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Bone Biocompatibility of Titanium and Zirconia Implants: An In Vitro Study

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Abstract

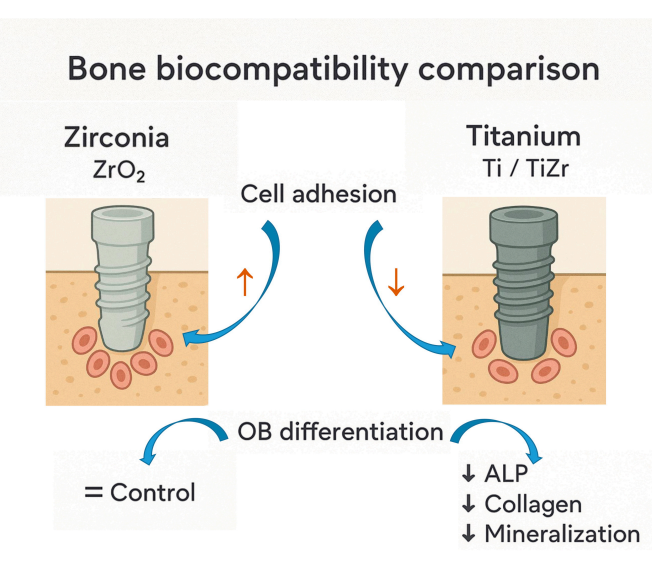
Background: Oral implants have become a viable option for the replacement of missing teeth. The success of oral implantation depends on the biocompatibility of the material with the surrounding bone, and on the effective osseointegration process, which generates a strong bond between the living bone and the implant. The aim of the present work was to evaluate the bone biocompatibility of titanium and zirconia implants, with a focus on the cellular events involved in the osseointegration process.

Materials and Methods: Murine preosteoblast (OB) cells cultured in the presence of the implants were used. Three types of dental implants were used: grade 4 titanium (Ti), 85% titanium/15% zirconium (Ti/Zr), and ceramic implants (ZrO_2). Alkaline phosphatase activity, extracellular collagen deposition, and matrix mineralization were measured as OB differentiation markers. OB adhesion to implants assays and scanning electron microscopy (SEM) analysis were carried out.

Results: The number of OBs adhered to ZrO_2 implant was significantly higher than that adhered to the Ti or Ti/Zr implants. SEM analysis showed that cell morphology was preserved among the OBs adhered to each device. The presence of ZrO_2 implants did not modify the OB differentiation markers evaluated compared to the control group, while Ti or Ti/Zr induced a one- to two-fold reduction in all bone markers studied.

Conclusion: In summary, zirconia implants exhibited better bone biocompatibility than titanium ones, as OB behavior was not altered compared to the control group. In contrast, the presence of titanium implants significantly reduced the ability of pre-OBs to differentiate into bone-forming cells.

Keywords: cell differentiation; implants; osteoblasts; titanium; zirconia



Graphical Abstract

Introduction

Oral implants have become a reliable treatment option for the replacement of missing teeth. An ideal implant material should be biocompatible, possess adequate toughness, and also have wear and fracture resistance [1]. Currently, metal-free treatment is a successful and predominant alternative in dentistry due to the aesthetic problems that metal restorations can produce. Zirconia-based implants were introduced to implant dentistry as an alternative to titanium implants. The current challenge in dental implantology is to develop a zirconia-based framework or surface treatment with bioactive, mechanical, and osteogenic properties that guarantees favorable early and long-term clinical results [2]. Due to its mechanical and aesthetic performance, and biocompatibility, zirconia has been applied to both dental implants and fixed dental prostheses. They offer an advantage over titanium implants, since there is no risk of discoloration or allergic hypersensitivity reactions [3]. Zirconia, particularly tetragonal polycrystalline zirconia, exhibits suitable biomechanical behavior when it is employed in dental implants. Recently, it was proposed that zirconia implants are a promising alternative to titanium with comparable osseointegration with soft tissue. Nevertheless, titanium tends to show a faster initial osseointegration process [4,5]. 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 physicochemistry of the surface, which are key factors for the osseointegration process at the bone tissue/implant interface [3].

