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Influence of a Nanoporous Zirconia Implant Surface of on Cell Viability of Human Osteoblasts

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Abstract

Purpose: The dense nonretentive surface of zirconia implants was modified into a nanoporous surface using selective infiltration etching surface treatment. The aim of this study was to investigate the influence of such a nanoporous modified zirconia surface on the attachment of human osteoblasts.
Materials and Methods: Human osteoblasts were cultured for 21 days on (i) selective infiltration etched zirconia (nanoporous surface), (ii) polished zirconia, (iii) polished titanium, or (iv) airborne particle abraded acid etched (SLA) titanium disks. After the culture period the following parameters were assessed: number of cells, the morphology of the cells, the attachment of the cells, alkaline phosphatase activity, and the level of total protein ($\alpha = 0.05$).
Results: Statistical analysis revealed a significantly higher cell count on the third ($F = 17.4, p < 0.001$) and eighth day ($F = 163, p < 0.001$) for nanoporous zirconia and SLA titanium surfaces compared to polished specimens. The number of cells (nanoporous zirconia $160 \pm 20/mm^2$, SLA titanium $133 \pm 15/mm^2$) and cell size (nanoporous zirconia $50.7 \pm 3\ \mu m$, SLA titanium $42.5 \pm 4\ \mu m$) were significantly higher than polished specimens. Nanoporous zirconia specimens demonstrated comparable alkaline phosphatase activity ($0.0036 \pm 0.0035\ ng/\mu l$) and intracellular protein content ($72.7 \pm 0.9\ ng/\mu l$) compared to other tested groups. Scanning electron microscopy revealed that cells attached on the polished surface using finger-like processes, whereas on the nanoporous surface, finger-like processes were not observed, as the cell membrane appeared to be in close proximity to the underlying surface.
Conclusion: The findings of this study suggest that a nanoporous zirconia surface favors cell growth and attachment compared to a polished surface. It was proposed that a nanoporous zirconia surface may improve clinical performance of zirconia implants.

Over the years, dental implants have demonstrated an increasing success rate and have become a trustworthy treatment option where failure is the exception.[1,2] For many decades titanium was the material of choice for the fabrication of dental implants and orthopedic devices.[3] Nevertheless, recent studies reported some cases of allergy in addition to elevated Ti serum levels for some patients.[4] Besides Ti, other materials were investigated, including alumina and zirconia polycrystalline ceramics, which offer superior mechanical properties, easier fabrication routes, reduced cost, and in general do not suffer from the grayish metallic color of the former.[5,6] Today, commercial zirconia implants and hip joint replacements are available on the market and are gaining more popularity; however, the dense nonretentive surface of zirconia prevents optimal osseointegration with this material.

In the last decade, attention has been focused on optimizing the surface of dental implants using different approaches in an attempt to optimize their performance. Surface properties, in terms of roughness, architecture, and chemical composition, and physical properties such as surface-free energy and electrical charge all play a significant role for successful osseointegration of dental implants. A combination of sandblasting and

acid etching (SLA) is currently one of the most widely used surface treatments for Ti implants. For zirconia, the choice remains between maintaining a relatively smooth as-milled surface (surface with landmarks characteristic of processing technique) or sandblasting as the suggested methods of surface treatments.[7,8] Nevertheless, new surface treatments such as laser application and surface coating have been evaluated.[9,10]

Selective infiltration etching is a new surface treatment designed to transform the relatively dense nonretentive surface of zirconia into a nanoporous surface by creating intergrain porosities. The technique differs from other surface treatment methods in that the entire surface of zirconia remains chemically the same and that the created surface roughness occurs on a nano-scale level without material loss and without increasing the microscopic surface roughness of the material. The aim of this study was to investigate the influence of a nano-rough zirconia surface on the attachment of human osteoblasts.

