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ORIGINAL ARTICLE

Osteoblast integration of dental implant materials after challenge by sub-gingival pathogens: a co-culture study *in vitro*

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Sub-gingival anaerobic pathogens can colonize an implant surface to compromise osseointegration of dental implants once the soft tissue seal around the neck of an implant is broken. *In vitro* evaluations of implant materials are usually done in monoculture studies involving either tissue integration or bacterial colonization. Co-culture models, in which tissue cells and bacteria battle simultaneously for estate on an implant surface, have been demonstrated to provide a better *in vitro* mimic of the clinical situation. Here we aim to compare the surface coverage by U2OS osteoblasts cells prior to and after challenge by two anaerobic sub-gingival pathogens in a co-culture model on differently modified titanium (Ti), titanium-zirconium (TiZr) alloys and zirconia surfaces. Monoculture studies with either U2OS osteoblasts or bacteria were also carried out and indicated significant differences in biofilm formation between the implant materials, but interactions with U2OS osteoblasts were favourable on all materials. Adhering U2OS osteoblasts cells, however, were significantly more displaced from differently modified Ti surfaces by challenging sub-gingival pathogens than from TiZr alloys and zirconia variants. Combined with previous work employing a co-culture model consisting of human gingival fibroblasts and supra-gingival oral bacteria, results point to a different material selection to stimulate the formation of a soft tissue seal as compared to preservation of osseointegration under the unsterile conditions of the oral cavity.

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INTRODUCTION

Biomaterial implants are indispensable in modern medicine for the restoration of function, but roughly 5% fail due to infection.[1] Biomaterial-associated infection is hard to cure, as the biofilm mode of growth protects adhering bacteria against the host immune system and antibiotic treatment. Bacteria can gain access to an implant surface at different points in time after implantation and through different routes.[2] A common route of infection is through peri-operative contamination,[3–4] which can give rise to clinical signs of infection years after implantation.[3–5] Alternatively, implants are at risk of becoming colonized by haematogenous spreading of bacteria from infection elsewhere in the body.[6] Depending on local antibiotic guidelines, implant patients receive antibiotics prior to dental treatment[7] in order to prevent haematogenous spreading of oral bacteria to an implant site. The oral pathway along which implants throughout the body can become infected raises the question of how dental implants can survive in an unsterile environment with infection rates that are roughly equal to the 5% seen in environments that are sterile by nature.[8–9]

The first line of defense of dental implants is the soft tissue seal surrounding the implants neck.[10] The soft tissue seal has to form immediately after implantation against the challenge of the oral microflora in a combat named 'the race for the surface'.[3] If bacterial colonization prevails over tissue integration, the implant is lost, while tissue integration offers the best protection of an implant against invading pathogens. Although an appealing concept, it has taken over 20 years before co-culture studies emerged enabling to study the simultaneous behaviour of tissue cells and bacteria on biomaterial surfaces. Co-culture studies have been performed under static conditions,[11] in macroscopic[12] or microfluidic[13] flow chambers and have superseded monoculture studies with either tissue cells or bacteria.[14–15] In a peri-operative contamination model, tissue cells have to integrate a bacterially contaminated implant surface,[16] while in a post-operative model cells covering an implant surface are challenged by bacteria.[17] Supra-gingival oral bacteria can easily contaminate the neck of an implant during implantation and have the ability to form biofilms on different implant materials including titanium (Ti), titanium-zirconium (TiZr) alloys and zirconium-oxides (ZrO_2; 'zirconia'), regardless of their roughness or hydrophobicity.[11] Also, all implant materials could be covered by human gingival fibroblasts for 80%–90% of their surface areas within 48 h of growth. Yet, human gingival fibroblasts lost the race for the surface against different supra-gingival bacterial strains on nearly all bacterially contaminated implant materials in a peri-operative contamination model, except on the smoothest Ti variants. Co-culture studies therewith demonstrate that

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smooth Ti surfaces provide the best opportunities for a soft tissue seal to form on bacterially contaminated implant surfaces, in line with results from the few clinical human studies carried out to this end.[18]

In peri-implantitis[19] the soft tissue seal is broken, which is accompanied by a compositional change in the local microflora, and anaerobic periodontopathogens, such as *Porphyromonas gingivalis* and *Prevotella intermedia*, have been identified as late colonizers from dental implants showing clinical signs of infection.[18,20] Although peri-implantitis initially involves the soft tissue seal, in more progressed states, it also involves an attack on the bone cells adhering to the osseointegratable part of an implant leading to bone loss and possibly loss of an entire implant. Osseointegration of dental implants is assured by a tight fit[21] and choosing materials with good osseointegrative surface properties. Ti has long been the material of choice,[22] but is unsuitable as an implant material in narrow interdental spaces or in case of severe maxillary resorption.[23–25] Ti alloys, especially TiZr alloys, possess improved mechanical strength, making them suitable for use as small-diameter implants.[26] ZrO_2 ceramics are also used as an alternative for Ti due to their high mechanical strength, chemical stability and resistance to corrosion, in addition to their aesthetic advantage of better matching the natural tooth colour.[27] Ti alloys and ZrO_2 have all been compared with Ti for their osseointegrative properties, either in miniature pigs[26,28–29] or rabbits[30] for periods of time up to 2 months. In general,[31] these studies are low-powered. A systematic review concluded that 'ZrO_2 may have the potential to be a successful implant material',[32] while osseointegration of Ti alloys may be called similar to Ti depending in part on the surface modification applied and resulting surface morphology and hydrophobicity.[28,33–34]

None of these studies have been geared towards comparing osseointegration of different implant materials upon challenging the interface between their osseointegratable part and bone with periodontal pathogens. While animal or clinical studies may well be impossible due to the necessary duration of such studies and statistical limitations, *in vitro* evaluation of these materials has only been done on the basis of monoculture studies with either bacteria[35–36] or cells[15,37–38] but never in a post-operative, co-culture model.[17] A post-operative, co-culture model distinguishes itself from a peri-operative model: in a post-operative model, an implant surface is fully covered by tissue cells after which tissue integration is challenged by bacteria, while in a peri-operative model tissue cells have to try to cover a bacterially contaminated implant surface.