An appropriate oral implant outcome depends on successful overlapping phases of tissue healing and regeneration at the implant-bone interface, to finally get 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. In 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 parameters have been shown to influence the behavior of osteoblasts in contact with titanium and zirconia implants [7-9]. Zirconia-treated surfaces are an appropriate substrate for the proliferation of osteoblastic cells [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 osteoblast cells [13]. Cell adhesion is higher on rough surfaces and depends on the cell type [3].

The aim of the present work was to evaluate the bone biocompatibility of titanium and zirconia implants, using murine osteoblast cell cultures. To that end, implants that possess different surface treatments were employed to study the cellular events involved in the osseointegration process.

Materials and Methods

Phenol-red free Dulbecco's modified Eagle's media (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 types of dental implants were used, based on their material composition: Ti (Tree-Oss® Anatomic, Tree-Oss, Buenos Aires, Argentina), Ti/Zr (Straumann® BLT, Straumann AG, Basel, Switzerland) and ZrO2 (Tree-Oss® CERAMIC, Tree-Oss, Buenos Aires, Argentina, and Straumann® PURE Ceramic, Straumann AG, Basel, Switzerland). All implants were 4 millimeters in diameter and 10 millimeters in length.

Animals

Neonatal (5-day old) Wistar rats were employed. The animals were provided with standard rat food, given water, and allowed to drink ad libitum. They were maintained on a 12-hour light/12-hour darkness 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 protocol used in this work 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 cells were then cultured at 37°C in a 5% CO_2 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. Cells up to passage 3 were used for all experiments.

Osteoblasts Cell Viability

In order to determine cell viability, the trypan blue exclusion staining technique was used [15]. The implants were placed separately in a 24-multiwell culture plate (NUNC). Subsequently, a suspension of 3.5×10^4 cells/well in DMEM supplemented

with 15% FBS and antimicrobials was added to each well. The plate was then incubated at 37°C in a 5% CO_2 atmosphere. After 72 hours of culture, 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 ratio of 1:1. The cell count was performed within 3 minutes in a Neubauer chamber, and the percentage of viability was calculated.

Osteoblasts Adhesion Assay

The total number of cells adhered to the implants was quantified. Briefly, the implants were placed in a 24-multiwell culture plate (one device per well), and OBs suspensions (3.5×10^4 cells/well in DMEM) were seeded into each well. After 72 hours of co-culture, implants were removed, washed with PBS, and the cells adhered to the devices were released using trypsin solution. The number of adhered cells was determined using a hematology analyzer (Mindray BC-5150). The results were expressed as the number of cells/cm^2.

SEM Analysis

Cells adhered to the implants were evaluated using SEM. Briefly, OBs were cultured for 72 hours into a 24-multiwell culture plate (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. Samples were then 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 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, cells were cultured for 11 days in a 24-multiwell culture plate (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: 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 images 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, cells were cultured for 14 days in a 24-multiwell culture plate (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 washed thrice 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, cells were cultured for 15 days in a 24--multiwell culture plate (NUNC) at a density of 3.5×10^4 cells/well in the presence of the implants. Once the incubation step was completed, implants were removed from the culture plate. Afterwards, the cell monolayer was washed with PBS and lysed with 100 µL of lysis buffer (0.2% TritonX-100, 150 mM NaCl, 3 mM $NaHCO_3$) for 10 min at 37°C. Aliquots of cell lysate were collected and ALP activity was measured employing 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.

Implant Roughness Measurement

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

Statistical Analysis

Results are expressed as mean ± standard deviation (SD) of four independent experiments (n=4) where each experimental condition was carried out in quintuplicate. Comparisons between means were made using one-way ANOVA followed by Tukey post hoc test. All statistical analyses were performed using IBM SPSS Statistics software for Windows, Version 23.0 (IBM Corp., Armonk, NY, USA). All p-values below 0.05 were considered statistically significant.

Results

The zirconia implants selected for this study possess different surface treatments; for example, pure zirconia stabilized with yttria through a process based on ultra-high pressure CIM technology. The arithmetic mean roughness obtained in these implants reached values of 2.249 ± 0.336 micrometers (Table 1). Meanwhile, when titanium was the predominant composition of the implants, the roughness showed extreme values in comparison with the ceramic ones (3.281 ± 0.473 and 1.637 ± 0.206 µm, Ti/Zr and Ti respectively, P<0.001, Table 1).

