

# Proliferation of human periodontal ligament mesenchymal cells on polished and plasma nitriding titanium surfaces

Rodrigo Alves Ribeiro<sup>1</sup>, Rodrigo Gadelha Vasconcelos<sup>1</sup>, Fernanda Ginani<sup>2</sup>, José Sandro Pereira da Silva<sup>1</sup>, Clodomiro Alves-Júnior<sup>3</sup>, Carlos Augusto Galvão Barboza<sup>2</sup>

<sup>1</sup>Area of Oral and Maxillofacial Surgery, Department of Dentistry, Federal University of Rio Grande do Norte, Natal, RN, Brazil

<sup>2</sup>Department of Morphology, Federal University of Rio Grande do Norte, Natal, RN, Brazil

<sup>3</sup>Department of Mechanical Engineering, Federal University of Rio Grande do Norte, Natal, RN, Brazil

## Abstract

**Aim:** To evaluate the proliferative capacity of mesenchymal cells derived from human periodontal ligament on polished and plasma-treated titanium surfaces. **Methods:** Eighteen titanium disks were polished and half of them (n=9) were submitted to plasma nitriding using the cathodic cage technique. Mesenchymal cells were isolated from periodontal ligament of impacted third molars (n=2) and cultured on titanium disks (polished and nitrided) and on a plastic surface as a positive control of cell proliferation. Cell proliferation was analyzed and growth curves were constructed for the different groups by determining the number of cells adhered to the different surfaces at 24, 48 and 72 h after plating. **Results:** Higher cell number was observed for the nitrided surface at 24 and 48 h. However, no statistically significant difference in cell proliferation was observed between the two different surface treatments ( $p>0.05$ ). **Conclusions:** We concluded that plasma nitriding produced surfaces that permitted the proliferation of human periodontal ligament mesenchymal cells. Associated to other physical and chemical properties, it is possible to assume the feasibility of plasma nitriding method and its positive effect on the early cellular events of osseointegration.

**Keywords:** biocompatible materials, titanium, cell proliferation, periodontal ligament.

## Introduction

Cell cultures have been extensively used in implantology to evaluate the effect of the substrate on the behavior of osteoblasts during osseointegration. Various biological events associated with bone healing on implant surfaces can be investigated separately using appropriate cell cultures, such as cell adhesion, proliferation and differentiation, as well as the production and mineralization of extracellular matrix. The use of these cultures provides cellular and molecular data that favor a nanostructural engineering approach in implant design and permits the testing of different hypotheses. Within this context, cell cultures offer unique insights into the process and phenomenon of osseointegration<sup>1</sup>.

*In vitro* models have been proposed in an attempt to better understand the early stages of osseointegration and how cells interact with surfaces modified by different methods. The functional and structural adhesion of bone tissue to the surface of load-bearing orthopedic implants seems to be a determinant factor for *in vivo* success<sup>2</sup>.

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### Correspondence to:

Carlos Augusto Galvão Barboza  
Avenida Senador Salgado Filho, 3000,  
CEP: 59072-970 – Lagoa Nova, Natal, RN, Brasil  
Phone: +55 84 94015955  
Fax: +55 84 32119207  
E-mail: cbarboza@cb.ufrn.br

There is consensus in the literature that the physicochemical properties of implant surfaces play a fundamental role in the success of osseointegration. Several surface treatments were developed to improve the molecular and cellular events that guide the early stages of this process<sup>3</sup>. However, the adequate parameters required to obtain an ideal surface are still not defined. In this respect, new surface modification techniques were studied<sup>4</sup>. Surface treatment permits the modification of the dental implants characteristics, such as chemical composition, morphology, topography and rugosity<sup>5</sup>.

Plasma nitriding, which consists of generating an electrical discharge in gas mixture containing low-pressure nitrogen, has been applied to modify biomaterials<sup>6</sup>. The basic concept in using ionic nitriding to improve titanium (Ti) surface properties is based on the possibility of forming nitrides or carbides below the alloy surface. Ti nitrides and carbides are brittle materials that improve tribologic surface properties, which means that they increase resistance to corrosion and surface roughness<sup>7</sup>.

The search for a titanium surface favoring cellular events that would permit faster and more effective osseointegration encouraged the present study. In this respect, this study evaluated the proliferation of mesenchymal cells derived from the periodontal ligament of human third molars on polished and plasma-nitrided titanium surfaces.

## Material and methods

The study was approved by the Ethics Committee of the Federal University of Rio Grande do Norte – UFRN (Process #080/2010).

Periodontal ligament mesenchymal cells were obtained from two healthy human teeth (impacted third molars). The teeth were extracted from patients with a surgical indication due to orthodontic reasons, who had no oral or systemic diseases. Grade II ASTM F86 titanium disks, 1.5 mm thick and measuring 15 mm in diameter, were used.

