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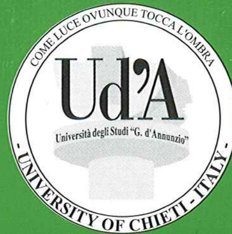

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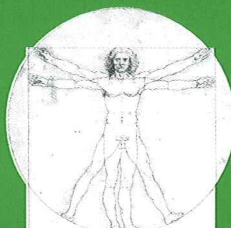

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ENHANCED BIOLOGICAL PERFORMANCE OF HUMAN ADIPOSE-DERIVED STEM CELLS CULTURED ON TITANIUM-BASED BIOMATERIALS AND SILICON CARBIDE SHEETS FOR ORTHOPAEDIC APPLICATIONS

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No Author has conflict of interest regarding the present data.

It is well known that the surface properties of biomaterials may affect bone-healing processes by modulating both cell viability and osteogenic differentiation. In this study we evaluated proliferation and osteogenic differentiation of human adipose-derived stem cells (hASCs) cultured on three prototypes of titanium disks and on thin layers of silicon carbide (SiC-PECVD), a material characterized by a high hardness and wear-resistance. Our data indicated that all the tested surfaces supported cell growth, in particular, hASCs seeded on both titanium treated by a double-step etching process (TIT) and titanium modified by two Anodic Spark Deposition processes (TAA) grew better respect to the ones cultured on titanium obtained by KOH alkali etching process on TAA (TAAK). Furthermore, hASCs well colonized SiC-PECVD surface, showing a quite similar viability to cells cultured on plastic (PA). TIT and TAA better supported osteogenic differentiation of hASCs compared to PA, as shown by a marked increase of both alkaline phosphatase activity and calcified extracellular matrix deposition; in contrast TAAK did not positively affect hASCs differentiation. SiC-PECVD did not alter osteogenic differentiation of hASC cells: indeed, ALP and calcium deposition levels were comparable to those of cells cultured on plastic. Furthermore, we observed similar results testing hASCs either pre-differentiated for 14 days in osteogenic medium or directly differentiated on biomaterials. Our study suggests that modifications of titanium surface may improve osteo-integration of implant devices and that SiC-PECVD may represent a valid alternative for the coating of prosthetic devices to reduce wear and metallosis events.

An increased number of orthopaedic and dental prosthetic surgery procedures motivated researchers to explore new biomaterials for bone implants (1). It is well recognized that surface properties of biomedical devices determine their clinical applications. In most cases, a surface modification is considered to be a prerequisite for better biocompatibility and/or for higher resistance of the implant to corrosion and wear.

Titanium and titanium alloys have been widely used as biomaterials in orthopaedic and dental surgeries due to their high mechanical and corrosion resistance, as well as their biocompatibility.

The clinical success of implants is related to their early osteo-integration (2), which depends on their surface properties. In order to improve the interaction between the surrounding bone tissue and titanium implant, the effects of surface topography on tissue response have been extensively studied to ameliorate the initial events that take place at the bone-implant interface, determining either the success or the failure of implants

Key words: Titanium, Silicon Carbide, human Adipose-derived Stem Cells, Osteoinduction, Osteointegration, Calcified matrix

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(3). Indeed, in terms of topographical quality, it is known that an increased implant-surface roughness significantly influences the osteoblastic response in vitro, improving cell adhesion and proliferation (3-6); other authors have reported the same effect on bone marrow mesenchymal stem cells (7-8).

The main problem with prosthetic joints lies in their wear and corrosion during long-term use. The debris formed as a consequence of this wear results in tissue inflammation, osteolysis and, finally, loosening of the implant (9). In order to reduce this phenomenon, new techniques of surface treatment and coating deposition have been developed to modify the biomaterials surface (10). There have been many studies during the last decade regarding innovative techniques to coat orthopaedic materials with hard coatings in order to obtain a hard and inert material to give an adequate protection to the implant and to decrease the wear rate of prosthetic devices (9, 11-12). Silicon carbide (SiC) may be considered a suitable material for these applications, due to its hardness and wear-resistance; moreover it has been shown that SiC particles do not give rise to any relevant inflammatory response and do not negatively affect bone growth (13).

Since in vitro analyses usually give useful results to correlate crucial substrate features with cell attachment, growth, differentiation and extracellular matrix production, we tested the properties of titanium (TIT), titanium modified by two consecutive anodic spark deposition processes (TAA), titanium obtained by KOH alkali etching process on TAA (TAAK) and SiC-Plasma Enhanced Chemical Vapor Deposition (SiC-PECVD), analyzing their effect on human adipose-derived stem cells (hASCs).These cells were chosen since they possess peculiar features, such as great proliferative and multi-differentiative potential (14-19), that make them useful for screening new biomaterials and suitable candidates in several tissue engineering applications. At last, since our future aim is to propose the use of hASCs in association with biomaterials in clinical applications, we also evaluated the difference to pre-differentiate or not cells towards the osteoblastic lineage before seeding them on biomaterials.