Table 1: Arithmetic mean roughness of each type of implant

Predominant composition of the implant	Micrometers (mean ± SD)
Ti/Zr	3.281 ± 0.473
Ti	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 50× objective lens and a total magnification of 1,070×, as described in the Methods section. Results represent the mean ± SD of six independent measurements (n=6). ** P=0.003 vs Ti/Zr. **** P<0.001 vs Ti.

To assess the biocompatibility of these devices with bone cells, OB adhesion to Ti, Ti/Zr and ZrO_2 implants was first evaluated by cell counting. Fig. 1 shows that the number of OBs adhered to ZrO_2 implant was significantly higher than that of the Ti or Ti/Zr devices (9469 ± 1042, 3338 ± 392, 2155 ± 220 cells/cm^2; ZrO_2, Ti/Zr, Ti respectively, P<0.001).

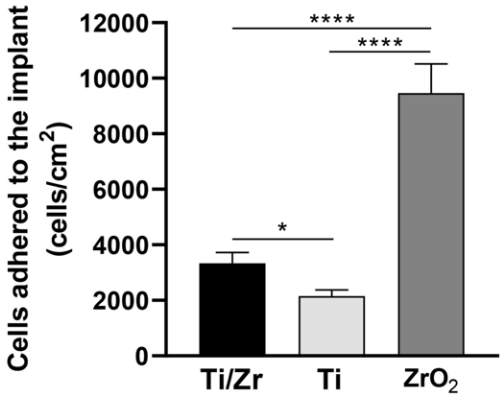


Figure 1: OB adhesion to Ti/Zr, Ti, and ZrO_2 implants. OBs were cultured for 72 h in a 24multiwell culture plate in the presence of the implants. Cell adhesion to the implants was analyzed by cell counting as described in the Methods section. Results represent the mean ± SD of four separate experiments (n=4) in which each experimental condition was performed in quintuplicate. *P=0.036; ****P<0.001.

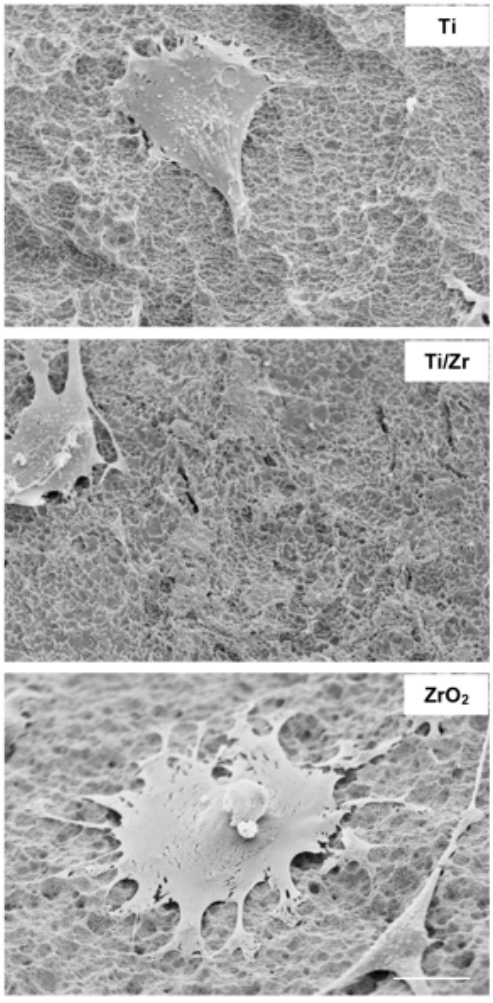


Figure 2: SEM analysis of OBs adhered to Ti, Ti/Zr, and ZrO_2 implants. Cells were cultured for 72 h in a 24-multiwell culture plate in the presence of the implants. Cell adhesion to the implants was visualized by SEM, as described in the Methods section. Images show representative fields of cells adhered to titanium and zirconia implants. Scale bar represents 20 μm (2500× magnification).

The data obtained also revealed that cell adhesion to Ti/Zr implants was slightly lower compared to that of Ti alone (P=0.036). There were no differences in the cell viability of the OBs cocultured with each device. SEM showed that the adhered cells exhibited typical OB-like shape and that the morphology was preserved among OBs adhered to each device. Fig. 2 shows SEM images of OBs adhered to each type of implant.