## Preparation of the titanium disks

Eighteen titanium disks were embedded in polyester resin and gradually polished with 220-, 360-, 400-, 600-, 1,200- and 2,000-grit silicon carbide sandpaper in running tap water, followed by polishing with an APL-2 polishing machine (series 212560, Arotec, Cotia, SP, Brazil) and cleaning with an OP-CHEM polishing cloth, colloidal silica (SiO<sub>2</sub>) suspension and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) 20 V until a final finishing resulted in a surface with a 0.04 µm roughness. Next, the samples were embedded and cleaned to remove contaminants that could interfere with the ion nitriding process. For this purpose, the same samples were washed in an ultrasound bath for 30 min, divided into three steps of 10 min each. Enzyme

detergent (EndoZime AW Plus, Planitrade, Porto Alegre, RS, Brazil) was used in the first wash, absolute alcohol in the second, and distilled water in the last wash. The samples were then dried at room temperature and stored in an appropriate container until the time for use.

Nine titanium disks were submitted to nitriding treatment using a previously described protocol<sup>8</sup>. The plasma atmospheres were set up using a gas flow of 15 sccm (Table 1).

Next, all samples were transferred to 24-well culture plates (2 cm<sup>2</sup>) and sterilized by gamma irradiation. The total irradiation dose per sample was 25 kGy, with a mean dose rate of 8.993 kGy/h (2 h and 46 min at a distance of 50 mm), and a Gammacell 220 Excel irradiator (MDS Nordion, Ottawa, ON, Canada) was used.

## Isolation of periodontal ligament cells

Mesenchymal cells were isolated from periodontal ligament of impacted third molars, as previously described by Vasconcelos et al.<sup>9</sup> (2012). Each tooth was immediately stored in a 50-ml Falcon tube containing alpha-MEM culture medium (Cultilab, Campinas, SP, Brazil), and then washed three times for 15 min each with alpha-MEM medium (Cultilab) supplemented with 10,000 IU/mL penicillin, 10,000 µg/mL streptomycin, 100 mg/mL gentamicin, and 250 µg/mL amphotericin B (all antibiotics were purchased from Gibco, Grand Island, NY, USA).

The periodontal ligament was removed by gently scraping the root surface with a scalpel, followed by enzymatic digestion with 3 mg/mL collagenase I (Gibco) and 4 mg/mL dispase (Gibco) for 1 h at 37°C. The solution was aspirated, filtered through a 70-µm filter (BD Falcon, Bredford, MA, USA), and centrifuged at 1,200 rpm for 5 min. The supernatant was discarded and the precipitated cells were resuspended in culture medium.

The periodontal ligament mesenchymal cells were cultured in 25-cm<sup>2</sup> bottles (TTP®, USA) in basic alpha-MEM medium (Cultilab) supplemented with 15% fetal bovine serum (Cultilab). The cultures were incubated at 37°C in a 5% CO<sub>2</sub> atmosphere and the medium was changed at intervals of 3 days until 70 to 90% confluence was reached.

Cell characterization was performed by expression of CD29/integrin β-1 (BD Bioscience, USA), a mesenchymal stem cell marker, by flow cytometry. Additionally, the multi-lineage differentiation potential of periodontal ligament cells was confirmed by culturing the cells in osteogenic and adipogenic differentiation media (StemPro® Differentiation Kits, Invitrogen, Carlsbad, CA, USA) for up to 21 days.

## Cell culture on titanium disks

After the third passage, the periodontal ligament cells were transferred to 24-well plates (TTP®, Trasadingen,

**Table 1.** Parameters of the plasma nitriding treatment.

Group	Plasma atmosphere	Treatment time	Plasma pressure	Plasma temperature	Total gas flow
Polished	-	-	-	-	-
Nitrided	20% N + 80% H	1 h	2.5 mbar	450°C/17.5 mA	15 sccm

Switzerland) at a density of  $2 \times 10^4$  cells per well. Eighteen titanium disks were used, nine for each group (polished - P and nitride - N). The same cell density was cultivated in wells without discs, as a positive control of cell proliferation. The disks are the same size of the well, so the growth area of disks and controls were the same.

### Analysis of cell proliferation

Cell proliferation was analyzed and growth curves were constructed for the different groups by determining the number of cells adhered to the titanium surfaces (polished and nitrided groups) at 24, 48 and 72 h after plating. The number of cells per well was obtained by counting viable cells in a hemocytometer using the Trypan blue exclusion method. Cell counts are reported as the mean of three samples per group for each time interval (24, 42 and 72 h). Differences were compared between groups by the Mann Whitney tests. A  $p$  value  $<0.05$  was considered to indicate statistical significance.

