

Acid and alkali etching of grit blasted zirconia: Impact on adhesion and osteogenic differentiation of MG63 cells *in vitro*

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There is a need for evaluating zirconia surface modifications and their potential impact on the biological response of osteogenic cells. Grit blasted zirconia discs were either left untreated or underwent acid or alkaline etching. Adhesion and osteogenic differentiation of MG63 cells was determined after one week of culture. The macro-scaled roughness of the grit blasted zirconia discs, independent of the surface treatment, was within a narrow range and only slightly smoother than titanium discs. However, the alkaline- and acid-etching led to an increase of the micro-roughness of the surface. The surface modifications had no effect on cell spreading and did not cause significant change in the expression of differentiation markers. Thus, in this respective setting, morphologic changes observed upon treatment of grit blasted zirconia discs with acid or alkaline do not translate into changes in MG63 cell adhesion or differentiation and are comparable to findings with anodized titanium discs.

Keywords: Zirconia, Osteogenic differentiation, Adhesion, Dental implants

INTRODUCTION

Dental implants are biomaterials that interact with the biological system of the jaw bone¹. Biomaterial research starts with *in vitro* testing for cytotoxicity before *in vitro* experiments on biofunctionality are performed². Cytotoxicity issues in implant dentistry are basically resolved. Biofunctionality research evaluates whether and how raw materials or surface properties affect specific cellular aspects which are relevant in the complex process of osseointegration². For example, adhesion and differentiation of MG63 osteoblast-like cells was studied on cells grown on unalloyed and alloyed titanium disks with smooth and rough microtopography³. Microarrays are now used to determine the impact of microtopography on the gene expression in MG63 cells⁴. However, information on biofunctionality is primarily available for titanium surfaces and cannot be extrapolated to other biomaterials⁵.

Ceramic implants made of zirconia already are available to a small segment of the global market. A few short-term clinical reports on zirconia implants are available⁶, but most evidence comes from preclinical studies in pigs^{7,8}, dogs⁹ and rabbits¹⁰. *In vitro* research on zirconia was initiated in an attempt to understand the role of wear particles in aseptic loosening of hip prostheses¹¹. Today, biofunctionality studies compare adhesion and differentiation of osteogenic cells on titanium and zirconia surfaces¹². Microarrays have also

been used to study MG63 grown on zirconia discs¹³, and the response of osteogenic cells to zirconia surface treatment by ultraviolet or laser irradiation has been reported^{14,15}. Still, studies comparing the impact of implant raw materials and surface modifications of biomaterials including titanium and zirconia are rare.

The authors are aware of one study evaluating the response of SAOS2 osteogenic cells grown on grit blasted zirconia, on grit blasted, alkaline-etched zirconia, or on grit blasted, acid-etched titanium¹⁶. Adhesion and osteogenic differentiation were more pronounced when SAOS2 cells were incubated on zirconia compared with titanium. The differences between zirconia with two different surface topographies were negligible, however. In that study, zirconia discs were etched in a hot alkaline salt bath. However, grit blasted zirconia discs can also be etched in acid salt baths¹⁷. Whether osteogenic cells are affected differently by acid or alkaline etching of grit blasted zirconia remains unknown. Thus, there is a demand for research into zirconia surface modifications caused by acid or alkaline etching and the potential impact on the biological response of osteogenic cells *in vitro*. Here we investigated the effects of acid or alkaline etching of grit blasted zirconia discs on the adhesion and osteogenic differentiation of MG63 osteoblast-like cells.

MATERIALS AND METHODS

Preparation of discs

Circular discs 16 mm in diameter were kindly provided by Dentalpoint AG (Zürich, Switzerland). The discs

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had the following characteristics: (i) commercially pure titanium grade 4, anodized, (ii) zirconia, grit blasted (GB) with corundum \varnothing 0.3 mm, 3.0 bar, (iii) zirconia, grit blasted, alkaline-etched (equimolar KOH and NaOH; 220 °C, 24 h¹⁶) and (iv) zirconia, grit blasted, acid-etched (hypophosphorous acid H₃PO₂, 80°C, <1 h). Ceramic discs were based on yttrium stabilized tetragonal zirconia polycrystals (Y-TZP). The grit blasted zirconia surface corresponded to the available “Zeramex” implants (Dentalpoint AG). The discs were sterilized by hot steam at 121°C. The SEM pictures of the surface are given in Fig. 1.

