

Original Article



Comparative in Vitro Evaluation of Osteoblast Responses to Titanium and Zirconia Dental Implants

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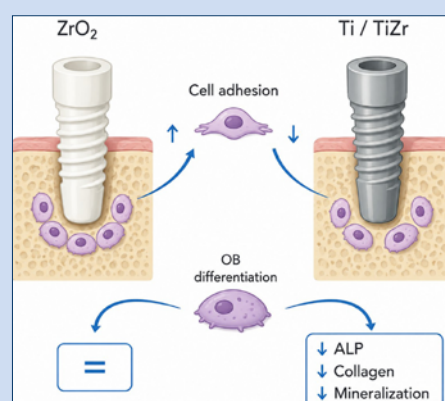
Abstract

Introduction: Successful dental implantation relies on effective osseointegration, a process initiated by osteoblast adhesion, differentiation, and extracellular matrix mineralization at the implant surface. This study compared the in vitro osteoblast responses to titanium- and zirconia-based dental implants to assess their potential influence on cellular events associated with osseointegration.

Methods: Primary murine preosteoblasts were cultured in the presence of three commercially available implant types: grade 4 titanium (Ti), titanium–zirconium alloy (Ti/Zr), and zirconia (ZrO₂). Osteoblast adhesion, viability, alkaline phosphatase (ALP) activity, extracellular collagen deposition, matrix mineralization, and cell morphology were evaluated using scanning electron microscopy (SEM).

Results: Osteoblast adhesion was significantly greater on ZrO₂ implants than on Ti or Ti/Zr implants, whereas cell viability and morphology were preserved in all groups. Exposure to ZrO₂ implants did not significantly affect ALP activity, collagen deposition, or extracellular matrix mineralization compared with control cultures. In contrast, Ti and Ti/Zr implants significantly reduced all the evaluated osteoblast differentiation markers. Integrated analysis of the biological parameters consistently showed that osteoblasts cultured in the presence of zirconia implants exhibited a cellular response more similar to that of control cultures than those exposed to titanium-based implants.

Conclusion: Under the experimental conditions of this in vitro study, zirconia implants promoted more favorable osteoblast responses than titanium-based implants. These findings suggest that zirconia surfaces may better support early cellular events involved in osseointegration.



Introduction

Oral implants have become a reliable treatment option for replacing missing teeth. An ideal implant material should be biocompatible and possess adequate toughness, wear resistance, and fracture resistance.¹ Currently, metal-free restorations have emerged as an attractive alternative in implant dentistry, mainly because they overcome the aesthetic limitations associated with metallic materials. Zirconia-based implants were introduced as an alternative to conventional titanium implants. The current challenge in dental implantology is to develop zirconia-based implant surfaces with bioactive, mechanical, and osteogenic properties that ensure favorable short- and long-term

clinical outcomes.² Owing to its favorable mechanical properties, aesthetics, and biocompatibility, zirconia has been widely applied in both dental implants and fixed prosthetic restorations. Compared with titanium, zirconia offers additional advantages because it does not cause gray discoloration of the peri-implant tissues and presents a negligible risk of allergic hypersensitivity reactions.³ In particular, yttria-stabilized tetragonal zirconia polycrystal exhibits suitable biomechanical properties for dental implant applications. Recent evidence suggests that zirconia implants represent a promising alternative to titanium, showing comparable soft tissue integration, although titanium may still exhibit faster early osseointegration.^{4,5}

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Since implant materials, including zirconia, will be in contact with different tissues, these materials must have optimal surface compatibility with bone and soft tissue. To that end, zirconia implants must be developed with rigorous control of the topography and physicochemical properties of the surface, which are key factors for the osseointegration process at the bone tissue/implant interface.³

Successful oral implantation depends on a series of overlapping phases of tissue healing and regeneration at the implant–bone interface, ultimately resulting in a dental implant that is “osseointegrated” with the alveolar bone. The osseointegration process can be divided into four stages. The first stage occurs immediately after implant placement and involves blood clot formation and the migration of platelets and inflammatory cells. The second stage involves the recruitment of neutrophils and macrophages to remove debris, bacteria, and damaged tissue. During the third stage, osteoblast progenitors migrate and differentiate into mature OBs in order to gradually form a mineralized matrix, thus bridging the implant and the bone. In the last stage, the newly formed bone undergoes remodeling, which involves the removal of old and damaged bone and its replacement with new mature bone.⁶