### Results

By flow cytometry, 97.2% of the periodontal ligament cells expressed CD29/integrin  $\alpha$ -1. After 21 days under osteogenic and adipogenic induction, the cells produced typical mineralized nodules and showed adipocyte morphology by light microscopy (Figure 1).

The growth curve of periodontal ligament-derived undifferentiated mesenchymal cells cultured on different surfaces is illustrated in Figure 2. Cell growth increased linearly on the control surface (plastic) over the period studied, since it is the gold standard surface for cell adhesion and proliferation.

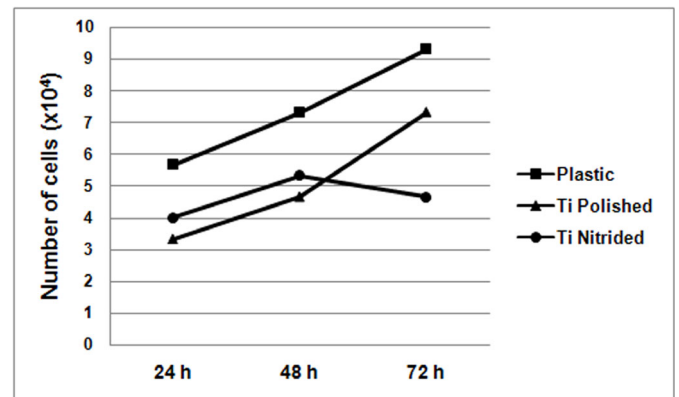
Comparing the studied titanium surfaces, higher number of cells was observed for the nitrided surface at 24 h. However, no statistically significant difference in cell proliferation was observed between the two different surface treatments (Table 2).

Nitrided surfaces favored cell proliferation at 48 h when compared with the polished surfaces. On the other hand, cell proliferation was increased at 72 h in the polished group, although no statistically significant difference was observed between polished and nitrided surfaces in both time intervals (Table 2).

**Table 2.** Analysis of cell proliferation for each time interval (24, 48 and 72 h) after plating in the different studied groups.

	Polished		Nitrided		$p$ value*
	Mean	SD	Mean	SD	
24 h	3.33	0.58	4.00	1.00	0.48
48 h	4.67	0.58	5.33	0.58	0.30
72 h	7.33	1.53	4.67	1.53	0.12

$p^*$ : Mann Whitney test.

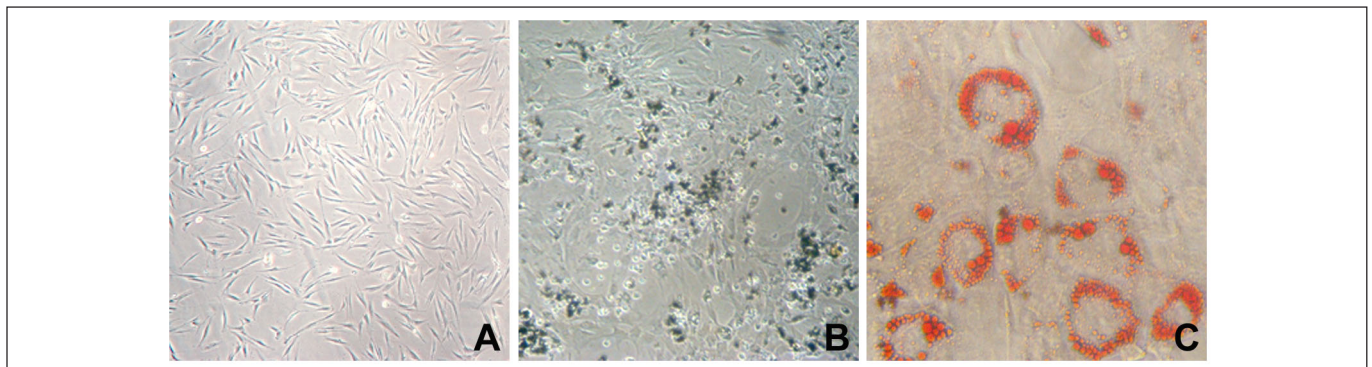


**Fig. 2.** Growth curve of periodontal ligament mesenchymal cells at different time intervals. The plastic surface was used as a positive control of cell proliferation.

### Discussion

Cell adhesion to titanium surfaces is known to be the key factor for osseointegration. Biocompatibility assays testing new titanium surfaces employ different types of cells (primary cells, clones, immortalized cells) that retain specific properties in terms of the degree of interaction with the microenvironment of the material. Immortalized cell lines that are able to express phenotypic characteristics of osteoblasts are generally used to study cell/material interactions, which are essential for the development of new materials<sup>10</sup>.

