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## Biocompatibility of the titanium-based implant surfaces: Effect of the calcium dihydrogen phosphate on osteoblast cells

MILENA R. KALUĐEROVIĆ<sup>1\*</sup>, JOACHIM P. SCHRECKENBACH<sup>2,3</sup> and  
HANS-LUDWIG GRAF<sup>1</sup>

<sup>1</sup>Department of Oral, Maxillary, Facial and Reconstructive Plastic Surgery, University Hospital of Leipzig, 04103 Leipzig, Germany, <sup>2</sup>ZL Microdent Co., Breckerfeld, Germany, 58339 Breckerfeld, Germany and <sup>3</sup>Department of Chemistry, Technical University of Chemnitz, 09107 Chemnitz, Germany

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**Abstract.** Investigated was the influence of the presence of calcium dihydrogen phosphate in acidic media on titanium-based implant surfaces (Ticer), used in clinical practice, and its white form (Ticer white), on osteoblast cells. Novel surfaces, denoted **M1** and **M2**, were obtained by immersing Ticer and Ticer white surfaces in calcium dihydrogen phosphate solution at pH 3.5. Scanning electron microscopy (SEM), energy-dispersive X-ray spectroscopy (EDS) and X-ray diffraction (XRD) investigations were performed to characterize the surfaces. Comparative results of interaction between investigated surfaces and human osteoblast cells from indirect biocompatibility (MTT and SRB assays), proliferation (DAPI assay) and mode of cell death (acridine orange/ethidium bromide (AO/EB) double staining) were found to be in good agreement, as well as findings from osteocalcin (OC) and bone sialoprotein (BSP) expression. Surfaces obtained by employing anodic plasma-electrochemical oxidation with spark discharges, without subsequent surface modifications were found to be more compatible. Soaking Ticer and Ticer white in phosphate solution resulted in toxic materials (**M1** and **M2**) which induced apoptosis and secondary necrosis in osteoblast cells.

**Keywords:** titanium implants; osteoblast cells; biocompatibility; morphology; bone sialoprotein; osteocalcin.

### INTRODUCTION

Novel dental implants are developed aiming to replace missing teeth, thus enabling patients normal functional and aesthetic recovery.<sup>1</sup> The surfaces of such materials stimulate osseointegration of the implant with the surrounding bone.<sup>2</sup> Furthermore, they are also utilized for maintenance and functional restoration of

\* Corresponding author. E-mail: milena.kaluderovic@medizin.uni-leipzig.de  
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the existing bone.<sup>3,4</sup> One of the important features of implants is their surface characteristics, primarily interactions of bone matrix and osteoblasts with biomaterials are influenced by surface topography, surface charge, components, chemical states as well as wettability.<sup>5,6</sup> Different implant surface modification methods (grit blasting, acid etching, anodization, plasma spraying or hybrid techniques)<sup>7-9</sup> might result in diverse and distinctive surface properties that could influence the host-to-implant response. These modifications improve bone-to-implant contact and therefore long-term success of dental implants.

Currently, in clinical use are metal-based implants, generally fabricated from titanium and its alloys (mainly Ti-6Al-4V) or ceramic materials. First two are the materials of choice for dental implants.<sup>10</sup> They possess high biocompatibility, high mechanical stability/passivity and modulus of elasticity.<sup>11,12</sup> However, their dark grayish color might cause aesthetic issues for patients, particularly in front teeth area. Ceramic materials express excellent aesthetics in dentistry (*e.g.*, veneers, inlays, onlays and crowns),<sup>13,14</sup> but on the other hand, they are often biologically inert and any interaction with the surrounding tissue is relatively slow. Moreover, alterations of zirconia-based implants usually lead to disruption of materials reflected in cracks and surface damage.<sup>15</sup>

In order to improve implant properties, several variations of implant surface alterations are important to mention, such as combinations of titanium and zirconia, *e.g.*, titanium/titanium dioxide-coated zirconia (an implant body made of zirconia; surface coating of titanium dioxide)<sup>16</sup> as well as zirconia-coated titanium/titanium dioxide (titanium body coated with zirconia).<sup>17-19</sup> However, titanium/titanium dioxide-coated zirconia implants neither solved the problem of mechanical stability of zirconia nor toxic potential of titanium ions, which might be released in the surrounding tissue. In the case of zirconia coated titanium/titanium dioxide, mechanical stability of titanium and the biological properties of zirconia remained as problems. Furthermore, binary titanium zirconium alloy, such as Roxolid™ (Straumann, Basel, Switzerland), showed tensile strength approximately 40 % greater than for titanium commercially pure implants (Ti cp, 680 MPa).<sup>20</sup>