MATERIALS AND METHODS

Biomaterials preparation

ISO 5832-2 commercially-pure grade-2 titanium was used to produce all metal samples used in this study. 12 mm diameter disks were punched from a 0.5 mm thick titanium sheet. Before any further treatments, all disks were acid pickled for 120 seconds in a 20% (v/v) of nitric acid with 3% (v/v) of hydrofluoric acid (Sigma-Aldrich, Milan, Italy). After pickling, all samples were cleaned by ultrasonic rinsing in acetone (RPE; Carlo Erba, Italy) for 5 min, then in distilled water for further 5 min.

Three different surface treatments were considered:

•TIT (acid etching treatment): titanium disks previously prepared were treated by a double-step etching process. An initial etching was carried out in a solution of 1 mol/L NaOH, mixed with 2% v/v H_2O_2 kept at 80°C. The second etching step consisted of an acid treatment performed at 28°C for 1 hour. After each etching step, specimens were rinsed 3 times for 5 min in distilled water by ultrasonic rinsing. Details of TIT surface modification treatment have been reported elsewhere (20).

•TAA: titanium disks surface was modified by the applications of two consecutive anodic spark deposition (ASD) processes carried out in different electrolyte solutions at different voltage ranges. The first ASD was performed in a solution containing phosphate anions ad calcium cations, and the second ASD was performed in a solution containing only calcium cations.

•TAAK: for the preparation of this material, previously treated TAA samples were soaked in a potassium hydroxide water solution at 60°C for 6 hours.

Full details of the TAA and TAAK surface modification treatment and sample preparation techniques have been reported elsewhere (21-22).

Silicon carbide sheets were obtained by plasma enhanced chemical vapor deposition (PECVD) technique, and kindly provided by Selex Galileo Avionica, Florence, Italy. The PECVD technology for the deposition of the SiC layer was performed under well-controlled conditions in order to obtain a highly homogeneous and porosity-free material, with a high polishing performance and a low micro-roughness grade.

Human adipose-derived stem cells (hASCs) isolation and culture

Subcutaneous adipose tissue was harvested from 4 healthy female donors (mean age 42±12 years, range 24-60 years) undergoing lipoaspiration, under written consent. Primary cultures of the stromal vascular fraction (SVF) were established as previously described (14). After isolation, cells were plated at a cellular density of 10^5 cells/cm^2 and cultured (37°C, 5% CO_2) in DMEM (Sigma-Aldrich) supplemented with 10% FBS (Fetal Bovine Serum, Sigma-Aldrich), 50 U/ml penicillin (Sigma-Aldrich), 50 μg/ml streptomycin (Sigma-Aldrich) and 2 mM L-glutamine (Sigma-Aldrich) (control medium, CTRL). At 80% of confluence, hASCs were detached by 0.5% trypsin/0.2% EDTA (Sigma-Aldrich), replated at 10^4 cells/cm^2 and cultured in CTRL or in osteo-inductive medium (OSTEO) consisting of CTRL supplemented with 10 mM glicerol-2-phosphate (Sigma-Aldrich), 10 nM dexamethasone (Sigma-Aldrich), 150 μM L-ascorbic acid-2-phosphate (Sigma-Aldrich) and 10 nM cholecalciferol (Fluka, Buchs, Switzerland) for 14 days (19). Then, CTRL hASCs were detached from tissue culture plates and seeded at 10^4 cell/cm^2 on two different sets of each biomaterial in a 24-wells plate: one maintained in CTRL medium and the other in osteogenic medium (OSTEO NPD, not pre-differentiated). Cells osteo-differentiated for 14 days on plastic were also detached and seeded at the same density on each biomaterial and maintained in osteogenic medium (OSTEO PD, pre-differentiated). Moreover, as negative controls, cells were maintained on tissue culture plates (PA, plastic) in all the different culture conditions (CTRL, OSTEO NPD and OSTEO

PD). Medium was changed twice a week.

hASCs adhesion and viability on biomaterials

By SEM analysis we evaluated adhesion and growth of hASCs on scaffolds. After 14 days of culture, samples were washed with 0.1 M sodium cacodylate buffer (Fluka) for 5 minutes at 4°C, fixed overnight at 4°C in glutaraldehyde (2% in 0.1 M sodium cacodylate buffer) and dehydrated through a series of graded ethanol and hexamethyldisilazane (Sigma-Aldrich) solutions. Samples were then mounted on aluminium stubs, sputter-coated with gold/palladium and analyzed with a scanning electron microscope (JSM-840A, Jeol Ltd., Tokyo, Japan).