Materials and methods

Specimen preparation

Sixty zirconia disks (19 mm diameter, 1 mm thick) were produced by cutting (Isomet 1000; Buehler, Lake Bluff, IL) and sintering CAD/CAM zirconia milling blocks (Cercon Base; Degudent GmbH, Hanau-Wolfgang, Germany). The sintered disks were polished using ascending grit silicon carbide polishing paper, 400, 600, 800, and 1200 grit, in a rotating metallographic polishing device (Ecomet; Buehler) under water cooling and a fixed load (300 g). Finally, the specimens were polished using diamond paste (1 and 0.5 μm) on a rotating rubber wheel. The average surface roughness and profilometry were performed using a traveling contact probe (S-J 400; Mitutoyo Corp, Tokyo, Japan) to assess proper polishing of all specimens ($R_a = 0.05$ μm). Half the zirconia disks were etched by selective infiltration to create a nano-rough surface (group 1) while the other half served as control (group 2). The selective infiltration etching technique creates a nano-rough surface by creating spaces between zirconia grains. A specific infiltration glass is coated on the surface and heated above its glass transition temperature. The molten glass infiltrates between zirconia grains and facilitates intergrain sliding movement. Upon washing the glass, a nano-rough surface is created. Step-by-step details are mentioned elsewhere.[11] Thirty titanium-aluminum vanadium disks (Ti 6-Al 14-V) received the same polishing procedure and served as golden reference (group 3), while another 30 titanium disks were sandblasted with alumina particles and acid etched (group 4 SLA) according to the manufacturer's instructions (Dyna Dental; Hoorne, The Netherlands). Thirty specimens were prepared for each of the four test groups ($n = 30$)

All specimens were ultrasonically cleaned in an ultrasonic bath (Sonorex RK 102; Bandelin Electronic GmbH, Berlin, Germany) for 30 minutes in each of the following successive solutions twice: acetone, 90% ethanol, demineralized water. They were then dried in a hot air convection oven. The specimens were sterilized in sealed envelopes at 300°C for 3 hours.

Cell culture technique

Each disk was placed in a single well in sealed culture flasks, and a drop of growth medium containing 5×10^3 human osteoblasts, obtained from cancellous bone of the femur, was applied to the center of each disk ($n = 30$). After a 1-hour incubation period, 500 μl of Earl's growth medium containing 10% fetal bovine serum, antibacterial (150 units/ml streptocycin), and antifungal agents was added to each well. The cells were kept at 37°C in a humidified incubator with 5% CO_2 (HEPA class 100 Model 121; Thermo Electric Corporation, West Chester, PA). The culture medium was refreshed every 48 hours, and this process was repeated until the cells reached a confluent state (21 days).[12] Cells cultured in empty wells served as a control.

Cell morphology and direct cell count

After 3 and 8 days, the disks ($n = 30$) were washed with a PBS solution twice, fixed in 4% formaldehyde in 0.1 M cacodylate buffer for 60 minutes followed by 1% osmium solution, and finally dehydrated in ascending concentrations of ethanol (60 to 100%). The specimens were finally gold sputter coated (S150B sputter coater; Edwards, Crawley, UK) and examined in a scanning electron microscope (XL20, Philips, Eindhoven, the Netherlands). Some specimens were examined directly with a stereomicroscope (Leica DMIL; Leica Microsystems, CMS GmbH, Wetzlar, Germany), and the cell count was performed on micrographs obtained from fixed positions at the center of each specimen using calibrated image analysis software (Cell A soft imaging solution, Olympus, GmbH, Munster, Germany). All cells in the field from each image were measured.

Alkaline phosphatase (AP) activity

After 21 days, alkaline phosphate activity was assessed by measuring the release of p-nitrophenol with a spectrometer (405 nm wave length using Wallac Victor®; EG&G, Turku, Finland). Briefly, assay buffer (diethanolamine 1M, MgCl 0.5 mM, Triton-100 0.1%, and para-nitrophenolphosphate 6 mM) was added to lysed cells (0.25 ml PIBA solution and five cycles of repeated freezing and thawing) and measurements were repeated every 10 minutes for a total period of 90 minutes ($n = 30$). The data obtained were compared to luminescence of a calibrated scale to quantify AP activity.[13]

Protein assay

After 21 days, protein concentration was assessed using a BCA assay ($n = 30$). Five microliter lysed cell solution was placed in 96-well plates, and 5-μl distilled water was added followed by 190 μl of protein assay solution according to the manufacturer's instructions. Data obtained were compared to an ascending scale of reference protein concentration to quantify protein content.[14]

