A number of surface treatment methods have been developed since the introduction of the concept of osseointegration in the 1960s. These include the large-scale manufacture of implants with texturized surfaces created using physical or chemical vapor deposition coatings, abrasive particle blasting, acid etching, thermochemical treatment, laser treatment, ionic implantation, and the use of plasma in a variety of techniques. Each method alters the material in a different way, either by adding or removing components of the surface or deeper portions of the material. Previous studies have shown that variations in surface texture or nanoscale topography features can affect the cell response to a titanium implant and that osteoblastic cell adhesion occurs more rapidly on rougher titanium surfaces. Moreover, a greater proliferation of extracellular matrix synthesis with subsequent mineralization has been observed with rougher surfaces. However, other studies on the cell response mechanism in implants do not agree with these findings. Thus, there is no consensus about the importance of the degree of roughness and its relationship to surface wettability, adhesion, and cell proliferation.

The initial interactions between the surface of the implanted biomaterial and the receptor tissue in vitro and in vivo have been the focus of many studies. In general, biomaterial researchers have concentrated on the study of titanium surfaces and their correlation with the physicochemical, chemical, and topographic properties of the implant surface, as well as their compatibility with human bone tissue.

Osteoblastlike Cell Adhesion on Titanium Surfaces Modified by Plasma Nitriding

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**Purpose:** The aim of this study was to evaluate the characteristics of various titanium surfaces modified by cold plasma nitriding in terms of adhesion and proliferation of rat osteoblastlike cells.

**Materials and Methods:** Samples of grade 2 titanium were subjected to three different surface modification processes: polishing, nitriding by plasma direct current, and nitriding by cathodic cage discharge. To evaluate the effect of the surface treatment on the cellular response, the adhesion and proliferation of osteoblastlike cells (MC3T3) were quantified and the results were analyzed by Kruskal-Wallis and Friedman statistical tests. Cellular morphology was observed by scanning electron microscopy.

**Results:** There was more MC3T3 cell attachment on the rougher surfaces produced by cathodic cage discharge compared with polished samples (P < .05). **Conclusions:** Plasma nitriding improves titanium surface roughness and wettability, leading to osteoblastlike cell adhesion.

**Key words:** cell growth, osteoblast adhesion, plasma nitriding, titanium

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Earlier studies of the effect of plasma on titanium surfaces indicated that this treatment is capable of improving cell adhesion by changing surface roughness and wettability (related to low contact angles of liquids), which decrease after plasma exposure.\textsuperscript{1,6,9} Plasma nitriding, which consists of generating an electrical discharge in a gas mixture containing low-pressure nitrogen, has been applied to modify biomaterials, notably titanium. The ions and neutral compounds that are formed collide with the titanium surface, resulting in the formation of nitride layers.\textsuperscript{14,15}

Titanium disks that were nitrided using a hollow cathode discharge showed stable nitride layers, with increased roughness and wettability in samples treated at 450°C and at a pressure of 150 Pa.\textsuperscript{15}

The aim of this study was to evaluate the surface characteristics, roughness ($R_a$), and wettability of commercially pure titanium disks modified by nitriding using two plasma techniques: planar and cathodic cage. The response of osteoblast-like cells to these disks was also evaluated.

**MATERIALS AND METHODS**

Seventy-eight grade 2 ASTM F86 titanium disks, 14 mm in diameter and 1.5 mm thick, were polished with 220-, 360-, 400-, 600-, 1,000-, 1,200-, and 2,000-grit silicon carbide sandpaper in running water and polished (Op-CHEM cloth, Struers) in colloidal silica ($\text{SiO}_2$) suspension with 0.1-μm particles (ATM) and hydrogen peroxide ($\text{H}_2\text{O}_2$) 20 V until a final finishing left the surfaces with a roughness of 0.04 μm. The samples were cleaned in an ultrasound bath with acetone for 10 minutes to remove contaminants, dried at room temperature, and conditioned in culture plates.