Several surface characteristics have been shown to influence the behavior of osteoblasts in contact with titanium and zirconia implants.^{7–9} Zirconia surfaces provide a suitable substrate for the proliferation of osteoblasts,¹⁰ exhibiting faster osseointegration than untreated surfaces.² In addition, they achieve good bone stability with no statistical differences in histological performance between zirconia and titanium implants.¹¹ To improve osseointegration of zirconia dental implants without impairing the original mechanical strength by crack initiation and partial phase transformation from tetragonal to monoclinic, roughening of the zirconia surface by sandblasting before the final sintering step was reported. The study provides evidence that roughening zirconia implants by sandblasting before sintering shows potential to improve the clinical performance of ceramic dental implants.¹² Smooth zirconia surfaces with a high tetragonal phase ratio provide the best surface conditions for interaction with osteoblasts.¹³ Cell adhesion generally increases on rough surfaces and depends on the cell type.³

The aim of the present study was to evaluate the bone biocompatibility of titanium and zirconia implants, using murine osteoblast cell cultures. For this purpose, implants with different surface treatments were employed to study the cellular events involved in the osseointegration process.

Methods

Materials

Phenol-red-free Dulbecco's Modified Eagle Medium (DMEM), penicillin-streptomycin, amphotericin B, trypsin/EDTA, trypan blue, Sirius red, and alizarin red S were purchased from Sigma Chemical Company (St Louis, MO, USA). Fetal bovine serum (FBS) was obtained from Natocor (Villa Carlos Paz, Córdoba, Argentina). Alkaline phosphatase (ALP) assay kit was purchased from Wiener

Lab Group (Rosario, Argentina). Dental implants were purchased from Straumann (Basel, Switzerland) and Tree-Oss (Buenos Aires, Argentina). Three commercially available dental implant types were evaluated according to their predominant material composition: Ti, Ti/Zr, and ZrO₂. All implants were 4 mm in diameter and 10 mm in length.

Animals

Neonatal (5-day-old) Wistar rats were employed. Animals had free access to standard laboratory chow and water. They were maintained on a 12-hour light/12-hour dark cycle. All procedures involving animals and their care were conducted at the Unit of Animal Care belonging to the Institution in accordance with the guidelines published in the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. The experimental protocol was reviewed and approved by the Animal Care and Use Committee of this Unit.

Osteoblast Cell Culture

Pre-OBs were obtained from 5-day-old neonatal Wistar rats as previously described.¹⁴ Briefly, calvarias were incubated at 37°C in PBS containing 4-mM EDTA for 10 min. The culture medium was discarded, and subsequently, the calvarias were rinsed in PBS and digested in PBS containing 200 IU/mL collagenase (type V) for 15 min. Cells released during the first digestion were discarded, while those released during the subsequent digestions were spun down and collected after centrifugation (300 × g for 10 min). These isolated cells were then cultured at 37°C in a 5% CO₂ atmosphere in DMEM supplemented with 15% FBS and antimicrobials (60 µg/mL penicillin, 10 µg/mL streptomycin, and 2.5 µg/mL amphotericin B) until 80% confluence. To induce cellular differentiation, the medium was replaced with fresh DMEM supplemented with 10% FBS, 0.05-g/L ascorbic acid, 10-mM β-glycerophosphate, and antimicrobials. Only cells up to passage 3 were used for all experiments.

Osteoblast Viability Assay

To determine cell viability, the trypan blue exclusion staining technique was used.¹⁵ The implants were placed separately in 24-well culture plates (NUNC). Then, a suspension of 3.5×10^4 cells/well in DMEM supplemented with 15% FBS and antimicrobials was added to each well. The plates were then incubated at 37°C in a 5% CO₂ atmosphere. After 72 hours of incubation, the implants were removed, and both the cells adhered to the implants and the cells in the monolayer were trypsinized. The cell suspension and the 0.4% w/v trypan blue solution were mixed at a 1:1 ratio. The cell count was performed within 3 minutes in a Neubauer chamber, and the percentage of viability was calculated.

