Plasma of Argon Accelerates Murine Fibroblast Adhesion in Early Stages of Titanium Disk Colonization

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Purpose: *This study was conducted to analyze how a cleaning treatment using plasma of argon would affect fibroblast growth on titanium disks at different time points to determine whether this treatment could enhance soft tissue healing around titanium dental implant abutments.* ***Materials and Methods:*** *Sixty sterile disks made of machined grade 5 titanium were divided into two groups; 30 disks were left untreated (control) and 30 were cleaned using plasma of argon (test). To simulate clinical conditions during soft tissue healing around titanium abutments, both groups were immersed in a culture of murine fibroblasts (L929) for 2, 8, or 48 hours. After preparation, they were stained using 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) to label the cellular nuclei and fluorescent phalloidin to label the cellular bodies. The nuclei were counted, and cellular bodies were analyzed with fluorescent microscopy and imaging analysis software. Analysis was performed at the three different time points.* ***Results:*** *Fibroblast adhesion for the test group was statistically significantly greater versus the control group at 2 and 8 hours but not at 48 hours. At 2 and 8 hours, the cellular bodies in the test group appeared flatter and more spread out, revealing more advanced cellular adhesion, compared to the cells observed in the control group. At 48 hours, the test and control specimens were nearly indistinguishable.* ***Conclusion:*** *The removal of organic and inorganic contaminants from the surfaces of titanium disks using plasma of argon accelerated fibroblast adhesion in the early stages of colonization (2 to 8 hours). This effect disappeared after 48 hours as a result of saturation. Clinically, abutment cleaning using plasma of argon might positively affect soft tissue healing in early stages.* Int J Oral Maxillofac Implants 2013;28:957–962. doi: 10.11607/jomi.2664

Key words: *abutments, cell colonization, fibroblast adhesion, glow discharge, plasma cleaning, titanium surface*

The initial interactions between cellular components and materials play an important role in the healing process around implants. These interactions are critically mediated by the qualities of the surface, as described in the literature.[1] Plasma treatment (gasses generally employed are air, oxygen, argon, and nitrogen) has been used for several decades to increase cellular adhesion to polymeric materials.[2] Plasma can change the chemical composition of a polymeric material surface, introducing polar functional groups in apolar and hydrophobic chains; this promotes the protein absorption that favors cellular adhesion.[3] Plasma has also been used to treat metallic materials; whereas there is almost no effect on the chemical composition of the material, it has been demonstrated that plasma modifies the contaminants that are inevitably present on the surface of high-superficial-energy materials such as metals.[4] Plasma treatment, which effectively removes the organic contamination on metals by interacting with the intrinsic components of the materials (titanium dioxide [TiO_2], in the case of titanium), creates a surface that is newly available for interaction with cells.[5] It must be highlighted that plasma treatment does not modify the topography (roughness) of the titanium, only its surface hydrophilic properties.

Several studies on this topic have been conducted since the 1980s, and increased cellular adhesion has been reported on high-energy surfaces (metals, ceramic) submitted to plasma treatment.[6–8] It was demonstrated that the effect of plasma treatment gradually decreases if the sample remained exposed to contaminants[9]; however, a lack of knowledge is present in the literature regarding the interaction over time between cells and treated metals.

The present study sought to verify the effects of treatment with plasma of argon on the interaction between sterile machined titanium disks and fibroblasts

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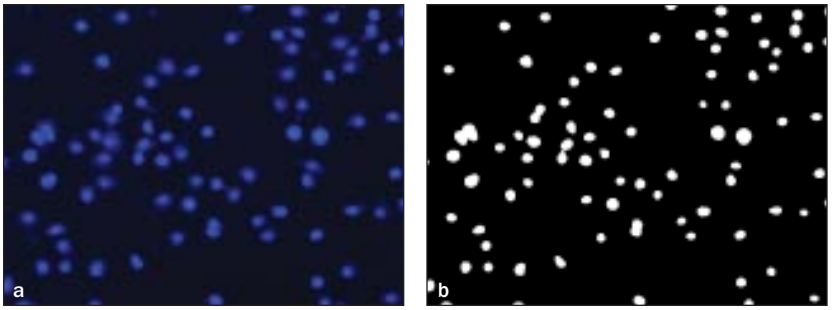


Figs 1a and 1b Images representing the same field *(a)* after DAPI staining and *(b)* after the process of elaborating for the count.

at different time points. The hypothesis was that the modification of abutment surfaces following treatment with plasma of argon could enhance soft tissue healing around titanium dental implant abutments.