Viability test was performed with hASCs at passage 3 cultured for 14 days either on tissue culture plates or on biomaterials: MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, 5 μg/ml in DMEM, Sigma-Aldrich) was added to cells and incubated for 4 hours at 37°C. The resulting formazan precipitate was then solubilized in DMSO 100% (Sigma-Aldrich) and absorbance was read at 570 nm (Wallac Victor II plate reader) (23).

Osteogenic differentiation assessment

Alkaline phosphatase activity (ALP) was determined on hASCs cultured for 14 days on biomaterials or on plastic. Cells were washed with PBS and lysed in 0.1% Triton X-100 (Sigma-Aldrich). ALP was quantified by incubating cellular lysates at 37°C with 1 mM p-nitrophenylphosphate (pNPP, Sigma-Aldrich) in alkaline buffer (100 mM diethanolamine and 0.5 mM $MgCl_2$, pH 10.5) (24). Enzymatic reaction was stopped with NaOH 1N and absorbance was read at 410 nm (Wallac Victor II plate reader). ALP activity was standardized on total protein content, determined by BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA) and expressed as ALP Units for μg of protein.

In order to evaluate calcified matrix deposition, after 21 days of culture cells were rinsed with PBS, fixed with ice-cold 70% ethanol for 1 hour and stained with 40 mM Alizarin Red-S (pH 4.1, Fluka) for 15 minutes. After washing, each sample was destained for 30 minutes with 10% cetylpyridinium chloride monohydrate (CPC, Sigma-Aldrich) in 0.1 M phosphate buffer (pH 7.0) and absorbance was read at 550 nm (Wallac Victor II plate reader) (25).

Statistical Analysis

Data are expressed as mean±SD and statistical analyses (Two-way ANOVA with Bonferroni's post test) were performed using GraphPad Prism v5.00 (GraphPad Software, San Diego, CA, USA). Level of significance was set at $p<.05$.

RESULTS

Biomaterials characterization

TIT showed a typical metallographically etched titanium surface, with the grain structure deeply corroded by the etching process (Fig. 1a), as previously described (20). Furthermore, the TIT surface treatment induced a particular topography and pattern modification of titanium in nanometric scale that may play a fundamental role for the improvement of biomaterial-tissue interaction.

TAA and TAAK treatments, performed using the anodic spark deposition technique and other chemical treatments, were able to properly modify the thickness, chemistry and morphology of the titanium oxide layer. A full chemico-physical characterization of TAA and TAAK surfaces has been already published (21-22). In synthesis, TAA was able to provide a micro-porous surface morphology (Fig. 1b) mainly composed by titanium oxide enriched of Ca and P ions. In TAAK, the final alkali etching treatment performed on the TAA surface induced a roughness in the nanometric scale (Fig. 1c),

The surface of SiC-PECVD was smooth and on a scale of 10 μm there was no evidence of large size irregularities (Fig. 1d).

hASCs adhesion and viability

CTRL and OSTEO hASCs were able to adhere and grow on the surface of the three different types of titanium and on SiC-PECVD, as shown by SEM pictures in Fig. 2a. Cell growth and viability were differently affected by biomaterials as demonstrated by MTT assay (Fig. 2b). Both OSTEO NPD and OSTEO PD cells cultured on the different biomaterials showed a significantly higher viability in comparison to CTRL hASCs cultured on the same surfaces ($p<.05$). We did not observe any significant difference in term of cell viability between OSTEO NPD and OSTEO PD cells, with the exception of OSTEO PD cells cultured on plastic (+57%, $p<.01$).

The viability of cells cultured in CTRL medium on all the tested titanium disks was significantly lower respect to cells on plastic ($p<.001$). The same trend was also observed for OSTEO PD cells cultured on TIT and TAA, in comparison with cells on PA ($p<.001$). Cell viability of OSTEO NPD hASCs was not affected by culture on TIT and on TAA, with MTT values similar to the ones observed for cells on PA. In all culture conditions, TAAK did not offer a good support for cell growth, negatively affecting hASCs viability; indeed, significant differences were found both compared to plastic and to the other types of titanium ($p<.001$).