Osteoblast Adhesion Assay

The total number of cells adhering to the implants was quantified. Briefly, the implants were placed in 24-well

culture plates (one device per well), and OB suspensions (3.5×10^4 cells/well in DMEM) were seeded into each well. After 72 hours of co-culture, the implants were removed, washed with PBS, and the cells attached to the implant surface were released using trypsin solution. The number of adhering cells was determined using a hematology analyzer (Mindray BC-5150). The results were expressed as cells/cm².

SEM Analysis

Cells adhering to the implants were evaluated using SEM. Briefly, OBs were cultured for 72 hours in 24-well culture plates (NUNC) at a density of 3.5×10^4 cells/well in DMEM in the presence of the implants. The implants were carefully washed with PBS, fixed in 2.5% glutaraldehyde (in PBS, pH = 7.2), and then rinsed twice with PBS. The samples were sequentially dehydrated in an increasing series of ethanol/acetone, critical point dried (Polaron E3000), and coated with gold (30 nm) in an Ion Sputter (Pelco 91000 model 3). The samples were finally examined at $\times 2,500$ magnification and photographed with a LEO Evo 40 XVP scanning microscope (Cambridge, England) at a potential of 7 kV.

Extracellular Collagen Deposition

Collagen content in OB culture matrix was determined by Sirius red staining, as previously described.¹⁴ Briefly, the cells were cultured for 11 days in 24-well culture plates (NUNC) at a density of 3.5×10^4 cells/well in the presence of the implants. The implants were removed, and the OB monolayers were fixed in Bouin's fluid (15:5:1 of picric acid, 35% formaldehyde, and glacial acetic acid). Sirius red solution (1 mg/mL) was added for 1 h under mild shaking. Subsequently, the supernatant was discarded, and the cells were washed with PBS. Representative micrographs of each condition were recorded using a digital camera (Tucsen MIchrome 6) coupled to an optical microscope (Nikon Eclipse TS100-F). For quantification, Sirius red fixed to the extracellular matrix was dissolved in 0.1-M NaOH, and the respective optical density (OD) was measured at 550 nm in a microplate reader (Biotek Synergy-HT). OD values were then normalized to the cellular protein content (Lowry method), and the results were expressed as OD/mg of protein.

Calcified Nodule Formation

Calcified nodule formation was analyzed by alizarin red S staining, as previously described.¹⁶ Briefly, the cells were cultured for 14 days in 24-well culture plates (NUNC) at a density of 3.5×10^4 cells/well in the presence of the implants. The implants were removed, and the OB monolayers were washed with PBS, fixed with 4% (w/v) paraformaldehyde for 10 min at room temperature, and subsequently washed with PBS. Then, 2% (w/v) alizarin red S solution was added for 30 min at room temperature, and the samples were washed three times with distilled water. After air-drying, representative images of each condition were recorded using a digital camera (Tucsen MIchrome 6 with Mosaic

2.3 software) coupled to an optical microscope (Nikon Eclipse TS100-F). For quantitative evaluation, alizarin red S in the calcified nodules was dissolved in 0.1-M NaOH for 24 h at room temperature, and then OD was measured at 540 nm in a microplate reader (Biotek Synergy-HT). OD was directly related to the total amount of mineralized extracellular matrix. The OD value was normalized to the cellular protein content (Lowry method), and the results were expressed as OD/mg of protein.

Alkaline Phosphatase (ALP) Activity Assay

ALP activity was quantified in cell lysates using a colorimetric endpoint assay. Briefly, the cells were cultured for 15 days in 24-well culture plates (NUNC) at a density of 3.5×10^4 cells/well in the presence of the implants. Once incubation was complete, the implants were removed from the culture plates. Afterward, the cell monolayer was washed with PBS and lysed with 100 μ L of lysis buffer (0.2% TritonX-100, 150 mM NaCl, 3 mM NaHCO₃) for 10 min at 37°C. Aliquots of cell lysate were collected, and ALP activity was measured using a commercially available kit (Wiener Lab Group, Rosario, Argentina), according to the protocol supplied by the manufacturer. ALP activity was normalized to the cellular protein content (Lowry method) in the cell lysate. The results were expressed as IU/mg of protein.

