THE EFFECT OF SIMULATED BODY FLUID ON THE GROWTH AND DIFFERENTIATION OF HUMAN MESENCHYMAL STEM CELLS CULTURED ON NANOSTRUCTURED Ti6Al4V SURFACES

[1]Eva FILOVÁ, [1,2]Barbora VOLTROVÁ, [3]Petra JAROLÍMOVÁ, [4]Roman MATĚJKA, [1]Věra LUKÁŠOVÁ, [3]Jaroslav FOJT, [3]Luděk JOSKA, [5]Matej DANIEL

[1]Institute of Experimental Medicine of the Czech Academy of Sciences, Prague, Czech Republic, EU, eva.filova@iem.cas.cz
[2]Charles University in Prague, Faculty of Science, Czech Republic, EU, barbora.voltrova@iem.cas.cz
[3]University of Chemistry and Technology Prague, Faculty of Chemical Technology, Prague, Czech Republic, EU, petra.jarolimova@vscht.cz, jaroslav.fojt@vscht.cz, ludek.joska@vscht.cz
[4]Institute of Physiology of the Czech Academy of Sciences, Prague, Czech Republic, EU, roman.matejka@fgu.cas.cz
[5]Czech Technical University in Prague, Faculty of Mechanical Engineering, Czech Republic, EU, matej.daniel@fs.cvut.cz

Abstract

TI6Al4V is a standard medical titanium alloy. In this study, we compared ground Ti6Al4V discs with nanostructured Ti6Al4V discs prepared using the anodic oxidation method at 30V. Subsequently, both the nanostructured and ground discs were exposed to simulated body fluid (SBF) for 7 days in order to form a hydroxyapatite-like layer. Human mesenchymal stem cells (hMSC) were seeded on the discs in osteogenic differentiation medium and cultivated for 21 days. hMSCs were tested for metabolic activity (MTS assay) and rate of proliferation. The synthesis of type I collagen was evaluated using immunohistochemical staining and confocal microscopy analysis. All samples supported cell adhesion and proliferation. The highest amount of type I collagen was found on nanostructured (N) and ground Ti6Al4V exposed to SBF (TiH) surfaces. Thus, SBF and anodic oxidation positively influenced the growth and osteogenic differentiation of hMSCs.

Keywords: Anodic oxidation, hydroxyapatite, mesenchymal stem cell, titanium alloy

1. INTRODUCTION

Proper bone implants should promote fast adhesion of cells, stimulate cell differentiation and support the formation of new bone tissue. Currently, titanium alloys are used as biocompatible materials capable of bone tissue integration [1,2]. Titanium and its alloys are widely used in orthopaedic and dental surgery. Despite the benefits of titanium alloys for such applications, there are still questions which need to be answered It is possible to modify the titanium surface in nanoscale using anodic oxidation in fluorides containing electrolytes [3,4]. This process leads to the formation of self-organised TiO_2 nanotube layer on the titanium surface. Such a change in the nano-topography of the material can improve cell adhesion which is crucial for the implants osseointegration [5]. Incubation in the simulated body fluid (SBF) forms a hydroxyapatite-like layer on the exposed surfaces. The deposited layer of hydroxyapatite by immersing Ti6Al4V in the simulated body fluid (SBF) was investigated previously [6,7].

This study was focused on the comparison of nanostructured and ground Ti6Al4V titanium alloys, modified by hydroxyapatite coating, on the cytotoxicity and differentiation capacity of seeded human mesenchymal stem cells (hMSCs). Nanostructures were fabricated using anodic oxidation. Hydroxyapatite surface modification of the titanium samples was realized by exposure to SBF.