Surface roughness

The roughness of the discs was investigated with a μ Surf confocal microscope (NanoFocus AG, Oberhausen, Germany) close to the center of the discs. The L20x objective was used and 6×1 stitching was applied in order to have an evaluated area of 4.14×0.74 mm. The

data processing was performed using the μ Soft Analysis XT software (NanoFocus AG, Oberhausen, Germany). From the measured profiles, spots where no signal was obtained were interpolated. A plane was subtracted from the measured profile for the topographies shown in Fig. 2. To determine the roughness parameters, more than 20 line profiles along the measurement were evaluated after applying a Gaussian filter. A cut-off distance of 0.8 mm according to DIN EN ISO 4287 and 4288 was chosen to determine the macro-roughness and a cut-off distance of 0.08 mm for the micro-roughness.

Cells

MG63 osteosarcoma cells are adherent and have a fibroblast-like morphology (American Type Culture Collection). Cells were cultured in a humidified atmosphere at 37°C in DMEM (Invitrogen Corporation, Carlsbad, CA, USA) containing 10% fetal calf serum (FCS; Invitrogen) supplemented with antibiotics (Invitrogen).

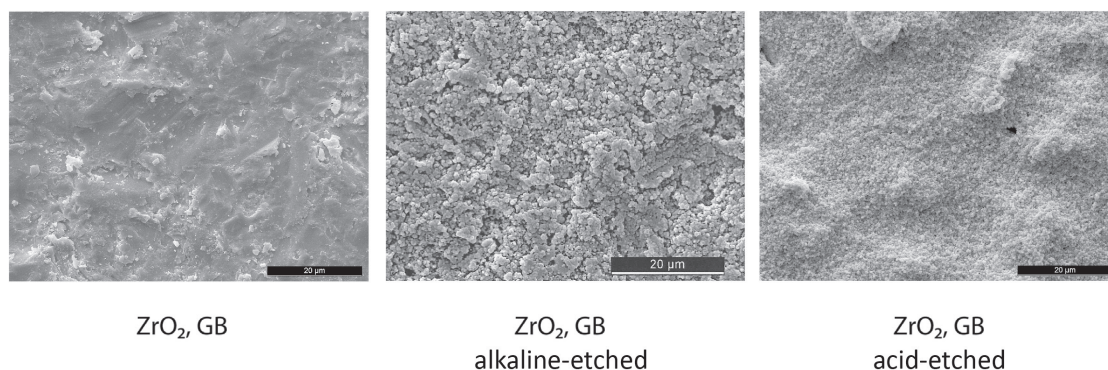


Fig. 1 Scanning electron microscope pictures of the zirconia discs.

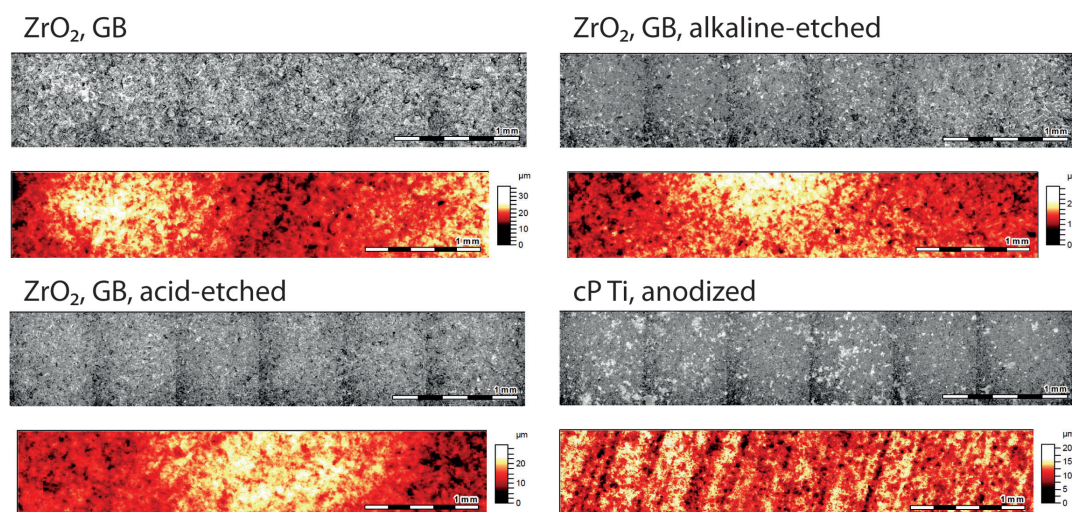


Fig. 2 Intensity of the confocal microscope image (top, grayscale) and flattened topography (bottom, colorplot) of the discs. On the intensity images, the five vertical darker areas are the borders of the six images, which were superimposed by the stitching of the confocal microscope.