According to Seo et al.<sup>11</sup> (2004), the human periodontal ligament contains cells that, like bone marrow stem cells, possess the potential to differentiate into osteoblasts, chondrocytes and adipocytes. In addition, these periodontal ligament stem cells can be used for regeneration of the



**Fig. 1.** Photomicrograph of periodontal ligament mesenchymal cells in the undifferentiated stage (A) and subjected to osteogenic (B) and adipogenic differentiation (C). (Light microscopy, A: 40x; B: von Kossa stain, 40x; C: Oil red stain, x100).

ligament itself and of cementum. This cell type was chosen in the present study because undifferentiated mesenchymal cells of the periodontal ligament have been reported to be responsible for both the regeneration of alveolar bone and the osseointegration of titanium implants placed immediately after tooth extraction from the alveolus<sup>12</sup>.

Studies have focused on the superficial properties of titanium as the main factor responsible for the adhesion and proliferation of cells with different phenotypes and the consequent formation of mineralized tissue at the implant interface<sup>13-14</sup>, although cell adhesion and proliferation have been shown to be similar on surfaces with different rugosities<sup>15-16</sup>. The present study evaluated the effect of surface modification of pure grade II titanium disks by plasma nitriding. This process is applied to modify materials used as dental implants and consists of depositing nitride onto metallic surfaces under nitrogen plasma<sup>6</sup>. The cathodic cage technique was used for plasma nitriding, in which the samples were surrounded by a titanium cage in order to enhance the effect of the plasma<sup>8</sup>. Plasma nitriding of metals is a well-established method that has numerous industrial applications since it results in peculiar physical and chemical characteristics of the materials, such as increased hardness and wear and oxidation resistance, in addition to proven biocompatibility<sup>17</sup>. Characterization of polished and nitrided surfaces used in this study was previously published by Da Silva et al.<sup>18</sup> (2011).

In the present study, periodontal ligament mesenchymal cells reacted differently to the tested surfaces. Cell proliferation was more pronounced on the plastic surface as expected, since plastic differs chemically from titanium and is the standard environment for cell culture. This behavior is frequently observed in surface biocompatibility studies<sup>8,19</sup>.

Rough surfaces seem to favor cell adhesion when compared with the polished ones<sup>20</sup>. Moreover, integrins are known to play a role in human periodontal ligament cells attachment to substrates and integrin receptors are necessary for attachment of fibroblasts to titanium substrates. The number or density of integrin receptors adhering the cell to the titanium surface varies depending on the type of titanium surface<sup>21</sup>.

Among the different titanium surfaces tested in the present study, the number of cells was higher on the nitrided surface at 24 h, indicating that the surface roughness obtained with this treatment favors cell adhesion, in agreement with the findings of previous studies<sup>18,22-23</sup>. These results suggest a positive effect of the cathodic cage nitriding on the capacity to generate homogenous textured surfaces due to the concentration of energized ions close to the samples. In addition, the surface of nitrided samples in the cathodic cage may contain a higher concentration of titanium nitride (TiN) residues due to the high density of ions on the samples, providing the topographic and chemical features necessary for the interaction of cells with a surface<sup>18</sup>.

The proliferation of undifferentiated mesenchymal cells derived from adult human periodontal ligament increased linearly in the polished group over the studied period. In the nitrided group, cell proliferation increased until 48 h, followed by a decline at 72 h. Similar results have been

reported by Da Silva et al.<sup>18</sup> (2011), who studied the proliferation of preosteoblastic MC3T3 cells on titanium surfaces subjected to plasma nitriding using the cathodic cage technique, demonstrating that different cell types behave similarly on the nitrided surface. The decline in the number of cells probably corresponds to the onset of cell differentiation characterized by a reduction in proliferative activity and modification in the extracellular matrix components produced in response to the surface treatment by plasma nitriding and the resulting increase of the surface roughness. In fact, a previous study with MG-63 cells showed that increasing Ti roughness decreases cell proliferation and increases cell differentiation<sup>24</sup>. Further studies are needed to prove this hypothesis.

The results confirmed the feasibility of the plasma nitriding method and its positive effect on the early cellular events of osseointegration. This method permitted to modify the titanium surface while maintaining the biocompatibility characteristics of the material<sup>22,25</sup>. The study of surface treatment techniques has shown that the nanoscale topographical features are particularly important for the development of strategies aiming to functionalize biomaterial surfaces with molecules that are known to promote cell adhesion<sup>26</sup>.

In conclusion, both polished and plasma nitriding surfaces permitted the proliferation of mesenchymal cells derived from the human periodontal ligament. In addition to previously reported physical and chemical properties, it may be suggested the use of plasma nitriding for improving the early cellular events of osseointegration.

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