MATERIALS AND METHODS

To simulate clinical conditions during soft tissue healing around titanium abutments, 60 sterile grade 5 titanium alloy (Ti-6Al-4V) smooth-surfaced disks (diameter, 4 mm; average roughness, 34.57 ± 5.79 nm; Sweden & Martina) were adopted for the present in vitro study. In fact, titanium presenting similar characteristics is usually adopted for manufacturing implant abutments. Cytotoxicity tests were performed to show that the titanium disks were not directly cytotoxic according to International Organization for Standardization 10993-5 (2009) to prevent any bias.[10]

The disks were divided into two groups. Thirty were left untreated and used as controls. The remaining 30 were used as the test group and underwent argon plasma treatment (10 W, 1 bar for 6 minutes) in a plasma reactor (Colibri, Gambetti Company). The test and control groups were then divided into three subgroups of 10 disks each to correspond to three different observation time points (2, 8, and 48 hours).

According to the protocol of Michaels et al,[11] the disks were put into polystyrene multiple-well sterile plates for cellular culture (Cell Star, Greiner Bio-One). Murine fibroblastic cells (L929, IZSLER) were put into the culture wells on treated (test) and untreated (control) disks. A cell suspension (1.5×10^5 cells/mL in 5 mL of Eagle's minimum essential medium mixed with 10% bovine fetal serum, L-glutamine, penicillin, and streptomycin; Gibco, Invitrogen) was introduced into sterile polystyrene 12-well plates (Cell Star, Greiner Bio-One) containing the disks. The viable cell density of each suspension was calculated by means of a TC10 Automated Cell Counter (Bio-Rad Laboratories). The plates were kept at 37°C in 5% carbon dioxide and a relative humidity of 98%.

At the different experimental times, the samples were removed and carefully washed with Dulbecco's phosphate-buffered saline (DPBS) (Invitrogen) to remove the nonadherent cells. All samples were fixed by immersion in 4% buffered formaldehyde at room temperature for at least 48 hours. The samples were observed under a fluorescence microscope (Motic AE 31, Motic Spain SLU) after the following steps were performed: *(1)* cellular membrane permeabilization using Triton X-100 1% solution, followed by rinses with DPBS; *(2)* blocking of nonspecific sites using 1% bovine serum albumin in DPBS; *(3)* fluorescent labeling of cellular bodies with phalloidin (Alexafluor 488, Life Technologies Italia), followed by rinsing in DPBS; and *(4)* nuclear staining with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) (Invitrogen), followed by rinsing with DPBS.

After the homogeneity of cell distribution had been demonstrated for every sample, images were obtained at ×200 magnification, and the number of cells in every subgroup was counted three times (Fig 1a). To facilitate additional measurements, images were then transformed into black and white (Fig 1b). Assessments were performed with image analysis software (ImageJ, US National Institutes of Health). The size of the image field was set at 560 × 420 µm, and cellular density (number of cells/unit of surface) was easily calculated. To quantitatively rate the effects of treatment, 10 repetitions of each measurement were made per subgroup.

The process of adhesion and colonization of the surfaces was also analyzed by observing the cellular bodies that had been stained by fluorescent phalloidin.

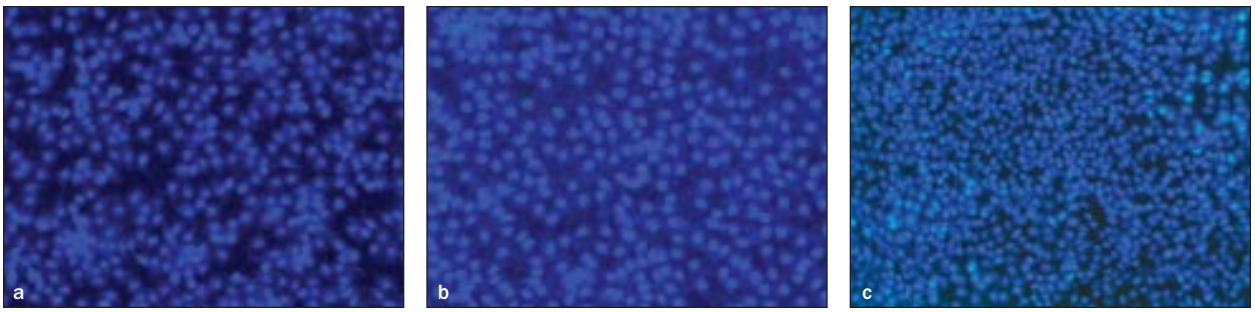
Statistical Analysis

Descriptive statistics, including means and standard deviations, were used to describe cell adhesion. Eventual differences between groups were analyzed using the Wilcoxon matched-pairs signed-ranks test.

