Biological Response of Soft Tissues to Three Abutment Materials Titanium, Zirconia, and Lithium Disilicate. An In Vitro Comparative Study

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TO CITE THIS ARTICLE
Nicolini M, Galdoporpora JM, Canellada A, Macchi R, Friedmann SM, Calvo Guirado JL, Ibanez JC. Biological Response of Soft Tissues to Three Abutment Materials Titanium, Zirconia, and Lithium Disilicate. An In Vitro Comparative Study.J Osseointegr 2024; 16(1): 72-80.

DOI 10.23805/JO.2024.624

KEYWORDS CAD/CAM, Titanium, Lithium Disilicate, Zirconia, Fibroblasts, Cell viability, X-ray fluorescence, Raman spectroscopy.

ABSTRACT

Aim The implant-abutment/emergence connection, and therefore the relationship with soft and hard tissues, is an important factor that determines the long-term success of dental implants in clinical practice. The purpose of this study was to compare the biological response of murine fibroblasts L929 when exposed to three materials: Titanium, Zirconia, and Lithium Disilicate (DSL), used as implant abutment materials.

Materials and methods Samples of titanium, Zirconia, and DSL were obtained. Prior to material characterization by X-ray fluorescence and Raman spectroscopy, the samples were sterilized in a steam autoclave at a temperature of 121 °C for 30 minutes. Murine fibroblasts L929 were seeded for cell viability measurement. The metabolic activity was measured at 24 hours and 48 hours of culture using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The results were analyzed using SPSS v. 20.0.

Results At 24 hours, an increase in viability was observed, although there was no significant difference among the three studied materials (p=0.564). At 48 hours vs. 24 hours, DSL showed the highest degree of cell viability (p=0.0003), followed by Zirconia (p=0.027), and then Titanium (p=0.056). The cell viability values for titanium, Zirconia, and DSL were 116%, 132%, and 172%, respectively. There was no evidence of cytotoxicity.

Conclusion Cell viability in response to the studied implant abutment materials could anticipate the biological response, the stability of the different materials in relation to soft tissues, and their connection. Other factors such as biomechanics and bacterial adhesion should be considered when choosing a material.

INTRODUCTION

Tissue integration post-implantation depends not only on the bone but also, and equally importantly, on the integration of the soft tissues where the prosthetic phase of the implants emerges. It is important that the cells receiving the implant allow for the appropriate genotypic expression of their functions and the expression of the biological principles that govern the interaction of cells and their molecular signals. The predictability of the outcome depends on the balance between peri-implant bone tissue and the behavior of the surrounding soft tissues.

Prosthetic abutments serve as intermediaries used as connecting structures between the implant and the future fixed prosthesis.

It should be noted that the development of prosthetic components in implantology has increased treatment possibilities, demanding not only function but also aesthetics. However, to achieve these results, it is important to consider that the function of the abutments has evolved from being a simple connection between the implant and the prosthesis to being a fundamental determinant in the final aesthetic and functional achievement of the rehabilitative treatment (1). Currently, there is a great dilemma regarding the selection of abutments, given the wide availability in the market and considering that the choice must provide the patient with the implant-supported restoration that best fits their case, both functionally and aesthetically. It should be mentioned that clinical complications have emerged, such as screw loosening,

abutment or implant fractures, and marginal bone loss due to overload and bacterial microleakage. These consequences are due to poor fit at the prosthesis or its components. Indeed, the ideal connection should function as a one-piece implant, avoiding the formation of a micro-gap at the implant-abutment interface (2). The nature of the abutment material is an important factor that affects the stability of the peri-implant mucosa and crestal bone (3-5). It has been shown that the abutment material is responsible for reducing crestal bone loss and soft tissue recession. Moreover, the composition of the abutments on implants is a key factor in the long-term survival and success of restorations (2,6). However, comparative studies regarding the biological behaviour of different abutments, especially those materials commonly used in clinical practice such as Titanium, Lithium Disilicate, and Zirconia, are scarce (7). The aim of this study was to evaluate the biological response of soft tissues to three emerging implant materials: Titanium, Zirconia, and Lithium Disilicate.

MATERIALS AND METHODS

Material characterization by X-ray fluorescence and Raman spectroscopy

X-Ray Fluorescence

X-ray fluorescence determinations were obtained using an R-XAS looper (Rigaku) equipment with a silicon detector at the Institute of Theoretical and Applied Physicochemical Research. Incident beams of 19 keV and 7 keV with a slit size of 10 mm x 0.4 mm were used. The samples were measured with the detector placed at a 90° angle to the incident radiation, and the sample was positioned with one of its faces at a 45° angle between the incident beam and the detector. Energy calibration was performed using characteristic fluorescence lines from various metal patterns (Si, Ti, and Zr).

