The Effects of Titanium Topography and Chemical Composition on Human Osteoblast Cell

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Summary

The objective of this study was to evaluate and compare titanium surfaces: machined (MA); sintered ceramic-blasted (HAS); sintered ceramic-blasted and acid-etched (HAS DE) and to determine the effects of surface topography, roughness and chemical composition on human osteoblast cell reaction. Titanium surface samples were analyzed with respect to surface chemical composition, topography, and roughness. The effects of material surface characteristics on osteoblasts was examined by analyzing osteoblast morphology, viability and differentiation. Osteoblasts cultured on these materials had attached, spread and proliferated on every sample. The viability of osteoblasts cultured on HAS and HAS DE samples increased more intensively in time comparing to MA sample. The viability of osteoblast cultured on HAS samples increased more intensively in the early phases of culture while for cells cultured on HAS DE the cells viability increased later in time. Alkaline phosphate activity was the highest for the cells cultured on HAS sample and statistically higher than for the MA sample. The least activity occurred on the smooth MA sample along with the rougher HAS DE samples. All the examined samples were found to be biocompatible, as indicated by cell attachment, proliferation, and differentiation. Titanium surfaces modification improved the dynamics of osteoblast viability increase. Osteoblast differentiation was found to be affected by the etching procedure and presence of Ca and P on the surface.

Key words

Surface characterization • Titanium • Osteoblast • Calcium • Phosphate

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Introduction

Modern dental implantology requires rapid implant loading with a prosthodontic appliance for a functional and aesthetic result. Osseointegration as a bond between the bone and the implant is an essential condition for successful implant loading, which is largely facilitated by the implant surface. Thus, various methods, have been developed to accelerate osseointegration, such as surface chemical modification, surface topography regulation, among others (Wu *et al*. 2015, Xavier *et al*. 2003, Nicolas-Silvente *et al*. 2020).

The development of bone-implant interface depends on the direct interactions of the bone matrix and osteoblasts with the implant. As the implant surface interacts with the bone it influences osseointegration by altering the adhesion, spreading, growth, viability and differentiation of the cells (Xavier *et al.* 2015, Barradas *et al*. 2012, Feng *et al* 2004, Sunarso *et al*. 2016, Rizo-Gorrita *et al*. 2020). The surface chemical composition and topography are known to alter these biological interactions (Xavier *et al*. 2003, Feng *et al*. 2004, Le Guehennec *et al*. 2008, Bretschneider *et al*. 2020, Lukaszewska-Kuska *et al.* 2018a, Lukaszewska-Kuska

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et al. 2018b, Lukaszewska-Kuska *et al.* 2019, Lukaszewska-Kuska *et al.* 2020). Titanium sandblasting increases the surface roughness, it also enriches the surface with blasting medium particles, while etching can remove part or all of the added by blasting particles (Xavier *et al*. 2003, Feng *et al*. 2004, Le Guehennec *et al*. 2008, Bretschneider *et al*. 2020, Lukaszewska-Kuska *et al.* 2018a, Lukaszewska-Kuska *et al.* 2020). For example surfaces roughness increases cells differentiation (Anselme and Bigerelle 2005, Kim *et al.* 2006) while Ca and P present on titanium surface improves cells viability (Zareidost *et al.* 2012).

The aim of this study was to compare osteoblastic cell behavior on various titanium surfaces with different chemical composition and topography in order to determine the influence of these combined surface features on cell reaction. Three sample groups were investigated: machined surface (MA); sintered ceramic-blasted (HAS); sintered ceramic-blasted and acid-etched (HAS DE). After characterizing the titanium surfaces, osteoblastic cell attachment, morphology, viability, and differentiation were studied using human osteoblast (NHOst) as test cells.

Materials and Methods

Sample preparation

Titanium discs were cut on a lathe from commercially pure class 4b rods (G&S Titanium Inc, USA) to produce specimens of 8 mm diameter and 1 mm thick. The titanium disc surface thus obtained was classified as-machined surfaced – MA. A machined surface blasted with a mixture of hydroxyapatite and tricalcium phosphate sintered in 1230 °C (HA 70 %; TCP 30 %) with particle size 300-600 μm at the pressure of 6 kPa was classified as HAS. A HA/TCP blasted surface treated with a mixture of acids (36 % HCl/96 % H_2SO_4 1:6) for 10 min in 25 °C was classified as HAS DE. After preparation all discs were ultrasonically washed in surfactant, followed by 2-propranol, disinfectant, and washed twice in distilled water and after packing sterilized by irradiation from an electron accelerator with a radiation dose of 25 kGy.

Sample surfaces analysis

The surface topography was analyzed using a scanning electron microscopy (Zeiss EVO 25, Carl Zeiss, Germany) with 6000× magnification. Surface roughness was evaluated with an optical profilometer (WYKO NT1100, Veeco Instruments, USA). The presence of Al, Ca, P and C on the surface was analyzed by EDS (Quantax AXS, Bruker, Germany).