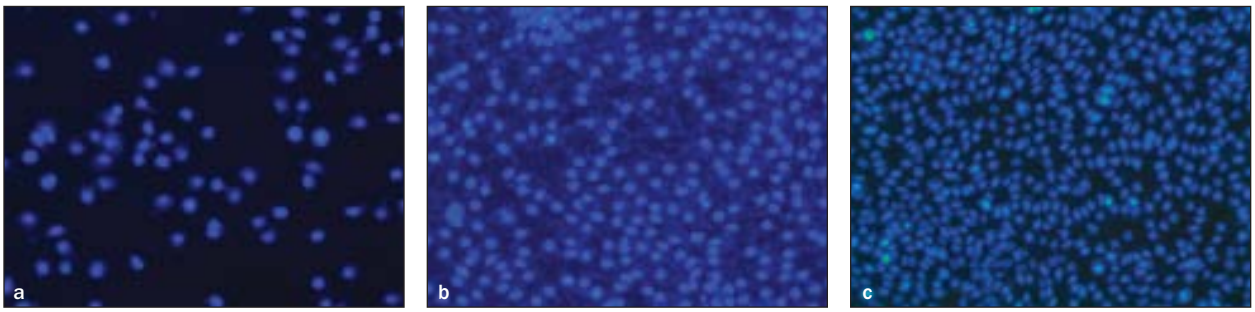
Table 1 Cellular Proliferation on Titanium Alloy Disks over Time

	Cell adhesion (cells/field)					
	2 h*		8 h†		48 h‡	
Sample	Control	Test	Control	Test	Control	Test
1	140	268	213	160	389	449
2	84	170	85	131	329	395
3	168	220	155	262	370	469
4	125	154	81	297	289	454
5	170	173	245	266	296	354
6	146	193	245	257	452	386
7	125	165	156	235	444	292
8	152	151	186	219	386	230
9	127	157	247	246	301	401
10	117	163	230	259	465	280
Mean	135	181	184	233	372	369
SD	26	37	64	51	67	84
Range	84–170	151–268	81–245	131–297	289–465	230–469

SD = standard deviation.
Wilcoxon test: *P = .0039; †P = .0488; ‡P = .9219.



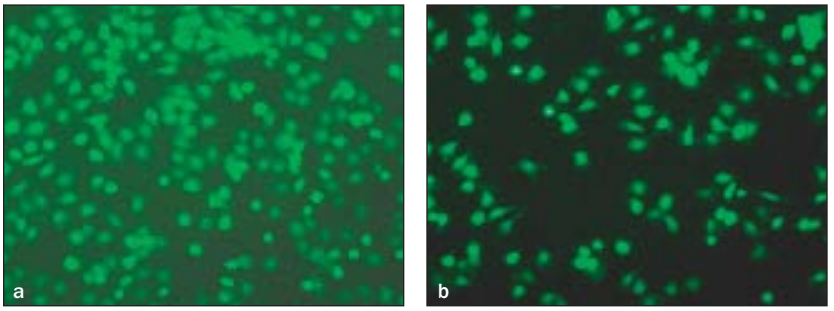
Figs 2a to 2c Typical images collected at *(a)* 2, *(b)* 8, and *(c)* 48 hours from the plasma-cleaned group (compare to Fig 3) (DAPI; ×200).