Raman Spectroscopy

Raman spectroscopy spectra were obtained using a Nicolet iS50 spectrometer at the Institute of Theoretical and Applied Physicochemical Research. Measurements were made using a 1064 nm excitation laser and a CaF2 filter for near-infrared. The spectra were obtained with a resolution of 4 cm-1 and 200 acquisitions.

Surface Roughness characterization (Ra) by confocal laser scanning microscopy

Traces were made on the unprocessed samples in six equidistant sectors for statistical comparison. Surface roughness (Ra) was determined using an Olympus OLS4000 LEXT confocal laser scanning microscope (Lext 3D Measuring laser microscope OLS4000). An UV laser light with a wavelength of 760 nm and a 50x objective, equivalent to a magnification of 1070x, was used for Ra measurement.

Cell proliferation on titanium surface compared to CAD/CAM systems (zirconia and LD2)

Cell Culture Preparation

The cell culture was performed at the Immunology Department of the Faculty of Pharmacy and Biochemistry, University of Buenos Aires. Murine fibroblasts L929 were seeded at a density of 1 x 104 cells per well, and the different materials were placed after adding 0.5 ml of DMEM. Metabolic activity was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay at 24 hours and 48 hours of culture. The method involved replacing a portion of the culture medium with 0.05 ml of a 0.5 mg/ml MTT solution in the culture medium. The samples were then incubated in a humid chamber with 5% carbon dioxide for 4 hours. Afterwards, the MTT solutions were removed, and 0.5 ml of absolute ethanol was added. The mixture was incubated at room temperature for 30 minutes. The solution was then centrifuged, and the absorbance of the ethanol supernatant, where the colour extraction occurred, was measured at 540 nm. A positive control was performed where cells were seeded on the plate surface without any biomaterial (baseline). The blank was done with a culture medium in the absence of cells. In all cases, the results were expressed as the mean ± SD of duplicate experiments as a percentage of viability compared to the positive control baseline at 24-hours.

Cell Viability Assay (Metabolic Activity) by MTT Reduction

MTT is a water-soluble compound belonging to the tetrazolium salt family and has a yellow colour.
When reduced, MTT is converted into a compound from the formazan family, which has a violet colour and is insoluble in water. To quantify it, it is usually dissolved in an organic solvent such as DMSO (dimethyl sulfoxide), and its colour (absorbance) is measured using a spectrophotometer at a wavelength of 570 nm. Cellular metabolic activity includes that of dehydrogenases. The action on MTT is mainly attributed to mitochondrial dehydrogenases, particularly succinate dehydrogenase, but cytosolic reductases or reductases from other subcellular compartments may also be involved. The resulting reduced coenzymes (NADH and NADPH) convert MTT to its formazan.

Procedure

This in vitro viability assay measures mitochondrial metabolism as an indirect measure of cell proliferation. To determine the viability of the Titanium, Zirconia, and Lithium Disilicate biomaterials, they were exposed to L929 murine fibroblasts for 24 hours and 48 hours. The

