considered a promising material among those available in the dental implant field, combining improved aesthetic and physical characteristics to excellent biological properties.

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