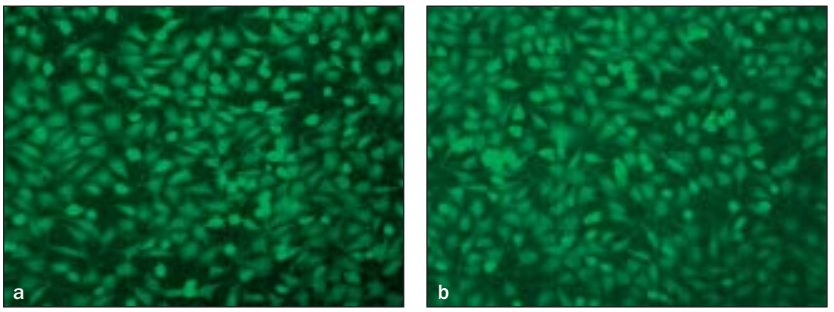
Figs 3a to 3c Typical images collected at *(a)* 2, *(b)* 8, and *(c)* 48 hours from the untreated group (compare to Fig 2) (DAPI; ×200).

RESULTS

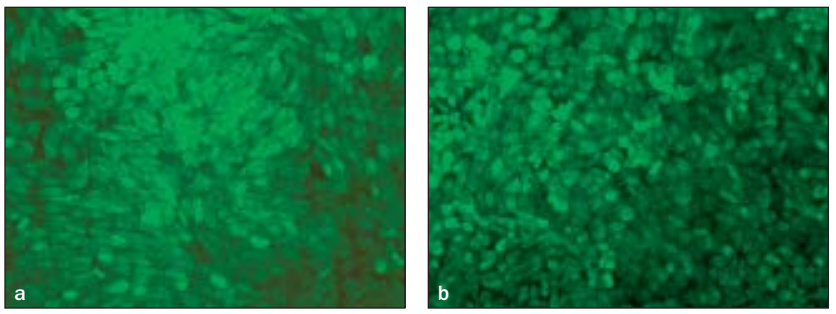
All data (average, standard deviations, and analysis of differences) regarding the number of cells per field for test and control group at each time point are reported in Table 1. In the test group, fluorescence microscopy showed mean fibroblast adhesion values of 181 (± 37), 233 (± 51), and 369 (± 84) cells/field at 2, 8, and 48 hours, respectively (Figs 2a to 2c). The control group presented mean fibroblast adhesion values of 135 (± 26), 184 (± 64), and 372 (± 67) cells/field at 2, 8, and 48 hours, respectively (Figs 3a to 3c). Statistical analysis of cell adhesion showed significant differences at 2 hours (P = .0039) and 8 hours (P = .0488) but not at 48 hours (P = .9219) (Table 1).



Figs 4a and 4b The cellular bodies were flatter and more spread out on the *(a)* treated than on the *(b)* untreated samples after 2 hours (fluorescent phalloidin; ×200).



Figs 5a and 5b Compared to Fig 4, differences in cellular bodies were more difficult to detect at 8 hours. *(a)* Treated samples; *(b)* untreated samples (fluorescent phalloidin; ×200).



Figs 6a and 6b The cellular bodies are flatter and the cells are thickly packed at 48 hours, colonizing the entire surface in the *(a)* test and *(b)* control groups. The cells in the test group appear compacted according to a spiraled organization, probably because of the initially improved adhesion to the substrate.

At 2 hours, the cellular bodies appeared flatter and more spread out, revealing advanced cellular adhesion in the treated samples (Figs 4a and 4b). The differences between test and control cells were more difficult to detect at 8 hours (Figs 5a and 5b). After 48 hours, all cellular bodies were flatter and the cells were thickly packed, colonizing the whole surface in both groups (Figs 6a and 6b).

DISCUSSION

Recent research has focused on promoting cell adhesion to titanium to shorten the time needed to achieve osseointegration. Several authors have suggested that chemical treatments[12] can influence osteoblast adhesion. In fact, physical treatment of the titanium implant surface can modify osteoblast adhesion, activating the surface and, thus, enhancing osteoblast adsorption on titanium.[13,14]

Other research efforts have explored soft tissue adhesion to the titanium abutments as key to esthetic success. To simulate clinical conditions of soft tissue adhesion to titanium abutments, grade 5 smooth-surface titanium disks and fibroblasts were used in the present study according to Hjalmarsson et al.[15] In fact, grade 5 smooth-surface titanium is routinely used to manufacture implant abutments.

Additionally, the mouse fibroblast cell line L929 was used because of its robust and established cell line, which ensured the required reproducibility and accuracy of the response. L929 cells have been demonstrated to adhere to most substrates (anchorage dependent) favoring screening cell adhesion. According to Michaels et al,[11] the present experimental design allows a clear vision of the process of fibroblast colonization of the titanium surface after the cleaning process using plasma of argon.