Calcium phosphates are also used as coatings on titanium and titanium alloy implants to improve osseointegration, thus combining the bioactivity of the calcium phosphates and the strength of the base metal.<sup>21,22</sup> Herein, the two novel titanium based-surfaces **M1** and **M2**, modifications of Ticer and white Ticer, respectively, were used for the assessment of osteoblast behavior in response to presence of calcium dihydrogen phosphate incorporated in the surface topography. **M1** and **M2** titanium-based materials were characterized by scanning electron microscopy, surface microanalysis and X-ray diffraction. Materials indirect toxicity, influence on proliferation and mode of cell death were investigated

using osteoblast cells, as well as bone sialoprotein (BSP) and osteocalcin (OC) production.

## EXPERIMENTAL

### *Material and methods*

All reagents used were of analytical grade. Ti cp specimens (round discs: diameter 6 mm, thickness 0.5 mm) were obtained as a kind gift from ZL Microdent, Germany.

Surface topography of the specimens was studied by a JEOL JSM 840 A scanning electron microscope (SEM). Energy dispersive spectroscopy (EDS) was used for the surface microanalysis in order to determine the concentration of the chemical elements. X-ray diffraction was used for the determination of solid state phases on the conversion layers.

### *Preparation of surfaces*

The reference materials Ticer and Ticer white, both with anodic conversion coatings, were prepared in the electrochemical cell from Ti cp (anode), using an electrical pulse generator as power supply.<sup>23,24</sup> Ticer and Ticer white coatings were generated in the solution of  $\text{Ca}(\text{H}_2\text{PO}_4)_2$  and 1.25 M NaOH electrolyte, respectively, as previously described in the literature.<sup>24,25</sup> Ticer and Ticer white specimens were separately immersed in  $\text{Ca}(\text{H}_2\text{PO}_4)_2$  solution ( $40 \text{ g L}^{-1}$ ) at pH 3.5, for 22 h yielding **M1** and **M2** surfaces, respectively. Afterwards, samples were subjected to further analyses. EDS results are given in Table I.

TABLE I. Concentrations, %, of elements present in investigated samples; for each sample 20 measurements were performed; figures indicate the range of percentage obtained

Sample	Ti	O	Na	Ca	P
Ticer	11–22	59–70	–	3–5	8–10
<b>M1</b>	12–23	60–71	–	3–5	8–10
Ticer white	28–31	66–70	2–4	–	–
<b>M2</b>	29–30	67–69	1–2	< 0.5	< 0.5

### *Biological experiments*

*Cell culture.* Ethics Committee of the University of Leipzig (No. 086-2008) approved all procedures employed in this study. Furthermore, procedures were conducted in agreement with the rules of the Declaration of Helsinki from 1975 (revised in 1983). Samples of the healthy human mandibular bone, without any clinical or radiographic pathological evidence, were attained from one male donor (undergoing lower wisdom tooth surgery) at the Department of Oral, Maxillary, Facial and Reconstructive Plastic Surgery at the University Hospital of Leipzig. Osteoblast cells were harvested from the bone sample by the method described in the literature<sup>17</sup> using 0.05 M sterile phosphate buffered saline (PBS, pH 7.4), penicillin/streptomycin at  $100 \text{ IU mL}^{-1}$  each (PromoCell, Heidelberg, Germany), and 0.25 % collagenase type IV ( $166 \text{ U mg}^{-1}$ ; Biochrom, Berlin, Germany). The cells were maintained in osteoblast growth medium (PromoCell) containing 10 % fetal bovine serum (PromoCell) in 5 %  $\text{CO}_2$  atmosphere at  $37 \text{ }^\circ\text{C}$ . The cells were grown to confluence in culture flasks (Greiner Bio-One, Frickenhausen, Germany) and the medium was changed twice a week.

