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BIOMEDICAL SCIENCES

Comparative analysis of the biocompatibility of endothelial cells on surfaces treated by thermal plasma and cold atmospheric plasma

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Abstract: In recent years, cold atmospheric plasma (CAP) is used for surface disinfection. However, little is known about its ability to improve biocompatibility of metallic surfaces when compared to thermal plasma methods. In this context, the study aimed to evaluate the response of human endothelial cells (Ea.hy926) on titanium surfaces treated by non-thermal plasma method and thermal plasma method under nitriding atmosphere. The wettability was characterized by the sessile drop method, the topography and roughness were evaluated by atomic force microscopy (AFM), and the microstructure by grazing angle X-ray diffraction (GIXRD). Endothelial cells were cultured and evaluated for morphology by scanning electron microscopy and viability by an MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay. CAP treatment reduced the contact angle of the Ti surface (13.43° ± 1.48; $p<0.05$), increasing hydrophilicity. Rz roughness was higher on the nitrided surface (220.44±20.30; $p< 0.001$) compared to the CAP treated surfaces (83.29 ± 11.61; $p< 0.001$) and polished (75.98 ±34.21a); $p<0.001$). The different applied plasma treatments created different titanium surfaces improving the biocompatibility of endothelial cells, however CAP results demonstrate its potential for biomedical applications, considering the low cost and ease of use of the technique, allowing surface treatments before clinical procedures.

Key words: Angiogenesis, cold atmospheric plasma, nitriding, titanium.

INTRODUCTION

Cold atmospheric plasma (CAP) research has advanced continuously since the beginning of this century, with its main applications in agriculture, health, and environment fields (Alves-Junior et al. 2021, Kim & Kim 2021, Silva et al. 2017). Concerning the health field, research on wound healing, blood clotting, disinfection of dental caries and alteration of mammalian cell functions displaying the potential for new therapies against cancer, among others, are already in full development or even in clinical use (Dubey et al. 2022, Dai et al. 2022, Alves-Junior et al. 2020, Shahbazi Rad et al. 2018, Yang et al. 2019).

Plasma applications aiming at bone and dental implant surface modifications, as well other implantable devices, are also frequently applied (Ujino et al. 2019). However, thermal plasma treatments, unlike CAP, frequently require low pressure conditions and, therefore, airtight chambers where the implantable devices are treated. In this regard, the nitriding method (thermochemical plasma treatment at low pressure), allows both plasma-surface interactions and ion diffusion to the applied material, such as titanium can form stoichiometric and non-stoichiometric titanium nitride (TiN) coatings with characteristics of a thin and hard film, which improves surface biocompatibility, leading to increased osteoprogenitor cell adhesion, proliferation and differentiation, as well as reduced bacterial adhesion and biofilm formation (Vitoriano et al. 2022, Del Castillo et al. 2021, Moura et al. 2019). This method, despite good results, is limited due to the need for vacuum conditions. In addition, processing is expensive due to the high costs of the vacuum equipment and its components, maintenance and the need for hermetically sealed chambers with dimensionally compatible with the part to be treated (Bárdos & Baránková 2008).

Thermal plasma is employed to promote metal surface modifications, mainly steels, through a combined process of material sputtering, adsorption and ion diffusion. This method applied plasma produced by an electrical discharge in a gaseous mixture inserted inside a hermetically sealed reactor, usually N_2+H_2. Active species and ions are then formed and collide presenting high energies on the metal surface, resulting in the sputtering of surface atoms, surface defects and the formation of unstable nitrides on the material surface. These different interaction effects result in positive topographically, chemically and structurally altered surfaces, capable of offering intrinsic property variability (Alves-Junior 2001). The CAP method, in turn, employs a high voltage (1-10 kV) and frequency (1-100 kHz), but low power, source. The produced plasma species can be transported to the application site by an inert gas jet (usually argon or helium), thus allowing a maximum distance between the source and the sample of up to 20 mm. Plasma jets can be obtained commercially in the form of small pens or brushes which are handled simply, safely and reliably (Alves-Junior et al. 2020, Braný et al. 2020). Thus, when comparing costs, ease of use, availability and practicality of both methods, the CAP method for environments such as operating rooms would undoubtedly be the most advantageous (Bernhardt et al. 2019). In the case of treatments with implantable devices, for example, this method can be applied immediately before implanting the device in the patient, by the health professional.