The samples were placed in a sealed stainless steel chamber (reactor) (Fig 1). Two techniques were used in the plasma reactor to compare the effects on the sample surfaces: planar and cathodic cage nitriding. The nitriding protocol used was developed by Alves et al.\textsuperscript{15,16}

Roughness ($R_a$) was measured on six samples from each group (group 1: polished titanium, group 2: 20% $\text{N}_2$:80% $\text{H}_2$ for 1 hour in the planar mode, and group 3: 20% $\text{N}_2$:80% $\text{H}_2$ for 1 hour in the cathodic cage) using a Surtronic 3 (Taylor-Hobson) profilometer with a cutoff of 0.25 mm. The measurements were made from three different directions, 120 degrees apart. The roughness values reported represent the mean of four sets of values.

After plasma treatment, three samples from each experimental group were submitted to wettability testing. An adjustable-volume digital micropipette, positioned perpendicularly and very close to a flat surface, was used to deposit 0.25 mL of a saline solution onto the surface of the sample. To standardize the test and because of the very small drop size, angle changes were monitored at 1, 30, and 60 seconds.

Next, adhesion of the cells was evaluated. Twenty-four–well culture plate samples (2 cm$^2$) were sterilized by gamma radiation. The total radiation dose per sample was 25 kGy, which was released at a mean dose of 8.9 kGy/h (166 min, 50 mm distant) in a Gammascell 220 Excel irradiator (MDS Nordion). For the adhesion experiments, $1 \times 10^{-4}$ cells/cm$^2$ per well were seeded from the MC3T3 line onto all plates after reaching passage 8 (P8). Twelve disks, four from each experimental group, were used. The cells were seeded in triplicate for all groups. Four wells were used as controls, and three samples were randomly selected from each group for cell morphology analysis.

**Figs 1a and 1b** The stainless steel chamber reactor used in the experiment.
Cell proliferation (growth) was evaluated (four disks for each group) by determining the number of cells that adhered to the samples at 24, 48, and 72 hours after plating, in triplicate. Thirty-six wells were counted, and the number of viable cells collected was obtained using a hemocytometer and the trypan blue exclusion test. The total number of cells was calculated as follows: (total counted number of cells) × dilution × 10⁴/(number of hemocytometers)². Finally, the viability of the cell population was obtained by dividing the number of viable cells by the overall number of cells and multiplying the result by 100.

Osteoblast morphology was also evaluated. One sample from each experimental group was fixed in 2.5% glutaraldehyde solution and 0.1 mol/L sodium phosphate buffer for 2 hours at room temperature. Postfixation was accomplished with 1% osmium tetroxide with the same buffer. The samples were then dehydrated using a graded series of ethanols, immersed in hexamethyldisilazane for 30 minutes, and air dried. The samples were gold-sputtered to a thickness of approximately 10 nm and analyzed with a JEOL 6100 scanning electron microscope. All the samples were photographed at a focal distance of 20 mm at 40× and 500× magnification, encompassing nearly the same area. A plastic sample (Thermanox) was also evaluated for comparison.

The existence of significant differences between each surface treatment was identified with a nonparametric analysis, which calculated the average of ranks and quartiles. Each cell count corresponded to the mean ± standard error of the mean (SEM) of four samples per group. The Friedman test was used to evaluate within-group data, and differences between groups were identified using the Kruskal-Wallis method if the data did not follow a normal distribution. The significance level was set at 5% (P ≤ .05), with 95% confidence.

### RESULTS

#### Roughness

The mean roughness (Ra) of the polished samples was lower than that of the plasma-treated samples (Table 1). However, the average values for the three samples were in the range of 0.04 to 0.09 µm, which is an acceptable value for mechanically polished samples. The results in Fig 2 show that the roughness of the polished samples was significantly different from the roughness of the samples exposed to plasma. In addition, the cathodic cage–nitrided samples presented with significantly (P = .01) different roughness in comparison with the other groups, suggesting a positive effect of this technique in altering the structure of titanium.