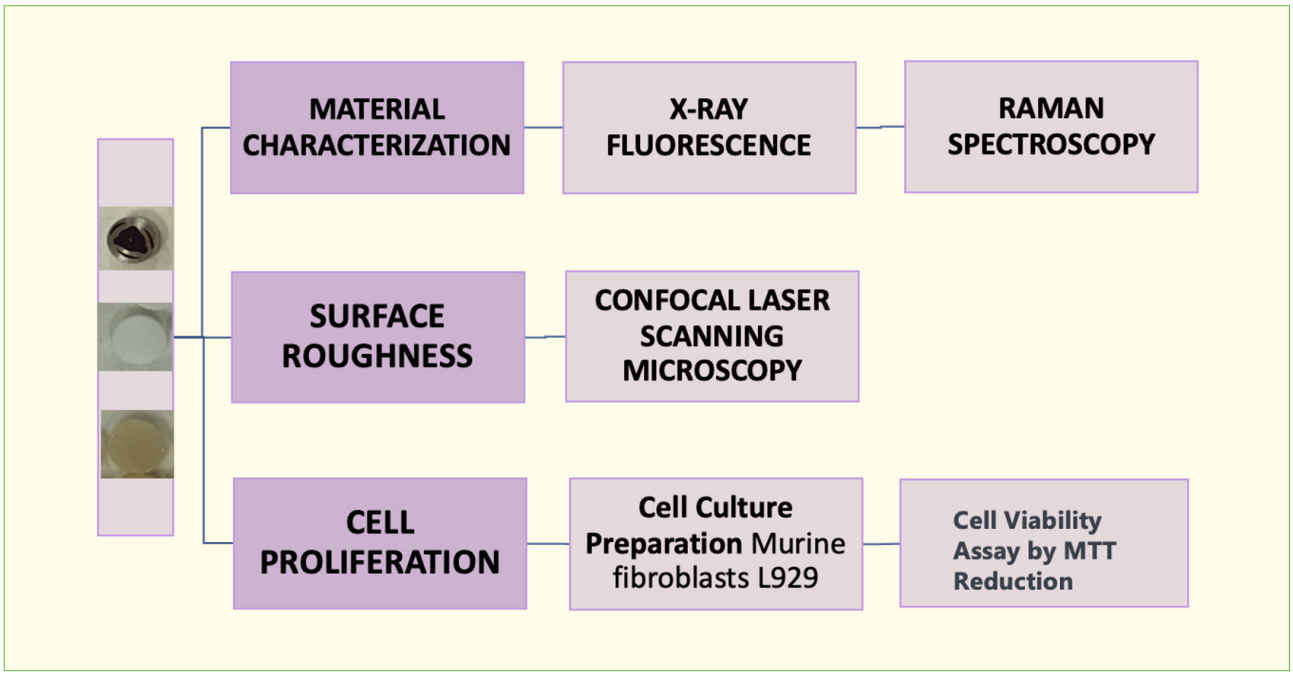


FIG. 1 Experimental Design

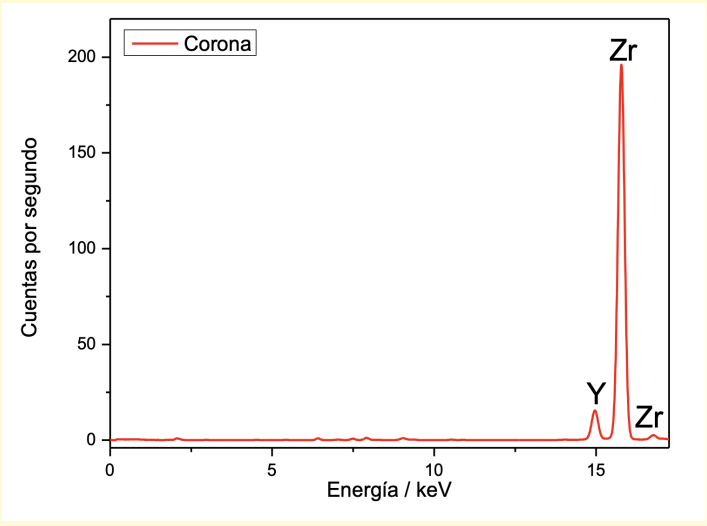


FIG. 2A X-ray fluorescence spectrum corresponding to the Titanium implant.

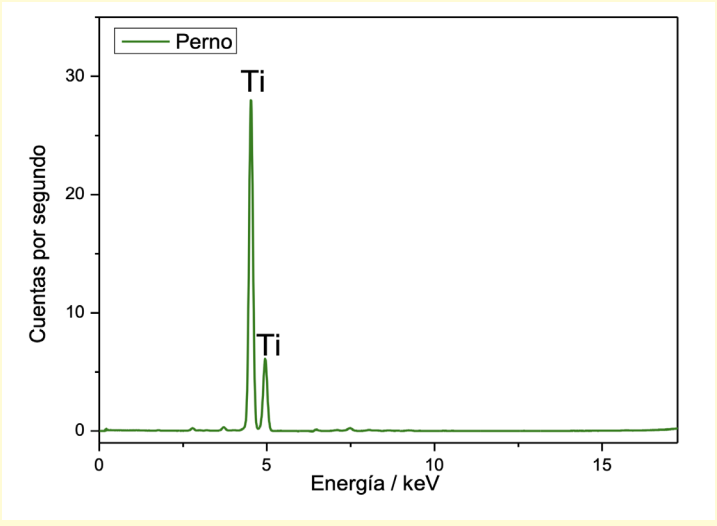


FIG. 2B X-ray fluorescence spectrum corresponding to the Zirconia sample.

behaviour of the materials can be predicted through the detailed colorimetric technique described above. An increase in mitochondrial metabolism indicates increased cell proliferation.

Statistical analysis

Results were expressed as mean ± SD. To determine the influence of the three materials on the cell line used, analysis of variance (ANOVA) was performed. Subsequently, Dunnett's or Student-Newman-Keuls multiple comparison tests were used. Paired t-test for paired samples. Differences between means were considered significant at $p < 0.05$. Statistical analysis was performed using SPSS v. 20.0 computer software (Chicago, IL, USA). Graphs were created using GraphPad Prism version 3.0 (GraphPad Software, San Diego, CA, USA).