Plasma treatments can also alter properties that influence biological responses, such as surface wettability (Baniya et al. 2020). This property influences the adsorption of important proteins during cell adhesion, proliferation and differentiation (Metwally & Stachewicz 2019). Thus, the use of biomaterials to promote tissue repair is intrinsically associated to the type of employed material, as well as protein adsorption and cell differentiation (Breithaupt-Faloppa et al. 2006). Endothelial cells are responsible for the angiogenesis process and are necessary during tissue repair to ensure good perfusion and tissue oxygenation, as this process involves bone remodeling and integration (Richarz et al. 2017). In this sense, endothelial cell responses can alter functional implant viability, which directly influences the tissue repair success of modified surfaces. So far, to the best of our knowledge, no studies have been carried out to investigate CAP effects in this regard. The CAP treatment is efficient in the production of functional groups that alter cellular metabolism, such as oxygen and nitrogen reactive species (RONS), as well as UV radiation, which are responsible for microorganism inactivation in different applications (Moldgy et al. 2020, Ahn et al. 2014).

CAP treatment applied to metallic surfaces is quite recent. These applications are mainly intended for the disinfection of surfaces, through the inactivation of microorganisms and inhibition of bacterial biofilm formation, with the first works dating back to 2014 (Mai-Prochnow et al. 2014). The biocompatibility of eukaryotic cells on CAP-treated surfaces has only been evaluated from 2017 onwards, but there are still very few published works on the effect of CAP treatment on the biocompatibility of metal surfaces (Berger et al. 2021, Echeverry-Rendón et al. 2017, Guo et al. 2019, Lee et al. 2022). However, none of them evaluated the biocompatibility of endothelial cells, despite their importance in angiogenesis, a fundamental phenomenon in any process of tissue regeneration (Richarz et al. 2017).

In this context, the present study aimed to evaluate the possibility of obtaining a good biocompatibility of endothelial cells on titanium surfaces treated by CAP in comparison with high temperature plasma methods.

MATERIALS AND METHODS

Titanium surface preparation

Commercially pure titanium discs (TiCP – grade II) 9 mm in diameter and 3 mm in thickness were sanded in increasing granulometries (220, 400, 600, 1500, 2000 MESH), polished with 30% colloidal silica and 70% H_2O_2 and subjected to ultrasonic washing with an enzymatic detergent, 70% alcohol and distilled water for 10 minutes each. After drying, the discs were packed in surgical grade paper, sterilized in an autoclave for 30 minutes at 120°C and separated into three groups: polished (untreated), treated by CAP and treated by thermal plasma nitriding.

Titanium surface treatment

The CAP was generated in a dielectric barrier with a helium gas discharge at 15 kV at 600 Hz for 15 minutes in five different disk areas (3 minutes per area), at 5 mm distance with 1L/minute of incident helium gas (Figure 1a).

Nitriding took place in a hermetically sealed chamber with grounded chamber walls (anode) and a polarized sample holder. Once