2. MATERIALS AND METHODS

2.1. Specimens preparation

Nanostructuring was realized using Potentiostat-Galvanostat IMP 88 PC-200V with PGU-AUTO Extern control unit (Jaissle). A silver/silver chloride reference electrode with 3 mol/L KCl (SSCE) was used and, glassy carbon bars were used as counter electrodes. The electrochemical measurement consisted of a potential ramp from the open circuit potential to the selected end potential 30V with a 100 mV/s sweep rate and potentiostatic exposure for 2200 s. An ammonium sulphate (1 mol/L) and ammonium fluoride (0.2 wt.%) solution was used as the electrolyte. All anodization experiments were carried out at room temperature. Following the electrochemical measurement, the samples were ultrasonicated in ethanol and deionised water for 10 minutes and then dried in air stream. Ground and nanostructured titanium (Ti6Al4V) discs were exposed in simulated body fluid (SBF) at temperature of 37 °C for 7 days in order to create a hydroxyapatite layer on the surface. In this study, 4 sets of flat round Ti6Al4V samples, with 16 mm diameter and height 4 mm, with different surface treatments were used (**Table 1**). Before treatment all samples were ground to the roughness of sandpaper P2500, degreased with ethanol and cleaned with distilled water in an ultrasonic bath. For the morphological characterization of samples, a Vega3 LMU scanning electron microscope (SEM, Tescan) with an Oxford Instruments EDS analyzer was used. The results of the elemental analysis by Energy-dispersive X-ray spectroscopy were evaluated using the Aztec software. The length and inner diameter of the nanotubes was evaluated from SEM images in ImageJ software. Data from four distant image fields from at least three samples were used for analysis.

Table 1 List of samples before and after exposition to simulated body fluid (SBF)

Samples	Name	SBF
Nanostructured Ti6Al4V	N	-
Ground Ti6Al4V	Ti	-
Ground Ti6Al4V exposed to SBF	TiH	+
Nanostructured Ti6Al4V exposed to SBF	NH	+

2.2. Cell culturing

hMSCs (ScienceCell, Carlsbad, CA), were cultivated in growth medium consisting of MEM Alpha medium (1X) (Gibco, Thermofisher Scientific, Waltham, MA, USA) supplemented with 10 % (v/v) fetal bovine serum (FBS), penicillin/streptomycin (100 IU/mL and 100 µg/mL, Sigma-Aldrich) in 5 % CO_2 and 37 °C. The hMSCs were seeded on Ti6Al4V scaffolds and control glass coverslip and control Tissue culture plast (TCP) at a density of 12,000 cells/cm^2. The samples were sterilized in collaboration with the Institute of Nuclear Physics of the Academy of Sciences of the Czech Republic using the Microtron MT25 cyclic particle accelerator with an electron beam of 9.8 MeV with a dose of 27 kGy. The cells were cultured in growth medium. After 3 days of cell culture, when the cells reached confluence, the cultivating medium was enriched by osteogenic differentiation additives: 40 µg/mL ascorbate-2-phosphate, 100 nM dexamethasone, and 10 mM glycerol-2-phosphate disodium salt. The osteogenic medium was changed twice a week.

2.3. Cell metabolic activity measurement

The metabolic activity of the hMSCs was determined using MTS assay (CellTiter96® AQueous One Solution Assay; Promega, Madison, WI, USA). To each scaffold, 800 µL of MTS solution in fresh medium was added. After 2 hours incubation, the absorbance of the metabolized solution (100 µL) was detected at 490 nm using Tecan Infinite® PRO 200 series reader (Tecan, Männedorf, SUI). The background absorbance (690 nm) was subtracted from the measured data, as well as the absorbance of media with MTS solution.

2.4. Cell proliferation

To determine the cell proliferation a fluorescence-based kit (Quant- iT™ PicoGreen® dsDNA Assay Kit; Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) was used. Media was aspirated from the samples after MTS assay and 1 mL of cell lysis buffer (10 mM L−1 Tris, 1 mM L−1 EDTA, 0.2 % v/v Triton X-100) was added, incubated 20 min in RT then frozen three times (-80°C) and thawed to facilitate the cell lysis and DNA release. Fluorescence intensity was detected using Tecan Infinite® PRO 200 series reader Tecan (λex = 485 nm, λem = 528 nm) and the DNA content was determined according to the λDNA calibration curve from the kit.