Cell cultures along with biochemical tests were conducted as previously described (Lukaszewska-Kuska *et al*. 2018a).

Cell culture

The human osteoblast cell line (NHOst-Osteoblasts OGM, cryopreserved ampule, Lonza, USA) was cultured as described previously (Lukaszewska-Kuska *et al*. 2018a). Briefly, the cells were cultured in Osteoblasts Basal Medium (Lonza, USA) supplemented with fetal bovine serum, ascorbic acid, hydrocortisone-21-hemisuccinate, beta-glycerophosphate gentamicin and amphotericin-B (Lonza, USA) in a humidified atmosphere containing 5 % CO_2 at 37 °C with daily changes of medium. The cells were detached with 0.25 % Trypsin/EDTA (Lonza, USA).

In-vitro cell response

24-well culture plates (Multidish 24 #144530, Roskilde, Denmark) were used. All tested samples along with polyester coverslip – Thermanox (Thermo Scientific, Denmark) used as controls were placed in the wells and 4×10^4 cells were seeded on every sample. Cells were cultured for 1, 3, 7 and 14 days.

Cell morphology

After three days of culture the osteoblasts were fixed with 2 % glutaraldehyde in PBS for 24 h, dehydrated in graded alcohols, dried and sputter-coated with gold. Specimen surfaces were examined by SEM and images in 250× and 5000× magnification were randomly chosen and recorded from each sample area.

Cell viability

After 1, 3, 7 and 14 days of cell culture on titanium discs and on plastic, viability test were performed. A solution of MTS (Promega, USA) and PMS (Promega, USA) was prepared, added to the medium and incubated. The absorbance of the resulting colored solution was measured (λ=490 nm), and the results were expressed as the sample absorbance.

Total protein concentration

After 14 days of culture on titanium discs and plastic discs, a Micro BCATM Protein Assay Kit (Thermo Scientific, USA) was used to determine the

protein concentration. Briefly, after the removal of the medium the cells were homogenized. A series of bovine albumin (Sigma Aldrich, USA) dilutions were prepared. BCA working agent was added to every sample of the diluted bovine serum and absorbance of the solution was measured. The protein concentration was determined and expressed in µg/ml with the aid of a calibration curve prepared from standard solutions of bovine serum dilutions.

Alkaline phosphate activity

After 14 days of culture on titanium and plastic discs, the medium was removed and the cells were homogenized. A BCATM Protein Assay Kit was used to assay the protein level. After determining the volume of 5 µg of protein sample, p-Nitrophenyl Phosphate Liquid Substrate System (Sigma Aldrich, USA) was added. After incubation followed by reaction termination the sample absorbance was measured and ALP activity was determined of a 50 µg/ml sample.

Statistical analysis

The results were expressed as a mean ± standard deviation of six-fold measurements. Comparisons of the mean values were performed with a one-way ANOVA Kruskal Wallis and Friedman tests depending on normality. The statistical significance was determined as $p \leq 0.05$ using STATISTICA PL 12.0 (SYSTAT Software, Inc.) software.

Results

Surfaces topography

The scanning electron microscope (SEM) images of the MA surface displayed parallel, systematic and oriented grooves as a result of the tooling process (Fig. 1a). The image of the HAS sample showed rough surfaces with depressions, moderately sharp-edged rims along with broad flatter areas with small spherical grains (Fig. 1b). For the HAS DE sample globular irregularities were less visible and the rims were slightly less sharp (Fig. 1c).