DNA assay

The protein assay and alkaline phosphatase activity data were normalized against the amount of DNA calculated from every specimen. One μl of lysed cell solution was added to a laser

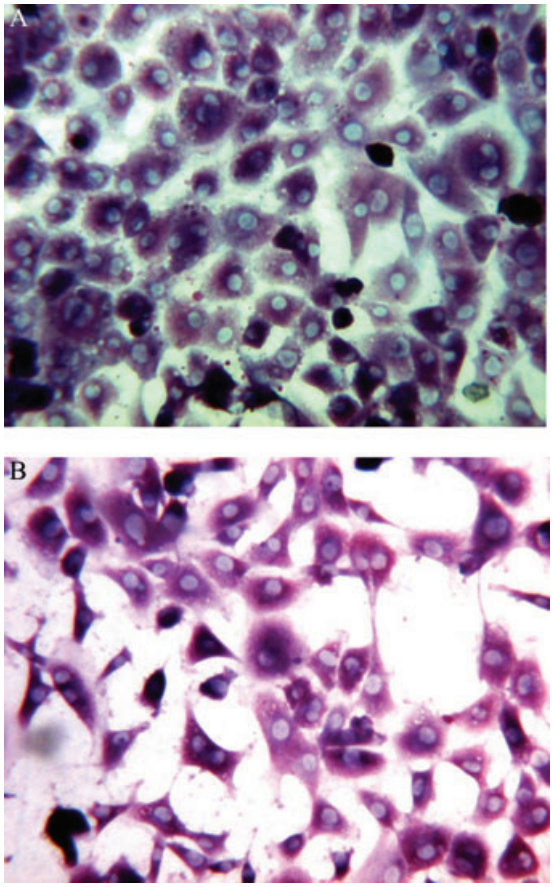

Figure 1 (A) Light microscope image demonstrating cell count and size of SIE zirconia specimen after 8 days. (B) Light microscope image demonstrating cell count and size of control zirconia specimen after 8 days.

spectrophotometer (NanoDrop® ND-1000; Wilmington, DE), and the amount of DNA was calculated (n = 30).[14]

Statistical analysis

One- and two-way ANOVA were used to analyze the data. A Bonferroni post hoc test was used for pair-wise comparisons ($\alpha = 0.05$).

Results

Cell morphology and cell count

Surface roughness of SLA titanium specimens ($R_a = 1.2 \pm 0.2$ μm) was higher than SIE zirconia ($R_a = 0.2 \pm 0.1$ μm) and both polished Ti and zirconia specimens ($R_a = 0.2 \pm 0.1$ μm). Statistical analysis revealed that SLA Ti and SIE zirconia specimens demonstrated significantly higher cell counts after 3 ($F = 17.4$, $p < 0.001$) and 8 days of culturing time ($F = 163$, $p < 0.001$) than did polished Ti and zirconia specimens (Fig 1). After 21 days, cells cultured on SLA Ti and SIE zirconia specimens achieved a confluent state, and cell count could not be accurately performed due to overlapping of the cells (Fig 2).