For experiments, 25,000 cells/cm² were plated into 24-well culture dishes containing circular discs.

Cell spreading assay

MG63 cells were seeded at a lower density onto circular discs pre-incubated with serum-containing medium for 5 min. Cells were allowed to adhere for 2, 5, and 24 h before unattached cells were removed by washing with PBS. Attached cells were fixed with 4% formalin and permeabilized with 0.1% Triton-X. Staining of actin was performed with TRITC-phalloidine (Invitrogen, Life Technologies). Images of attached cells were taken with a fluorescence microscope (Olympus BX51). A total of 50 randomly selected cells from each sample were outlined and the area was determined by morphometric analysis (ImageJ software, NIH Image, Bethesda, MD, USA).

Gene expression analysis

Cellular RNA was isolated on day 7 after exposing cells to ascorbic acid (50 µg/mL) and 1.25(OH)₂ vitamin D3 (100 nM) of culture using an RNAqueous-Micro Kit containing DNase I (Ambion, Life Technologies). RNA was quantified (Nanodrop 2000c; Thermo Scientific, Waltham, MA, USA) and 10 ng of total RNA was used per sample well. Reverse transcription (RT) was performed with a high-capacity cDNA RT-kit (Applied Biosystems, Foster City, CA) and PCR was done with TaqMan's universal PCR Master Mix (Applied Biosystems) on a 7500 Real-Time PCR System (Applied Biosystems). Probes of alkaline phosphatase, collagen type I and osteocalcin were manufactured by the TaqMan® Gene Expression Assays service (Applied Biosystems). The mRNA levels were calculated by normalizing to the housekeeping gene GAPDH using the ΔCt method.

Alkaline phosphatase activity

Enzymatic activity was determined on day 7 after exposing cells to ascorbic acid (50 µg/mL) and 1.25(OH)₂ vitamin D3 (100 nM) using a colorimetric assay. MG63 cells were washed with PBS and subjected to ultrasonic homogenization in 0.1% Triton X-100. Enzymatic activity was calculated based on the conversion of p-nitrophenylphosphate (Sigma, 1 mg/mL in diethanolamine buffer). The reaction was stopped with 0.1 M EDTA in 1 M NaOH. The absorption was measured at 405 nm in a microplate reader. Enzymatic activity was normalized for DNA, the latter being determined by binding to Picogreen reagent Quant-iT™ PicoGreen® dsDNA Reagent (Invitrogen). Fluorescent readings were

performed at an excitation wavelength of 480 nm and an emission reading of 520 nm (Infinite 200; Tecan Group Ltd., Männedorf, Switzerland). Experiments were performed in triplicate, with two experiments.

Statistical analysis

Experiments were repeated at least twice, with three replicates each, and data are reported as the mean and standard deviation. ANOVA was used to detect effects of surface modifications on cell behavior. A *p* value ≤ 0.05 was considered significant.

RESULTS

Physical characterization

As a base material, zirconia was roughened by grit blasting, leaving some corundum particles on the surface (Fig. 1). With the acid and alkaline etching further material including the corundum particles was removed, leading to an etched surface structure. In case of the alkaline etching, the grain boundaries were strongly corroded and laid open (Fig. 1). We next examined the impact of surface modifications on the roughness parameters. As shown in Table 1 and Fig. 2, both the macroscopic and the microscopic roughness of the zirconia discs, independent of additional surface treatment, were within a narrow range, with R_a values of 1.4 to 1.6 µm and 0.7 µm respectively. The zirconia discs were slightly smoother than titanium discs. However, the basic and the especially the acid-etching led to a slightly higher micro-roughness compared to the sandblasted surface. But because of the limited optical resolution, the smallest structures in the sub-micrometer range can not be resolved with the confocal microscopy.