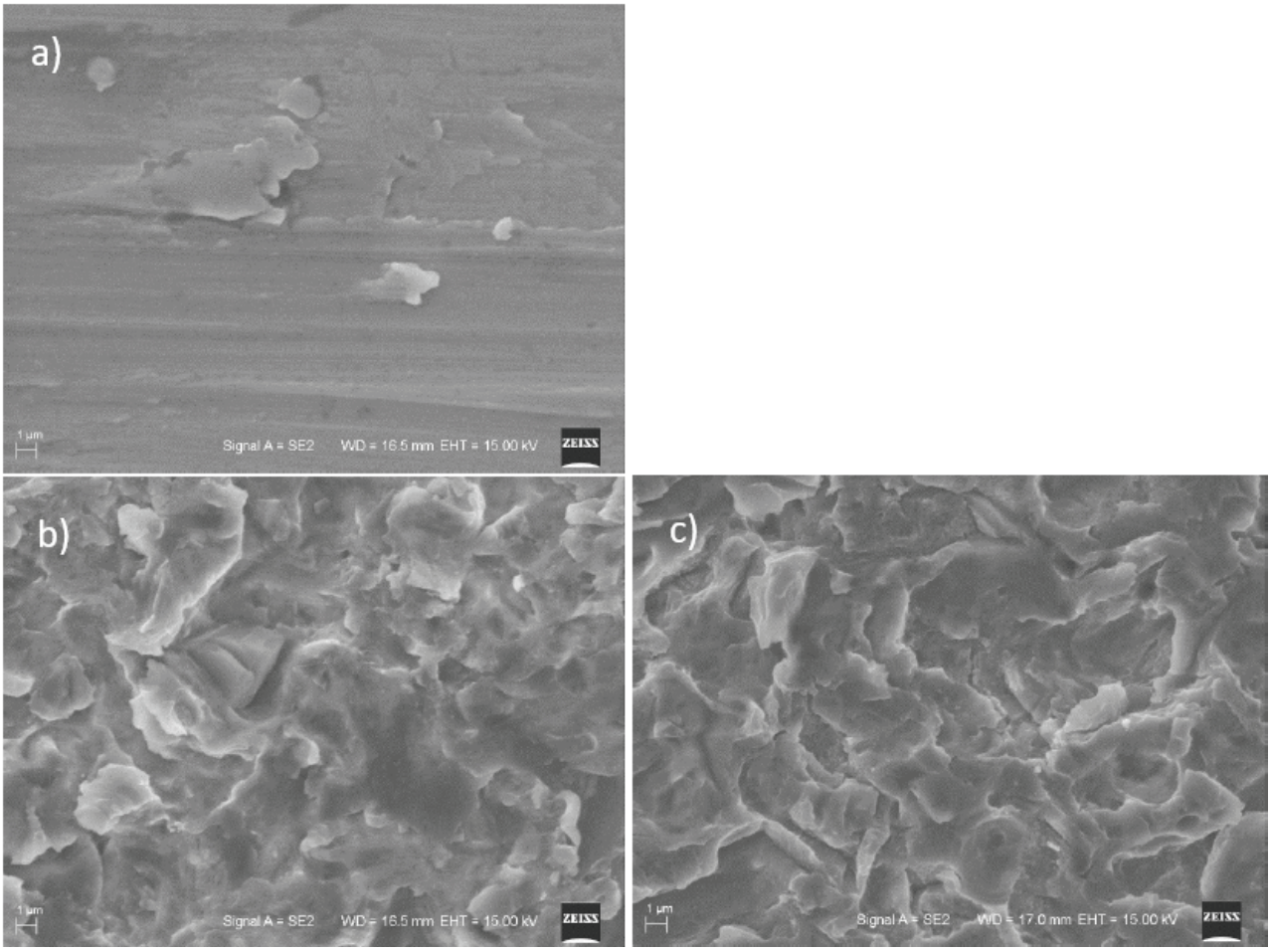

Fig. 1. Scanning electron micrographs of the tested titanium discs (**a**) MA sample, (**b**) HAS sample, (**c**) HAS DE sample. Original magnification 6000×; bar=1 µm.

Surface chemistry

Energy dispersive spectroscopy (EDS) was used to analyze the presence of phosphorus and calcium surface deposition from the grit-blasting procedure. For the machined sample, EDS indicated the presence of titanium and carbon (Fig. 2a). EDS analysis of the HAS sample revealed peaks from titanium, calcium, phosphorus, oxygen, carbon, and aluminum (Fig. 2b) while for the HAS DE sample only peaks from titanium, oxygen and carbon were present (Fig. 2c).