2.5. Immunohistochemical staining of type I collagen

On day 14, the samples were fixed with 70 % frozen methanol, washed with phosphate buffered saline (PBS) and incubated in 3 % FBS in PBS/0.1 % Triton-X for 30 min at RT. Primary monoclonal antibody against type I collagen (M-38-C, DSHB, USA, dilution 1:20) was added, and incubated overnight at 2°C-8°C. After 3 washes with PBS/0.05 % Tween, and one in PBS, the samples were incubated with secondary antibody Alexa Fluor 488-conjugated anti-mouse antibody for 45 min, RT (4408S, Cell Signalling Technologies, dilution 1:400). Cell nuclei were stained with 5 µg/mL propidium iodide in PBS for 5 min, RT (Sigma Aldrich, USA) and twice washed in PBS. The cells were visualized using a confocal microscope ZEISS LSM 5 DUO at λexc=488 nm and λem =505-550 nm for Alexa Fluor 488, and λex =560 nm, λem >575 nm for propidium iodide). The intensity of fluorescence was evaluated from 8 - 9 photomicrographs per sample group using MATLAB.

2.6. Statistical analysis

Quantitative data are presented as mean ± standard deviation. The results were evaluated statistically using one-way analysis of variance (ANOVA), normality of the data was tested, and Student-Newman-Keuls Method was used as a post-hoc test. Fluorescence intensity was evaluated using Multifactorial ANOVA. The level of significance was set at 0.05.

3. RESULTS

Prepared nanostructured Ti6Al4V discs were evaluated using SEM. According to obtained images, nanotubes of diameter of 70 nm (SD ± 16 nm) were fabricated on the surface of the discs (**Figure 1**). The elemental composition of the samples was measured using EDS (**Table 2**). The metabolic activity of seeded hMSC (**Figure 2A**) on four different titanium alloys was evaluated using MTS metabolic assay on days 1, 7, 14 and 21. On day 1, the highest metabolic activity was measured in groups Ti and TiH. In the following days the metabolic activity rose steadily and no differences were seen between the different titanium discs (**Figure 2**). Further differences were seen only on days 7 and 21 between all tested groups and the TCP control group that is specifically modified to support cell growth. These finding are in agreement with a study that confirmed biocompatibility of the titanium alloys [8]. However, a study by Saha et al. showed improved metabolic activity of MG63 cells on nanostructured Ti6Al4V discs [9]. The rate of hMSCs proliferation was evaluated using a PicoGreen kit on days 1, 7, 14, and 21. Both Ti and TiH samples showed a significantly higher amount of DNA (**Figure 2B**) compared to both nanostructured samples on day 1. This suggests that nanostructured surface did not improve cell adhesion regardless of the presence of hydroxyapatite layer.

Table 2 Samples composition

Samples	Ti [at. %]	Al [at. %]	V [at. %]	Ca [at. %]	P [at. %]
N	88.6	6.9	4.5	/	/
Ti	89.1	6.6	4.3	/	/
TiH	87.7	6.1	3.8	1.5	0.9
NH	78.1	4.2	1.9	9.7	6.1

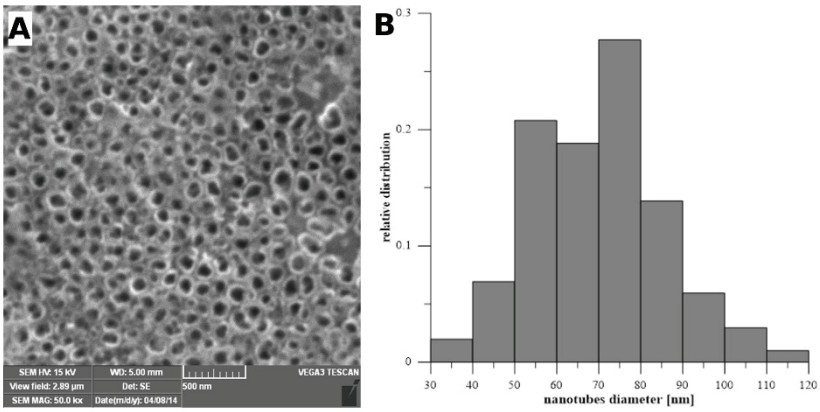

Figure 1 SEM image of nanostructure and histogram of nanotube inner diameters created on Ti6Al4V samples by anodic oxidation at 30V