Biological characterization: cell adhesion

To investigate the possible impact of the surface modifications on the response of osteogenic cells, we used the widely established MG63 osteosarcoma cell line^{3,4,11,13}. Titanium and the zirconia surface, independent of the surface modification, allowed cell adhesion as early as 2 h after seeding, when cells still had a round shape. Cell spreading was more pronounced after 5 h and after 24 h, and all cells showed the characteristic appearance of adherent cells, with an expanded cytoskeleton (Fig. 3a). Morphometric analyses confirmed these observations. As shown in Fig. 3b, cell spreading after 24 h was no visibly affected by the surface modifications.

Table 1 Arithmetic average roughness R_a of the discs according to ISO 4287

Sample	ZrO ₂ GB	ZrO ₂ GB & alkaline-etched	ZrO ₂ GB & acid-etched	Ti, anodized
Macro R _a [µm]	1.37 (0.09)	1.40 (0.08)	1.54 (0.08)	1.76 (0.09)
Micro R _a [µm]	0.66 (0.03)	0.68 (0.04)	0.72 (0.03)	0.85 (0.03)

The macro-roughness values were determined according to ISO 4288 with a cut-off distance of 0.8 mm. The micro-roughness was determined with a cut-off distance of 0.08 mm allowing leveling out the macro-roughness of the sandblasted surfaces.

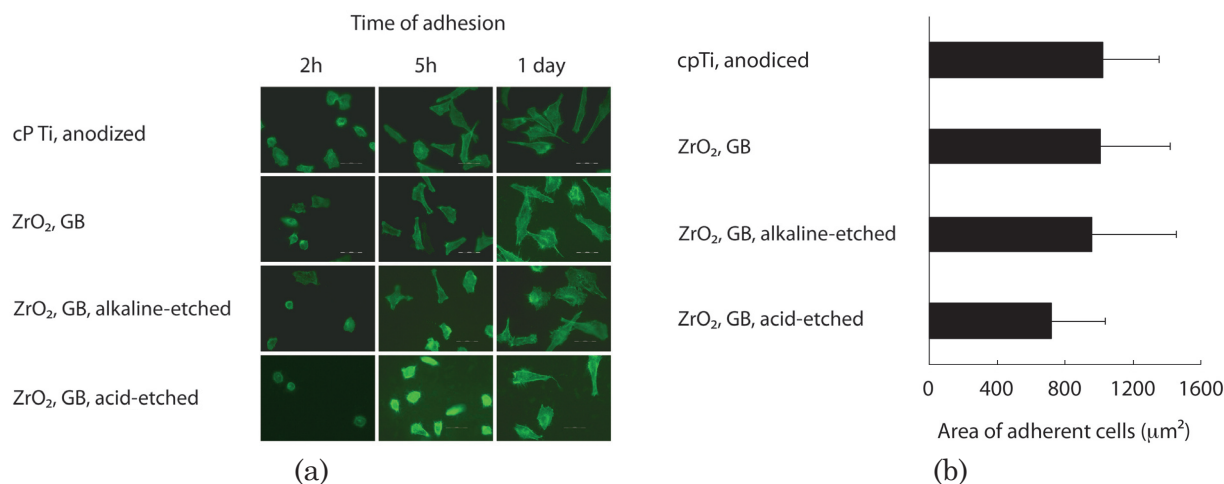


Fig. 3 Cells spreading assay.

MG63 cells were allowed to adhere for 2, 5, and 24 h on the on the indicated discs. (a) Fluorescence staining of actin with phalloidine determines the morphology and the area of the attached cells. (b) Data represent the area of the attached cells based on morphometric analysis of fluorescence staining of actin with phalloidine. Data are given as mean and standard deviation. No statistical significance was observed.

Biological characterization: osteogenic differentiation

Alkaline phosphatase, collagen type I and osteocalcin are established marker genes of osteogenic differentiation¹⁸. Based on this evidence and the findings that modification of implant surfaces can change the osteogenic differentiation of adherent cells^{3,15,16,19-21}, we investigated whether acid etching and alkaline etching can change the expression of the marker genes for osteogenic differentiation. To this end, the corresponding mRNAs were examined by quantitative RT-PCR. Neither of the surface modifications caused a significant change in the expression of alkaline phosphatase, collagen type I, or osteocalcin (Fig. 4). In line with these findings, the enzymatic activity of the alkaline phosphatase was not altered in response to growing cells on different surfaces, including the titanium discs (Fig. 5). Thus, osteogenic differentiation of MG63 cells occurs independent of modifications to the surfaces of zirconia discs and was comparable to findings with titanium discs.