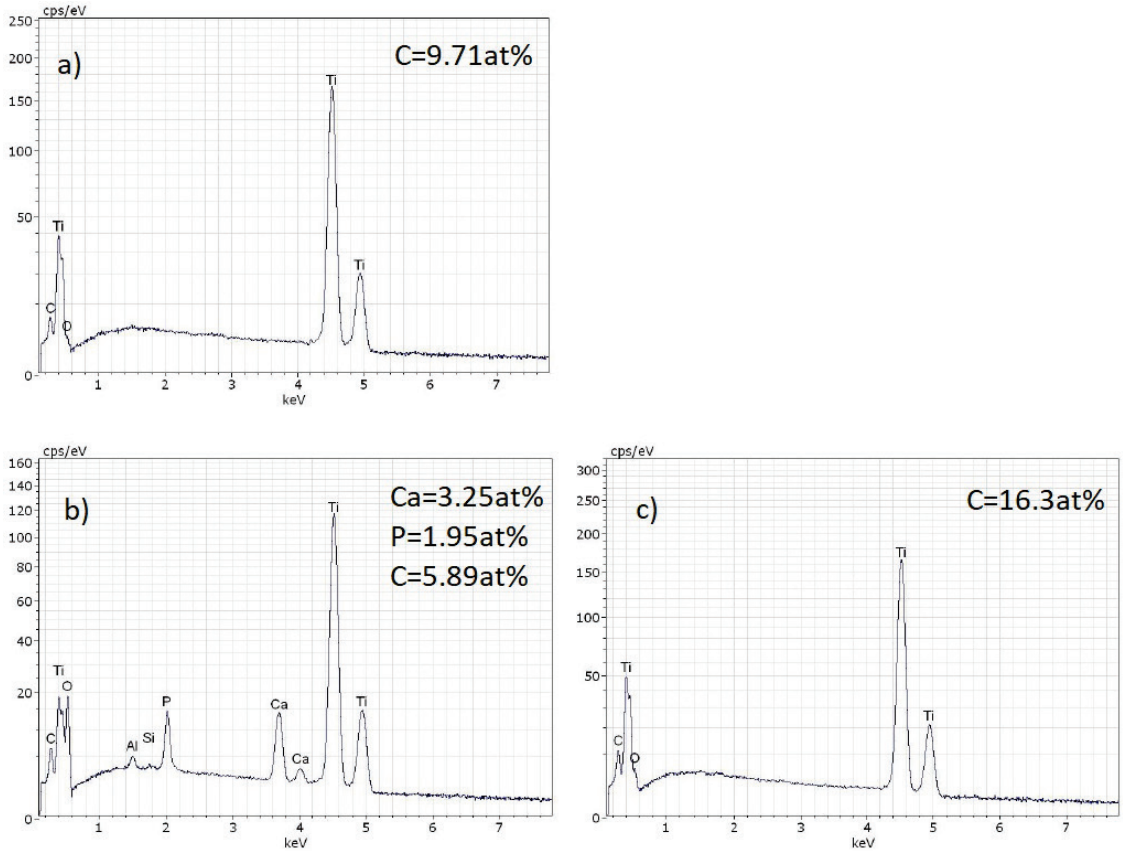

Fig. 2. Energy dispersive spectroscopy (EDS) of (**a**) MA sample, (**b**) HAS sample and (**c**) HAS DE sample.

Surface roughness analysis

Surface roughness analysis as indicated by amplitude parameters showed that sample HAS DE (Sa=614.26 nm) had considerably rougher surfaces than sample MA (Sa=181.91 nm). The percentage of additional surface area contributed by the texture compared to an ideal plane in the measured area – Sdr also revealed that the samples could be divided into two categories. One with developed surfaces with Sdr ranging from 79.06 % for HAS DE to 117 % for HAS and the other with Sdr=7.13 % for MA sample.

Similar differences were evident in the MA samples with a texture aspect ratio – Str identified as anisotropic while remaining samples were identified as isotropic. The above surface roughness parameters are presented in Table 1.

Table 1. Arithmetic mean of surface ordinates (Sa), surface development (Sdr), surface texture indicator (Str) measured for the tested titanium discs ± SD.

Sample	MA	HAS	HAS DE
Sa (nm)	181.91 ± 55.77	621.00 ± 12.88	614.26 ± 57.14
Sdr (%)	7.13 ± 4.15	117.02 ± 3.29	79.06 ± 6.38
Str (none)	0.12 ± 0.09	0.84 ± 0.01	0.79 ± 0.04

Cell morphology

SEM examination of osteoblasts cultured on materials for three days revealed that cells had attached, spread and proliferated on every sample (Fig. 3a-f).

On the MA sample osteoblasts were flatter, with a greater cell surface and in closer contact with the sample surface comparable to the cells cultured on the rougher titanium samples. They were also oriented according to grooves formed during machining (Fig. 3a-b). The osteoblasts were not oriented according to surface features on the other samples but grew parallel to each another. They also represented a smaller cell surface and less extensive contact with sample surface (Fig. 3c-f).