### *Experimental settings*

The 96 well plates were used for cytotoxicity and eight chamber slides for all other experiments. Cells were seeded at a density of  $4,000 \text{ cells mL}^{-1}$  ( $2,000 \text{ cells per well}$ ) in 96 well plates or chamber slides.<sup>26</sup> Two reference materials (Ticer – clinically-employed and

Ticer white) and two novel materials (**M1** and **M2**) were examined, all prepared as sterile round discs with 6.0 mm diameter and thickness of 0.5 mm. As a control, cells cultivated on glass were used. Each experiment was performed in triplicate. For each immunocytochemistry assay or morphological analysis experiments one eight chamber slide with the osteoblast cells was removed from the incubator on days 3, 5, 7 or 10 and the cells were fixed in paraformaldehyde (4 % in PBS) for 15 min and rinsed in PBS or double stained with AO/EB, respectively. All images were visualized using motorized Zeiss Axiophot2 fluorescence microscope (Zeiss, Oberkochen, Germany). Sections were screened at 200× magnification and analyzed using Image J software (version 1.43u for Windows).

#### *Cytotoxicity*

MTT and SRB colorimetric assays were employed for determination of an indirect cytotoxicity.<sup>27-29</sup> Extracts for the indirect cytotoxicity tests were obtained by immersing materials in osteoblast growth medium supplemented with 10 % fetal bovine serum (extraction vehicle) for 120 h at 37 °C, without agitation. The ratio between the sample surface and the volume of the extraction vehicle was 3 cm<sup>2</sup> mL<sup>-1</sup>. Extracts obtained at pH 4.2 (**M1**) and 4.7 (**M2**). The osteoblast cells were incubated with pure extract (100 %) and two dilutions (50 and 20 %) or osteoblast growth medium supplemented with 10 % fetal bovine serum (control). After 72 h, metabolic activity (MTT) of cells and total protein amount (SRB) were determined. Absorbance was measured at 570 nm using a 96 well plate reader (Tecan Spectra, Crailsheim, Germany). Results are presented as percentage absorption value (S) in comparison to the control group.

#### *Proliferation (DAPI assay)*

Cell nuclei were stained with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI, Serva, Heidelberg, Germany) and quantification of cell proliferation was performed as earlier described.<sup>26</sup>

#### *Morphological analysis (AO/EB double staining)*

Osteoblast cell death induced by investigated surfaces was determined using acridine orange (AO) and ethidium bromide (EB) double staining according to the literature.<sup>30-32</sup> Briefly, after 3, 5, 7 or 10 days of osteoblast growth on investigated surfaces, eight chamber slides with target cells were stained with AO/EB mixture (3 µg mL<sup>-1</sup> AO and 10 µg mL<sup>-1</sup> EB in PBS), and visualized with fluorescence microscope (Zeiss, Oberkochen, Germany). Apoptosis, autophagy and necrosis can be easily distinguished using AO/EB double staining assay.<sup>31,33</sup> Only AO can cross the plasma membrane of viable and early apoptotic cells. In the apoptosis condensation of chromatin as dense green areas or membrane blebbing can be clearly detected.<sup>30,34</sup> AO stains the autophagosomes from orange to red indicating autophagy,<sup>33,35,36</sup> while EB can enter only the cells with disrupted membrane and such necrotic cells are stained red.<sup>30,34</sup>

#### *OC and BSP expression*

Immunocytochemical evaluation of osteocalcin (OC) and bone sialoprotein (BSP) was performed as recently described using 10 % normal goat serum (Vector, Burlingame, Calif., USA), primary antibodies against OC (monoclonal, mouse-anti-human; Acris, Hiddenhausen, Germany) and BSP (monoclonal, mouse-anti-human; Immundiagnostik AG, Bensheim, Germany), secondary antibody goat-antimouse-Cy3 (Jackson Immuno Research, West Grove, Pa., USA), 4 % bovine serum albumin (Serva) and for counterstaining DAPI (Serva).<sup>25</sup>

### Statistics

Data from the repeated experiments are presented as the mean and standard deviation. The significance of the differences between various treatments was assessed using ANOVA software, followed by the Student-Newman-Keuls test. Differences were considered significant if the  $p$  value was lower than 0.05.