DISCUSSION

Modification of the surfaces of implant materials can change the adhesion and the expression of genes related to osteogenic differentiation *in vitro*^{3,19}. Recently, the impact of alkaline etching of grit blasted zirconia on adhesion and osteogenic differentiation of the osteosarcoma cell line SaOS2 was tested¹⁶. Corresponding studies with acid etching of grit blasted zirconia have not been performed. Thus the novelty of our study is that we report on both surface modifications alkaline and acid etching. The work of the present report shows that both acid etching and alkaline etching changed the surface characteristics of grit blasted zirconia, leading to an increased micro-roughness. Moreover, the visible changes in surface

characteristics did not provoke changes in the adhesion or the osteogenic differentiation of MG63 cells. In view of these findings, we conclude that the indicated surface modifications of already grit blasted zirconium are not necessarily linked with changes in behavior of osteogenic cells *in vitro*.

The work of the present report supports previous findings that surface modifications of zirconia implant materials has a negligible effect on the adhesion and osteogenic differentiation of SaOS2 cells¹⁶. Our data are also in agreement with findings showing that acid etching of grit blasted titanium had no substantial impact on proliferation and collagen production of MC3T3-E1 cells²² or on osteogenic differentiation of MG63 cells²¹. Thus, the present results provide additional support for the view that modifications of already grit blasted surfaces, even if they might have an impact on the process of osseointegration *in vivo*, do not necessarily have an impact on cell behavior *in vitro*.

Considering the importance of comparing the cellular response to titanium with the response to zirconia, the lack of research into the issue up until now is surprising. We found no changes in cell adhesion or osteogenic differentiation when cells were grown on grit blasted anodized titanium or grit blasted acid-etched zirconia. Our data are in agreement with findings comparing acid-etched zirconia and titanium surfaces with regard to cell adhesion and osteogenic differentiation of primary osteogenic cells¹². Acid-etched titanium was found to be superior to alkaline-etched zirconia with regard to adhesion of SaOS2 cells¹⁶; however, we used anodized titanium discs, which might explain the discrepancy. Microarrays revealed differences in gene expression of MG63 cells grown on titanium versus zirconia surfaces²³. It is possible that the material properties of titanium