Since osseointegration depends on the differentiation of the osteoprogenitor cells adhered to the implant into mature OBs [17], the effect of the devices on pre-OB differentiation was analyzed. To that end, ALP activity, extracellular collagen deposition, and calcium nodule formation were selected as markers for maturation, differentiation, and mineralization, respectively. ALP activity was measured at 15 days of implants-OB coculture. As can be seen in Fig. 3, ZrO_2 did not alter ALP activity compared to control cells. However, the presence of Ti or Ti/Zr implants induced a twofold reduction (P<0.001) in the enzyme activity. Furthermore, the ALP activity elicited in the presence of Ti/Zr implant was significantly lower compared to the Ti implant (60,60± 6.94, vs. 94.80 ±9.10 10-3 IU/mg protein respectively, P=0.002).

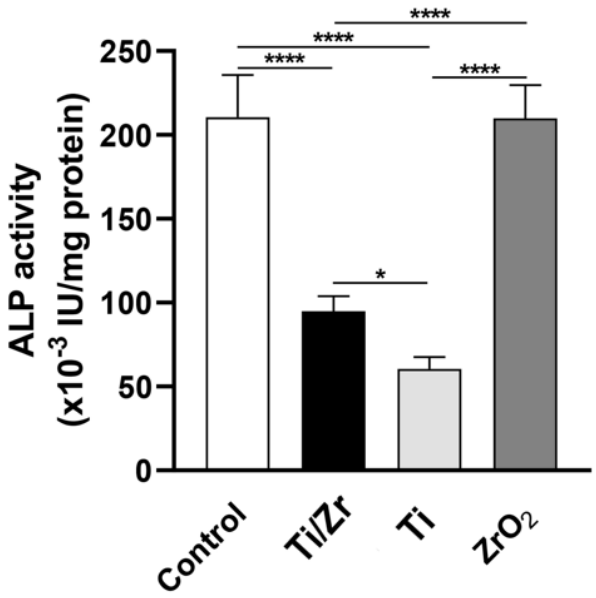


Figure 3: ALP activity in OBs cultured in the presence of Ti/Zr, Ti, and ZrO_2 implants. Cells were cultured for 15 days in a 24-multiwell culture plate in the absence (control) or presence of the implants. ALP activity in cell lysates was measured as described in the Methods section. Results represent the mean ± SD of four separate experiments (n=4) in which each experimental condition was performed in quintuplicate. *P=0.002; ****P<0.001.

Fig. 4 shows the results of extracellular collagen content determination. ZrO_2 implants did not affect the ability of OBs to secrete collagen into the bone matrix. No significant differences were detected between the control and the ZrO_2 experimental group. Nevertheless, 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=0.005 and P<0.001 respectively). Fig. 4 also includes microphotographs of each experimental condition after Sirius red staining.

To evaluate extracellular matrix mineralization, the deposition of calcification nodules was analyzed (Fig. 5). After 14 days of implant-OB co-cultures, the devices were removed and the monolayers were stained with alizarin red-S. The presence of ZrO_2 implants did not modify the OB's capability to mineralize (17.53 ± 0.15 vs. 17.00 ± 0.62 OD units, ZrO_2 vs. C, respectively; Fig. 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 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, Fig. 5).