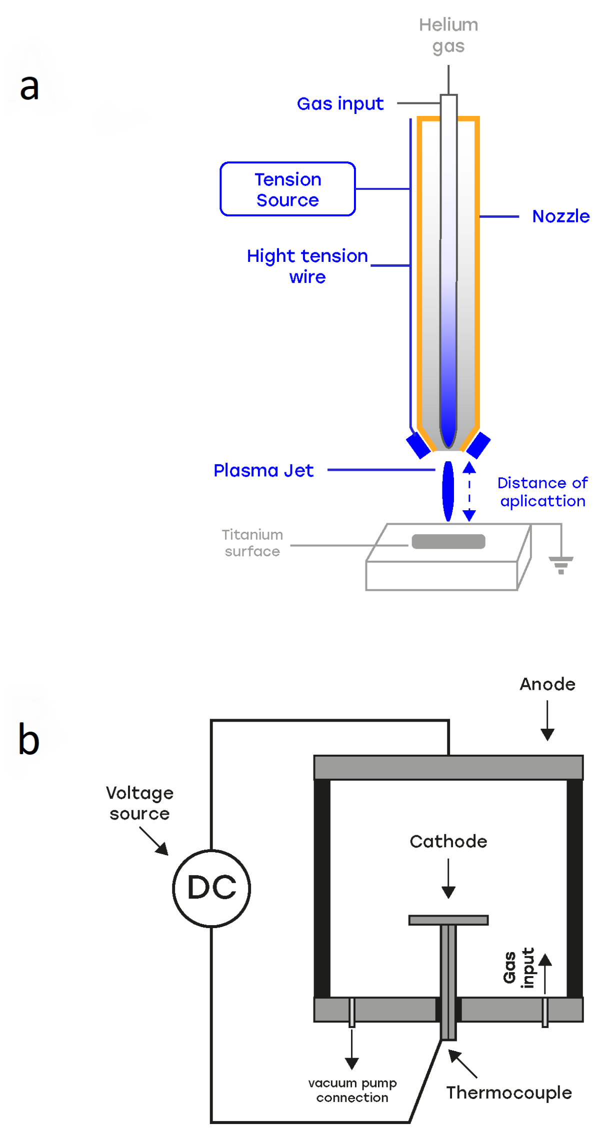

Figure 1. Schematic diagram of the apparatus used for plasma generation. (a) Cold atmospheric plasma (CAP) device applying plasma on titanium surfaces. (b) Low pressure plasma nitriding treatment with an airtight chamber.

the sample was placed in the sample holder, the reactor was closed and pumped to the residual lower limit of pressure of 2.7 Pa, followed by a 24 sccm hydrogen gas flow introduced for sample cleaning. This pre-treatment was carried out at 900 V at temperature up to 120° C for 30 min (typical cleaning condition applied in our laboratory) to clean the samples, to remove impurities and oxide films from the titanium surface. A pressure of 100 Pa was used for the plasma nitriding treatment, as an abnormal discharge regime is guaranteed under these conditions, *i.e.*, the voltage varies linearly with the current. Nitrogen gas was added to partially replace hydrogen up to a pressure of 200 Pa and N_2/H_2 flux ratio of 36 sccm/24 sccm at 450° C for 1 h (Figure 1b)

Titanium surface characterization

Wettability was evaluated by the sessile drop method, applying 20 µl of distilled water on the disks and obtaining an image of the formed drop (Braz et al. 2022). Roughness was determined through atomic force microscopy (AFM) in the non-contact mode, obtaining average roughness (Ra), maximum peak height profile (Rp), and maximum profile height per length (Rz) values. Crystalline microstructures were analyzed by grazing angle X-ray diffraction (Shimadzu XRD-6000), at a fixed incidence of 1° and scanning angle of 2° Theta.

Cell culture

Human endothelial Ea.hy926 strain cells (CRL-2922™, ATCC) were cultured in DMEM High Glucose medium (Dulbecco's Modified Eagle's Medium) modified with 4 ml of L-glutamine, 4500 mg/L of glucose, 1 ml of sodium pyruvate and 1500 mg/L of sodium bicarbonate and 10% of fetal bovine serum (SFB, Cultilab, SP, Brazil), 100 IU/ml of penicillin and 100 µg of streptomycin (Cultilab, SP, Brazil). The cells were incubated at 37° C in a 5% CO2 chamber with culture medium changes every 48 hours.

The cells were then cultured for 4 h on the Ti surfaces and fixed in 2.5% glutaraldehyde in phosphate buffer pH 7.4 (PBS) for 24 h followed by post-fixing in 1% osmium tetroxide for 4 h, dehydration employing increasing ethanol concentrations and metallization in 12 nm gold (Quorum – Q150RES). Images were obtained using a scanning electron microscope (SEM-SXX 550 Superscan, Shimadzu Corporation, Tokyo, Japan).