Surface Roughness Analysis

The roughness measurement was carried out using a scanning laser confocal microscope (OLYMPUS Lext 3D) with a $\times 50$ objective lens (total magnification: $\times 1070$). The lowest and highest points of each specimen were selected for three-dimensional surface reconstruction. Three-dimensional images were processed using OLYMPUS OLS4100 software. Six horizontal lines were drawn over the image, and roughness parameters were determined according to ISO4287.

Statistical Analysis

Data were presented as the mean $\pm$ standard deviation (SD) of four independent experiments ($n = 4$), each performed in quintuplicate. Differences among groups were analyzed using one-way ANOVA followed by post hoc Tukey tests. All statistical analyses were performed using IBM SPSS 23.0 (IBM Corp., Armonk, NY, USA). A two-sided P -value < 0.05 was considered statistically significant.

Results

Because implant surface topography influences osteoblast behavior, the surface roughness of each implant type was first characterized (Table 1). Among the three implant types, Ti implants exhibited the highest arithmetic mean roughness, whereas Ti/Zr implants showed the lowest values. Zirconia implants displayed intermediate roughness values (Table 1). Statistically significant differences were observed between the three implant surfaces ($P < 0.001$).

To evaluate the early osteoblast response to the different implant materials, cell adhesion was first quantified. Figure 1 shows that osteoblast adhesion was significantly

greater on zirconia implants than on Ti or Ti/Zr implants (9469 ± 1042 , 3338 ± 392 and 2155 ± 220 cells/cm², respectively; $P < 0.001$). Cell adhesion to Ti/Zr implants was also significantly lower than that observed on Ti implants ($P < 0.05$). Cell viability remained unchanged in all experimental groups. SEM analysis confirmed that adherent cells maintained the typical polygonal morphology of osteoblasts regardless of implant type (Figure 2).

Because successful osseointegration depends on osteoblast differentiation,¹⁷ alkaline phosphatase (ALP) activity, extracellular collagen deposition, and calcium nodule formation were assessed as representative markers of osteoblast maturation, differentiation, and extracellular matrix mineralization, respectively. ALP activity was measured at 15 days of implants-OB coculture. As shown in Figure 3, ZrO₂ implants did not significantly modify ALP activity compared with control cultures. However, the presence of Ti or Ti/Zr implants significantly reduced ALP activity ($P < 0.001$). Furthermore, ALP activity elicited in the presence of a Ti/Zr implant was significantly lower than that elicited by a Ti implant (60.60 ± 6.94 vs. 94.80 ± 9.10 10⁻³ IU/mg protein, respectively, $P < 0.01$).

Figure 4 shows that extracellular collagen deposition was preserved in cultures exposed to zirconia implants and did not differ from control cultures. In contrast, the presence of Ti or Ti/Zr implants significantly reduced collagen deposition (24% and 33% below the control for Ti and Ti/Zr, respectively; $P < 0.01$ and $P < 0.001$, respectively). Figure 4 also includes microphotographs of each experimental condition after Sirius red staining.

To evaluate extracellular matrix mineralization, calcification nodule deposition was analyzed (Figure 5). After 14 days of implant-OB co-cultures, the devices were removed, and the monolayers were stained with alizarin red S. No differences in extracellular matrix mineralization were detected between control cultures and those exposed to zirconia implants (17.53 ± 0.15 vs. 17.00 ± 0.62 OD units, ZrO₂ vs. C, respectively; Figure 5). On the other hand, a statistically significant reduction was observed in the mineralization of those OBs cultured in the presence of Ti or Ti/Zr implants compared to the control group (15.00 ± 0.12 or 14.63 ± 0.15 , vs. 17.00 ± 0.62 OD units, Ti or Ti/Zr vs. control, respectively; $P < 0.001$, Figure 5).