## RESULTS AND DISCUSSION

### Modification and characterization of materials

Ticer and Ticer white were generated by the anodic plasma-electrochemical oxidation with spark discharges in the solutions of  $\text{Ca}(\text{H}_2\text{PO}_4)_2$  and  $\text{NaOH}$  electrolytes as previously described.<sup>25</sup> Materials **M1** and **M2** were obtained by immersing Ticer and Ticer white in  $\text{Ca}(\text{H}_2\text{PO}_4)_2$  solution at pH 3.5. SEM topography of the **M1** and **M2** along with Ticer and Ticer white are shown in Fig. 1. The basic morphology of the Ticer surface appeared built up from a porous film network with irregular shapes, which resemble in some regions enlarged pores. Ticer white surface showed clusters of knob-like structures. The sample **M1** surface corresponds to the Ticer, while **M2** corresponds to Ticer white surface topography. Based on the results, the modification by immersion in the  $\text{Ca}(\text{H}_2\text{PO}_4)_2$  solution ( $40 \text{ g L}^{-1}$ ) at pH 3.5, the surface topography of the respective materials was unmodified.

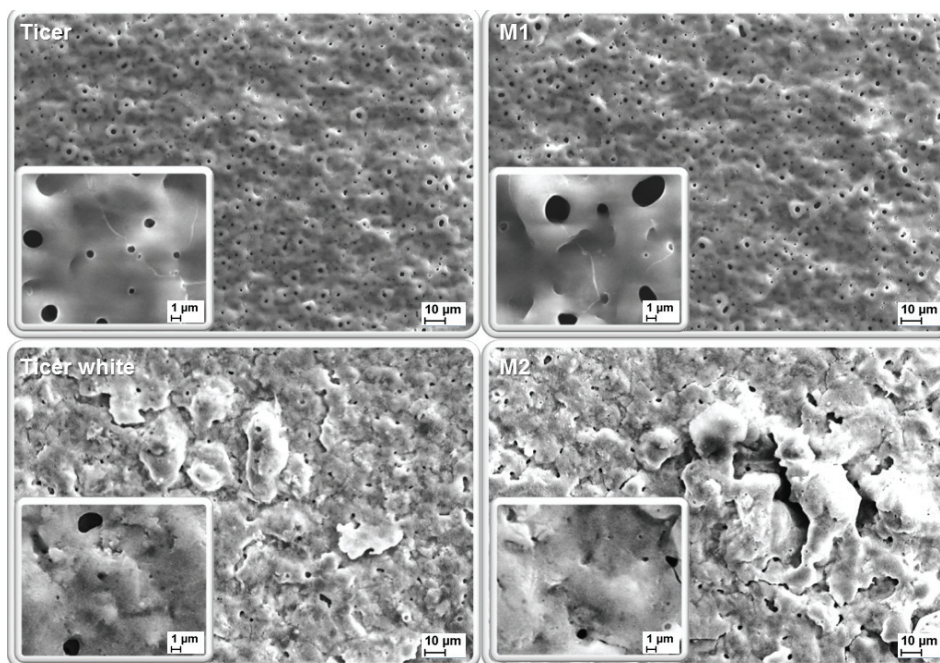


Fig. 1. SEM images of the investigated topographies.

Energy dispersive spectroscopy (EDS) was employed for the surface micro-analysis in order to determine the concentration of the chemical elements. As expected, Ticer surface consisted of oxygen, titanium, calcium and phosphorus, while Ticer white had main composition of oxygen, titanium and sodium.<sup>25</sup> **M1** and **M2** resembled composition of Ticer and Ticer white, respectively, without significant differences (Table I), except for sodium on **M2**. Namely, less sodium is present on **M2** surface, than on Ticer white which might be due to the leakage from the Ticer white surface into the immersion solution. It is assumed that immersing such surfaces in the electrolyte they might absorb ions as sponges. However, it was proven by SEM that could occur exceptionally in the energetically favourable areas observed as crystalline depositions on the surface. In the preparation process of Ticer, a thin layer comprising of small amounts of calcium and phosphorous is formed. Immersing materials in the same electrolyte did not improve further enrichment of the mentioned elements on their surface. Therefore, incorporation of calcium and phosphorus was attained through the plasma-chemical reactions.