Observations at each time point of the study reported a growing number of cells on both types of

samples. This constant growth reached its saturation point at 48 hours, in agreement with the observations of Furuhashi et al.[16] The observation outcomes of the first two time points of the present study show that it is possible to accelerate the effects of plasma treatment in the first moments of cell-material interaction, ie, during the process of cellular adhesion. Indeed, major effects of the treatment were reported after 2 and 8 hours. According to the present data, cellular adhesion seemed to be accelerated by the use of plasma treatment in the short term, when fibroblasts came into direct contact with the titanium surface.[6,7,11,12] In fact, the removal of every low-energy contaminant from the surface clearly implies an increase in the cellular adhesion kinetics. The reported data, however, demonstrated that this effect was not present after 48 hours; in fact, no statistically significant difference was found at 48 hours after treatment between the test and control groups. The lack of difference in terms of cells could be related to the decrease of adhesion power in the test group, as reported by Swart et al.[5] However, this phenomenon could also be related to other factors. According to the literature,[16–18] the cells colonized both surfaces to the point of saturation of the space available. Considering the dimension of the field of observation, cellular density had reached 1,500 cells/mm^2 at 48 hours. Incidentally, these data confirm the expected absence of phenomena of toxicity to titanium, either treated or untreated.[19]

From a clinical point of view, reported data may lead one to suppose that plasma cleaning treatment of titanium abutments could provide favorable results in terms of soft tissue healing. In fact, positive wound-healing responses around metallic implants depend on critical control of the surgical and restorative approaches used in dental implant treatment. One clinical parameter is the role of a clean, sterile oxide surface on implant abutments.[5] In the early stages of wound healing, faster fibroblast adhesion to a plasma-cleaned titanium abutment at the supracrestal level could lead to stronger fixation of the connective tissue collar, which may prevent epithelial downgrowth. In fact, to avoid bacterial penetration that could jeopardize either initial healing or long-term behavior of implants, the formation of an early and long-standing effective barrier capable of biologically protecting the peri-implant structures is mandatory. The establishment of this soft tissue barrier is a critical part of tissue integration and is fundamentally the result of wound healing, which must establish an effective interface between living tissues and a foreign body.

If the interaction of a biomaterial with its environment is governed largely by surface properties, the chemical characterization of the surfaces possesses great importance. Titanium abutments, in fact, have demonstrated surface oxidation, while preparation by a technician or the eventual sterilization process could result in chemical or physical contamination of the surface.[20] A recent prospective proof-of-principle human study, in fact, demonstrated that a microscopically modified implant collar (8- to 12-µm microgrooves created with a laser) allowed direct supracrestal connective tissue attachment.[21] Accordingly, modifications to the abutment surface may also be effective in preventing the commonly observed crestal bone loss and stabilizing the supracrestal soft tissue attachment.[22]

On the other hand, as demonstrated by Amoroso et al,[23] bacterial adhesion to implant surfaces may be influenced by material surface roughness and surface free energy parameters. This event could complete the direct fibrocollagenous adhesion to titanium in the early stage of soft tissue healing, mitigating its longitudinal positive effect. However, as demonstrated by Canullo and Götz,[24] preoperative and postoperative antiseptic treatment of the peri-implant mucosa and prevention of contact of the abutment with saliva or skin during prosthetic procedures should minimize bacterial competition.

Despite the positive clinical preliminary results, however, clinical and histologic trials should be performed to confirm whether the improved fibroblast adhesion produced by plasma of argon cleaning procedures may lead to better formation of a collagenous connective tissue in the clinical setting.

CONCLUSIONS

Plasma of argon treatment on titanium disks immediately before exposure to a suspension of L929 murine fibroblastic cells significantly increased the speed of cellular adhesion compared to untreated control disks. Cellular density on treated disks was significantly superior after 2 and 8 hours, whereas at 48 hours the density was equivalent to that on control (untreated) disks because of the complete colonization of the available surface. Observations of the cellular morphology confirmed the increase of the speed of adhesion. This study confirmed that plasma of argon may be advantageously adopted for abutment cleansing to favor peri-implant tissue healing around implant abutments.

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The authors reported no conflicts of interest related to this study.

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