Finally, to facilitate the comparison of the different biological endpoints, all the variables were standardized and analyzed together using an integrated mathematical

model (Figure 6). This analysis showed that osteoblast responses in the zirconia group closely resembled those of the control cultures, whereas Ti and Ti/Zr implants consistently reduced all evaluated parameters. Indeed, the overall osteoblast response of OBs cultured on a Ti/ZrO₂ implant was 42% and 52% higher than that of bone cells cultured on Ti or Ti/Zr implants, respectively ($P < 0.05$). Regarding the surface roughness of dental implants, no linear association was detected between roughness and the variables analyzed ($P < 0.05$). Overall, these findings indicated that zirconia implants provided a more favorable in vitro environment for osteoblast adhesion and differentiation than titanium-based implants.

Discussion

The present study compared the in vitro osteoblast responses to commercially available titanium-, titanium-zirconium-, and zirconia-based dental implants. Our findings indicated that zirconia implants promoted more favorable osteoblast responses than titanium-based implants, as osteoblast adhesion and differentiation markers remained comparable to those of the control cultures. In contrast, titanium-based implants were associated with significant reductions in osteoblast differentiation markers under the experimental conditions used.

Osseointegration is the process of creating a strong bond between living bone and the surface of an implant.¹⁸ It is a dynamic process involving a series of cellular and molecular events that lead to a direct structural and functional connection between the implant surface and bone.¹⁹ Osteoprogenitor cells adhere to the implant surface, proliferate, and differentiate into mature OBs, which can produce a collagen-rich matrix that mineralizes and becomes new bone that bridges the implant to the skeletal tissue.

Implant surface topography plays a key role in regulating osteoblast behavior and subsequent bone formation. The number of osteoblasts adhering to the implant surface is considered a critical determinant of successful osseointegration, as micro- and nano-scale roughness increases the available surface area for cell attachment. This is essential for initiating osseointegration and improving implant stability.²⁰ In the present study, significant

Table 1. Arithmetic mean roughness of each type of implant

Predominant composition of the implant	Micrometers (mean $\pm$ SD)
Ti	3.281 ± 0.473
Ti/Zr	1.637 ± 0.206 *
ZrO ₂	2.249 ± 0.336 *, §

The roughness measurement was carried out using a scanning laser confocal microscope (OLYMPUS Lext 3D) with a $\times 50$ objective lens and a total magnification of $\times 1,070$, as described in the Methods section. Results represent the mean $\pm$ SD of six independent measurements ($n = 6$).

* $P < 0.001$ vs. Ti

§ $P < 0.001$ vs. Ti/Zr

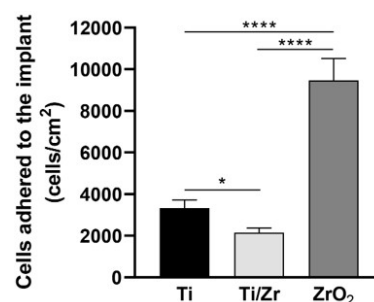


Figure 1. OB adhesion to Ti, Ti/Zr, and ZrO₂ implants. OBs were cultured for 72 h in 24-well culture plates in the presence of the implants. Cell adhesion to the implants was analyzed by cell counting as described in the Methods section. The results represent the mean $\pm$ SD of four separate experiments ($n = 4$), in which each experimental condition was performed in quintuplicate (* $P < 0.05$; **** $P < 0.001$)