X-ray diffraction was used for the determination of solid state phases of **M1** and **M2**. **M1** consisted of X-ray amorphous titanium oxides as well as calcium phosphates, analogously to Ticer. Surface of **M2** comprised of TiO<sub>2</sub> modification, rutile and monoclinic phase of sodium titanium oxides, Na<sub>2</sub>Ti<sub>6</sub>O<sub>13</sub>,<sup>37</sup> comparably to Ticer white.<sup>25</sup>

#### *Indirect cytotoxicity*

Using MTT and SRB assays, the indirect toxicity tests of investigated materials on osteoblast cells were performed (Fig. 2). Consistent with previous results,<sup>25</sup> the extracts obtained from Ticer and Ticer white showed no significant difference in the cell viability compared to osteoblasts grown in completed medium ( $p < 0.05$ ). Explicitly, such extracts did not influence respiration or total protein production of osteoblasts. Furthermore, no significant difference was detected among the extract dilutions and the control. The results obtained from the MTT and SRB are in a good agreement. On the contrary, extracts obtained from **M1** and **M2** materials efficiently disturbed both mitochondrial functions as well as protein production, manifested as decreased survival of the osteoblasts (Fig. 2) in MTT and SRB assays, respectively. Disturbance was the most pronounced in the pure extract and its intensity decreased with dilution (100 → 50 → 20 %). Generally, the production of proteins was affected more than the respiration of the cells. More toxic was the extract derived from **M1** than from **M2** surface. Presumably, their acidity might be a reason for the toxic effects (extracts pH 4.2 and 4.7 for **M1** and **M2**, respectively).

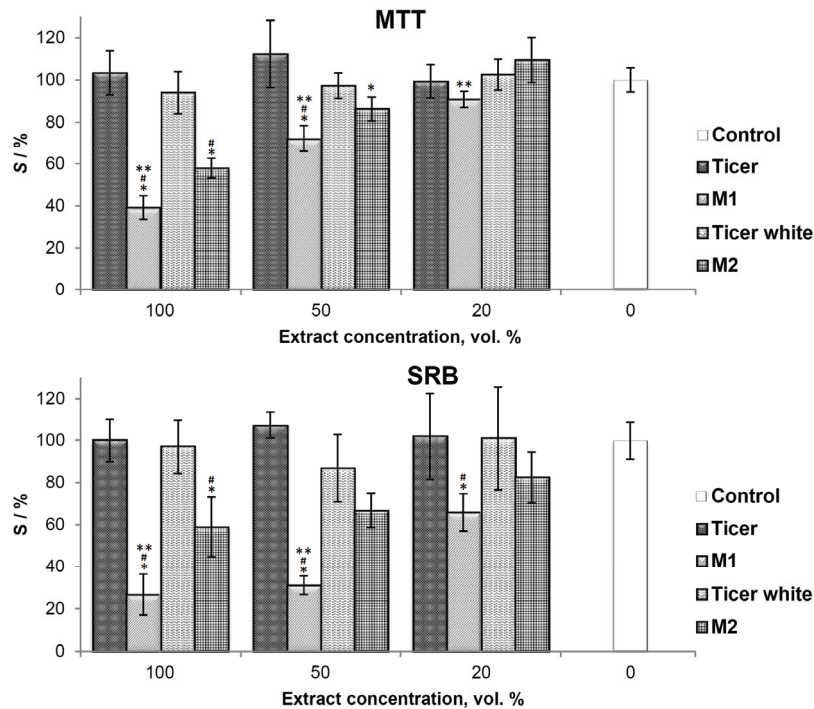


Fig. 2. Cytotoxicity evaluation of surfaces using human osteoblast cells at different extract dilutions (100, 50 and 20 %) by the means of MTT and SRB assays ( $S$  – survival).  
\*, # and \*\*  $p < 0.05$  vs. control/Ticer, Ticer white or **M2**, respectively.

### Cell proliferation

Osteoblast cells were seeded on the implant surfaces of Ticer, Ticer white, **M1** and **M2** and allowed to proliferate up to 10 days. Proliferation was assessed using the DAPI assay; results were normalized to control cells and presented in Fig. 3. Overall, up to 10 days of culture a decrease in proliferation was observed. However, from the literature it is known that the low cell proliferation rate is a sign of a more differentiated cellular phenotype in the culture.<sup>38,39</sup> Also, topographies which stimulate cell proliferation are mostly inadequate for cell differentiation.<sup>40</sup> Furthermore, it is well known that faster and improved osseointegration is noticeably promoted by rough topographies.<sup>41–43</sup> Considering that cells in the control sample were able to proliferate, while those grown on rough surfaces of Ticer and Ticer white were starting differentiation process toward osteocytes, as recently shown by our group,<sup>35</sup> such behaviour observed in this study was reasonable.

Even though **M1** and **M2** are similar in topography, roughness, chemical element composition and surface solid state phases to Ticer and Ticer white surfaces, respectively, they showed a different influence on human osteoblast cells.