The aim of this study was to compare the surface coverage of osteoblasts cells on different dental implant materials after growth in absence and presence of a challenge with two anaerobic sub-gingival periodontopathogens (*P. intermedia* ATCC 49046 or *P. gingivalis* ATCC 33277) in a post-operative, co-culture model. The implant materials include differently modified Ti surfaces, as well as TiZr alloys and ZrO_2, as previously compared in a peri-operative, co-culture model involving human gingival fibroblasts and a variety of different supra-gingival strains.[11]

MATERIALS AND METHODS

Implant materials

All implant materials have been evaluated before in a peri-operative, co-culture model[11] and were received under a Materials Transfer Agreement from Institut Straumann AG (Basel, Switzerland) as 5 mm diameter discs with a thickness of 1 mm. Implant materials comprise Ti (cold-worked, grade 4), TiZr alloy (15% (*m/m*) Zr) and ZrO_2 (ZrO_2 with Y-TZP), modified according to different procedures, indicated as:

- P: mirror-polished with finally a 0.04 μm SiO_2 suspension and cleaned with a Deconex (a commercial detergent for metal cleaning obtained from Borer Advanced Cleaning Solutions, Zuchwil, Switserland) solution, followed by concentrated nitric acid and water.
- M: ground to mimic the machined part of the implant and washed with Deconex solution, followed by concentrated nitric acid and water.
- MA: ground and then acid-etched with a boiling mixture of concentrated HCl and H_2SO_4 (or hot hydrofluoric acid in case of ZrO_2) and rinsed with concentrated nitric acid and water.
- modMA: ground and acid-etched with HCl/H_2SO_4, as described above, but rinsed with water only under N_2 protection and directly stored individually in glass containers, filled with an isotonic NaCl solution, protected by N_2 filling.

M, MA, modMA and P modifications were all applied to Ti, whereas TiZr alloy and ZrO_2 were only modified according to M and modMA (TiZr alloy) or MA (ZrO_2). M, MA and P modified discs were individually packed in aluminum foil and sealed in plastic. All discs were sterilized in their respective packaging by γ-irradiation at 25–42 kGy. Surface properties of the different implant materials are summarized in Table 1.

Bacterial strains and culturing

Two anaerobic, sub-gingival pathogens known to be involved in periodontitis, *P. intermedia* ATCC 49046 and *P. gingivalis* ATCC 33277 were used in this study. Strains were streaked onto blood agar plates and incubated for 48 h anaerobically (85% N_2, 10% H_2 and 5% CO_2) at 37 ℃. One colony was inoculated into 5 mL brain heart infusion broth (BHI; Oxoid, Basingstoke, UK) medium (with 5 $g \cdot L^{-1}$ yeast, 5 $mg \cdot L^{-1}$ hemin and 10 $mg \cdot L^{-1}$ menadion) and grown for 24 h under anaerobic conditions. Bacteria were counted in a Bürker-Türk counting chamber and the bacterial culture was diluted with sterile adhesion buffer (50 $mmol \cdot L^{-1}$ potassium chloride, 2 $mmol \cdot L^{-1}$ potassium phosphate, 1 $mmol \cdot L^{-1}$ calcium chloride, pH 6.8) to a suspension with a concentration of 10^4 or 10^6 bacteria per mL.

Bacterial adhesion and biofilm formation in monoculture studies

Discs of each implant material were placed in 48-well plates and 10 μL droplets of a bacterial suspension (10^6 bacteria per mL) were placed on each disc under anaerobic conditions at 37 ℃ for 1 h. Subsequently, the bacterial suspensions were removed by dipping the discs three times in sterile adhesion buffer after which discs with adhering bacteria were inserted into modified culture medium (for details see section below on 'Osteoblast cells culturing and harvesting') and

Table 1 Physical-chemical properties of differently modified Ti, TiZr alloys and ZrO_2 implant surfaces involved in this study

	Physical properties		Chemical composition/%				
Material	R_a/μm	θ_w/°	C	Ti	Zr	$O_{in\ oxide}$	O_{other}
Ti-P	0.01	55	52	11	–	28	9
Ti-M	0.07	92	52	11	–	25	11
Ti-MA	0.66	81	44	14	–	31	10
Ti-modMA[a,b,c]	0.73	2	42	11	–	27	9
TiZr alloy-M	0.13	72	51	10	2	27	10
TiZr alloy-modMA[a,b]	0.24	23	41	9	2	26	10
ZrO_2-M	0.09	53	50	–	12	21	13
ZrO_2-MA	0.77	82	45	–	16	28	11

R_a, surface roughness; θ_w, water contact angle.
Data taken from Zhao *et al.*[11].
[a] Contains about 5% Na and Cl.
[b] Contains 2%–4% N.
[c] Contains 2% Ca.

biofilm formation was allowed for 96 h under anaerobic conditions at 37 ℃. Growth medium was exchanged after 48 h.

After 96 h of growth, the discs were dipped three times in sterile adhesion buffer and biofilms were stained with a vitality staining solution, containing 3.34 mmol·L^{-1} SYTO 9 and 20 mmol·L^{-1} propidium iodide (live/dead stain, *Bac*Light; Invitrogen, Breda, The Netherlands) in sterile ultrapure water. Staining was done in the 48-well plates for 15 min in the dark at room temperature. Next, biofilms were examined with a confocal laser scanning microscope (CLSM, Leica DMRXE with a confocal TCS SP2 unit) equipped with a water objective (HCX APO L 40.0 × 0.80 W) using 488 nm excitation and emission filters of 500–550 nm and 605–720 nm to reveal live or dead bacteria, respectively. Images were taken over the depth of a biofilm in sequential steps of 0.8 μm. Subsequently, the stacks of images acquired were analysed for the total biofilm volume per unit area with the program 'COMSTAT'.[39] In short, the expression 'total biovolume' refers to the volume occupied per unit substratum area by dead and live bacteria in a biofilm. Accordingly, its unit is μm^3 per μm^2. Biovolume is calculated from the number of green (live bacteria) and red (dead bacteria) in an image stack multiplied by the voxel size (cubic pixel size).