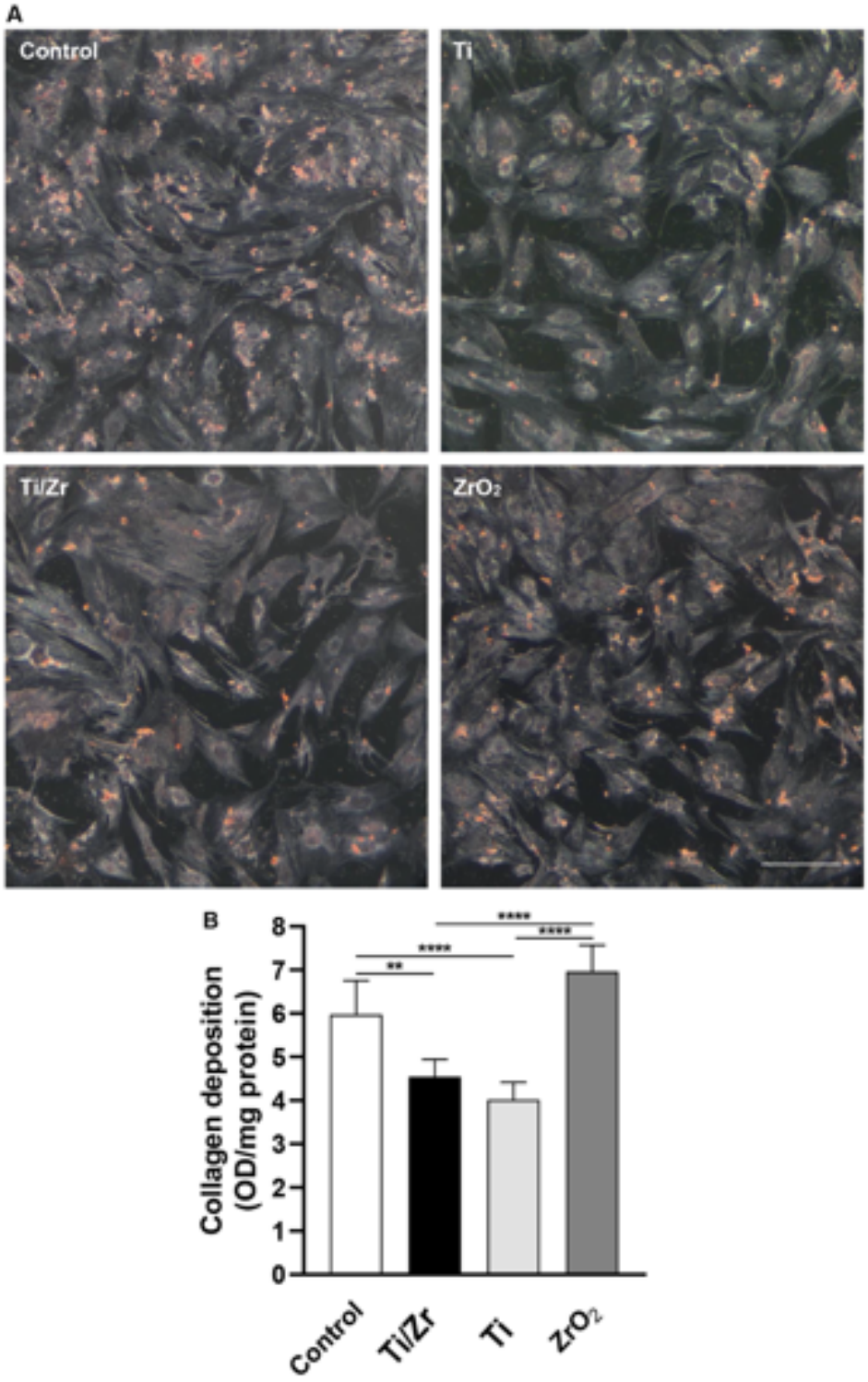


Figure 4: Extracellular collagen deposition in OB-implant co-cultures. Cells were cultured for 11 days in a 24-multiwell culture plate in the absence (control) or presence of the implants. Collagen deposition was measured as described in the Methods section. (A) Images show representative fields after Sirius Red staining. Scale bar represents 60 μm. (B) Quantification data. Results represent the mean ± SD of four separate experiments (n=4) in which each experimental condition was performed in quintuplicate. $^{**}P$=0.005; $^{****}P$<0.001