Cell viability

Cells were grown at a $5x10^5$ concentration on the polished discs and treated in 24-well plates for 24 h, followed by incubation in MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) for 3 h. The formazan crystals synthesized by MTT reduction were then dissolved with 1 ml of absolute ethanol in each well for 15 min and 100 µL from each well were transferred to a 96-well plate for analysis by absorbance spectrophotometry at 570 nm in a microplate reader.

Statistical analyses

Data normality was evaluated by the Shapiro-Wilk test. The contact angle and roughness results were submitted to a One Way ANOVA followed by Tukey's test, and a one-way analysis of variance test (ANOVA) was performed for cell viability, considering $p< 0.05$ as significant.

RESULTS

CAP- and low temperature plasma nitriding-treated surface characterizations

The wettability results obtained through the evaluation of the contact angle indicated that CAP-treated surfaces exhibited a significantly lower contact angle (13.43° ± 1.48) compared to

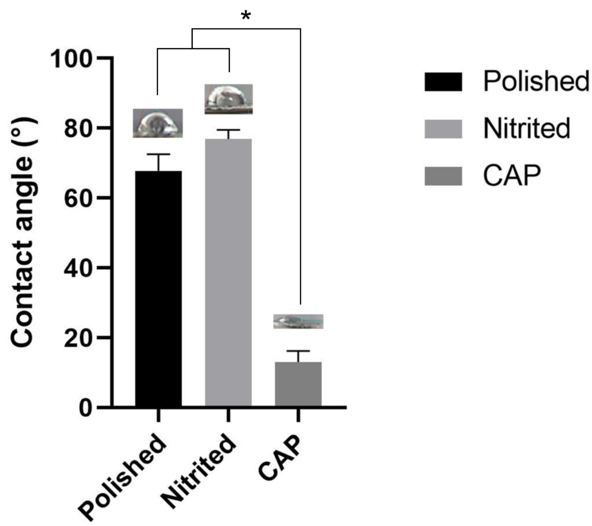

Figure 2. Wettability analysis through the sessile droplet method. (*) p<0.0001.

the nitrided (76.88° ± 2.68) and polished (67.83° ± 4.7, p<0.0001) surfaces (Figure 2).

The analyzed roughness parameters Ra, Rp, Rz and the Rp/Rz ratio provided information on treated surface morphology (Table I). In this regard, the roughness parameters of the CAP-treated surface were only slightly altered compared to the polished surface, in contrast to the nitrided surface, which displayed a 4-fold higher Ra compared to the polished sample. Both surfaces presented Rp/Rz>0.5 values, implying in the formation of sharp peaks on the treated surfaces (Figure 3a-b). However, the nitrided surface exhibited a more uniform distribution regarding disk roughness peaks (Figure 3c).

The grazing angle X-ray diffraction results indicate that the sample treated by low pressure plasma incorporated nitrogen into its crystalline structure (TiN interstitial solid solution), while the CAP-treated samples did not undergo microstructural alterations, denoted by XRD peak similarities to that of the polished surfaces (Figure 4).

Biocompatibility of CAP- and plasma nitriding-treated surfaces

Human endothelial cells (Ea.hy926) on CAP-treated surfaces exhibited a fibroblastoid morphology with the emission of cytoplasmic extensions (Figure 5a), similar to the morphology observed on the polished surfaces (Figure 5b). The emission of cytoplasmic extensions was not significant on the plasma nitrided surface (Figure 5c). A significant increase in the viability of human endothelial cells was observed on the CAP-treated surface when compared to the polished surface (Figure 6).

DISCUSSION

Metallic surface modifications employing plasma to improve/enhance their biocompatible properties have been applied in the health field with promising results, especially in the cardiology and orthopedics areas (Becerikli et al. 2021, Oikawa et al. 2021, Shim et al. 2018). However, due to a diversification of commercial devices used to obtain plasma, modified surface biocompatibility and characterizations are required. Therefore, our study aimed to evaluate titanium surfaces treated by both CAP and plasma nitriding and their implications regarding human endothelial cell biocompatibility.