Osteoblast cells culturing and harvesting

U2OS osteosarcoma cells (ATCC HTB-94) were routinely grown in Dulbecco's modified Eagle's medium/low glucose (DMEM/LG) supplemented with 10% (*V/V*) fetal bovine serum, 0.2 mmol·L^{-1} ascorbic acid-2-phosphate (DMEM/LG-complete) at 37 ℃ in a humidified 5% CO_2 atmosphere. At 70%–80% confluence the U2OS cells were passaged using a trypsin-elhylene diaminetetraacetic acid (EDTA) solution (Invitrogen, Breda, The Netherlands). Thus, grown cells were used in all mono- and co-culture studies. Importantly, in co-culture studies, U2OS osteosarcoma cells were initially grown in DMEM/LG-complete in 5% CO_2, pursuing survival of U2OS osteoblasts and simultaneous growth with oral sub-gingival anaerobes in a modified culture medium under anaerobic conditions.

To develop such a modified culture medium suitable for bacterial growth and survival of U2OS osteoblasts under anaerobic conditions, bacterial (BHI^+) and cellular growth media (DMEM/LG-complete) were combined in different ratios and the growth of *P. intermedia* ATCC 49046, *P. gingivalis* ATCC 33277 or U2OS osteoblasts were monitored over time in monocultures.

With respect to U2OS osteoblast growth, suspensions of cells (3×10^4 mL^{-1} in DMEM/LG-complete) were added in 48-well plates made of tissue culture polystyrene. After growth for 24 h at 37 ℃ in a 5% CO_2 atmosphere, survival of the U2OS osteoblasts was determined under anaerobic conditions at 37 ℃ after replacing DMEM/LG-complete by modified culture media with different ratios of DMEM/LG-complete and BHI^+. After 24 or 48 h, the morphology of the U2OS cells was assessed using phase-contrast microscopy, yielding the conclusion that cells survived anaerobic conditions in modified culture medium with maximally 10% BHI^+ added for at least 48 h (see Figure 1).

With respect to the growth of the sub-gingival anaerobes in modified culture medium, bacteria of each strain were first grown in 5 mL BHI^+ for 24 h, after which 0.5 mL of these cultures was used to inoculate 4.5 mL of modified culture media with different ratios of DMEM/LG-complete and BHI^+ for 24 or 48 h under anaerobic conditions at 37 ℃. After 48 h, numbers of bacteria per mL (see Figure 2) of the different cultures were compared. Both sub-gingival anaerobes showed limited growth in DMEM/LG-complete, while bacterial growth increased with increasing percentage of BHI^+. Since U2OS osteoblasts could not survive in media with higher percentages of BHI^+ than 10%, a modified culture medium was chosen that consisted of DMEM/LG-complete with 10% BHI^+ added. In the remainder of this study, therefore, DMEM/LG-complete with 10% BHI^+ will be denoted as 'modified culture medium'.

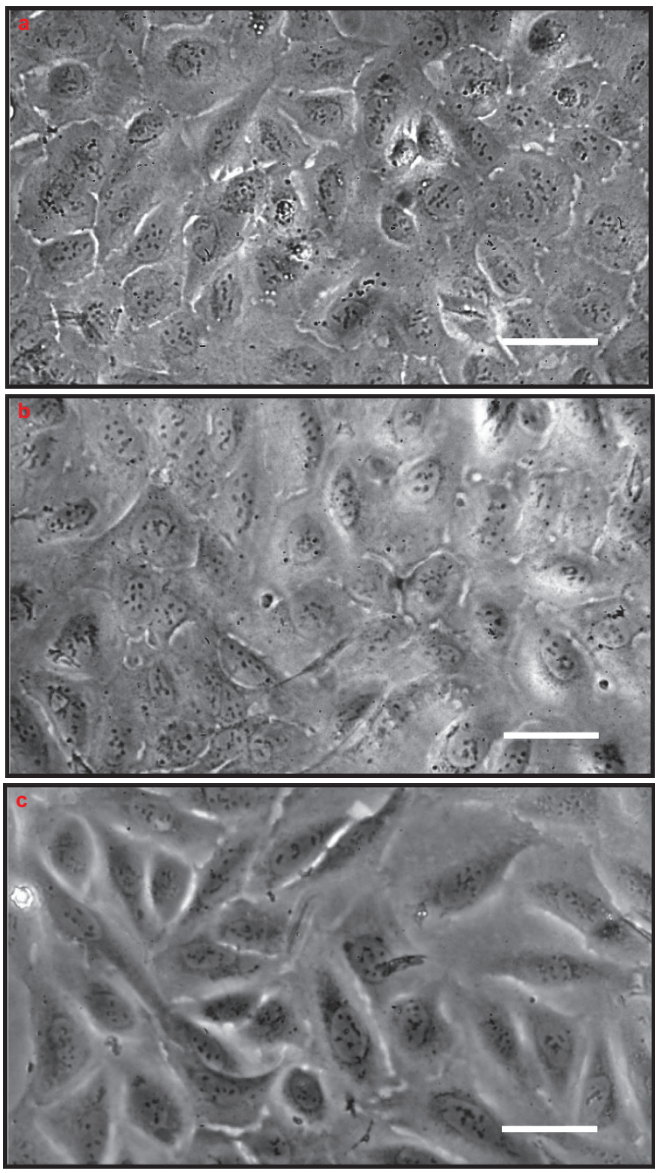

Figure 1 Phase contrast micrographs of U2OS osteoblasts initially grown aerobically with pursued further growth under anaerobic conditions. (**a**) U2OS osteoblasts initially grown in 100% DMEM/LG-complete at 37 ℃ in a 5% CO_2 atmosphere for 24 h in tissue culture polystyrene wells; (**b, c**) pursued further growth for 24 or 48 h in modified culture medium with 10% BHI^+ added under anaerobic conditions. Bar markers indicate 50 μm. BHI, brain heart infusion; DMEM/LG, Dulbecco's modified Eagle's medium low glucose.