Cells entirely colonized SiC-PECVD surface forming a monolayer (Fig. 2a). CTRL and OSTEO PD hASCs cultured on SiC-PECVD showed cell viability values similar to those of hASCs on PA, while we observed a great increase in cell growth when OSTEO NPD cells were cultured on SiC-PECVD in comparison to OSTEO NPD cells on PA (+58%, $p<.01$).

hASCs osteogenic differentiation

Both on plastic and on biomaterials, OSTEO NPD and OSTEO PD hASCs showed a significant increase of ALP activity in comparison to CTRL cells ($p<.05$), with

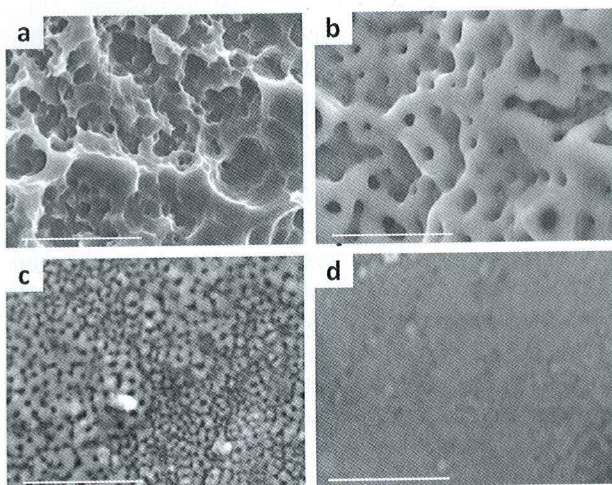


Fig. 1. *SEM microphotographs of the analyzed biomaterials. The three types of titanium surfaces (a, TIT; b, TAA; c, TAAK) and SiC-PECVD (d) (scale bar 10 μm).*

the exception of OSTEO PD cells cultured on TIT and on TAAK (Fig. 3a). ALP activity in CTRL hASCs was just mildly affected by culture on biomaterials: although ALP levels of cells cultured on TIT, TAA, TAAK and SiC-PECVD were higher than those of cells on PA, these increases were not significant (Fig. 3b, PA=1, bar not shown). Only TIT and TAA showed an osteoinductive effect on differentiated hASCs in comparison to cells on plastic: we observed a significant increase in ALP levels in OSTEO NPD cells on TIT (+125%, $p<.001$) and both in OSTEO NPD and in OSTEO PD hASCs on TAA (+78%, $p<.01$ and +48%, $p<.05$, respectively) in comparison to PA (Fig. 3b).

Both on plastic and on titanium biomaterials, calcium deposition was not significantly increased by culture in osteogenic medium, except for OSTEO PD cells cultured on TIT (+119%, $p<.001$, Fig. 3c); however, this lack of a marked increase for TIT and TAA can be partially related to the high level of calcium deposition in CTRL hASCs in comparison to CTRL cells on PA and to the great inter-donor variability. On the contrary, osteo-differentiated cells on SiC-PECVD showed significant increases in calcium deposition in comparison to CTRL hASCs (+61% and +64% for OSTEO NPD and OSTEO PD hASCs respectively, $p<.05$, Fig. 3c)

Regarding the osteoinductive effect of biomaterials, TIT was able to upregulate calcium deposition both in CTRL (+54%, $p<.001$) and in OSTEO hASCs (+28%, $p<.001$ and +69%, $p<.001$ for OSTEO NPD and OSTEO PD cells, respectively, Fig. 3d) respect to cells on PA; for TAA a significant increase of calcified matrix production was observed just in CTRL hASCs in comparison to PA (+82%, $p<.001$, Fig. 3d, PA=1, bar not shown).

We did not observe any osteo-inductive effect either for TAAK or for SiC-PECVD: in particular, culture on TAAK seemed to negatively affect osteogenic differentiation, whereas the behaviour of cells maintained on SiC-PECVD was completely similar to the one of hASCs cultured and differentiated on plastic (Fig. 3b and 3d).

Moreover, we did not find any significant difference in term of ALP activity and calcium deposition between OSTEO NPD and OSTEO PD cells, except for ALP level increase in OSTEO NPD cells on TIT (+146% respect to OSTEO PD hASCs, $p<.001$, Fig. 3a).