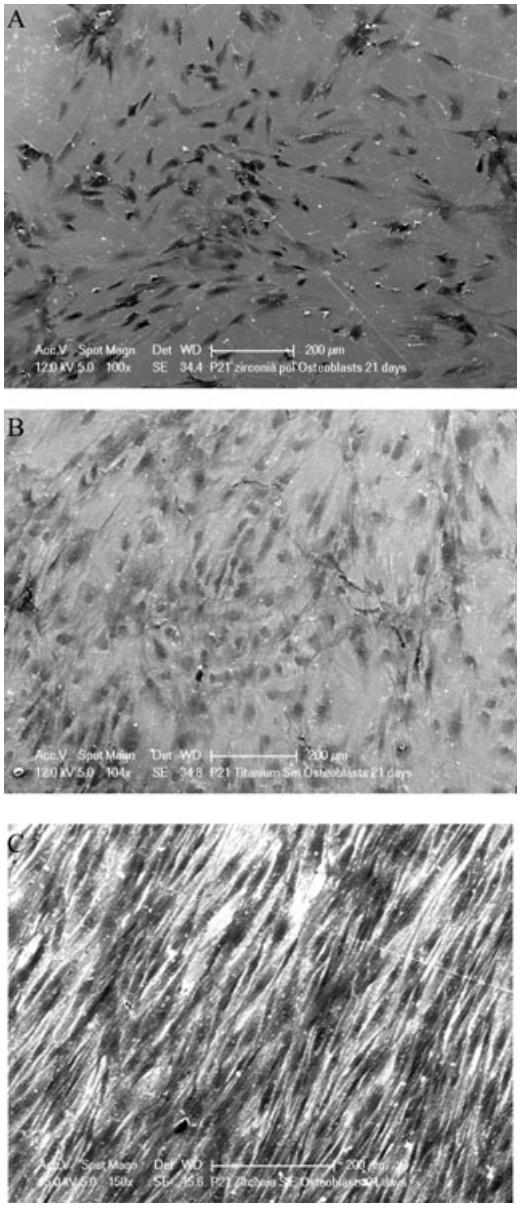

Figure 2 (A) SEM image, 100×, demonstrating the cell count in the observed field over the polished zirconia implant. (B) SEM image, 104×, demonstrating cell count in the observed field on a polished titanium specimen.(C) SEM image, 150×, demonstrating the elongated appearance of the cultured cells on selective infiltration etched zirconia (21 days).

SEM images revealed that the cell size, measured as the average length of two perpendicular axes was larger on the SIE-treated zirconia and on SLA Ti disks than on polished specimens (Fig 3) (Table 1).

On polished Ti and zirconia surfaces, cell attachment took place by extending finger-like processes, which anchored the cells to the surface of the implant material, while there was an obvious gap between the cell body and the underlying surface, which could be due to fixation and examination under

Table 1 Cell size, count, AP activity, and intracellular protein assay of tested groups

Test group (n = 20)	Cell size (μm) day 1	Day 3 cell count (/mm^2)	Day 8 cell count (/mm^2)	AP activity (ng/μl)	Intracellular protein (ng/μl)
Polished titanium	31.7 ± 5^A	42.3 ± 7^A	85 ± 9^B	0.0058 ± 0.0016^A	73.73 ± 1.7^A
SLA titanium	42.5 ± 4^B	40.5 ± 12^A	133 ± 15	0.0064 ± 0.0017^A	73.79 ± 1.8^A
Polished zirconia	30.7 ± 4^A	29.2 ± 5^B	98 ± 12^B	0.0044 ± 0.0029^A	72.65 ± 2.3^A
SIE zirconia	50.7 ± 3^B	40.8 ± 7^A	160 ± 20	0.0036 ± 0.0035^A	72.7 ± 0.9^A
Empty flask	27.5 ± 3^A	27.4 ± 6^B	60 ± 11	0.0050 ± 0.0077^A	71.17 ± 2.2
	($F = 129$, $p < 0.001$).	($F = 17.4$, $p < 0.001$)	($F = 163$, $p < 0.001$)	($F = 1.4$, $p < 0.25$)	($F = 4.2$, $p < 0.003$)

No statistically significant differences were observed for groups with similar superscript letters.