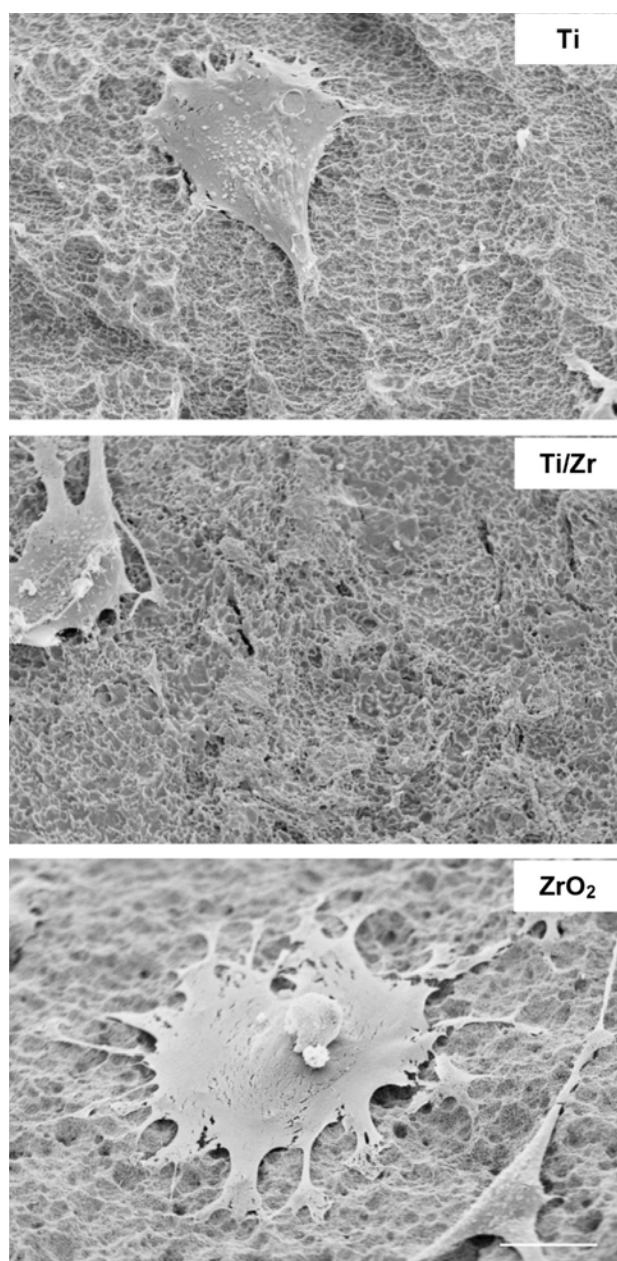


Figure 2. SEM analysis of OBs adhered to Ti, Ti/Zr, and ZrO₂ implants. Cells were cultured for 72 h in 24-well culture plates in the presence of the implants. Cell adhesion to the implants was visualized by SEM, as described in the Methods section. Images show representative fields of cells adhered to titanium and zirconia implants. Scale bar represents 20 μ m ($\times 2500$ magnification)

differences in arithmetic roughness were observed between the three implant types, with zirconia implants exhibiting intermediate roughness values between Ti and Ti/Zr devices. Despite these differences, zirconia implants showed approximately threefold greater preosteoblast adhesion than titanium-based implants. Adherent cells retained their typical polygonal morphology and osteoblastic phenotype. Similar findings have been reported for human osteosarcoma cells cultured on chemically identical but topographically distinct zirconia surfaces (Herath et al, 2015).²¹

Osteoblast differentiation is characterized by the intracellular expression of hormonal receptors and the ability to synthesize bone-specific proteins that are secreted

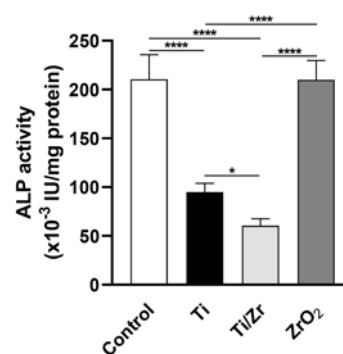


Figure 3. ALP activity in OBs cultured in the presence of Ti, Ti/Zr, and ZrO₂ implants. Cells were cultured for 15 days in 24-well culture plates in the absence (control) or presence of the implants. ALP activity in cell lysates was measured as described in the Methods section. The results represent the mean $\pm$ SD of four separate experiments ($n=4$), in which each experimental condition was performed in quintuplicate (* $P<0.01$; **** $P<0.001$)