#### Wettability

The lowest liquid-titanium contact angle was obtained using the cathodic cage technique, and this surface was characterized by hydrophilic and polar behavior (Fig 3, Table 1). The worst wettability was observed for the planar plasma–treated sample, as demonstrated by the value of the contact angle. The dispersion was very low for all samples.

#### Cell Adhesion

The MC3T3 cells responded differently, depending on the topography and chemical composition of the exposed surfaces (Table 1). Cell adhesion was higher in the control group. Plastic (Thermanox), the standard medium for cell cultivation, has a smoother surface and is chemically different from titanium. Significant differences in cell adhesion were found for the titanium surfaces after 24 hours (Fig 4); adhesion was more pronounced on the surfaces modified by cathodic cage–plasma nitriding.

<table>
<thead>
<tr>
<th>Disk</th>
<th>Adhesion*</th>
<th>Proliferation*</th>
<th>Contact angle (deg)</th>
<th>Roughness (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (plastic)</td>
<td>173,000</td>
<td>687,000</td>
<td>15.04</td>
<td>0.04</td>
</tr>
<tr>
<td>Polished</td>
<td>37,333</td>
<td>192,000</td>
<td>48.85</td>
<td>0.06</td>
</tr>
<tr>
<td>Planar</td>
<td>56,000</td>
<td>258,667</td>
<td>11.62</td>
<td>0.09</td>
</tr>
<tr>
<td>Cathodic cage</td>
<td>66,667</td>
<td>173,333</td>
<td>0.71</td>
<td>0.03</td>
</tr>
</tbody>
</table>

*Adhesion was evaluated 24 hours and proliferation 72 hours after cell seeding.
Cell Proliferation

Cell growth for the control group increased linearly during the evaluation period. Cell proliferation showed a similar behavior to that found for cell adhesion, and there were no statistically significant differences between polished and nitrided surfaces, regardless of the treatment. The cells showed significant proliferation after 72 hours, with a slight difference in the planar group between 24 and 72 hours (Fig 5, Table 1).

Dendritic projections and filopodia could also be seen randomly distributed over the rough surfaces, representing focal adhesion points resulting from surface roughness. In addition, the cells were closer to one another (Figs 6b and 6c).

DISCUSSION

Several studies have examined the effects of specific surface treatments on the composition and structure of the oxide layer of titanium-based materials. The treatments result in topographic surface alterations on a particular scale, represented mainly by surface roughness and in changes in chemical composition and surface free energy.\(^{1,4}\)
This study focused on modifications of pure titanium (grade 2) surfaces by glow-discharge plasma nitriding. The technique consists of exposing titanium to a gas atmosphere containing a mixture of 20% N₂ and 80% H₂ at low pressure and ionized by a continuous current. This physical treatment is used for the modification of dental implant materials and consists of depositing nitride onto metallic surfaces under nitrogen plasma. Compared with more conventional techniques, it has the following characteristics: environmental cleanliness, low treatment temperature, short treatment time, possible nitriding of equipment parts, better control, uniformity of layer thickness, and lower cost.¹⁵

In the present study, nitriding was performed using the hollow cathode planar technique, wherein the disks are supported in an ordinary sample holder. The authors also used the cathodic cage technique,¹⁶ in which the samples are enclosed in a titanium cage to enhance the effect of the plasma.