Adhesion, spreading and growth of U2OS osteoblasts in monoculture studies

Discs of each implant material were placed in 48-well plates and 1 mL of a cell suspension (3×10^4 mL^{-1}) in DMEM/LG-complete was added. Cells were grown 24 h at 37 ℃ in a humidified atmosphere with 5% CO_2. Then, DMEM/LG-complete was replaced by modified culture medium and put for 48 h under anaerobic conditions at 37 ℃. Subsequently, discs with adhering cells were prepared for immunocytochemical staining to assess U2OS osteoblast spreading and number of adhering cells. For fixation, growth medium was removed and

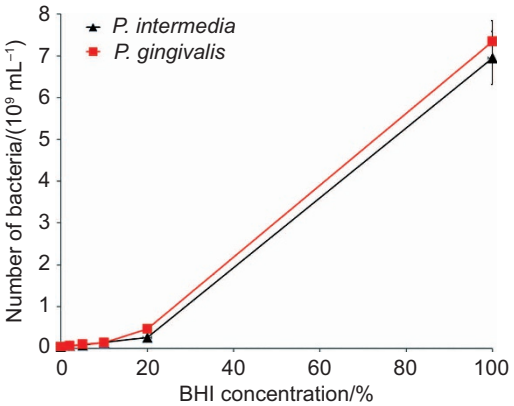

Figure 2 Growth curve of *P. gingivalis* and *P. intermedia* as a function of the percentage BHI$^+$ in modified culture medium. *P. gingivalis* and *P. intermedia* were grown anaerobically in a mixture of BHI$^+$ and DMEM/LG-complete, anaerobically. 0% BHI means 100% DMEM/LG-complete. BHI, brain heart infusion; DMEM/LG, Dulbecco's modified Eagle's medium low glucose.

replaced by 3.7% paraformaldehyde in cytoskeleton stabilization buffer (0.1 mol·L^{-1} Pipes, 1 mmol·L^{-1} ethylene glycol tetraacetic acid, 4% (*m/m*) polyethylene glycol 8000, pH 7.0). Adhering cells were permeabilized with 0.5% Triton X-100 in phosphate-buffered saline (PBS; 10 mmol·L^{-1} potassium phosphate, 0.15 mol·L^{-1} NaCl, pH 7.0) for 3 min, and stained with 2 mg·mL^{-1} phalloidin- tetramethylrhodamine (TRITC) and 4 mg·mL^{-1} 4′,6-diamidino-2-phenylindole (DAPI; both from Sigma-Aldrich, St. Louis, MO, USA) in PBS with 1% bovine serum albumin for 30 min in the dark at room temperature. After that, the discs were incubated in PBS with 1% bovine serum albumin for 5 min. Subsequently, discs were examined with fluorescence microscopy (Leica DM4000; Leica Microsystems, Wetzlar, Germany). Images (five images on different locations) were taken and the number of adhering cells per unit area and the average area per spread cell were determined using Scion image software to yield the total coverage of the substratum surface by U2OS osteoblasts. All experiments were performed in triplicate on each implant surface.

Challenging osteoblast layers with anaerobic sub-gingival bacteria in a co-culture model

A previous study with a staphylococcal strain challenging U2OS monolayers on poly(methyl methacrylate) has shown that initial coverage by tissue cells needs to be above a threshold coverage of above 40%,[17] while furthermore too high a bacterial challenge concentration kills all adhering cells, impeding comparisons of different materials. Therefore, U2OS monolayers were grown for 24 h to a surface coverage of 80%–100% in DMEM/LG-complete at 37 ℃ in a humidified atmosphere with 5% CO_2 in tissue culture polystyrene wells, representative of a near-fully osseointegrated implant. DMEM/LG-complete was replaced with modified culture medium and challenged with one of the two oral sub-gingival pathogens in modified culture medium at a bacterial concentration of 10^4 or 10^6 bacteria per mL for 48 h, after which the morphologies of the adhering cells were examined by phase-contrast microscopy. U2OS cells did not demonstrate any morphological change in response to a bacterial challenge at 10^4 mL^{-1} for either *P. intermedia* ATCC 49046 or *P. gingivalis* ATCC 33277, but at 10^6 mL^{-1} near complete cellular detachment was observed after challenge with *P. intermedia* ATCC 49046 (Figure 3). After challenge with

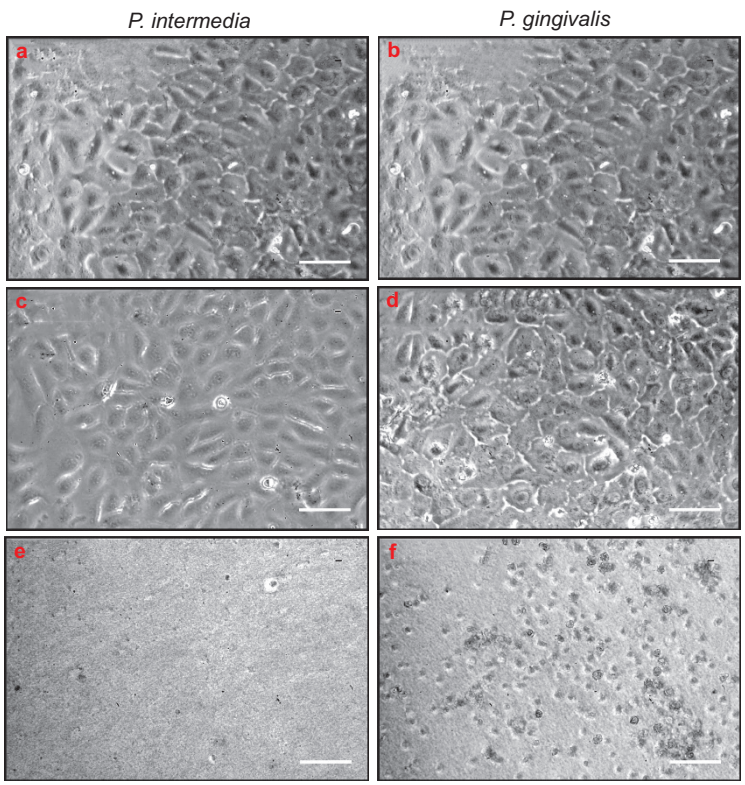

Figure 3 Phase-contrast micrographs of U2OS osteoblasts challenged with different concentrations of sub-gingival pathogens. U2OS osteoblasts initially grown in 100% DMEM/LG-complete at 37 ℃ in a 5% CO_2 atmosphere for 24 h in tissue culture polystyrene wells, and after pursued further anaerobic growth for 48 h in modified culture medium in absence and presence of different concentrations of *P. intermedia* ATCC 49046 or *P. gingivalis* ATCC 33277. (**a, b**) No bacteria; (**c, d**) $\times 10^4$ mL^{-1}bacteria; (**e, f**) $\times 10^6$ mL^{-1} bacteria. Bar markers indicate 25 μm. DMEM/LG, Dulbecco's modified Eagle's medium low glucose.