RESULTS

Characterization of the materials used X-ray fluorescence

Figure 2a shows the X-ray fluorescence spectrum

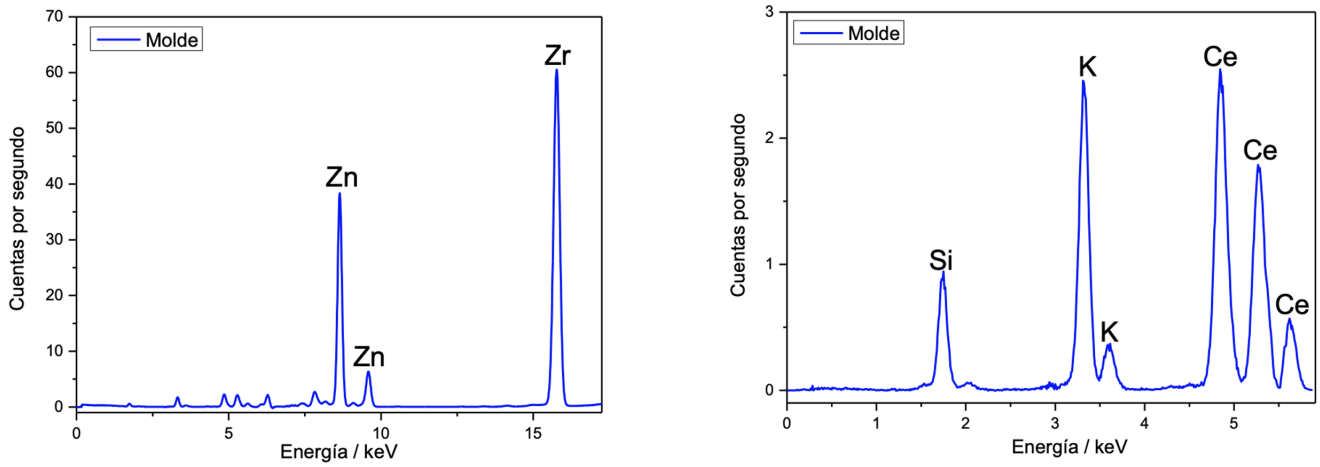


FIG. 2C X-ray fluorescence spectrum corresponding to the Lithium Disilicate sample.

corresponding to Titanium. It can be observed that the sample consists entirely of Titanium. This is evident from the presence of only the lines corresponding to the α and β emissions of this element.

In Figure 2b, the spectrum corresponding to the Zirconia sample is shown. The main element is Zr (86 to 93%). However, the presence of Yttrium in a smaller proportion (6.5 to 8%) was also detected. The other oxides present in the sample were not detected by X-ray fluorescence.

The left side of Figure 2c shows the measurement corresponding to the Lithium Disilicate mold, obtained using the same incident energy as the previous samples for a simple comparison. While this sample was expected to be composed mainly of Lithium Disilicate, the obtained spectrum indicates that it is predominantly composed of Zirconia (possibly forming Zirconia) with a significant presence of zinc. In addition to these elements, the spectrum reveals the presence of other elements. To better identify them, another spectrum was obtained using a lower incident energy (7 keV), and the result is presented in the right side of Figure 2c. The presence of silicon (Si), likely originating from the disilicate, is observed, along with the presence of cerium (Ce) and potassium (K).

Raman Spectroscopy The spectrum of Titanium is shown in Figure 3a. A high-intensity peak at 145 cm-1 corresponding to the stretching of the Ti-O bond is observed, along with two lower-intensity peaks at 450 and 720 cm-1. Based on the presence of these peaks, it is estimated that the titanium oxide is in its anatase form.

The Raman spectrum for the Zirconia sample is shown in Figure 3b. It exhibits the characteristic signals of zirconium oxide with a tetragonal crystal structure, which are observed at 147, 266, 320, 467, and 647 cm-1.

FIG. 3A
Raman Spectrum for Titanium sample.

FIG. 3B
Raman Spectrum for Lithium Zirconia sample.