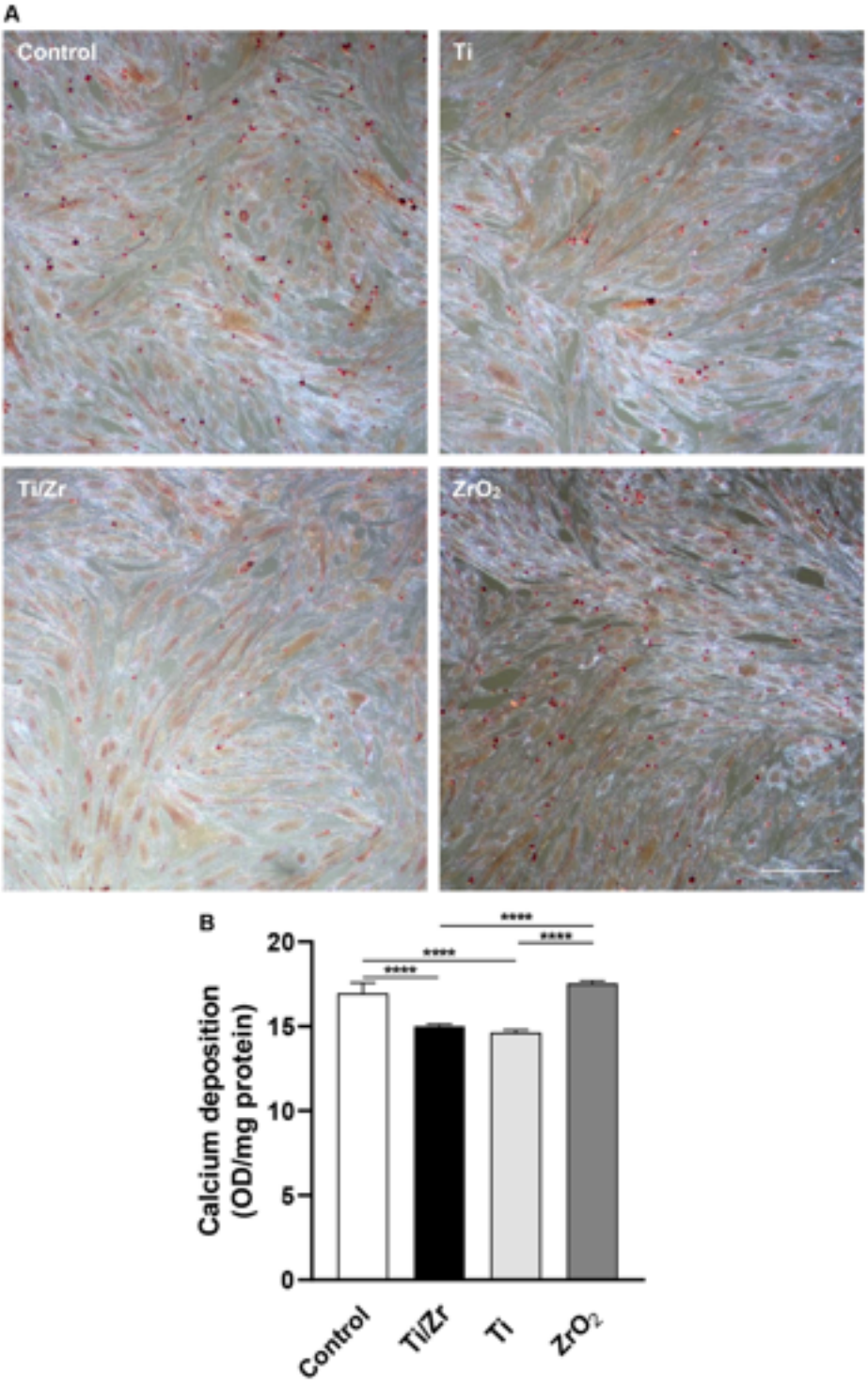


Figure 5: Calcified nodule formation in OB-implant co-cultures. Cells were cultured for 14 days in a 24-multiwell culture plate in the absence (control) or presence of the implants. Calcified nodule formation was measured as described in the Methods section. (A) Images show representative fields after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

Finally, the integrated analysis of the performance of all studied osteoblast variables, grouped by material using mathematical models, is presented in Fig. 6.

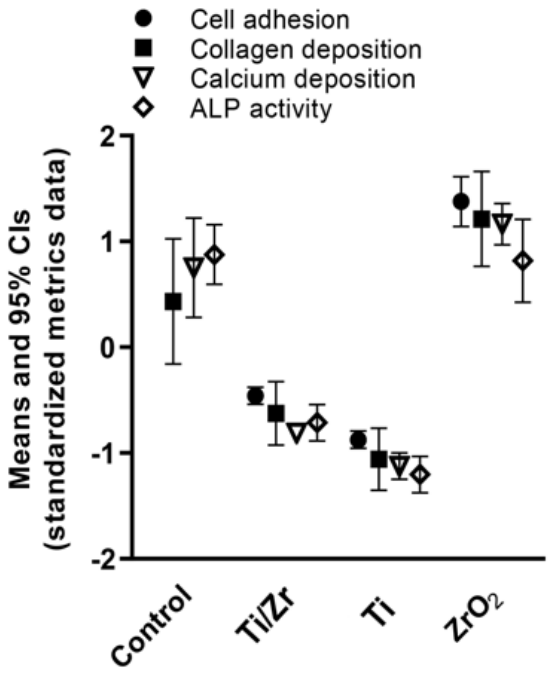


Figure 6: Model output of standardized variable data. The data show the means and 95% confidence intervals (CIs) for the variables evaluated across the experimental conditions. The data were standardized prior to analysis using each variable's global mean and its standard deviation.