P. gingivalis ATCC 33277, adhering U2OS rounded up in response to bacterial challenge (see also Figure 3). Accordingly, a bacterial challenge concentration of 10^4 mL^{-1} was chosen for the experiments.

Next, U2OS cells were grown in a humidified 5% CO_2 atmosphere to a surface coverage of 80%–100% on all material surfaces and growth continued in absence and presence of a challenge with either *P. intermedia* ATCC 49046 or *P. gingivalis* ATCC 33277 under anaerobic conditions, as described above. After 48 h, U2OS cells were fixed, stained with phalloidin-TRITC and DAPI and analysed as described above for surface coverage and cell numbers. All experiments were performed in triplicate on each implant materials.

Statistical analyses

Biovolumes by the different bacterial strains and U2OS cells interaction with the different implant materials in monoculture or co-culture experiments are presented as means with standard deviations. All statistical comparisons between multiple groups were performed using one-way analysis of variance (ANOVA), with Tukey's multiple comparison tests to compare each material variant with the other variants in the same material group. To analyse differences in surface coverage and cell adhesion number in absence and presence of a bacterial challenge, surface coverage and adhesion numbers were compared by two tailed Student's *t*-test. To analyse the magnitude of these differences on variants within the same material group, one-way ANOVA and Tukey's multiple comparison tests were carried out. In addition, the average differences in surface coverages and cell numbers grown in absence or presence of a bacterial challenge were compared between the group of four Ti variants and the other groups of materials using Mann–Whitney tests. A *P*-value <0.05 was regarded as statistically significant. GraphPad Prism 6 statistical software was used for all analyses.

RESULTS

Biofilm formation on different dental implant materials in monocultures

The biovolumes of *P. intermedia* and *P. gingivalis* across the different materials are summarized in Figure 4. *P. intermedia* biofilms were generally of equal thickness or thinner than *P. gingivalis* biofilms on the same implant material. For *P. intermedia*, significantly ($P <$ 0.05) less biofilm was found on the smoother surfaces compared to rougher variants from the same material (compare Table 1). For *P. gingivalis* a less pronounced effect of surface roughness was seen.

Adhesion, spreading and growth of U2OS osteoblasts in monocultures

U2OS osteoblasts showed similar surface coverage on all implant surfaces (no significant differences across materials and their variants) with surface coverages of between 80% and 100% after 24 h growth in DMEM/LG-complete in a 5% CO_2 atmosphere and 48 h continued growth in modified culture medium under anaerobic conditions (see Figure 5a). Differences existed in the number of cells adhering (see Figure 5b) and the lowest number of cells was found on TiZr alloy-modMA and ZrO_2-M, indicating that the spread area per cell was highest on relatively rough (R_a = 0.24 μm) and hydrophilic (θ_w = 23°) TiZr alloy-modMA (1 667 μm^2) and smoother (R_a = 0.09 μm), but more hydrophobic (θ_w = 53°) ZrO_2-M (1 156 μm^2).

Co-culture of U2OS osteoblasts with anaerobic sub-gingival bacteria on different dental implant materials

Figure 6 illustrates the effects of a U2OS osteoblast monolayer grown in absence and presence of a challenge with *P. intermedia* ATCC 49046. As can be seen, high numbers of U2OS osteoblasts adhere in absence of a bacterial challenge both on Ti-P (R_a = 0.01 μm; θ_w = 55°) and ZrO_2-MA (R_a = 0.77 μm; θ_w = 82°). Despite this similarity between both materials in surface coverage and number of adhering cells in absence of a bacterial challenge, U2OS osteoblasts show a considerably less surface coverage in presence of a bacterial challenge on Ti-P than on ZrO_2-MA, where cell surface coverage was almost similar in absence and presence of a bacterial challenge.

Quantitative effects of growing U2OS osteoblast layers on the different materials under bacterial challenge can be seen by comparing the coloured columns in Figure 5 with the black ones. Both the presence of *P. intermedia* and *P. gingivalis*, significantly ($P < 0.05$) decreases U2OS osteoblast surface coverage on the Ti variants, but no reductions due to growth in presence of a bacterial challenge are seen on TiZr alloys and ZrO_2 variants (Figure 5a). Note, however, that

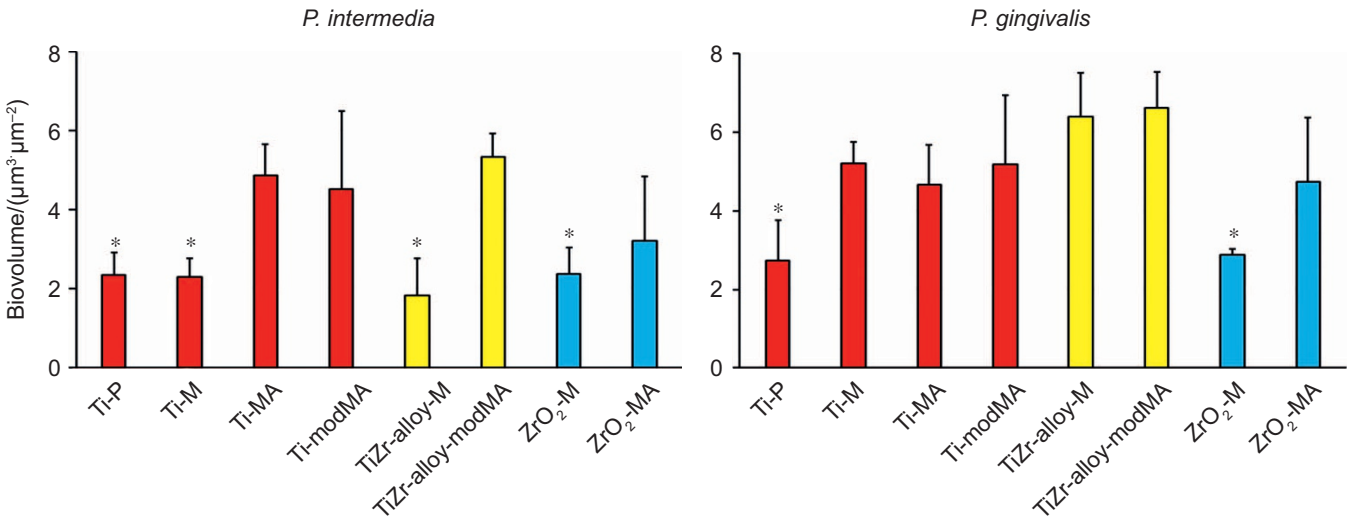

Figure 4 Biovolumes of *P. intermedia* ATCC 49046 and *P. gingivalis* ATCC 33277 biofilms after 96 h of growth on different implant surfaces. Error bars represent standard deviations over three experiments with different discs of each material and separately cultured bacteria. *Significantly ($P < 0.05$) different from all other surfaces in the same group of materials.