Table I. **Roughness parameters (nm) of the analyzed titanium surfaces.**

Treatment	Ra	Rp	Rz	Rp/Rz
Polished	6.71 ±1.86	106.65 ±43.18	75.98 ±34.21[a]	0.70 ±0.04
CAP	3.06 ± 0.73	119.28 ± 18.14	83.29 ± 11.61[b]	0.70±0.09
Nitrided	27.72±1.43	140.14±26.33	220.44±20.30[c]	0.63±0.08

[(a-c)] **difference between treatments p< 0.001. One Way ANOVA followed by the Tukey test.**

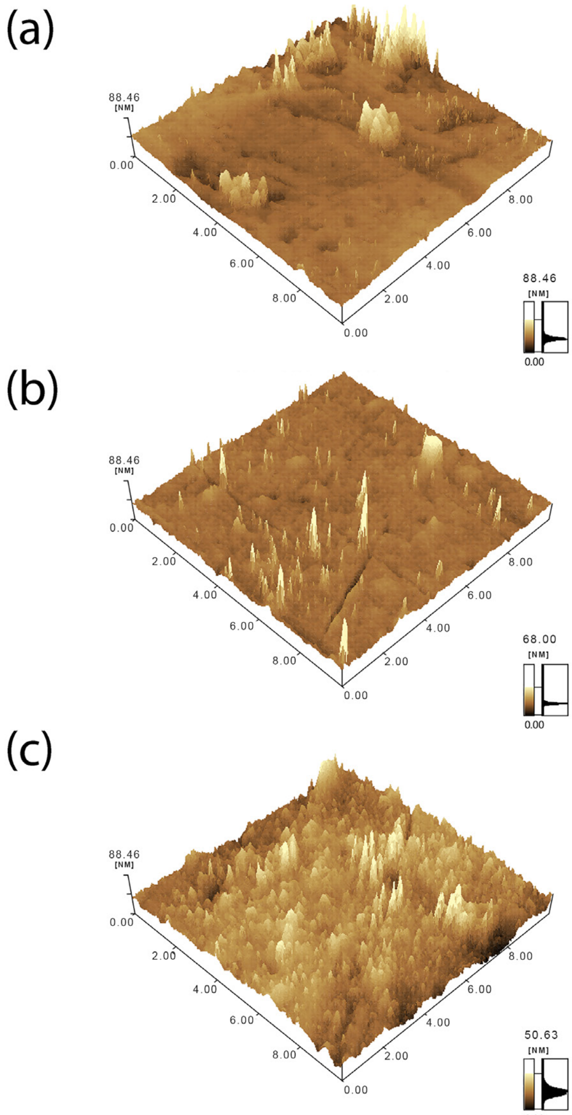

Figure 3. Nanometer roughness profile of titanium surfaces. (a) Polished surface profile. (b) CAP-treated surface profile. (c) Low pressure plasma nitrided surface.

The incorporation of different functional groups by plasma treatments on the Ti surface is directly associated with surface wettability (Kuźmicz-Mirosław et al. 2022). However, the titanium surface in the CAP treatment exhibited a reduced contact angle, making the surface highly hydrophilic when compared to previous results reported for this type of plasma in different materials, namely (Šrámková et al. 2020, Wang et al. 2019, Yan et al. 2022, Dong et al. 2020).

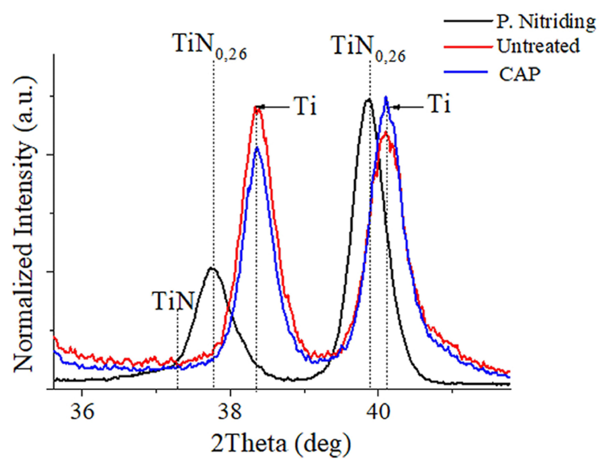

Figure 4. Diffractogram of titanium surfaces at a 2° Theta grazing angle.