In order to plot the four variables together, they were standardized to the same scale by subtracting the mean from each observation and dividing by the standard deviation. Thus, the four standardized variables had a mean of 0 and a SD of 1. Regarding the whole OB markers evaluated in this study, the mathematical model shows that OBs in contact with ZrO_2 implants exhibited similar behavior to control cells. In contrast, OBs cultured in the presence of Ti or Ti/Zr devices displayed significant reduction in all parameters compared to control cells. Indeed, it was observed that the performance of OBs cultured on Ti/ZrO_2 implant was 42% and 52% higher than that of bone cells cultured on Ti or Ti/Zr implants, respectively (P=0.055). Regarding the surface roughness of dental implants, no linear association was detected between roughness and the variables analyzed (P<0.05). Overall, the analysis suggests that, ZrO_2 implants exhibited better features for osseointegration.

Discussion

The present study provides evidence that zirconia implants exhibit better bone biocompatibility than titanium ones, since OB behavior was not altered compared to the control group. In contrast, the presence of titanium implants significantly reduced the ability of pre-OBs to differentiate into bone-forming cells.

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

The surface topography of the implant alters adherent cellular responses, leading to greater bone formation. The number of attached osteoblasts is crucial. Micro- and nano-scale roughness increases the available osteoblast attachment sites, leading to more osteoblasts attaching to the implant. This is essential for initiating osseointegration and improving implant stability and

successful integration [20]. Our results demonstrated that significant differences in arithmetic roughness were detected between zirconia and titanium devices, and that zirconia implants exhibited a 3-fold increase in adhered pre-OBs. The adhered cells retained their typical polygonal shape and morphology, and exhibited an osteoblastic-like phenotype. Similar results were reported using human osteosarcoma cell lines and different zirconia surfaces, which were chemically identical but topologically different [21].

OB differentiation involves the intracellular expression of hormonal receptors as well as the capability to synthesize specific bone proteins that are released into the extracellular space to form the bone matrix and allow for its subsequent mineralization [22]. Here, it was shown that the differentiation process of pre-OBs into OBs was not altered by the presence of zirconia implants. ALP activity, extracellular collagen deposition, and matrix mineralization were sustained in concomitant cultures of OBs and zirconia devices. Other authors have reported that primary bovine osteoblasts were able to attach, proliferate, and differentiate on zirconia surfaces in both in vitro and in vivo animal models, exhibiting biocompatibility similar to that of titanium implants [23,24]. Furthermore, under bone morphogenic protein stimulation, zirconia had a more pronounced effect on the proliferation and differentiation of the MC3T3 cell line compared with titanium [25].

To date, it has been stated that titanium remains the gold standard for manufacturing oral implants, and zirconia implants hold promise for the future. However, the incidence of zirconia-induced mechanical failures cannot be ruled out [1], and further in vitro and in vivo clinical studies are necessary.

Conclusion

In summary, under the conditions studied in this work, a better bone biocompatibility of zirconia compared to titanium is suggested, which could represent a benefit for the osseointegration process. However, additional investigations are required to fully comprehend the clinical relevance of these data.

Authors' Contributions

Conceptualization: Germán Mirotti, Pablo Hernán Cutini

Data curation: Marisa Julia Sandoval

Formal analysis: Pablo Hernán Cutini

Investigation: Germán Mirotti, Pablo Hernán Cutini

Methodology: Germán Mirotti, Pablo Hernán Cutini, Juan Carlos Ibañez

Resources: Germán Mirotti

Supervision: Marisa Julia Sandoval

Validation: Marisa Julia Sandoval

Writing–original draft: Mario Sezin

Funding acquisition: Juan Carlos Ibañez, Virginia Laura Massheimer

Writing–review & editing: Juan Carlos Ibañez, Virginia Laura Massheimer

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Competing Interests

The authors declare that they have 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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