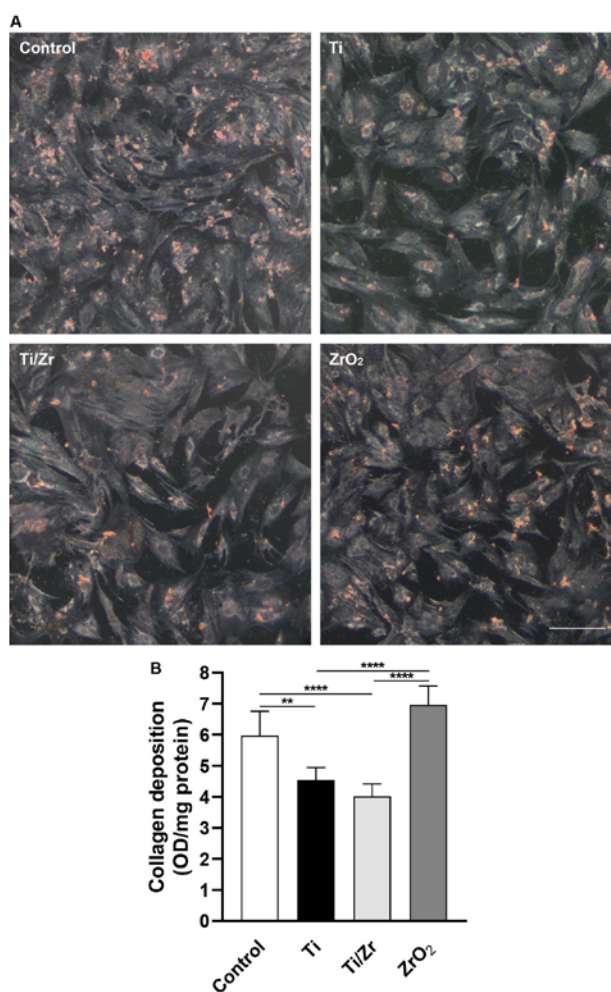


Figure 4. Extracellular collagen deposition in OB-implant co-cultures. Cells were cultured for 11 days in 24-well culture plates in the absence (control) or presence of the implants. Collagen deposition was measured as described in the Methods section. (A) Images show representative fields after Sirius Red staining. Scale bar represents 60 μ m. (B) Quantification data. The results represent the mean $\pm$ SD of four separate experiments ($n=4$), in which each experimental condition was performed in quintuplicate (** $P<0.01$; **** $P<0.001$)

into the extracellular space, where they contribute to bone matrix formation and subsequent mineralization.²² Our findings showed that the differentiation of preosteoblasts into mature osteoblasts was not affected by the presence of zirconia implants, as ALP activity, extracellular