Novel samples, **M1** and **M2** influenced significantly lower osteoblast proliferation than Ticer and Ticer white. Explicitly, significant differences were observed for **M1** from day 5 and for **M2** from day 3 of incubation. Based on the results of indirect cytotoxicity assays, it was clearly shown that both **M1** and **M2** surfaces possess potential to disturb cell respiration and protein production, such findings seem to be rather pointed to cell death than to cell differentiation. Because of that mode of cell death, production of BSP and OC was also investigated.

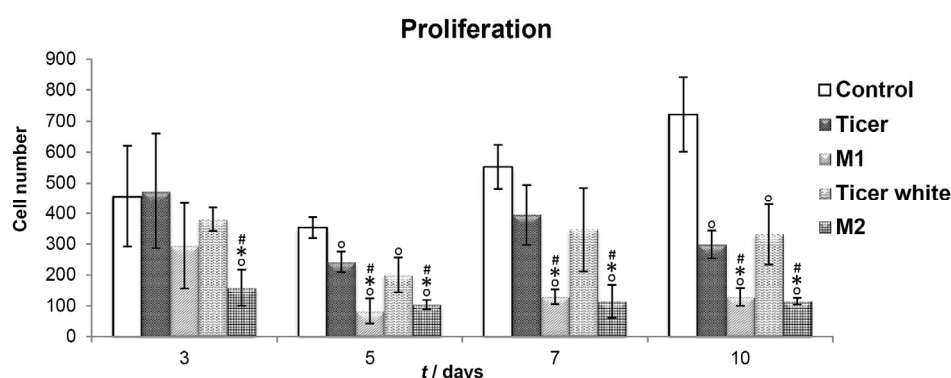


Fig. 3. Proliferation of osteoblasts on different surfaces. °, \* and #  $p < 0.05$  vs. control, Ticer, or Ticer white, respectively. Only cells in the control sample show an increase in proliferation over the time period.

#### AO/EB double staining

Recently, we reported that Ticer and Ticer white induced autophagic dependent PI3/Akt signalling pathway in human osteoblast cells.<sup>35</sup> Ticer white accelerated differentiation process of osteoblasts in comparison to Ticer. Osteoblast cells in rounded configuration divide at lower rate than those that are flattened and well spread.<sup>44</sup> In agreement with those results, AO/EB double staining showed activation of autophagy in the osteoblasts grown on Ticer and Ticer white, seen as yellow to orange autophagic vesicles (Fig. 4). Morphology of the cells changed from flattened to polygonal shaped, indicating differentiation of osteoblasts, as previously reported.<sup>35</sup> Apoptosis was detected marginally as in the control cells, while necrotic process was not triggered in osteoblasts by the stated surfaces at all. On the other hand, **M1** and **M2** surfaces caused massive apoptosis, noticed by the presence of apoptotic bodies on day 3 (Fig. 4). Furthermore, from day 5 the same surfaces triggered secondary necrosis or activation of necrotic pathway, seen as red nuclei or red cytoplasm with disrupted membrane (Fig. 4). Therefore, results from AO/EB double staining confirmed assumptions raised from indirect cytotoxicity and proliferation assays.