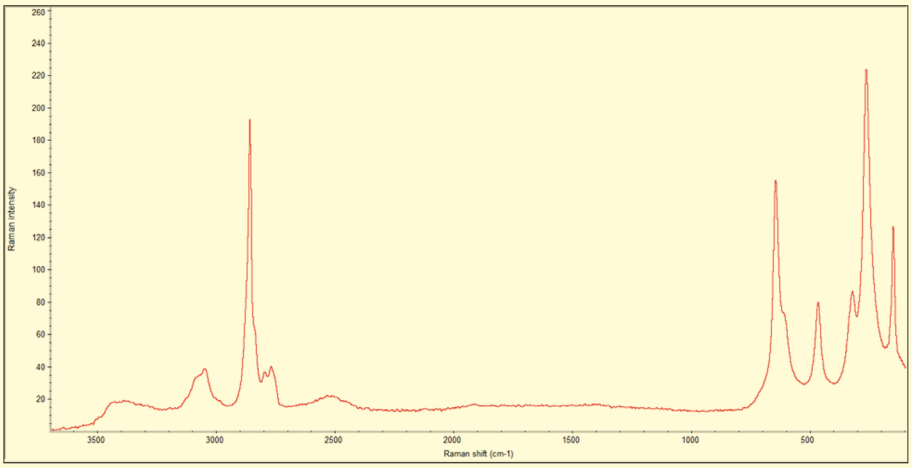
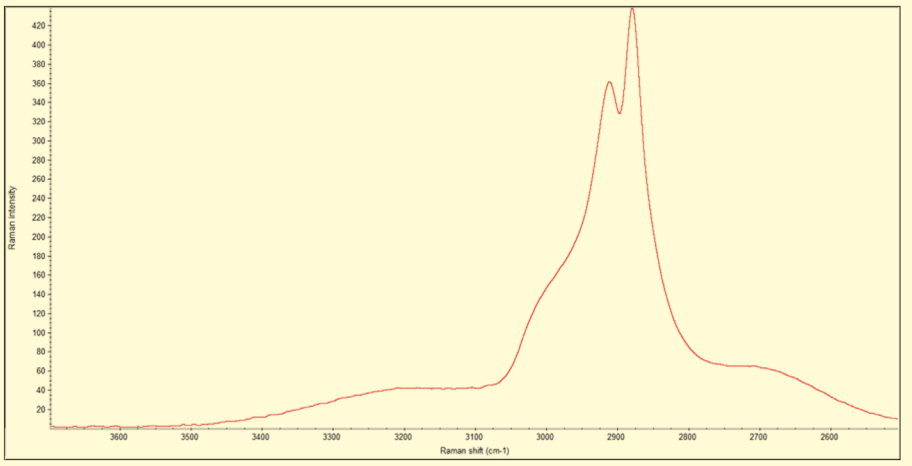


FIG. 3C
Raman Spectrum for Lithium Disilicate sample.



The bands corresponding to Yttrium are not detected due to its low presence.
The spectrum for the Disilicate sample is shown in Figure 3c. It exhibits a split peak at 2900 cm-1, which corresponds to the stretching vibration of the Si-O-Si groups. The peak corresponding to Cerium or Zirconia is not observed.

Measurement of surface roughness (Ra) by Confocal Laser Scanning Microscopy

The images of the three samples revealed a Ra (mean ± SD) of 0.617 ± 0.06, 0.444 ± 0.05, and 0.635 ± 0.14 (p=0.03) for Titanium, Zirconia, and Lithium Disilicate, respectively.

Cell Proliferation

As a result of the exposure of Titanium, Zirconia, and Lithium Disilicate to L929 Murine Fibroblasts for 24 and 48 hours, respectively, Figures 5a and 5b were obtained. At 24 hours, no significant difference was observed among the three materials studied (p=0.564). At 48 hours, a significant change can be observed in all three materials. The Ti, Zir, and DSL samples showed significant differences compared to the basal level and among themselves (p=0.0006). Regarding the comparative study of each sample, the growth between 48 hours and 24 hours showed the highest degree of cell viability for Lithium Disilicate (p=0.0003). In the case of Titanium samples, a small increase was observed (p=0.056), and for Zirconia samples, cell viability was significantly different (p=0.027). The cell viability values for Titanium, Zirconia, and DSL were 116%, 132%, and 172%, respectively.

DISCUSSION

The present study compared the biological response of three materials - Ti, Zir, and DSL - used as implant abutments in clinical practice (8-10). The materials were characterized, and subsequently, mitochondrial metabolism was evaluated as an indirect measure of cell growth or proliferation and, therefore, the effect of the material on soft tissues.
Regarding the nature of the materials used, the characterization of the materials by X-ray fluorescence spectroscopy and Raman spectroscopy showed a unique component in the Titanium sample. In contrast, the Zirconia sample (Zirconium oxide) revealed, through