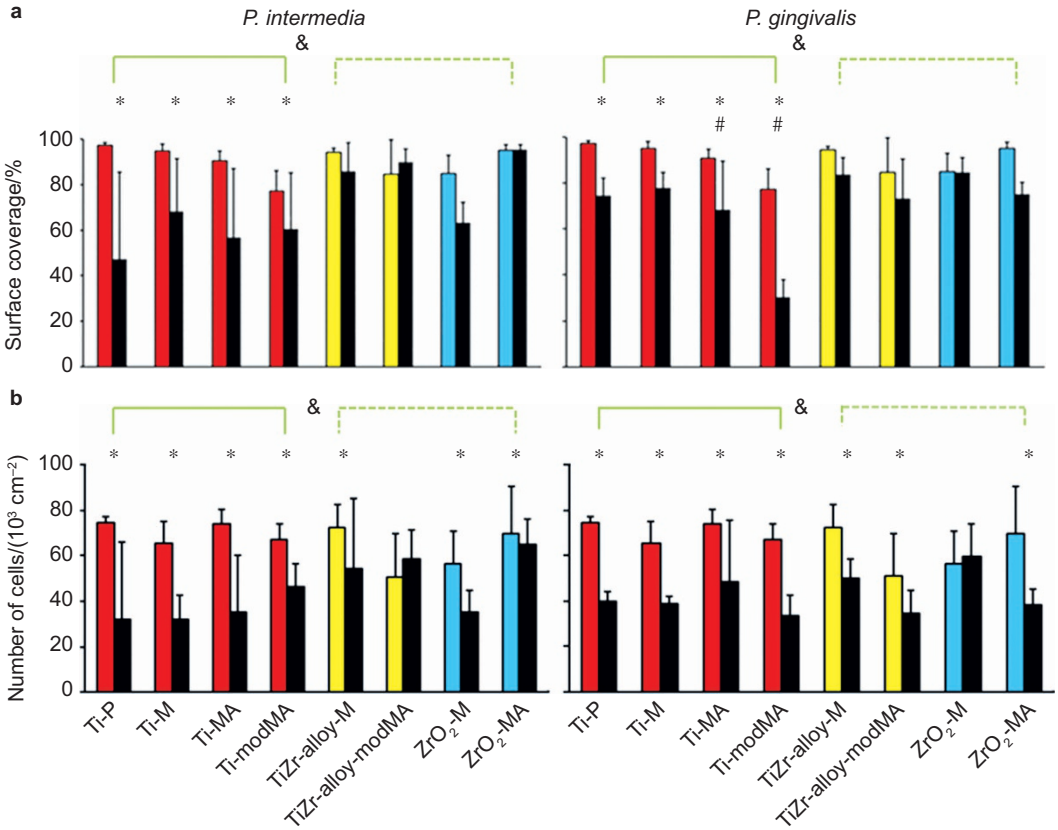

Figure 5 U2OS osteoblast surface coverage and cell number on different implant materials grown in absence (coloured columns) or presence (adjacent black columns) of a challenge by sub-gingival oral bacteria. (**a**) Surface coverage; (**b**) number of cells. Error bars indicate standard deviations over triplicate experiments with separately grown cells and bacteria. * Significant ($P < 0.05$) differences in surface coverages and cell numbers in absence versus presence of a bacterial challenge (black and colored columns respectively) on the same material. # Magnitudes of the differences in surface coverages and cell numbers in absence versus presence of a bacterial challenge on the same material are significantly ($P < 0.05$) higher than on the other Ti variants. & Magnitudes of the differences in surface coverages and cell numbers in absence versus presence of a bacterial challenge on the same material are significantly higher ($P < 0.05$) than in the other groups of materials, indicated by the green lines.

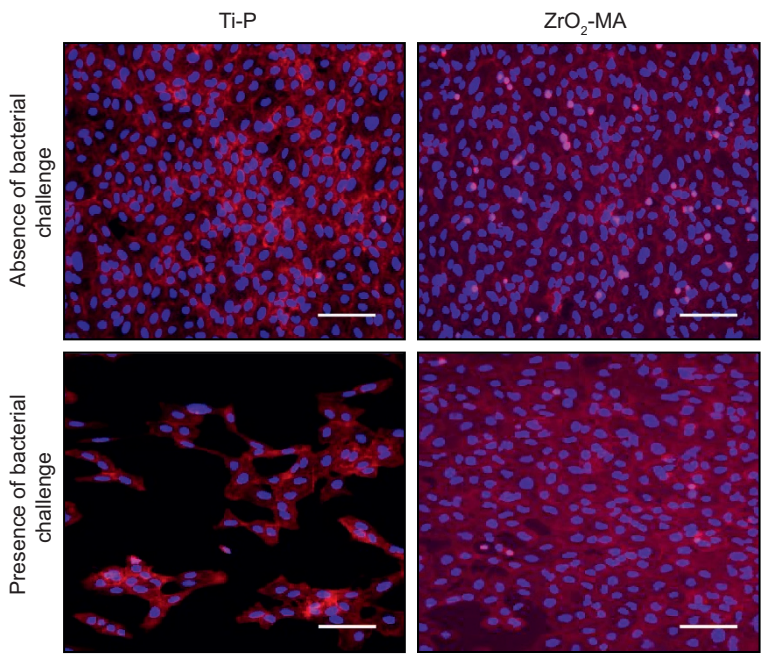

Figure 6 Example of immune-cytostained images of U2OS osteoblasts grown on Ti-P and ZrO2-MA surfaces. U2OS osteoblasts were grown for 24 h in DMEM/LG-complete at 37 ℃ in a humidified atmosphere with 5% CO_2 and subsequently grown for 48 h in anaerobic conditions in modified culture medium in absence and presence of a challenge with *P. intermedia*. Bar markers indicate 100 μm. DMEM/LG, Dulbecco's modified Eagle's medium low glucose.

these decreases were less pronounced but still significant ($P < 0.05$) for *P. gingivalis* than for *P. intermedia*. In line, the numbers of adhering U2OS cells generally decreased most upon a bacterial challenge on the Ti variants ($P < 0.05$), irrespective of the strain involved (Figure 5b).