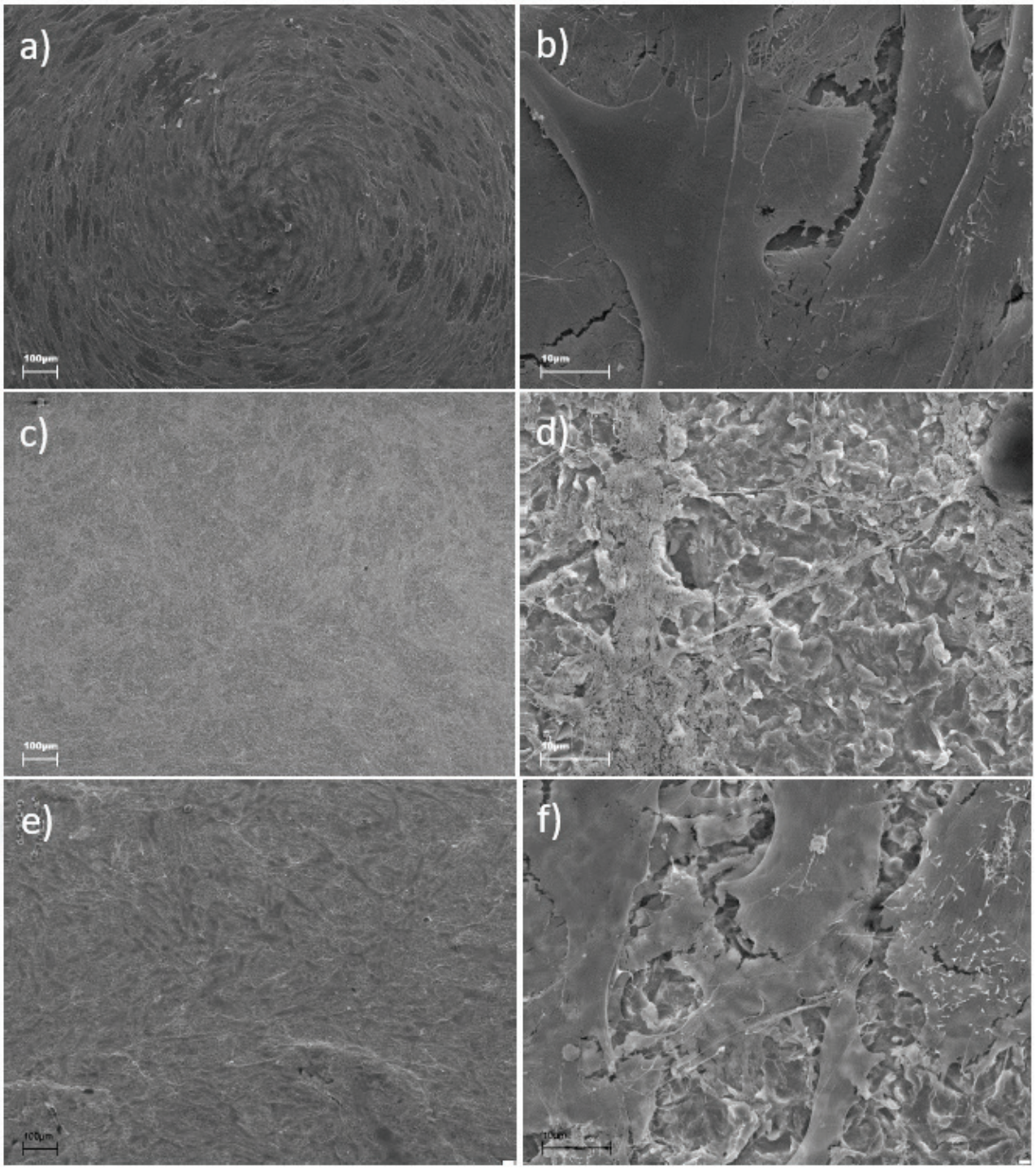

Fig. 3. SEM observation of NHOst cells morphology after 3 days of culture on (**a**) MA 250× magnification, bar=100 μm, (**b**) MA 5000× magnification, bar=10 μm, (**c**) HAS 250× magnification, bar=100 μm, (**d**) HAS 5000× magnification, bar=10 μm, (**e**) HAS DE 250× magnification, bar=100 μm, (**f**) HAS DE 5000× magnification, bar=10 μm.

Cell viability

The osteoblast viability expressed as mitochondrial activity (MTS) after 1, 3, 7 and 14 days is presented in Fig. 4. No statistically significant differences were observed between the examined groups. All samples showed a statistically significant increase in cell viability between day 1 and 14 (plastic $p<0.05$, MA $p<0.05$, HAS and HAS DE $p<0.001$). For the HAS sample, osteoblast viability increased significantly during the first three days ($p<0.05$) and between day 1 and 7 ($p<0.01$) while for the HAS DE samples the increase occurred latter, between day 3 and 14 of culture ($p<0.05$) and between day 1 and 7 ($p<0.01$).

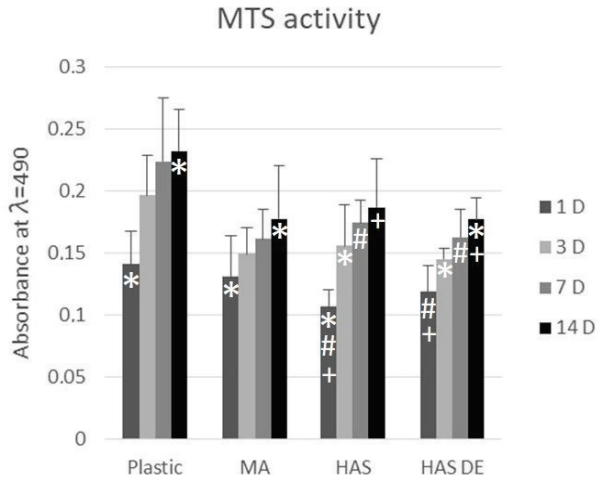

Fig. 4. The viability of NHOst cultured cells on plastic and titanium for 1, 3, 7 and 14 days expressed as sample absorbance at λ=490 nm. * indicates the two groups with statistically significant difference $p<0.05$; # indicates the two groups with statistically significant difference $p<0.01$; + indicates the two groups with statistically significant difference $p<0.001$.