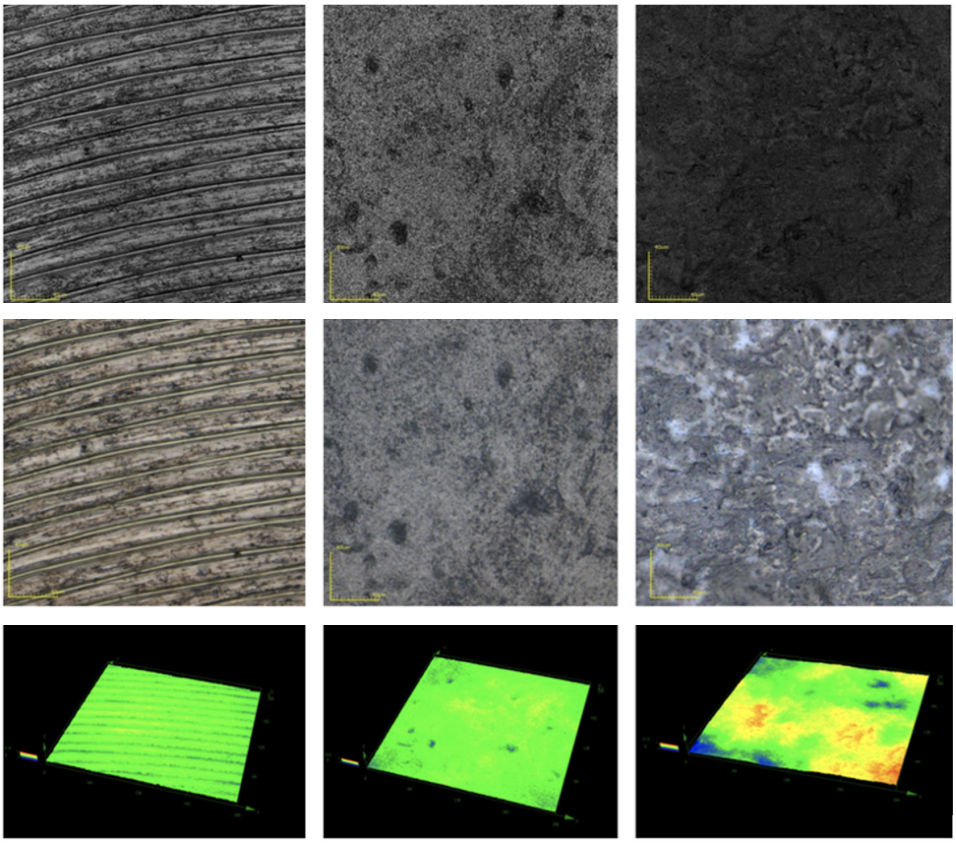


FIG. 4
From left to right; Titanium, Zirconia, and Lithium Disilicate discs. Bottom: 3D images of the samples.

X-ray fluorescence spectroscopy, the presence of 95% zirconium oxide and approximately 5% yttrium oxide (magnesium, cerium, and calcium). This component allows stabilizing the tetragonal structure at room temperature and resisting fracture propagation. Additionally, the DSL showed the presence of other components with important properties, such as Zn with a slightly antimicrobial effect, Ce that allows UV radiation protection (photocatalysis), and the Si-Zir combination responsible for the material's strength. The composition of these elements will likely impact the biological response.

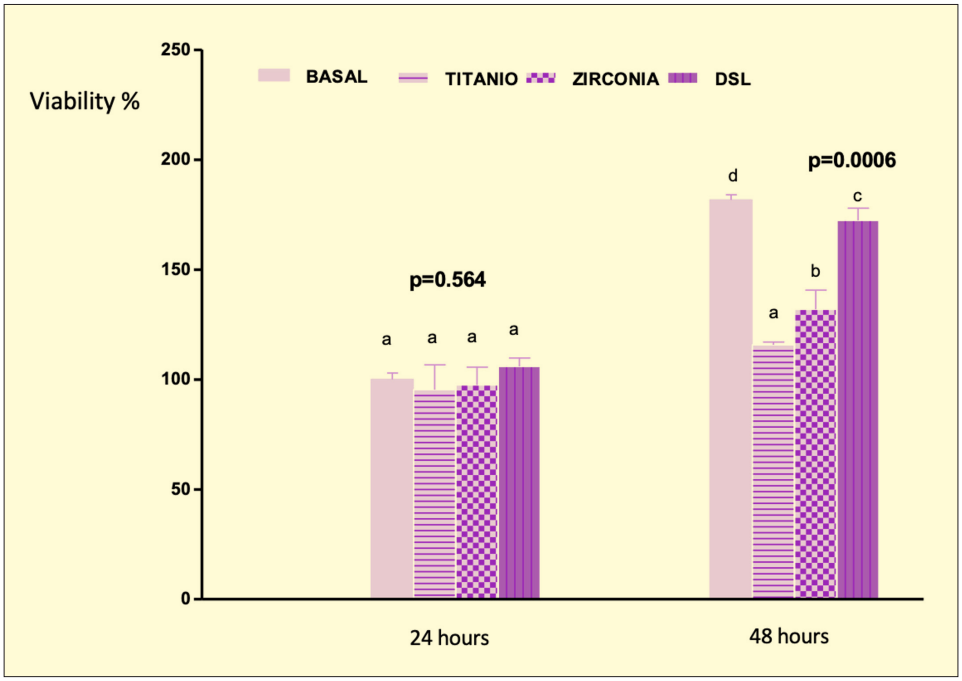


FIG. 5A
Percentage of cell viability compared to the basal level at 24 and 48 hours.