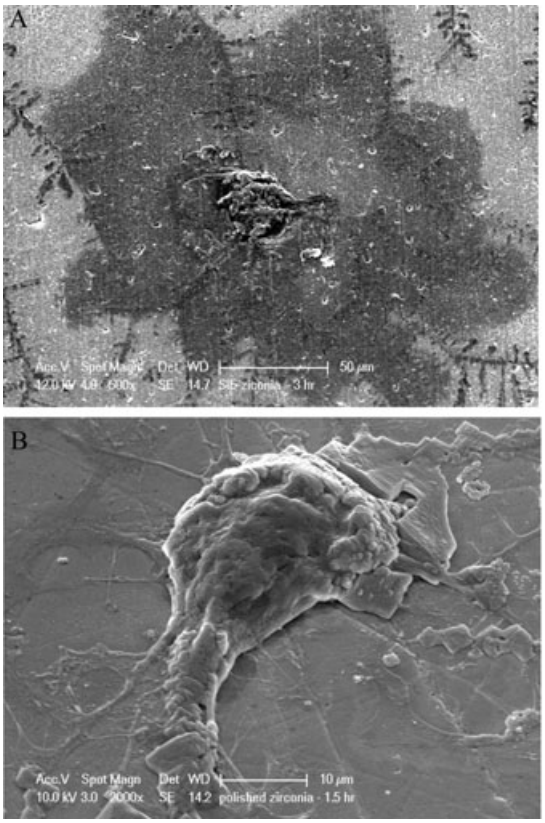

Figure 3 (A) SEM image, 500×, of an osteoblast cultured on selective infiltration etched zirconia. The cell demonstrated maximal spreading and appeared flat. (B) SEM image, 2000×, of an osteoblast cultured on polished zirconia. The cell attachment occurred by extending finger-like processes, while there was an observable gap between cell body and the implant.

vacuum. Similar findings were observed for cells cultured on empty flasks. On SIE-treated specimens, the cell membrane established good contact with the nanoporosities created on the structured surface as the cell membrane appeared in close proximity to the implant surface, which was observed for most of the examined specimens (Fig 4).

The level of alkaline phosphatase activity was similar for all samples tested (Table 1). The level of protein content was also comparable between all tested groups except for osteoblasts cultured in empty flasks, which demonstrated lower protein content (Table 1).

Discussion

Effect of surface roughness on cell attachment and cell morphology

In the present study, the nanoporous zirconia surface improved cell growth as indicated by a higher cell count and larger cell size compared to polished zirconia and titanium specimens and SLA Ti specimens. Selective infiltration etching created a nanoporous zirconia surface without increasing the surface roughness of the treated zirconia ($R_a = 0.2 \pm 0.2$ μm), which is a direct advantage compared to airborne particle abrasion associated with introducing surface and subsurface damage, leading to compromising of the abraded zirconia fatigue resistance.[15,16]

Micro-roughness produced by airborne particle abrasion (sandblasting) in combination with acid etching is one of the most widely used surface treatments for Ti implants.[7] Recent studies concerned with attachment of osteoblast cells to implant materials reported that a micro-rough surface ($R_a < 1$ μm) favored cell growth and attachment compared to a smooth surface, as the cultured cells appeared smaller and more rounded on polished surfaces compared to larger and more flattened cells cultured on micro- and nano-rough surfaces, which is in direct agreement with the findings of this study.[16-20] Increasing the surface roughness using larger particles did not also improve cell attachment and viability, indicating that excessive roughness did not improve cell attachment.[13]

Cell attachment on polished specimens appeared to depend on the formation of cytoplasmic extensions as finger-like processes. These structures seemed to anchor the cells to the underlying surface. Next to this interaction, the cell membrane was observed to be fused with the nanoporous surface. This spreading of the cells suggests that surface topography on a nano-scale influences cell attachment.[21,22] In a recent study, cells grown on polished surfaces attained a longitudinal pattern where those grown on rough surfaces were flattened after 24 hours of culture time.[23] Oates et al observed that surface roughness of implant material influences cell attachment and morphology of the cultured cells due to interaction of different integrin subunits with the surface.[24] Schneider et al reported a correlation between