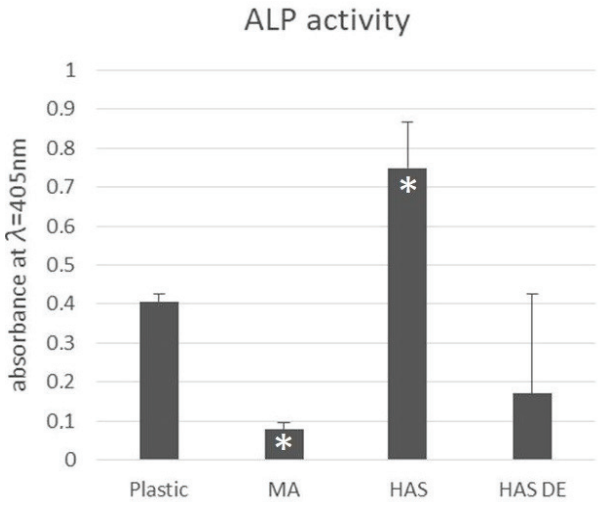

Fig. 5. Alkaline phosphate activity normalized to protein content expressed as sample absorbance at λ=405 nm of cells cultured on plastic and titanium samples for 14 days.* indicates the two groups with statistically significant difference $p=0.001$.

Alkaline phosphate activity

Alkaline phosphate activity (ALP) of NHOst normalized to protein content is presented in Fig. 5. ALP activity was highest for the HAS sample, and statistically significantly higher than for the MA sample ($p=0.001$). The lowest results were noted for the smooth MA sample along with the rougher HAS DE samples.

Discussion

Surface chemical composition along with surface topography are known to alter biological reactions (Xavier *et al*. 2003, Feng *et al*. 2004, Le Guehennec *et al*. 2008, Bretschneider *et al*. 2020, Lukaszewska-Kuska *et al*. 2018a, Lukaszewska-Kuska *et al*. 2018b, Lukaszewska-Kuska *et al*. 2019, Lukaszewska-Kuska *et al*. 2020, Wang *et al*. 2020). Aluminum in the form of Al_2O_3 is a common grit-blasting material used to increase titanium surface roughness. During the process, aluminum is incorporated into the blasted surface and enriches its chemical composition. There are different opinions of the effects of aluminum on biological response. Some authors claim a possible competitive action with Ca, which may impair bone matrix mineralization and as a result bone formation, although this phenomenon is reported to occur in a dose-dependent manner (Canabarro *et al*. 2008). The negative effect on the mineralization process may also be caused by other constituent ions. For example Ti as titanium ions can also inhibit the growth of hydroxyapatite (HA) *in vitro* (Esposito *et al*. 1998). In a human study concerning serum concentration of titanium and aluminum in patient with cementless primary total hip replacements serum concentrations of titanium were elevated approximately twofold in the patients who had a loose implant while no significant differences in terms of serum concentration of aluminum were observed among groups with a loose and integrated prosthesis (Jacobs *et al*. 1991). There are also reports on reduced osteoblast and osteoclasts activities, but this phenomenon is caused by high doses of aluminum (Jeffery *et al*. 1996). On the other hand, there are also reports of accelerating the rate of osteoprogenitor cell differentiation and the formation of bone nodules by aluminum resulting in greater osseointegration (Bellows *et al*. 1999, Yurttutan *et al*. 2018). Other studies show that residual aluminum oxide particles on the implant surface do not affect the osseointegration of titanium dental implant (Piatelli *et al*. 2003, Gehrke *et al*. 2018). Thus the effects of aluminum

on the biological response, in general, remains ambiguous.