DISCUSSION

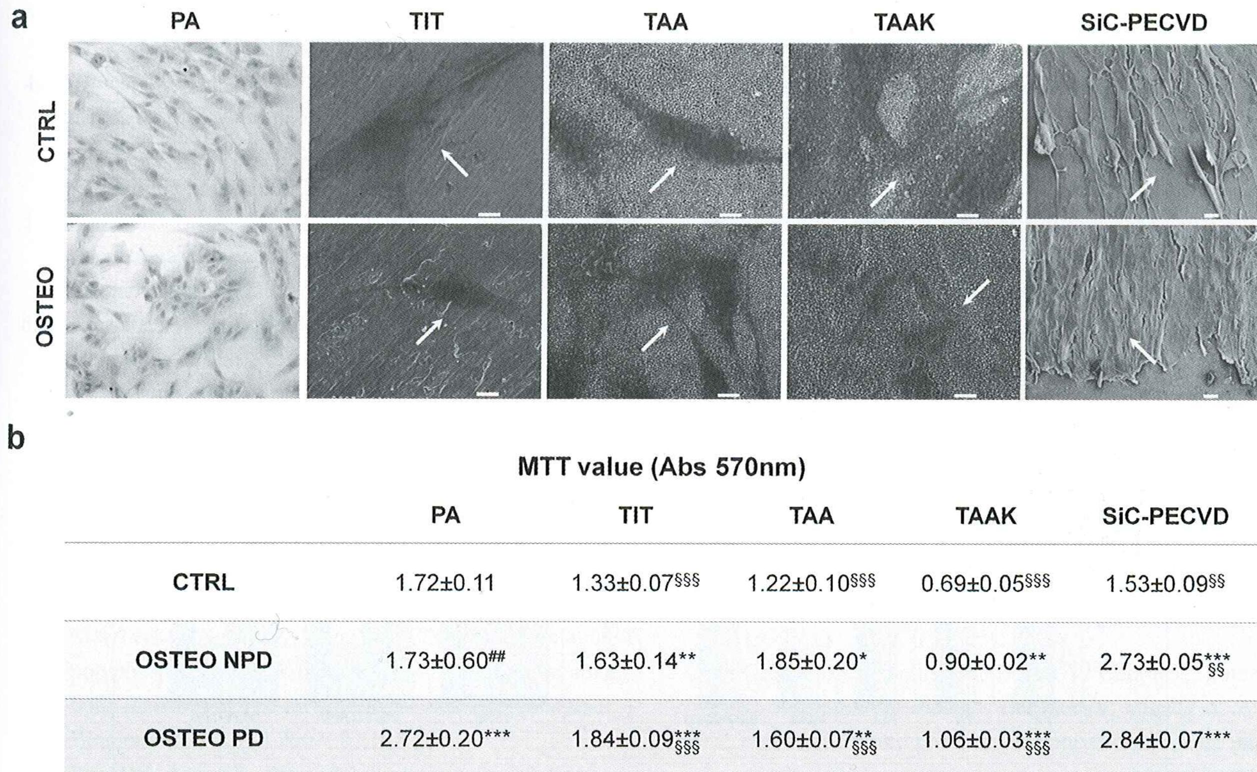
Several efforts have been made in the orthopaedic field to modify biomedical materials surface properties according to the specific requirements of their clinical applications, in order to ameliorate their performance in term of both osteointegration and wear resistance.

The peculiarity of titanium surface, considering its use for an implantable device, is the possibility of controlling its surface cleanness, decontaminating it from impurities and contaminants, such as machining oils, right before sterilisation at the end of the industrial manufacturing process.

In this study we have evaluated the effects of different acid etching and anodic spark deposition processes on the properties of titanium as a support for in vitro cell proliferation and osteogenic differentiation. In particular, we used hASCs since this cellular model has been previously shown to possess a marked osteogenic potential that, together with its easy availability, makes these cells useful and promising tools for orthopaedic applications.

Since it is also well known that scratches and wearing process negatively affect the life span of metal prosthetic devices, we tested the properties of SiC-PECVD as a biocompatible material. SiC, obtained by PECVD technique, is an amorphous material, fundamentally composed by Si, C and O that grants a high grade of surface cleanness, thus reducing the presence of contaminants.

Its great resistance and hardness make SiC-PECVD a promising candidate for the coating of some portions of the implant that need good wear-resistance and low friction, such as femoral head and acetabular components of the hip prosthesis. Due to its hardness and resistance, SiC-PECVD is not subjected to any extensive particles



MTT value (Abs 570nm)

	PA	TIT	TAA	TAAK	SiC-PECVD
CTRL	1.72 ± 0.11	$1.33\pm0.07^{\S\S\S}$	$1.22\pm0.10^{\S\S\S}$	$0.69\pm0.05^{\S\S\S}$	$1.53\pm0.09^{\S\S}$
OSTEO NPD	$1.73\pm0.60^{\#\#}$	$1.63\pm0.14^{**}$	$1.85\pm0.20^{*}$	$0.90\pm0.02^{**}$	$2.73\pm0.05^{***}_{\S\S}$
OSTEO PD	$2.72\pm0.20^{***}$	$1.84\pm0.09^{***}_{\S\S\S}$	$1.60\pm0.07^{**}_{\S\S\S}$	$1.06\pm0.03^{***}_{\S\S\S}$	$2.84\pm0.07^{***}$