Thus, CAP increases surface wettability, possibly due to the incorporation of polar groups derived from atmospheric air on the titanium surface (Dong et al. 2020).

Hydrophilic surfaces favor protein adsorption from the extracellular matrix and allow for cell adhesion (Moura et al. 2019). This implies in morphological cell changes, accelerating the surface integration process and reducing the risk of infection by microorganisms (Yan et al. 2022). The hydrophilic surface obtained by the CAP process directly increased the viability of the assessed human endothelial cells in only 24 hours, exhibiting good Ti cell adhesion, with expressive filopodia and cell membrane extensions adhered to the surface indicated by the morphology analyses conducted through scanning electron microscopy (SEM).

Among the treatments employed, only nitriding produced significant topographic changes on the Ti surfaces in relation to the control. Plasma nitriding produces roughness on metallic surfaces through the sputtering process (Moura et al. 2019, Fontoura et al. 2021). In CAP, a sputtering process does not occur, so changes in roughness are not significant (Lee et al. 2019). In the present study, plasma nitriding

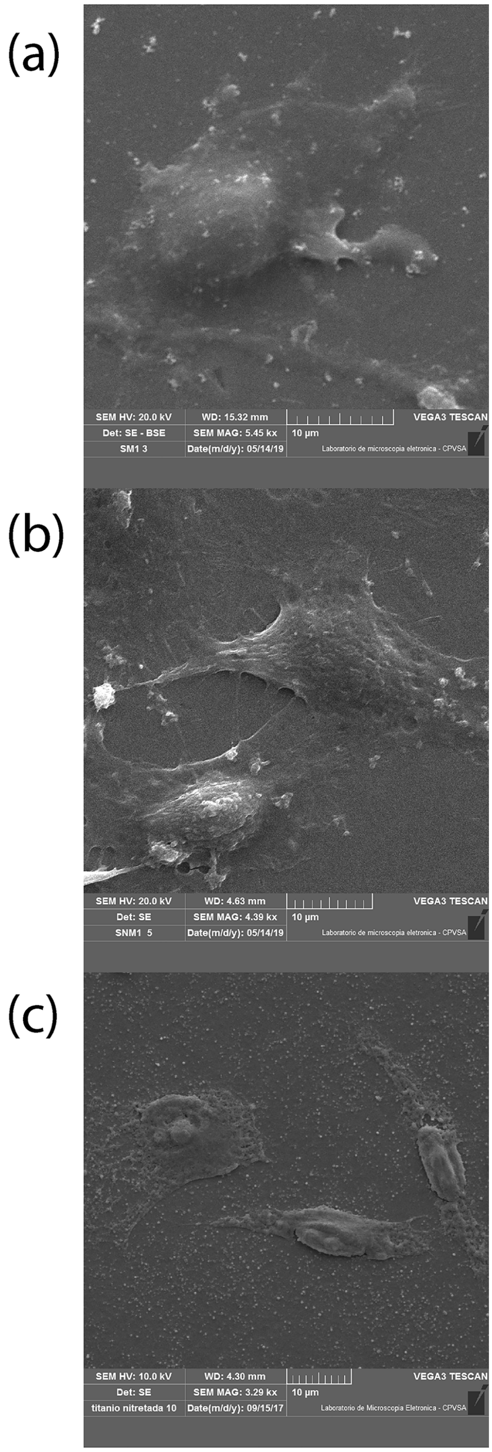

Figure 5. Electrophotomicrographs of Ea.hy926 human endothelial cells on titanium surfaces. (a) Fibroblastoid cell emitting cytoplasmic extensions on a CAP-treated. (b) Fibroblastoid cell emitting cytoplasmic extensions on a polished surface. (c) Elongated cells on low-temperature plasma nitrided titanium surfaces.

produced a roughness of a nanometric nature, with sharp and homogeneous surface peaks, mainly due to the integration of nitrogen ions in the composition of the titanium surface, as demonstrated in the XRD analysis. While, on the CAP surfaces, no significant differences were observed in relation to the polished surfaces (control). Titanium surfaces presenting nanoroughness peaks exhibit increased adhesion of osteoblastic cells (Gongadze et al. 2011, Moura et al. 2019) although nanoroughness can also make it difficult for endothelial cells to adhere to the titanium surface, as the natural microenvironment of these cells requires smooth surfaces that mimic the endothelium (Braz et al. 2020). This corroborates the increased viability of human endothelial cells noted on the CAP-surfaces compared to the cell viability obtained by nitriding.