Since the aluminum influence on the bone response is ambiguous, different materials can be used for titanium grit blasting. Calcium phosphates (CPCs) are a group of materials that can be used, in place of aluminum, for roughening titanium surface. Research shows that CPCs, i.e. hydroxyapatite and tricalcium phosphates (TCP) support osteoblast adhesion and proliferation along with the stimulation of new bone formation by influencing bone marrow stromal cells and preosteoblasts differentiation into mature (Feng *et al*. 2004, Sunarso *et al*. 2016, Mastrogiacomo *et al*. 2006, Bin Sulaiman *et al*. 2013, Surmanev *et al*. 2014, Trávníčková and Bačáková 2018, Zofkova 2018, Zofkova and Blahos 2017, Zofkova *et al.* 2017). Calcium in the form of Ca^{2+} ions by providing positively charged surfaces facilitates fibronectin and vitronectin adsorption, cell attachment-promoting proteins, which act as osteoblasts ligands (Feng *et al*. 2004). Ca^{2+} ions also mediate cell-cell conjunction and cell-cell communication improving cell adhesion and cell activity (Feng *et al*. 2004, Anselme 2000). They also increase differentiation markers such as ALP activity and bone-like nodule formation (Barradas *et al*. 2012, Sunarso *et al*. 2016). PO_4^{3-} ions are also capable of adsorbing proteins and osteoblasts but with the help of Ca^{2+} ions which work in chemical synergism with PO_4^{3-} ions (Barradas *et al*. 2012). Both Ca^{2+} and PO_4^{3-} ions influence attachment, proliferation, ALP activity, and bone-nodule formation but their influence is more significant when they act together synergistically improving the cytocompatibility of the titanium surfaces (Sunarso *et al*. 2016).

Along with titanium surfaces grit-blasting, acid-etching can be used to improve surface texture. This process can also be used to remove residual components imbedded in the titanium surface during the blasting procedure. Although titanium etching does not cause a cytotoxic cellular effect and, by altering surfaces texture, improves cell adhesion it also increases the surface carbon concentration and decreases the surface oxygen concentration which leads to a decline in surface energy that impairs osseointegration by a deteriorated regulation of the inflammatory response (Orsini *et al*. 2000, Hotchkiss *et al*. 2017). Titanium etching also causes a release of constituent ions with the Ti impairing mineralization process (Hotchkiss *et al*. 2017). However acid etched titanium surface was also reported as suitable candidate for healing into the bone tissue due to high osteoblast proliferation, the highest production of osteogenesis markers and low production of inflammatory cytokines and due to the most intensive blood clot formation (Kubies *et al*. 2011).

Surface topography, apart from surface chemical composition, influences bone response towards endosseous implants (Jokstad *et al*. 2003, Wennerberg and Albrektsson 2009). Moderately rough surfaces (Sa>1-2 μm) show a stronger bone response than smooth, minimally smooth and rough surfaces (Wennerberg and Albrektsson 2009). One explanation may be that friction of smooth surfaces may be too small for proper retention and for rough surfaces only peak contact with the bone will occur (Wennerberg and Albrektsson 2009). Another explanation for a poor bone response towards smooth surfaces is the possibility of cell flattening preventing cell nutrition, for rough surfaces, on the other hand, cells recognize them as smooth since distances between peaks and valleys are relatively large (Wennerberg and Albrektsson 2009). Increased surface roughness also results in an overall increase in pro-inflammatory cytokine release impairing bone healing (Olivares-Navarrete *et al*. 2013). Also in long-term observations both smooth and rough implant surfaces present negative clinical results. For implants with a rough surface, a recurrent marginal infection may lead to clinical failure while for those with a smooth surface lower degree of bony contact might increase the risk of overloading and also lead to clinical failure (Esposito *et al*. 1998). An optimal surface for cell reaction would be a surface, which mimics the natural architecture of natural tissue with irregularities matching the size of extracellular matrix molecules and extracellular parts of cell adhesion receptors improving osteoblasts adhesion, growth and differentiation (Vandrovcová and Bačáková 2011).

As mentioned above, chemical and topographical properties have been shown to influence the osteoblast reaction in this study. In term of cells viability surface features of all the examined samples were biocompatible. More favorable surface for osteoblasts viability were HAS and HAS DE surface. The viability increased more dynamically for those rougher samples comparing to MA. The same conclusion was drawn by Wennerberg in the paper where she stated that smooth and minimally rough surfaces show a weaker bone response than rough surfaces (Wennerberg and Albrektsson 2009). Osteoblast viability increased more intensively in the initial phases in the case of HAS samples, with a developed interfacial area ratio

around 110 %. The influence of Ca, P, or C on osteoblasts viability was not evident.

By blasting the surface with particles, the surface composition may be altered and thus, affect the biocompatibility (Xavier *et al*. 2003). Such an improved material characteristic can be observed in the ALP activity diagram where surfaces with incorporated Ca and P during the blasting procedure, improved cell differentiation. A positive reaction for a surface enriched with Ca and P has also been concluded by other authors (Barradas *et al*. 2012, Sunarso *et al*. 2016). For HAS sample, ALP activity is even higher than for the control, plastic material. The surfaces roughness does not seem to influence cells differentiation since ALP activity for smooth MA sample is not statistically different than for rough HAS DE sample. This result is in contrast with the previous studies, which stated that increased surfaces roughness increases alkaline phosphate activity (Anselme and Bigerelle 2005, Kim *et al*. 2006, Rausch-fan *et al*. 2008). Maybe the negative influence of etching is stronger than the positive effect of increased roughness.