The gas composition affects the surface changes on titanium. In another study, samples nitrided with 80% N₂ for 1 hour showed a weaker interaction between the titanium surface and plasma nitrogen than samples treated in 20% N₂ and were not able to form titanium-nitrided compounds, possibly as a result of a combination of the short treatment time and weak surface passivation as a consequence of the low H₂ concentration.¹⁵ That suggests that the conditions used in the present investigation represent the optimum H₂ concentration needed to deposit nitrides on titanium surfaces in a short time, or at least in the shortest possible time using this technique. Although both parameters influence the creation of a chemi-

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**Fig 6** Scanning electron microscopy of osteoblastlike cell morphology on different surfaces at 24 hours. (a) Polished surface, (b) planar hollow cathode surface, and (c) cathodic cage surface.
cally modified surface, the titanium nitride (TiN) compounds are more dependent on exposure time than they are on H₂ concentration.¹⁵

Titanium alloys polished with alumina and submitted to plasma nitriding show TiN phases that are dependent on gas mixture and exposure time. Layer hardness and corrosion resistance are higher when the layers are treated at 900°C for 4 hours with a mixture of nitrogen and hydrogen.⁵ This fact favors the use of plasma nitriding, since reduced corrosion is crucial for titanium biocompatibility.

In this study, roughness was analyzed by contact profilometry. This method, despite its widespread use in surface characterization studies, has limitations in terms of its scale resolution (microns). Nevertheless, the roughness values obtained for the polished samples and those exposed to plasma were significantly different.

The surface with the highest roughness value (Rₐ = 0.13 μm) showed the lowest liquid-titanium contact angles. This fact may be related to the roughness of the surface and the regularity of the profile pattern, both of which reduce the possibility of retaining contaminants in the microdeformations of the surface. Indeed, during the treatment phase, the reactor was fed with a flow of H₂ for around 30 minutes at low pressure. Hydrogen gas was then used to passivate and activate the surface by removing the oxide layer and any impurities that might interfere with the nitriding process.

Studies of surface characteristics attribute the way osteoblasts adhere to and proliferate on titanium mostly to wettability,¹⁰,¹¹ and, in general, hydrophilic surfaces offer better conditions for cell adhesion than hydrophobic surfaces. However, the surface wettability of a biomaterial should not be attributed solely to hydrophilicity, because a number of other factors play roles, such as chemical composition, crystallographic structure, surface treatment type, and even the liquids used in measurements. Nevertheless, a close relationship can be observed between titanium roughness and wettability and, in the present study, the surfaces nitried by cathodic cage showed a reduction in contact angle, confirming that a surface with better wettability exhibits better cell adhesion. Thus, plasma nitriding proved to be an effective technique for modifying titanium surfaces in terms of increased roughness and increased wettability.

Owing to the limited availability of in vivo testing to investigate material biocompatibility, in vitro models with cell cultures are frequently used to evaluate potential osseointegration of biomaterials. These models create well-controlled and accessible microenvironments that provide consistent data for analysis,¹²,¹³ granting a better understanding of the initial stages of the osseointegration phenomenon and how cells interact with modified surfaces.

It is known that cell adhesion to titanium surfaces is vital for the osseointegration of metallic prostheses and dental implants and that the cellular response is influenced by surface conditions, such as type and purity of the material, roughness, wettability, and surface energy. Many reports have focused on surface energy and wettability as important parameters for cellular response, although roughness also plays a direct role.³,¹⁰,¹¹ Studies have shown that surfaces with different roughnesses may show a similar response with respect to cell adhesion and proliferation⁷,¹⁷ and that titanium surface roughness has been directly associated with osteoblast adhesion and the consequent development of mineralized tissue at its interface with the implant.¹⁸,¹⁹

The mean roughness of a surface is not, in itself, a definitive parameter for a direct assessment of the cell response to the topography of a particular material. Different treatments can produce similar Rₐ values, which will not necessarily result in similar clinical performance. Combined topographic parameters should be analyzed for a better correlation with wettability and alterations in cell morphology after adhesion.⁸

Biocompatibility surface tests can be performed with different types of cells (eg, primary, clone, immortalized). Osteoblastic cells enable the study of cell-material interactions and are essential for the development of new materials. Many studies have been conducted with immortalized cell lines with the potential of expressing phenotypical osteoblast characteristics, such as SaoS2, MG-63, L929, ROS17/2.8, and MC3T3.²⁰–²²