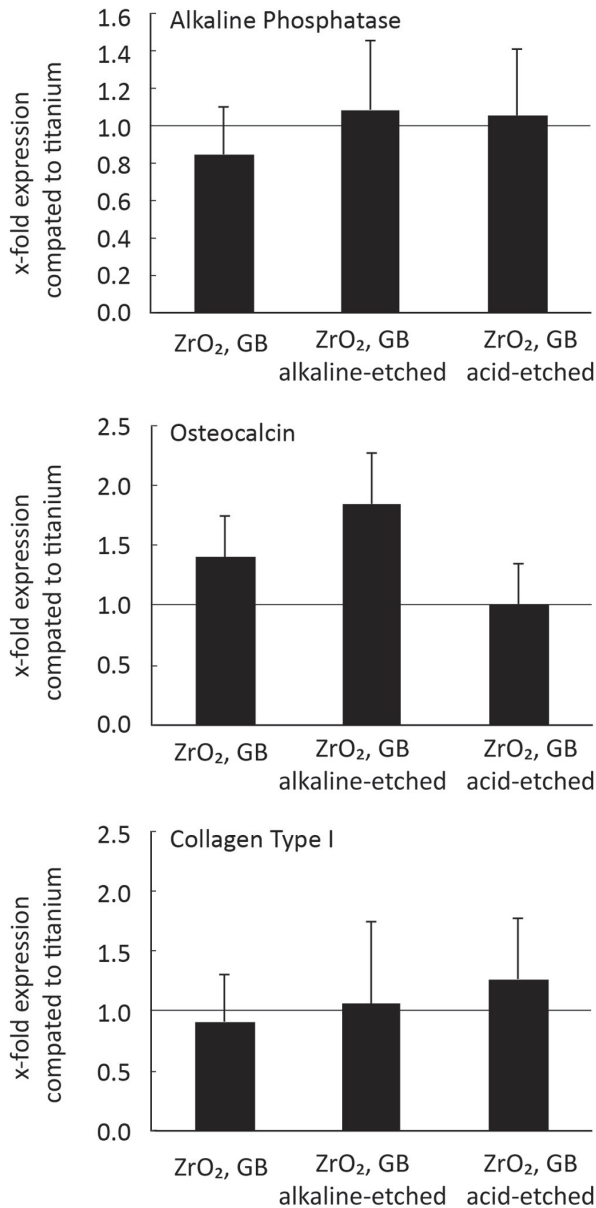


Fig. 4 RT-PCR of parameters of osteogenic differentiation. MG63 cells were grown on the indicated discs for 7 days. Total RNA was prepared and subjected to quantitative reverse transcription-PCR analysis. Expression levels of alkaline phosphatase, collagen and osteocalcin were normalized to beta-actin. Data obtained with cells grown on zirconia discs were further normalized for data obtained on titanium discs. Graphs represent the data of five independent experiments. Data are mean and standard deviation. ANOVA did not reach the level of significance.

and zirconia alone are not sufficient to provoke different cellular behavior *in vitro*.

The reasons for the discrepancy in the findings are likely to be complex, and include variations of the raw

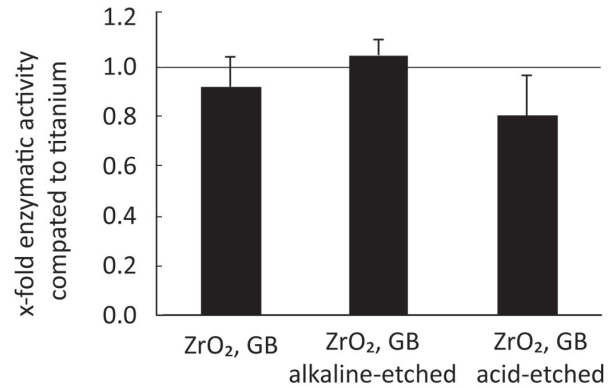


Fig. 5 Alkaline phosphatase activity. MG63 cells were grown on the indicated discs for 7 days. Enzymatic activity was determined in cell lysates using with a colorimetric assay. Enzymatic activity was normalized for total cellular DNA. Graphs represent the data of two independent experiments. Data are mean and standard deviation. No statistical significance was observed by ANOVA.

materials, the surface modification, the cells, and the *in vitro* model *per se*. Thus, our findings do not rule out that primary bone-derived cells or other cell lines, including the SaOS2 cells, behave differently from MG63 cells. Moreover, other experimental parameters—including the presence of serum or the observation periods—might affect the overall outcome. Gene expression analyses were carried out by 7 days culture only. Prolonged culture periods might affect the osteogenic differentiation, for example because the surface modifications might change matrix synthesis, which in turn affects cell differentiation, providing room for further research. In addition, the two biofunctionality assays—cell adhesion and osteogenic differentiation—represent only a small part of the overall process of osseointegration. Future *in vitro* studies should therefore include a larger spectrum of biofunctionality assays with osteogenic cells. Moreover, research on the impact of surface modifications on the formation of blood clots²⁴⁾ and aspects of macrophage activity²⁵⁾ might provide greater insight into the biofunctionality of the biomaterial we have used in the present study.

In vitro studies provide a rapid and cost-effective method to predict the performance outcomes of biomaterials *in vivo*. The *in vivo* environment is complex, however, and exploring the synergistic combination of defined *in vitro* and *in vivo* tests is mandatory prior to use of biomaterials in humans. Thus, our data do not rule out that surface modifications of zirconia implants affect the performance of the implants *in vivo*—and therefore no general conclusions should be drawn based on *in vitro* models alone. We are currently testing study implants made from zirconia with the indicated surface modifications in a minipig model.

In conclusion, acid etching and alkaline etching removes the grit particles and produce an etched surface structure with a slightly increased micro-roughness on zirconia. However, the *in vitro* response of MG63 cells to these changes, when related to adhesion and osteogenic differentiation, was comparable. Moreover, the *in vitro* response of the cells to anodized titanium and the zirconia discs was rather similar. This *in vitro* study is a necessary and logical step in the strategy of biomaterial testing, where *in vitro* studies precede preclinical research before implants are released for clinical use.

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