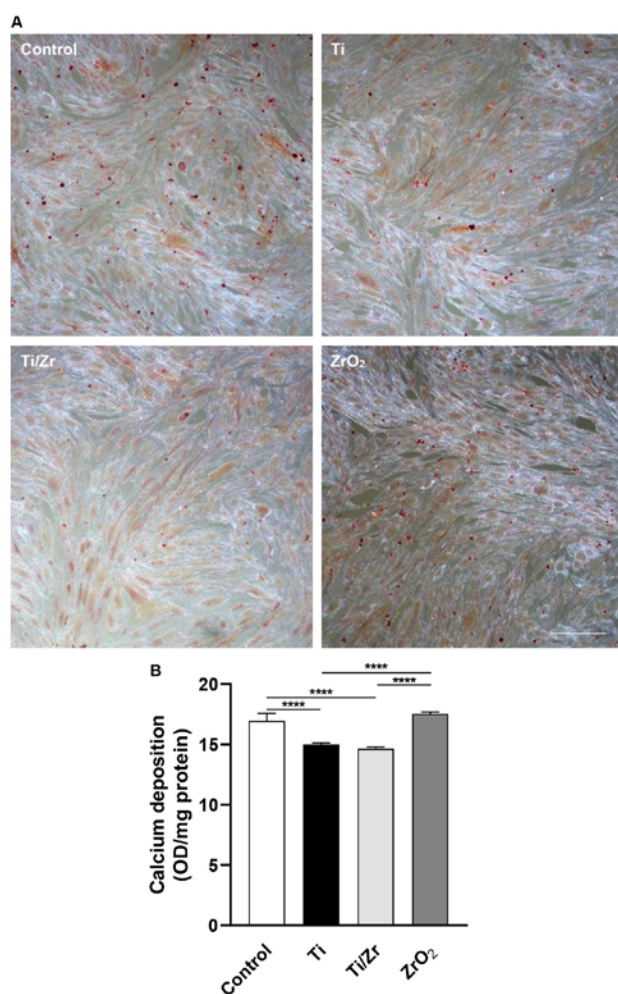


Figure 5. Calcified nodule formation in OB-implant co-cultures. Cells were cultured for 14 days in 24-well culture plates in the absence (control) or presence of the implants. Calcified nodule formation was measured as described in the Methods section. (A) Images show representative fields after alizarin red-S staining. Scale bar represents 70 μ m. (B) Quantification data of calcified nodules after alizarin red-S staining. The results represent the mean $\pm$ SD of four separate experiments ($n=4$), in which each experimental condition was performed in quintuplicate (**** $P<0.001$)

collagen deposition, and matrix mineralization remained comparable to those of the control cultures. Similar observations have been reported in primary bovine osteoblasts cultured on zirconia surfaces, which exhibited attachment, proliferation, and differentiation comparable to those observed on titanium implants in both in vitro and in vivo animal models.^{23,24} Furthermore, under bone morphogenetic protein stimulation, zirconia promoted greater proliferation and differentiation of MC3T3 cells than titanium.²⁵

Titanium remains the gold standard for manufacturing dental implants, whereas zirconia has emerged as a promising alternative because of its favorable biological properties. Nevertheless, the long-term clinical performance and mechanical reliability of zirconia implants require further investigation.¹ Therefore, although the present findings suggest more favorable osteoblast responses to zirconia under the experimental conditions used, additional in vivo and clinical studies are needed before these observations can be extrapolated to clinical practice.

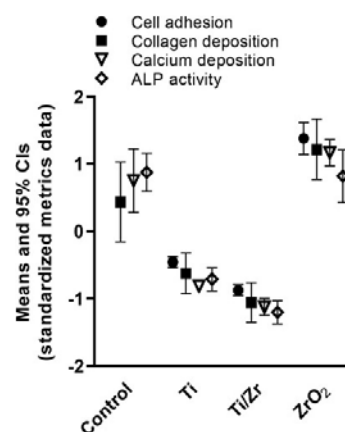


Figure 6. Model output of standardized variable data. The data show the means and 95% confidence intervals (CIs) for the variables evaluated across the experimental conditions. The data were standardized prior to analysis using each variable's global mean and its standard deviation

Conclusion

Under the experimental conditions of this in vitro study, zirconia implants promoted osteoblast adhesion and maintained osteoblast differentiation markers more effectively than titanium-based implants. These findings suggest that zirconia may provide a favorable surface for early cellular events associated with osseointegration. However, this study has some limitations that should be considered when interpreting the results. First, the experiments were performed using an in vitro murine culture model, which cannot fully reproduce the complex biological environment of peri-implant bone healing in vivo. Second, only early osteoblast adhesion and differentiation markers were evaluated, whereas other biological processes involved in osseointegration, such as immune responses, angiogenesis, mechanical loading, and long-term bone remodeling, were beyond the scope of this work. In addition, only three commercially available implant types were analyzed, and differences in surface characteristics or manufacturing processes may also have influenced cell behavior. Therefore, although the findings suggest favorable osteoblast responses to zirconia implants under the experimental conditions used, further in vivo and clinical studies are required to determine their clinical relevance.

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Not applicable.

Authors' Contribution

Conceptualization: Pablo Hernán Cutini, Germán Mirotti
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Competing Interests

The authors declare no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

Ethical Approval

All the procedures involving animals were carried out in accordance with the guidelines published in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The Institutional Animal Care and Use Committee of the Department of Biology, Biochemistry and Pharmacy (Universidad Nacional del Sur, Bahía Blanca, Buenos Aires, Argentina), reviewed and approved the protocol used in this study. Protocol ID: 175/2021.

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