In the present study, MC3T3 cells reacted distinctively depending on the topography and chemical composition of exposed surfaces. Cell adhesion was greater in the control group, as has often been observed in surface biocompatibility tests.²³ Among the different titanium surfaces, adhesion was most pronounced on the roughest surface, indicating that its microarchitecture favors cell accommodation. These results suggest a positive effect of the cage in yielding homogenous texturized surfaces. This is likely to occur in the presence of a high concentration of energized ions near the samples, since the processing parameters were the same (temperature, reactor pressure, and treatment time) for both plasma techniques. Moreover, the samples nitried in the cathodic cage may have had more TiN compounds at the surface, thus promoting chemical-cell interactions.

Some studies of various methods of preparing titanium and stainless steel surfaces show that neither roughness amplitude nor material composition influences cell adhesion, except in pure titanium substrates treated with sandblasting. However, there are statistically significant differences between the preparation process, morphology, and chemical
composition of the resulting surface layer.\textsuperscript{16,21} Furthermore, there seems to be a relationship between surface roughness and cell adhesion, and osteoblasts showed stronger adhesion to rougher surfaces up to a certain limit. In fact, studies of MG-63 cells derived from human fibroblasts show that increasing titanium roughness beyond a certain point decreases cell proliferation and increases cell differentiation.\textsuperscript{24}

Cell proliferation was similar in all studied samples, which is an indication of the nontoxicity of the TiN film.\textsuperscript{23} The results indicate that adhesion is more closely related to the surface characteristics of titanium than cell growth because, as the cells tend to converge, the overlaid layers have little contact with the original topography. For the time length studied, no statistically significant differences were found between the groups.

Osteoblast morphology varied according to the surface pattern of the titanium. Dendritic projections and small filopodia were observed. However, it was not possible to clearly identify differences in cell morphology among the various surfaces.

Titanium has a polycrystalline structure; its physical properties depend on the distribution and orientation of the crystals. Preosteoblastic MC3T3 cells exposed to titanium-aluminum-vanadium samples with two crystallographic orientations revealed that cell adhesion and proliferation are affected by the three-dimensional orientation of the crystals and that the type of process used to alter the surface determines this modification.\textsuperscript{21}

Studies focusing on cell behavior on new titanium surfaces, such as those of Faghihi et al.,\textsuperscript{21} Wirth et al.,\textsuperscript{24} and Chien et al.,\textsuperscript{25} have shown wide variations in experimental design, including sample preparation method, sterilization, and cell type; this makes it difficult to identify the actual role that each factor plays in the results. In fact, different preparation methods might produce similar surface characteristics that induce different cellular responses. Furthermore, it may be inferred from the experimental work developed that there are difficulties in controlling plasma nitriding equipment. Mild variations in gas flow or internal reactor pressure may alter the surface characteristics of the samples. Therefore, rigid control of the entire process is imperative for obtaining homogenous samples and reliable results.

Process productivity can also be considered a critical issue, given that the working temperature is only obtained nearly 45 minutes after system startup and that the samples can be removed only after the reactor cools, bringing the overall nitriding cycle time to about 3 hours. The size of the sample holder is also an important limitation to the number of disks that can be simultaneously exposed to plasma. Clearly these limitations could be overcome with the development of industrial-scale nitriding equipment.

**CONCLUSIONS**

From this study carried out with pure titanium (grade 2) samples modified by plasma nitriding, it can be concluded that:

- Plasma nitriding had a positive effect on the initial cellular events leading to cell adhesion and proliferation, in that it was capable of producing surfaces with hydrophilic characteristics, especially those nitrided with the cathodic-cage technique.
- Both plasma nitriding techniques were capable of modifying both the topography and characteristics of the surface layer while maintaining biocompatibility characteristics (in this case, compatibility with preosteoblastic cells).
- Surface wettability was greater in the rougher samples.
- Cell adhesion was influenced by chemical composition and substrate surface roughness.

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