The etching procedure was found to decrease osteoblast differentiation which can be caused by the removal of incorporated Ca and P during the blasting procedure. This can also be caused by increasing carbon contaminants during etching. Titanium with increased carbon content has higher contact angles and lower surface energy (Xavier *et al*. 2003). Titanium surfaces with high surface energy present an increased rate of osseointegration and reduced healing times due to the improved regulation of the inflammatory response generated by the high-energy surface (Xavier *et al*. 2003). However, reduced surface energy due to etching impairs osteoblast differentiation (Xavier *et al*. 2003). Also an increase in trace element impurities from Ti was observed after titanium etching which can impair osteoblast differentiation (Xavier *et al*. 2003). Acid treatment also decreased the surface development ratio – Sdr that could also be the reason for the decrease of osteoblast differentiation.

As for surface orientation, it does not seem to influence cell viability nor their differentiation but influences their morphology. Osteoblasts cultured on isotropic titanium surfaces were oriented according to surface features while in case of anisotropic surfaces they were aligned with respect to one another, which is in agreement with other authors' observations (Anselme and Bigerelle 2005, Anselme and Bigerelle 2006, Bigerelle *et al*. 2002, Dorocka-Bobkowska *et al*. 2017). Titanium surface roughness expressed as arithmetical mean height along with developed interfacial area ratio also influence osteoblast morphology. In the case of smoother surfaces, osteoblasts were flatter, with a greater cell surface and in closer contact with the sample surface compared to cells cultured on rougher titanium samples. Similar observations were made by other authors (Anselme and Bigerelle 2005, Anselme and Bigerelle 2006, Dorocka-Bobkowska *et al*. 2017, Batzer *et al*. 1998, Rebl *et al*. 2012).

Different blasting materials used for surface roughening result in different surfaces texture and compositions. For exempla, titanium surface blasted with Al_2O_3 is rougher than blasted with sintered ceramic materials. Blasting with Al_2O_3 also enriches the titanium surface with Al as described in our previous work (Lukaszewska-Kuska *et al.* 2018a). Such surface also improves osteoblasts viability with even higher results than for HAS surface, although these differences are not statistically significant. Alumina blasted surface also statistically significantly improves ALP activity comparing to the plastic samples ($p=0.03$) but this activity still is significantly lower than for the HAS sample ($p=0.0002$). Such results could be explained by the beneficial influence of Ca and P of HAS sample or by its lower amplitude roughness parameters. Also, etching of alumina blasted surface causes a statistically significant decrease of osteoblasts differentiation comparing to alumina blasted surface ($p=0.006$). The same decrease can be observed for the HAS DE surface comparing to the HAS sample suggesting the unfavorable influence of titanium etching on osteoblasts differentiation in spite of blasting medium (Lukaszewska-Kuska *et al.* 2018a).

While comparing sintered ceramics used in this study with a particle size of 300-600 μm with non sintered ceramics used in our previous study having the particle size <300 μm differences in titanium surface were noted along with differences in osteoblast viability and ALP activity (Lukaszewska-Kuska *et al.* 2018a). Comparing cells viability in 1, 3, 7 and 14 day of culture, titanium blasted with non sintered ceramic along with titanium blasted with non sintered ceramic and etched reached higher results after 3 days compared to surfaces blasted and blasted and etched with sintered ceramics. This fact could not be explained either by surfaces roughens nor by its chemical composition. After 7 and 14 days of culture, the results for titanium blasted with sintered and non sintered ceramics were comparable.

While comparing the rate of cell viability increase for titanium samples blasted with sintered and non sintered ceramics, it was greater for rougher, richer with Ca and P non sintered ceramic sample and occurred between days 3 and 14. Results of osteoblasts differentiation expressed in ALP activity were higher for titanium blasted with sintered ceramics than for titanium blasted with non sintered ceramics. Sample blasted with non sintered ceramic and etched had lower ALP results as sample only blasted with non sintered ceramic although those differenced were not statistically significant. Sample blasted with sintered ceramic and etched noted lower results of ALP activity from the group of sintered and non sintered ceramic blasted materials. The decrease in osteoblast differentiation for etched samples can be noted for both sintered and non sintered ceramic blasted surfaces what can be connected with Ca and P withdraw during the etching procedure along with C increase.

All examined samples, regardless of the topography and surface chemical composition, were found to be biocompatible, because they allowed cell attachment, proliferation, and differentiation. The surface chemical composition influenced the cell reaction in a different manner than surface topography. The rough HAS and HAS DE samples were more conducive to osteoblast viability with the surface development ratio improving cell viability in the early phases. Osteoblast differentiation was influenced by the etching procedure and improved by the presence of surface Ca and P.

Conflict of Interest

There is no conflict of interest.

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