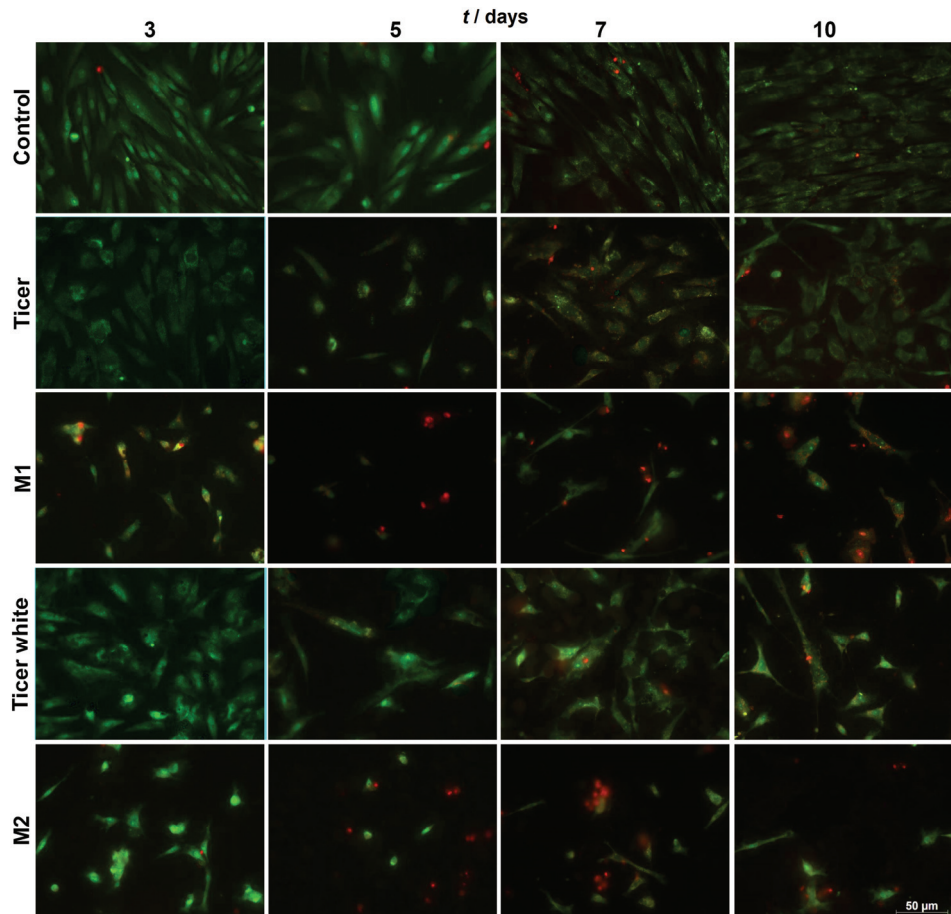


Fig. 4. Morphology of osteoblast cells grown on investigated surfaces during time.

#### *Osteocalcin and bone sialoprotein expression*

In order to assess the regulation of osteoblastic OC and BSP grown on Ticer, Ticer white, **M1** and **M2** immunocytochemical evaluation of the noncollagen bone proteins was performed (Fig. 5). BSP is highly upregulated in the early stage of bone maturation.<sup>45,46</sup> It is considered a marker for the osteoblast phenotype and promoting *in vitro* and *in vivo* mineralization,<sup>47,48</sup> while OC, major non-collagenous protein, gets incorporated in the bone matrix during bone formation.

Ticer and Ticer white surfaces upregulated expression of OC and BSP in osteoblast cells. The general upregulation trend was found to be similar as in the recent study.<sup>25</sup> Those findings are in accordance with other studies of modified surfaces, which elevated expression of the bone associated proteins.<sup>49</sup> Considering osteoblast proliferation in the control, as well as Ticer and Ticer white sur-

faces, it can be stated that lower number of the cells provoked significantly higher secretion of OC and BSP than the higher number of cells in the control. Contrary, **M1** and **M2** influenced downregulation of OC and BSP, less than one magnitude of quantity of secreted proteins in comparison to Ticer and Ticer white (day 10, see Fig. 5). That could be due to lower proliferation of cells since **M1** and **M2** are causing death of osteoblasts rather than osteoblastic differentiation.