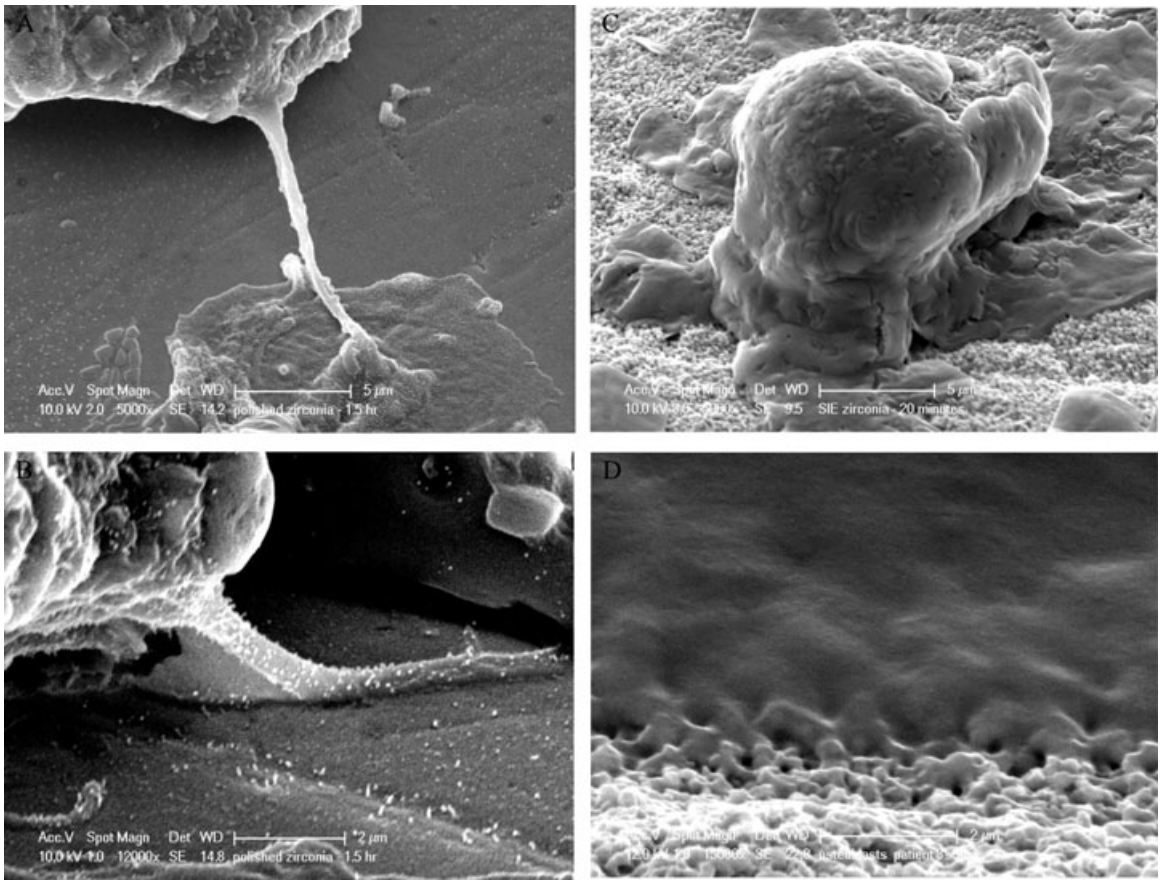

Figure 4 (A) SEM image, 5000×, of the same specimen demonstrating focal attachment between the cultured cell and the polished zirconia surface. Observe the presence of a cement-like material where the cell inserted the finger-like process. (B) High power SEM image, 12,000×, of the same specimen demonstrating finger-like process that extends from the cell body over the polished implant surface. (C) SEM image, 5000×, of an osteoblast cultured on selective infiltration etched zirconia (20 minutes). The cell membrane began to spread on the nanoporous surface of the implant. (D) SEM image, 15,000× demonstrating direct contact between the cell membrane (upper half) with the nanoporous selective infiltration etched zirconia.

cell attachment and surface architecture through regulation of gene expression, which could open a pathway for controlling osteoblast cell attachment to dental implants.[25]

Alkaline phosphate activity, a differentiation marker for cells of the osteogenic lineage, is used in several studies to compare activity of cells cultured on different surfaces.[16,26,27] In the present study, alkaline phosphate activity was comparable for all tested groups, indicating that the type of the substrate material and its surface roughness did not result in upregulation of the measured enzyme activity, which is in agreement with several studies.[28,29] On the other hand, differences in alkaline phosphate activity were observed for cells cultured on Ti implants with different surfaces (SLA and plasma spraying), indicating that alkaline phosphatase activity is material dependent.[18,20]

All tested specimens revealed an almost identical level of intracellular protein content, which is related to normalizing the data against the DNA content of each test group, thus excluding cell count from influencing the data.[30] By employing isotope labeling of amino acids, over 400 proteins were associated with marked changes in expression influenced by type of implant materials.[31] Findings indicate further possibilities to study biocompatibility of implant materials on a molecular level.[32]

Conclusion

The selective infiltration etching technique improved cell count and cell size of cultured human osteoblasts, which could improve performance of zirconia implants.

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