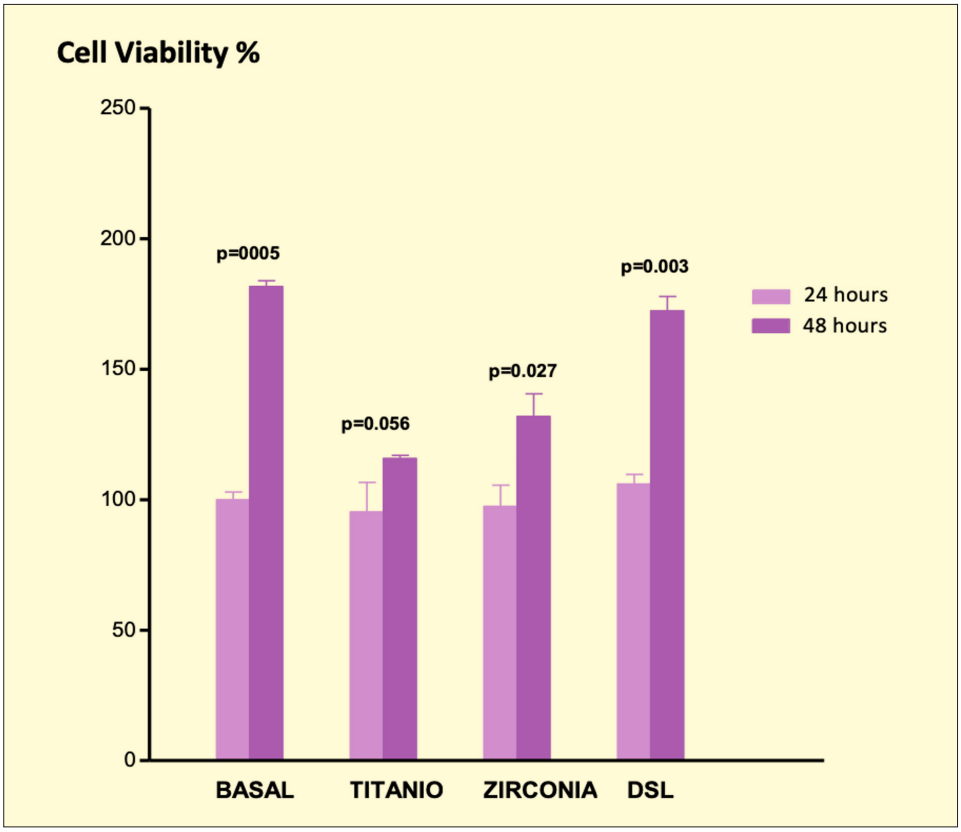


FIG. 5B
Comparison of the percentage of cell viability for each sample at 48 hours versus 24 hours.

Studies conducted by other authors have shown that Zirconia has suitable mechanical properties similar to stainless steel, such as high flexural and tensile strength, high abrasion and corrosion resistance, and low thermal conductivity. The survival rate and fracture load after mastication simulation of Zirconia abutments were found to be similar to Titanium abutments (11). These physical-chemical and biomechanical properties should be considered in further studies.

Since physical-chemical modifications on the material surfaces would alter their tomography, it would be difficult to distinguish which surface property is affecting cellular behaviour. Therefore, this study was designed using Titanium, Zirconia, and Lithium Disilicate samples with untreated contact surfaces obtained for analysis.

However, it was considered that the measurement of surface roughness (Ra) would be highly useful, and taking into account the characteristics of the comparison patterns, all samples demonstrated being within the range of medium roughness (Ra=0.61µm, Rz=3.35µm, Rmax=4.05µm, λc=0.8mm). The results showed that the Ra of Zirconia was close to the lower value of the medium roughness range, while Titanium and DSL had Ra values with no significant differences and within the average range.

Undoubtedly, the integration with soft tissues will depend on the nature of the material used for the abutment/emergent. Therefore, the choice of material is of utmost importance because the reaction of cells and tissues to a foreign body depends on the properties of the material and its behaviour in the exposed environment (12).

This in vitro study conducted on murine L929 fibroblast culture demonstrated adequate biocompatibility of the three materials at the studied time points. Other studies conducted in vitro, but using human gingival fibroblast culture, showed similar results using Zirconia (13) and Titanium (11), despite the presence of some roughness and moisture in these materials.