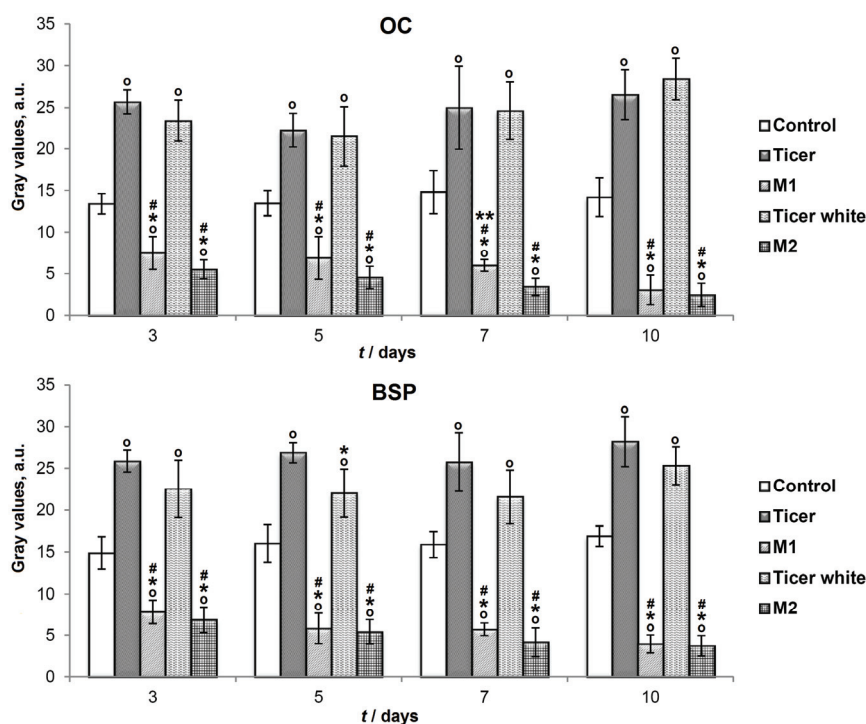


Fig. 5. OC and BSP expression of osteoblast cells induced by different surfaced in the function of the time. °, \*, # and \*\*  $p < 0.05$  vs. control, Ticer, Ticer white or **M2**, respectively.

#### CONCLUSIONS

Two novel **M1** and **M2** surfaces obtained by immersing dental implant surfaces Ticer, clinically used, and Ticer white in calcium dihydrogen phosphate under acidic conditions displayed similar topography, roughness, chemical elements composition and surface solid state phases as parent materials. **M1** and **M2** surfaces exhibited *in vitro* activity on osteoblast cells differently from the Ticer and Ticer white. While Ticer and Ticer white caused no toxicity but rather provoked differentiation of the cells, that reflected through presence of autophagy and upregulation of OC and BSP, **M1** and **M2** induced massive apoptosis and necrosis of the osteoblasts. Survived cells expressed downregulation of OC and BSP. Therefore, changes in the cell response to similar topologies (Ticer and **M1**;

Ticer white and **M2**) herein are not only correlated to material pattern, but also to the solution in which such materials were preserved ( $\text{Ca}(\text{H}_2\text{PO}_4)_2$ , pH 3.5). Such solution might modify sufficiently materials to trigger changes in activation of signaling pathways (induction of apoptosis, necrosis, and autophagy; OC and BSP production), cell shape and cytoskeletal organization as well as differentiation. Toxicity of **M1** and **M2** surfaces on osteoblast cells was caused by induction of apoptosis and secondary necrosis.

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## ИЗВОД

## БИОКОМПАТИБИЛНОСТ ПОВРШИНА ИМПЛАНТАТА НА БАЗИ ТИТАНА: УТИЦАЈ КАЛЦИЈУМ-ДИХИДРОГЕНФОСФАТА НА ЋЕЛИЈЕ ОСТЕОБЛАСТА

МИЛЕНА Р. КАЛУЂЕРОВИЋ<sup>1</sup>, ЈОАЧИМ Р. SCHRECKENBACH<sup>2,3</sup> и HANS-LUDWIG GRAF<sup>1</sup>

<sup>1</sup>Department of Oral, Maxillary, Facial and Reconstructive Plastic Surgery, University Hospital of Leipzig, 04103 Leipzig, Germany, <sup>2</sup>ZL Microdent co, Breckerfeld, Germany, 58339 Breckerfeld, Germany и

<sup>3</sup>Department of Chemistry, Technical University of Chemnitz, 09107 Chemnitz, Germany

Испитиван је утицај присуства калцијум-дихидрогенфосфата у киселој средини на површини имплантата Ticer на бази титана, који се користи у клиничкој пракси, као и његове беле форме (Ticer white) на ћелије остеобласта. Нове површине **M1** и **M2** добијене су урањањем површине Ticer као и Ticer white у раствор калцијум-дихидрогенфосфата при pH 3,5. Површине су окарактерисане коришћењем SEM, EDS и дифракције X-зрака. Резултати интеракције испитиваних површина и хуманих остеобласт ћелија, и то индиректна биокompatibilност (MTT и SRB тест), пролиферација (DAPI тест) и тип ћелијске смрти (акридин-оранж/етидијум-бромид (АО/ЕВ), двоструко бојење) показују добру сагласност, као и резултати експресије остеокалцина (OC) и коштаног сијалопротеина (BSP). Нађено је да су ефикасније површине оне које су добијене поступком анодне плазма-електрохемијске оксидације под варничним пражењем без накнадне модификације. Урањањем Ticer и Ticer white површина у фосфатни раствор добијени су токсични материјали (**M1** и **M2**) који су индуковали апоптозу и секундарну некрозу код ћелија остеобласта.

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