Fig. 2. *Morphology, adherence and viability of hASCs grown on biomaterials. (a) CTRL and OSTEO PD hASCs cultured for 14 days on plastic (haematoxylin-eosin staining, light microscopy, 20X) and on TIT, TAA, TAAK and SiC-PECVD (SEM microphotographs, white arrows indicate cells, scale bars 10 μm). (b) Viability of CTRL, OSTEO NPD and OSTEO PD hASCs evaluated by MTT assay after 14 days of culture either on plastic or on biomaterials (mean±SD, n=3) (BIOMATERIAL vs PA: $^{\S}p<.05$, $^{\S\S}p<.01$ and $^{\S\S\S}p<.001$; OSTEO vs CTRL: $^{*}p<.05$, $^{**}p<.01$ and $^{***}p<.001$; OSTEO NPD vs OSTEO PD: $^{\#\#}p<.01$).*

formation and is characterized by a lower wear rate compared to the metals commonly used in prosthetic devices, thus the use of this material could be beneficial to reduce metallosis events, that are known to be potential causes of implant failure (26).

hASCs viability was mildly reduced by culture these cells on titanium disks: indeed, we observed a general mild decrease in cell viability of hASCs maintained for 14 days on TIT and TAA both in CTRL and OSTEO PD conditions compared to PA. This reduction in cell viability might be related to an initial lag phase needed for cells to adapt to the new surface conditions.

Modifications performed to obtain TAAK had a strong negative influence on cell viability, with a significant reduction compared both to all the other titanium disks and to plastic. These data are partially in contrast with the ones previously published (27), where it was shown that the human osteoblast cell line MG-63 was able to well adhere and colonize TAAK surface. This discrepancy could depend on the intrinsic difference between the two types of cells, since primary cells like hASCs are known to be more susceptible to any variation of culture environment. Furthermore, these two studies differ in the time points chosen to evaluate cell viability: indeed, here we determined hASCs viability after a more extended period of culture, thinking that a prolonged culture period could be more predictive about the behaviour of hASCs in combination with these materials in view of a possible future clinical use.

SiC-PECVD provided an optimal surface for cell adhesion and growth: viability of CTRL and OSTEO PD hASCs was very similar to that of cells cultured on plastic, whereas we even noticed significantly higher values for OSTEO NPD hASCs on SiC-PECVD respect to plastic.

These data, together with those previously published by Santavirta et al. (28), demonstrate the cyto-compatibility of SiC-PECVD and allow to suggest its use as a coating for prosthesis devices.