On day 7, a significant difference in the amount of DNA was detected again on the samples Ti and TiH compared to sample N. No significant differences were detected on day 14. However, on day 21, a higher amount of DNA was found on Ti and TiH samples compared to N and TCP group. Thus, the results implied that nanostructures formed, by anodic oxidation, decreased the rate of cell proliferation in comparison to ground titanium alloys. However, the exposition of samples to SBF had no effect on the rate of cell proliferation. This is in contrast to several studies where it was proved that the nanostructured surface of the Ti6Al4V improved cell proliferation [9,10]. Similarly, Deligianni et al. reported that nanorough surfaces of Ti6Al4V showed increased adsorption of total proteins from FBS, and the amount of adsorbed fibronectin on surfaces with higher roughness compared to lower roughness. This finding correlated with number of cells adhered on the surface [1]. On titanium surfaces modified with nanotubes, the highest protein adsorption was found on samples containing nanotubes with the diameter of about 30 nm and the lowest with the diameter of 80 nm. Similarly, hMSCs proliferation correlated with the rate of protein adsorption.

Type I collagen, an extracellular protein, is a marker of osteogenic differentiation. The measurement of type I collagen (**Figure 3**) on day 14 revealed the highest amount of this extracellular protein on the samples N and TiH. Therefore, we conclude that formed nanostructures on the surface of Ti6Al4V discs had the same effect on collagen type I production as the exposition of the ground titanium alloy to SBF. Moreover, on all the samples, there was a statistically higher intensity detected in comparison to the glass control. Those results are in agreement with the study of Filova et al. [5]. Similarly, surfaces with higher roughness of Ti6Al4V showed higher alkaline phosphatase activity of hMSCs compared to surfaces with lower roughness [1]. Higher nanotube diameter on titanium samples improved osteogenic markers compared to smaller diameter [11].

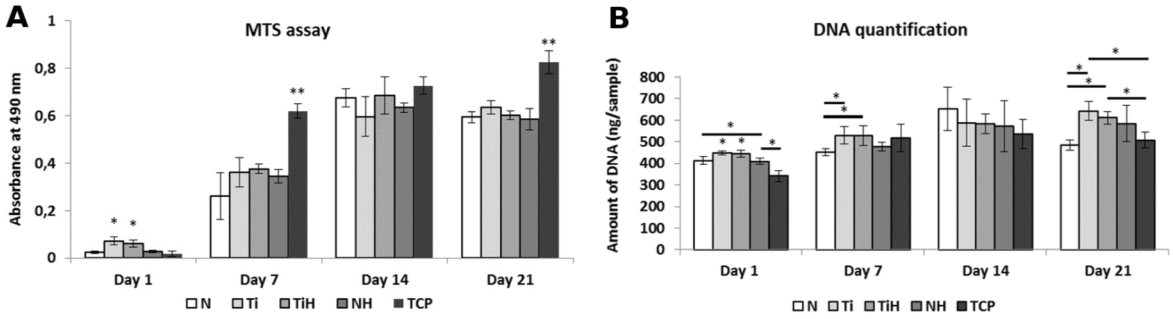

Figure 2 (A) Metabolic activity of the hMSCs measured by MTS assay (B), the proliferation of the hMSCs determined using the PicoGreen assay. Statistical difference with P < 0.05 marked as * **and P**< 0.001 as ****.**

In a human body, apatite layer is formed on the implant surfaces. Through this layer, bone is binding to the implant surface [7]. Thus, the properties of the formed layer may affect cell adhesion, growth and differentiation. Modification of titanium surfaces by hydroxyapatite layer (HAp) has been tested using different techniques, e.g. electrophoretic deposition, electrophoretic deposition, plasma spraying, phase-transited lysozyme coating etc. [12,13]. They provide HAp of slightly different composition, morphology, crystallinity, and different stability. HAp modified titanium surfaces showed higher osteoconductivity and osteoinductivity than non-modified surfaces [14]. In our study, we used sample incubation in SBF solution. The surfaces of TiH and NH were completely covered with HAp-like layer. TiH did not negatively affect hMSC proliferation compared to N samples. On the other hand, type I collagen significantly decreased in NH samples although it amount was higher than on Ti samples which was probably influenced by decreased roughness.