DISCUSSION

Osseointegration of dental implants is assured by a tight fit and osseointegrative material selection. Material selection for optimal osseointegration, however, also involves the requirement of material surface properties on which bone cells can effectively withstand a pathogenic challenge. Clinical studies to evaluate the infection resistance of different dental implant materials are scarce and not seldom under-powered.[31] In this paper, we rely on a post-operative, co-culture model consisting of U2OS osteoblasts and sub-gingival, anaerobic periodontopathogens to demonstrate that adhering U2OS osteoblasts are more readily displaced from differently modified Ti surfaces by challenging sub-gingival pathogens than from TiZr alloys and ZrO_2 variants. TiZr alloys and ZrO_2 variants are thus to be preferred over Ti for the osseointegratable part of dental implants.

Monocultures on biofilms formation show that *P. gingivalis* biofilms are thicker than of *P. intermedia*, with *P. intermedia* preferring rough surfaces over smooth ones with little or no preferences for a specific surface chemistry or hydrophobicity of the implant material. In a comparative study[40] of *P. gingivalis* and *P. intermedia* adhesion to smooth Ti surfaces extending over a 22 h time period, *P. gingivalis* was found to adhere in lower numbers (4.0×10^8 cm^{-2}) than *P. intermedia* (6.4×10^8 cm^{-2}), which is opposite to what is observed in the current study after growth. However, this difference is less than a factor of two, which makes it extremely small in view of the fact that bacterial doubling times in a biofilm are in the orders of several tens of minutes.[41]

U2OS osteoblasts grew equally well on all materials when in monoculture. Hydrophobicity and roughness of the different materials did not show any difference in surface coverage of U2OS cells, except for the very hydrophilic Ti-modMA exhibiting the lowest surface coverage. However, surface coverage depends on the number and spread area of adhering cells and the equal surface coverages observed across the different materials comes into existence through different pathways. U2OS osteoblasts spread best on a relatively rough and hydrophilic TiZr alloy variant and relatively smooth and hydrophobic ZrO_2 variant. This is in line with results on spreading of human osteoblast-like MG63 cells, for which it was concluded that spreading depended on an interplay between surface roughness and materials chemistry.[42] In summary, a materials preference for the osseointegratable part of a dental implant cannot be derived from monoculture studies with neither bacteria nor cells, emphasizing the need for a proper co-culture model.

A co-culture model in which anaerobic bacteria are grown together with tissue cells has never been employed before and could only be realized by first growing a layer of U2OS osteoblasts on a material in a humidified 5% CO_2 atmosphere and then changing the conditions to anaerobic as required for co-culturing with anaerobic bacteria. U2OS osteoblasts remained to look morphologically healthy despite oxygen deprivation (see Figure 6) and in fact oxygen is not a prerequisite for tissue cells to survive.[43] Hypoxia is known to reduce osteoblast alkaline phosphatase activity and expression of mRNAs for alkaline phosphatase and osteocalcin. Transmission electron microscopy revealed that collagen fibrils deposited by osteoblasts cultured in 2% O_2 were less organized and less abundant than in 20% O_2 cultures. Importantly, hypoxia did not increase the apoptosis of osteoblasts.[43] This is an important property of osteoblasts that allowed us to challenge them in a co-culture model under anaerobic conditions with sub-gingival oral pathogens.

In our anaerobic co-culture model, adhering U2OS osteoblasts as on the osseointegratable parts of an implant, on average withstand a challenge by sub-gingival pathogens better on TiZr alloys and ZrO_2 variants than on Ti variants (see Figure 7, left panels) regardless of roughness indicating an overriding effect of materials chemistry.[42] Challenges with either of the two periodontopathogens have about the same negative impact on the coverage of the implant surfaces by osteoblasts, despite the fact that in monoculture studies *P. gingivalis* forms more extensive biofilms than *P. intermedia*. Hypothetically, we propose that U2OS osteoblasts adhere more strongly to TiZr alloys and ZrO_2 than to pure Ti. Osteoblasts-like cell adhesion in monoculture has been found to be enhanced on ZrO_2 compared to pure Ti and also the gene expression of integrin β1, ERK1/2 and *c-fos* was higher on ZrO_2 than on Ti,[44] although other monoculture studies showed no difference in initial cell response to Ti and ZrO_2.[45–46] Previous work[11] employing an aerobic, peri-operative, co-culture model consisting of human gingival fibroblasts and supra-gingival oral bacteria, pointed out that a soft tissue seal as needed on the neck part of an implant, develops more readily on bacterially contaminated, smooth Ti surfaces (see also Figure 7, right panels). Therewith our previous[11] and present co-culture studies on dental implant materials indicate that different requirements should be set to the materials properties for the neck versus the osseointegratable part of a dental implant.

Whereas we previously argued that our conclusion on material selection for the implant neck on basis of infection-resistance coincided with the few, low-powered clinical human study results available,[18] this remains to be demonstrated for the conclusion drawn from our co-culture study on the osseointegratable part. Favourable conclusions on all implant materials evaluated in this study can be found in the literature. A systematic review[32] concluded that there were no differences in the rate of osseointegration between the different implant materials in animal experiments, while scientific clinical data for ceramic implants in general and for ZrO_2 implants in particular were considered insufficient to recommend ceramic implants for routine clinical use. *In vivo* studies in miniature pigs[28] and New Zealand rabbits[30] concluded that TiZr alloy (sandblasted and acid-etched) implant surfaces had a similar potential for clinical applications as clinically proven Ti surfaces (sandblasted and acid-etched).[28] Another study in miniature pigs[26] concluded that TiZr alloy with a hydrophilic, sandblasted and acid-etched surface showed similar or even stronger bone tissue responses than the Ti control, while another study in miniature pigs suggested that unloaded, sandblasted ZrO_2 and sandblasted and acid-etched Ti implants osseointegrated comparably within a healing period of four weeks.[29] Unfortunately, no studies on these different materials have been found geared towards a comparison of the infection-resistance of osseointegration.