Plasma can alter the chemical composition of films produced on metallic surfaces (Braz et al. 2022). Nitriding plasma resulted in nitrogen incorporation and the formation of a solid solution, allowing for thermally activated nitrogen atom diffusion in the titanium lattice, accelerated by surface bombardment and the formation of surface defects. CAP treatment, on the other hand, did not alter the microstructure of the titanium surface, but was able to keep the basic surface elemental structure stable, probably because the process is short and occurs at room temperature, making atomic diffusion impossible. However, it has been previously demonstrated that the CAP technique could incorporate molecules of gases present in the atmosphere, such as nitrogen and oxygen, on the titanium surface. The incorporation of these molecules increases the polar oxygen groups, such as hydroxyl, carbonyl, and carboxyl groups, resulting from the direct interaction of the plasma with the surface (Modic et al. 2019). These formed groups, mainly the OH groups,

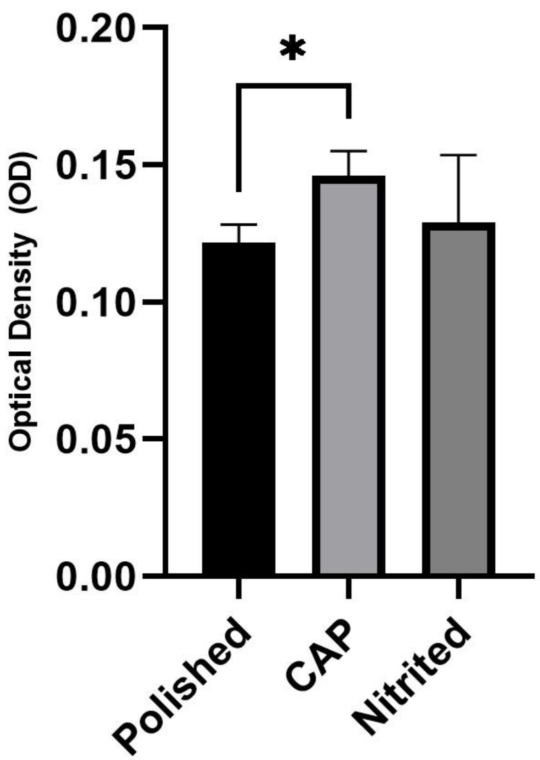

Figure 6. Endothelial cell viability on a CAP-treated surface and following low temperature nitriding. (*) p<0.05.

contribute to increase the hydrophilicity of the titanium surface (Wu et al. 2019). Surfaces with hydrophilic characteristics positively influence the adhesion, communication, and proliferation of cells on a surface. This is due to the fact that the surface interacts better with organic compounds, such as integrins, which are involved in the cell/surface interaction process (Berger et al. 2021). In the present work, CAP did not alter the roughness of the titanium surfaces, but there was a significant increase in the wettability of the Ti surface, as demonstrated in the contact angle test. Therefore, certainly wettability influenced the adhesion and viability of endothelial cells on CAP-treated surfaces.

CONCLUSION

The different plasma treatments applied herein promoted different modifications to the investigated titanium surfaces. these modifications were able to improve the biocompatibility of endothelial cells. the cap results are paramount for biomedical applications, especially considering application conditions. commercial cap devices are small, easily handled and can be used under normal ambient temperature and pressure conditions, allowing for surface treatments immediately prior to clinical procedures.

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AS and JKB: investigation, writing – review & original. GM and VS: investigation. JV: formal analysis, validation. AA and DN: writing – review & original, visualization. EL and HR: supervision, resources. CAB: metodology, supervision, writing – review & editing. CAJr: funding acquisition, supervision, resources, writing – review & editing. CEB: conceptualization, data curation, project administration, writing – review & editing.