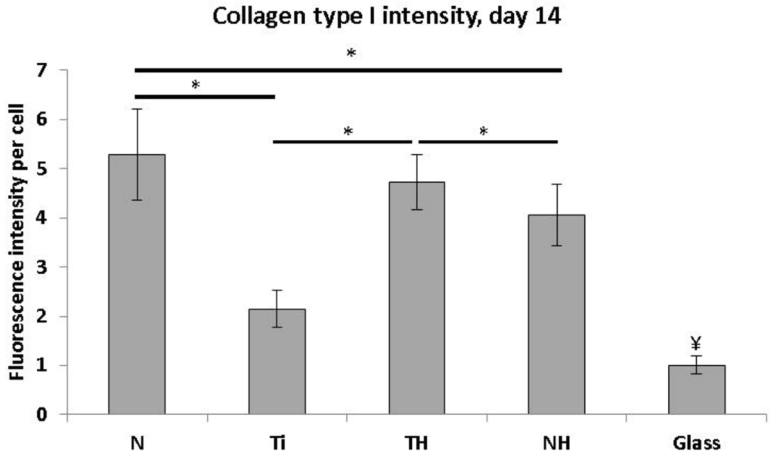

Figure 3 Type I collagen intensity on diverse Ti6Al4V discs. Statistical difference with P < 0.05 marked as *****, ¥ mean this group showed the lowest fluorescence intensity statistically.

4. CONCLUSION

Following the surface modification of Ti6Al4V discs (Ti) by anodic oxidation, the nanotubes with a diameter of 70 nm ± 16 nm were formed (N). Subsequently, the hydroxyapatite-like layer was deposited on both the nanostructured and ground titanium surfaces (NH, TiH) by the discs exposure in SBF. Our results clearly demonstrated that all four kinds of Ti6Al4V discs supported hMSCs metabolic activity and growth. Significant differences in the fluorescence intensity of collagen type I revealed that both modifications, nanostructuring of the surfaces and exposition to the SBF, positively influenced the osteogenic differentiation of seeded hMSCS.

ACKNOWLEDGEMENTS

The project was supported by the Czech Science Foundation project Nr. 16-14758S and by the Ministry of Education, Youth and Sports of the Czech Republic under NPU I: LO1508.

REFERENCES

[1] DELIGIANNI, D.D., KATSALA, N., LADAS, S., SOTIROPOULOU, D., AMEDEE, J. and MISSIRLIS, Y.F. Effect of surface roughness of the titanium alloy Ti-6Al-4V on human bone marrow cell response and on protein adsorption. Biomaterials. 2001. vol. 22, no. 11, pp. 1241-1251.

[2] BORDJI, K., JOUZEAU, J.Y., MAINARD, D., PAYAN, E., NETTER, P., RIE, K.T., STUCKY, T. and HAGE-ALI, M. Cytocompatibility of Ti-6Al-4V and Ti-5Al-2.5Fe alloys according to three surface treatments, using human fibroblasts and osteoblasts. Biomaterials. 1996. vol. 17, no. 9, pp. 929-940.

[3] CHRZANOWSKI, W., SZEWCZENKO, J., TYRLIK-HELD, J., MARCINIAK, J. and ZAK, J. Influence of the anodic oxidation on the physicochemical properties of the Ti6Al4V ELI alloy. Journal of Materials Processing Technology. 2005. vol. 162-163, pp. 163-168.