In summary, this *in vitro* co-culture study suggests that for the osseointegratable parts of dental implants, TiZr alloys and ZrO_2 variants are the preferred materials with respect to maintaining osseointegration under conditions of pathogen challenge. Previously, we demonstrated that for the implant neck, smooth Ti was the material of choice with respect to rapid formation of an effective soft tissue seal in the presence of bacterial contamination. Differential requirements for the different parts of a dental implants have never been proposed from the perspective of infection control but may lead to a new generation of dental implants with reduced clinical infection rates.

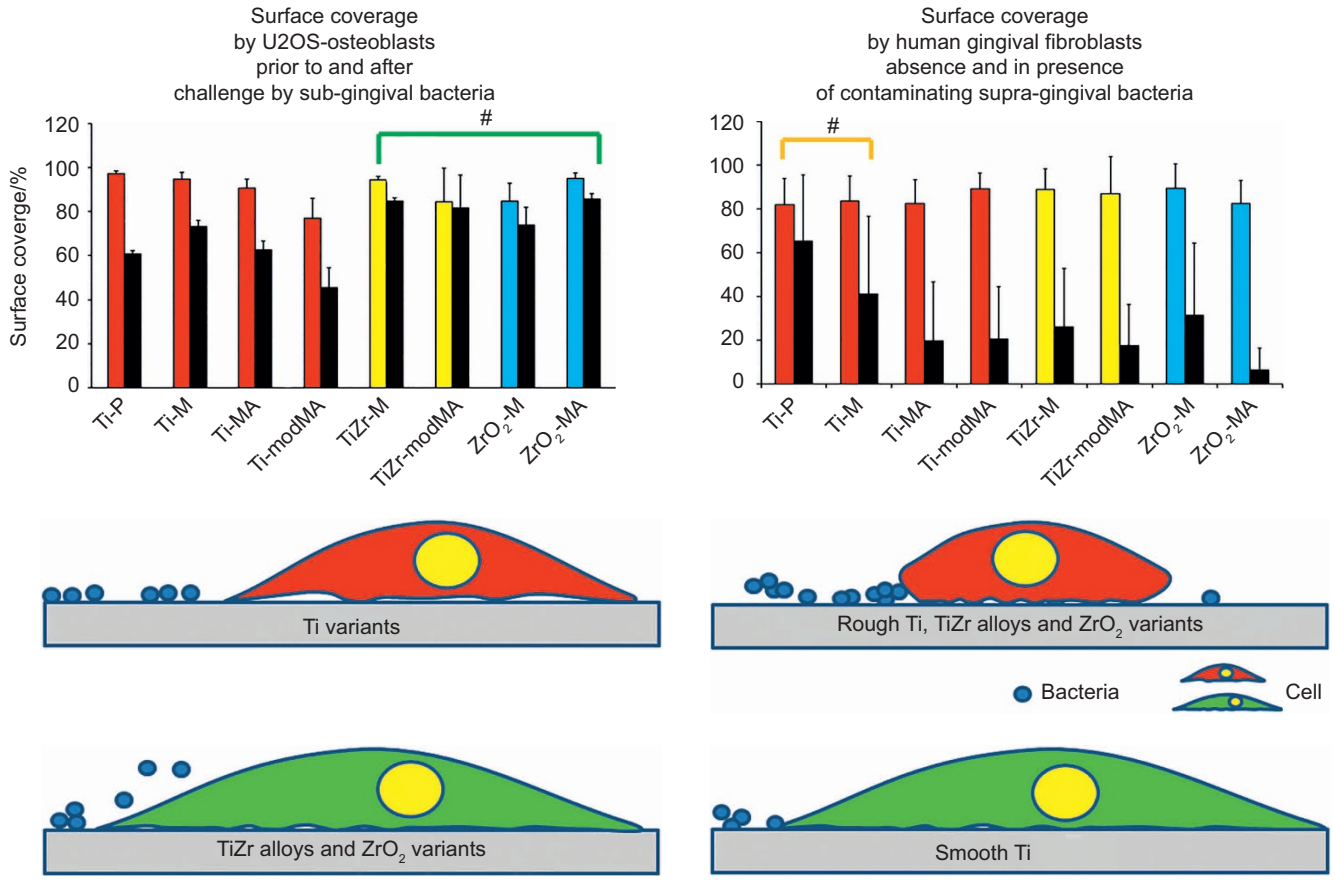

Figure 7 Battles occurring on the osseointegratable part of a dental implant between an existing layer of adhering U2OS osteoblasts and challenging periodontopathogens (left panel) and on the neck of an implant between gingival fibroblasts and contaminating oral bacteria adhering to the implant surface (right panel). Left panel: a comparison of the surface coverage by U2OS cells on dental implant materials grown prior to (coloured bars) and after (black bars) of a challenge by sub-gingival, oral bacterial strains. Data represent averages over *P. intermedia* ATCC 49046 and *P. gingivalis* ATCC 33277. On TiZr alloys and ZrO_2 variants, adhering osteoblasts withstand a challenge by sub-gingival bacteria more effectively than on Ti variants, regardless of surface roughness (green coloured, well-spread cell versus red cell), making TiZr alloys and ZrO_2 variants most suitable for the osseointegratable part of an implant. Right panel: a comparison of the surface coverage by human gingival fibroblasts on dental implant materials grown in absence (coloured bars) and presence (black bars) of contamination by adhering supra-gingival, oral bacteria (*Streptococcus oralis* J22, *Streptococcus mitis* BMS, *Streptococcus salivarius* HB and *Staphylococcus aureus* ATCC 25923). Data represent averages over the four strains indicated (data taken with permission from Zhao *et al.*[11]). Displacement of contaminating supra-gingival bacteria from smooth Ti variants (green coloured, well-spread cell versus red, rounded-up cell) is easier than from the other implant materials included in this study, making smooth Ti variants most suitable for the neck of an implant. #These materials on average perform better than the other materials with respect to a bacterial challenge of an existing cellular layer or integration of a material in the presence of contaminating bacteria.

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