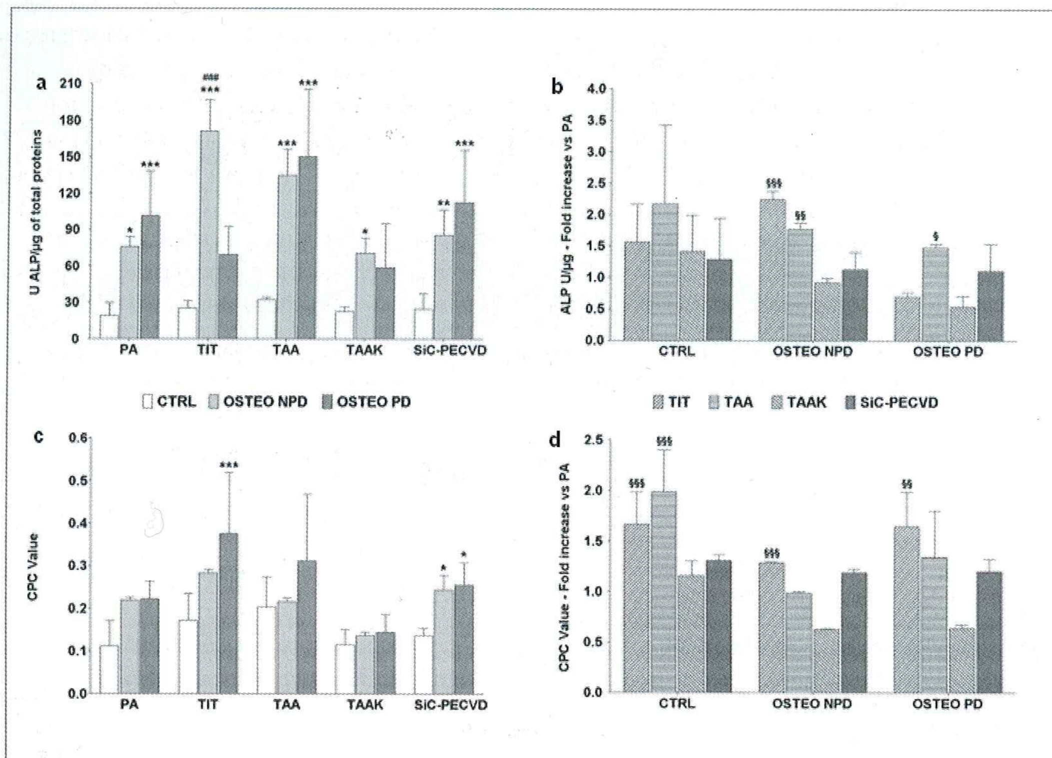


Fig. 3. *Effect of osteogenic medium on differentiation of hASCs. ALP activity (a) and calcified extracellular matrix deposition (c) in CTRL, OSTEO NPD and OSTEO PD hASCs cultured respectively for 14 and 21 days on PA, TIT, TAA, TAAK and SiC-PECVD (mean±SD, n=4). Effect of biomaterials on the expression of osteogenic markers. comparison of ALP activity (b) and calcified matrix deposition (d) of hASCs cultured on TIT, TAA, TAAK and SiC-PECVD respect to PA expressed by fold increase, where PA was arbitrarily fixed at 1 (PA bar not shown, n=4). (OSTEO vs CTRL: *p<.05, **p<.01 and ***p<.001; OSTEO NPD vs OSTEO PD: ###p<.01; BIOMATERIAL vs PA: §p<.05, §§p<.01 and §§§p<.001).*

In our study the most performing biomaterials, in term of osteo-inductive properties, were TIT and TAA, as shown by the increase of both calcium deposition and alkaline phosphatase in comparison to plastic, whereas TAAK reduced the ability of hASCs to differentiate towards the osteoblastic lineage.

Levels of markers for osteo-differentiation of hASCs cultured on SiC-PECVD were comparable to those of cells grown on plastic; this could be probably due to the great smoothness of SiC-PECVD, where the lack of a tridimensional microstructure did not provide any positive stimuli for the osteogenic differentiation of hASCs.

All together our results demonstrate that some surface modifications may lead to an improvement of the biomaterial performance, and that in vitro studies are useful to provide preliminary informations about the hypothetical behaviour of biomaterials in vivo. Further investigations in animal models will provide additional response about the effectiveness of these modified biomaterials for clinical applications

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REFERENCES

1. Sollazzo V, Pezzetti F, Scarano A, Piattelli A, Bignozzi CA, Massari L, Brunelli G, Carinci F. Zirconium oxide coating improves implant osseointegration in vivo. Dent Mater 2008; 24:357-61.
2. Le Guehennec L, Soueidan A, Layrolle P, Amouriq Y. Surface treatments of titanium dental implants for rapid osseointegration: review. Dent Mater 2007; 23:844-54.