[4] VELTEN, D., BIEHL, V., AUBERTIN, F., VALESKE, B., POSSART, W. and BREME, J. Preparation of TiO(2) layers on cp-Ti and Ti6Al4V by thermal and anodic oxidation and by sol-gel coating techniques and their characterization. Journal of Biomedical Materials Research. 2002. vol. 59, no. 1, pp. 18-28.

[5] FILOVA, E., FOJT, J., KRYSLOVA, M., MORAVEC, H., JOSKA, L. and BACAKOVA, L. The diameter of nanotubes formed on Ti-6Al-4V alloy controls the adhesion and differentiation of Saos-2 cells. Int J Nanomedicine [online]. 2015. vol. 10, p. 7145-7163 [viewed 2018-09-25]. Available from DOI: 10.2147/IJN.S87474.

[6] EL-WASSEFY, N.A., HAMMOUDA, I.M., HABIB, A.N., EL-AWADY, G.Y. and MARZOOK, H.A. Assessment of anodized titanium implants bioactivity. Clinical Oral Implants Research. 2014. vol. 25, no. 2, pp. e1-e9.

[7] TAKADAMA, H., KIM, H.-M., KOKUBO, T. and NAKAMURA, T. XPS study of the process of apatite formation on bioactive Ti-6Al-4V alloy in simulated body fluid. Science and Technology of Advanced Materials. 2001. vol. 2, no. 2, pp. 389-396.

[8] GALLARDO-MORENO, A.M., PACHA-OLIVENZA, M.A., SALDAÑA, L., PÉREZ-GIRALDO, C., BRUQUE, J.M., VILABOA, N. and GONZÁLEZ-MARTÍN, M.L. In vitro biocompatibility and bacterial adhesion of physico-chemically modified Ti6Al4V surface by means of UV irradiation. Acta Biomaterialia. 2009. vol. 5, no. 1, pp. 181-192.

[9] SAHA, S., KUMAR, R., K. PRAMANIK, K., and BISWAS, A. Interaction of osteoblast -TiO2 nanotubes in vitro: The combinatorial effect of surface topography and other physico-chemical factors governs the cell fate. Applied Surface Science. 2018, vol. 449, pp. 152-165.

[10] SHOKUHFAR, T., HAMLEKHAN, A., CHANG, J.-Y., CHOI, C.K., SUKOTJO, C. and FRIEDRICH, C. Biophysical evaluation of cells on nanotubular surfaces: the effects of atomic ordering and chemistry. Int J Nanomedicine [online]. 2014. vol. 9, pp. 3737-3748 [viewed 2018-09-20]. Available from DOI: 10.2147/IJN.S67344.

[11] DING, X., ZHOU, L., WANG, J., ZHAO, Q., LIN, X., GAO, Y., LI, S., WU, J., RONG, M., GUO, Z., LAI, C., LU, H. and JIA F. The effects of hierarchical micro/nanosurfaces decorated with TiO2 nanotubes on the bioactivity of titanium implants in vitro and in vivo. Int J Nanomedicine [online]. 2015. vol. 10, pp. 6955-6973 [viewed 2018-09-25]. Available from DOI: 10.2147/IJN.S87347.

[12] YANG, J., ZHANG, K., QUE, K., HOU, S., CHEN, Z., LI, Y., WANG, Y., SONG, Y., GUAN, B., ZHANG, W., ZHU, D., LI, C., WANG, D., GENG, P. and ZHANG, X. Surface modification of titanium with hydroxyapatite layer induced by phase-transited lysozyme coating. Materials Science and Engineering. C, Materials for Biological Applications. 2018. vol. 92, pp.206-215.

[13] MANO, T., UEYAMA, Y., ISHIKAWA, K., MATSUMURA, T. AND K. SUZUKI, K. Initial tissue response to a titanium implant coated with apatite at room temperature using a blast coating method. Biomaterials. 2002. vol. 23, no. 9, pp. 1931-1936.

[14] LIN, L., CHOW, K.L. and LENG, Y. Study of hydroxyapatite osteoinductivity with an osteogenic differentiation of mesenchymal stem cells. Journal of Biomedical Materials Research A. 2009. vol. 89, no. 2, pp. 326-335.