Furthermore, other authors have studied both in vitro (14) and in vivo (15) the biological behaviour of cells at the material interface. Tete et al. (14) evaluated the proliferation of human gingival fibroblasts in vitro on samples of Zirconia and DSL with and without polishing; after 24 hours, no differences were observed in terms of viability and production levels of type I collagen. Kohal et al. (15) investigated the histological response (osseointegration) to sandblasted Titanium and Zirconia implants, subsequently treated with or without acid etching, and demonstrated similar dimensions of peri-implant tissues in an animal model in monkeys. Similarly, Buser et al. (16) in an experimental study in dogs using Titanium with and without surface roughness did not observe differences in the biological response of soft tissues.

Regarding the biological response of the materials used, the present study evaluated cell viability on Titanium,

Zirconia, and DSL, showing different behaviour at 24 hours compared to 48 hours.
Indeed, at 24 hours, the MTT assay showed similar growth for all three materials. Consistent with the present study, Kwon et al. in 2014 (17), working with human gingival fibroblast cultures, demonstrated in vitro that the biocompatibility of Zirconia was comparable to that of Titanium.
However, other authors demonstrated in vitro using human gingival fibroblast cultures a different response of DSL and Zirconia (14), possibly due to the less favourable behaviour of DSL in its monolithic form.
Regarding the response at 48 hours, the present study showed a highly significant increase in cell growth for DSL, slightly less for Zirconia, and even less for Titanium, as observed through the MTT assay. It is important to note that all materials used - Titanium, Zirconia, and DSL - exhibited surface growth, indicating that the cells were able to adhere to the materials. While it could be inferred that there might be a slowdown in the biological response in the Titanium samples at 48 hours, it can be stated that none of the materials caused a halt in growth, nor were there any signs of cytotoxicity. Long-term studies will shed light on their different behaviour.
The findings regarding cell viability in response to the studied abutments are important because they anticipate the biological response, which is crucial for the subsequent sealing around the dental implant as it predicts the stability of the different materials in relation to soft tissues (3,18). According to studies conducted by Buser et al. (16) in an animal model in dogs, this stability would determine the connection with soft tissue and osseointegration/marginal bone resorption.
There is evidence that the epithelial tissue that attaches to the implant abutment does so through the internal basal lamina and hemidesmosomes, similar to the attachment established in natural teeth (19). However, the attachment to the epithelium includes the internal basal lamina and hemidesmosomes formed only in the lower part of the peri-implant epithelium, around the implant abutment. In contrast, in natural teeth, these connections are widely distributed along the epithelial-tooth junction interface (20). Therefore, the epithelial connections formed around the implant abutment are more vulnerable than those formed around natural teeth due to their limited distribution area.
In addition to the sealing mentioned earlier, there are other important factors to consider, such as the different orientation of collagen fibers (12) and the lower degree of vascularization compared to natural teeth (14), which contribute to the increased vulnerability of the implant. Indeed, the stability of the implant-abutment connection and, consequently, the relationship with the surrounding hard and soft tissues are determining factors for the success of dental implants in clinical practice (8,21). Biological phenomena that occur once osseointegration is established include the response of soft tissues in terms of cell viability, absence of cytotoxicity, and synthesis of collagen fibers, located in the peri-implant connective tissue (22). These factors are essential for the subsequent sealing around the dental implant. This sequence of events has been documented both in vitro and in vivo. In an in vitro study, Kwon et al. (17) using human gingival fibroblast cultures showed similar cell viability results for Zirconia at 24 and 48 hours, despite using a different cell culture. Additionally, these authors observed the production of a collagen-rich extracellular matrix, osteopontin, and TGFß1. Similarly, Welander et al. (59), in an in vivo experimental model in dogs using Titanium and Zirconia abutments, observed fibroblast growth and similar collagen content.
The focus of the present study has been on the investigation of the nature of Titanium, Zirconia, and Lithium Disilicate used as implant abutments and their potential biological response in soft tissues. This study was conducted in vitro using murine L929 fibroblast cultures. Special attention has been given to the possible biological implications that would arise from this connection in terms of its relationship with the health of soft tissues.
However, due to the limitations of this in vitro study, further research is proposed to analyze the physical-chemical and biomechanical properties of Titanium, Zirconia, and Lithium Disilicate and evaluate their potential in terms of long-term cell proliferation and adhesion as implant abutments, as well as their response to soft tissues.

CONCLUSIONS

Cellular viability in the face of the studied implant abutments could anticipate the biological response and stability of soft tissues towards different materials.
This study allowed ruling out the presence of cytotoxicity in the three materials used as implant abutments.
When selecting an implant abutment, it is necessary for the dentist to be familiar with the nature of the materials and their biological response.

Acknowledgements
Microscopy Laboratory, Faculty of Mathematics, Astronomy, Physics and Computing (FAMAF), National University of Córdoba Faculty of Dentistry, University of Buenos Aires Immunology Laboratory, Immunology Department, Faculty of Pharmacy and Biochemistry, University of Buenos Aires.

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