3. Anselme K. Osteoblast adhesion on biomaterials. Biomaterials 2000; 21:667-681.
4. Bowers KT, Keller JC, Randolph BA, Wick DG, Michaels CM. Optimization of surface micromorphology for enhanced osteoblast responses in vitro. Int J Oral Maxillofac Implants 1992; 7:302-10.
5. Keller JC, Schneider GB, Stanford CM, Kellogg B. Effects of implant microtopography on osteoblast cell attachment. Implant Dent 2003; 12:175-179
6. Marinucci L, Balloni S, Becchetti E, Belcastro S, Guerra M, Calvitti M, Lilli C, Calvi EM, Locci P. Effect of titanium surface roughness on human osteoblast proliferation and gene expression in vitro. Int J Oral Maxillofac Implants 2006; 21:719-725.
7. Ozawa S, Kasugai S. Evaluation of implant materials (hydroxyapatite, glass-ceramics, titanium) in rat bone marrow stromal cell culture. Biomaterials 1996; 17:23-29.
8. Maekawa K, Yoshida Y, Mine A, Fujisawa T, Van Meerbeek B, Suzuki K, Kuboki T. Chemical interaction of polyphosphoric acid with titanium and its effect on human bone marrow derived mesenchymal stem cell behaviour. J Biomed Mater Res A 2007; 82:195-200.
9. Roy RK, Lee KR. Biomedical applications of diamond-like carbon coatings: a review. J Biomed Mater Res B Appl Biomater 2007; 83:72-84.
10. Sharma S, Patil DJ, Soni VP, Sarkate LB, Khandekar GS Bellare JR. Bone healing performance of electrophoretically deposited apatite-wollastonite/chitosan coating on titanium implants in rabbit tibiae. J Tissue Eng Regen Med 2009; 3: 501-511.
11. Lappalainen R, Anttila A Heinonen H. Diamond coated total hip replacements. Clin Orthop Relat Res 1998; 352:118-127.
12. Wimmer MA, Loos J, Nassutt R, Heitkemper M Fischer A. The acting wear mechanisms on metal-on-metal hip joint bearings: in vitro results. Wear 2001; 250:129-139.
13. Aspenberg P, Anttila A, Konttinen YT, Lappalainen R, Goodman SB, Nordsletten L, Santavirta S. Benign response to particles of diamond and SiC: bone chamber studies of new joint replacement coating materials in rabbits. Biomaterials 1996; 17: 807-812
14. Zuk PA, Zhu M, Mizuno H, Huang J, Futrell JW, Katz AJ, Benhaim P, Lorenz HP, Hedrick MH. Multilineage cells from human adipose tissue: implications for cell-based therapies. Tissue Eng 2001; 7:211-228.
15. Zuk PA, Zhu M, Ashjian P, De Ugarte DA, Huang JI, Mizuno H, Alfonso ZC, Fraser JK, Benhaim P, Hedrick MH. Human adipose tissue is a source of multipotent stem cells. Mol Biol Cell 2002; 13:4279-4295.
16. De Ugarte DA, Morizono K, Elbarbary A, Alfonso Z, Zuk PA, Zhu M, Dragoo JL, Ashjian P, Thomas B, Benhaim P, Chen I, Fraser J, Hedrick MH. Comparison of multi-lineage cells from human adipose tissue and bone marrow. Cells Tissues Organs 2003; 174:101-109.
17. de Girolamo L, Sartori MF, Albisetti W, Brini AT. Osteogenic differentiation of human adipose-derived stem cells: comparison of two different inductive media. J Tissue Eng Regen Med 2007; 1:154-157.
18. de Girolamo L, Sartori MF, Arrigoni E, Rimondini L, Albisetti W, Weinstein RL, Brini AT. Human adipose-derived stem cells as future tools in tissue regeneration: osteogenic differentiation and cell-scaffold interaction. Int J Artif Organs 2008; 31: 467-479.
19. de Girolamo L, Lopa S, Arrigoni E, Sartori MF, Baruffaldi Preis FW, Brini AT. Human adipose-derived stem cells isolated from young and elderly women: their differentiation potential and scaffold interaction during in vitro osteoblastic differentiation. Cytotherapy 2009; 11: 793-803.
20. Giordano C, Sandrini E, Busini V, Chiesa R, Fumagalli G, Giavaresi G, Fini M, Giardino R, Cigada A. A new chemical etching process to improve endosseous implant osseointegration: in vitro evaluation on human osteoblast-like cells. Int J Artif Organs 2006; 29:772-780.
21. Sandrini E, Chiesa R, Rondelli G, Santin M, Cigada A. A novel biomimetic treatment for an improved osteointegration of titanium. JABB 2003 ; 1:33-42.
22. Giordano C, Chiesa R, Sandrini E, Cigada A, Giavaresi G, Fini M, Giardino R. Physical and biological characterizations of a novel multiphase anodic spark deposition coating to enhance implant osseointegration. J Mater Sci Mater Med 2005; 16:1221-1229.
23. Kingham PJ, Kalbermatten DF, Mahay D, Armstrong SJ, Wiberg M, Terenghi G. Adipose-derived stem cells differentiate into a Schwann cell phenotype and promote neurite outgrowth in vitro. Exp Neurol 2007; 207:267-274.
24. Bodo M, Lilli C, Bellucci C, Carinci P, Calvitti M, Pezzetti F, Stabellini G, Bellocchio S, Balducci C, Carinci F,Baroni T. Basic fibroblast growth factor autocrine loop controls human osteosarcoma phenotyping and differentiation Mol Med 2002; 8: 393-404.
25. Halvorsen YD, Franklin D, Bond AL, Hitt DC, Auchter C, Boskey AL, Paschalis EP, Wilkison WO, Gimble JM. Extracellular matrix mineralization and osteoblast gene expression by human adipose tissue-derived stromal cells. Tissue Eng 2001; 7:729-741.
26. Saikko V, Paavolainen P, Slätis P. Wear of the polyethylene acetabulum cup. Metallic and ceramic heads compared in a hip simulator. Acta Orthop Scand 1993; 64:391-402.

27. Sandrini E, Morris C, Chiesa R, Cigada A, Santin M. In vitro assessment of the osteointegrative potential of a novel multiphase anodic spark deposition coating for orthopaedic and dental implants. J Biomed Mater Res B Appl Biomater 2005; 73:392-399.

28. Santavirta S, Takagi M, Nordsletten L, Anttila A, Lappalainen R, Konttinen YT. Biocompatibility of silicon carbide in colony formation test in vitro. A promising new ceramic THR implant coating material. Arch Orthop Trauma Surg 1998; 118:89-91.