Plasma of argon increases cell attachment and bacterial decontamination on different implant surfaces

~~Atmospheric-pressure dielectric-barrier-discharge plasma of Argon influences cell attachment and bacterial decontamination: in vitro preliminary results~~

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Abstract

Aim: ~~To test the effects of Argon atmospheric-pressure dielectric-barrier-discharge (APDBD) to disinfect different implant surfaces.~~ This *in vitro* study tests the effects of Argon atmospheric-pressure dielectric-barrier-discharge (APDBD) on different implant surfaces with regards to physical changes, bacterial decontamination and osteoblast adhesion.

Material and methods: Seven hundred twenty disks with 3 different surface topographies - machined (MAC), plasma sprayed (TPS) and zirconia-blasted and acid etched (ZRT)- were tested in this experiment. Bacterial adhesion tests were performed recurring to a simplified biofilm of *Streptococcus mitis*. Bacteria were incubated in presence of the sample, which were subsequently either left untreated as controls or treated with APDBD for 30, 60 and 120 seconds. Samples were then metalized, prior to the acquisition of images recurring to a scanning electronic microscope (SEM). Protein adsorption, surface wettability and early biological response were determined for both treated (120") and untreated implant surfaces. For depicting the eukaryotic cell behavior, pre-osteoblastic murine cells were used. Cells ~~adherent at 12 minutes~~ were conveniently stained and nuclei were counted. Cell Viability was assessed by a chemiluminescent assay, at 1, 2 and 3 days.

Results: On all treated samples, values of the contact angle measurements were lower than 10°. The untreated samples showed values of contact angle of 80°, 100° and 110°, respectively for MAC, TPS and ZRT. The plasma of Argon disinfection significantly increased the protein adsorption on TPS and ZRT~~. No significant effect was achieved on the machined titanium disks. Bacteria adhesion was greatly reduced even after 60 seconds of Argon treatment. The number and the cell spreading area of osteoblasts adherent at 12 minutes significantly increased in all treated surfaces.~~ The protein adsorption on TPS and ZRT was significantly increased after the plasma of Argon disinfection. However, no significant effect was noted on the MAC disks. The number and the cell spreading area of osteoblasts adherent significantly increased in all treated surfaces. Nonetheless, Argon treatment did not influence the osteoblast proliferation and viability at different time-points. Bacteria adhesion was significantly reduced even after 60 seconds of Argon treatment.

Conclusions: ~~Preliminary data suggest that APDBD disinfect implant surface with potential to promote osteoblasts attachment and spreading.~~ Preliminary data showed that argon atmospheric-pressure dielectric-barrier-discharge disinfect implant surface with potential to promote osteoblast attachment and spreading, suggesting this maybe a possible approach to decontaminate peri-implantitis contaminated implant surface.

Introduction

Peri-implantitis ~~or implant infection~~ is a biologic complication involving soft and hard tissues around implants. The prevalence of this disease is estimated to be ~~A recently published study presented the prevalence of this disease ranging between~~ 12% and 14% ~~in the private practice. Similar prevalence rate were also reported in the University environment~~ [12]. In order to successfully treated the disease, the contaminated surface has to be disinfected.

Different methods/techniques were proposed to minimize or even completely remove biofilm from these contaminated surfaces [3]. Chemical and air-abrasive treatments have been shown to be able to disrupt biofilm [4,5]. However, chemical cleaning solution in combination with mechanical debridement ~~is ineffective to remove~~ has been shown to be incapable of removing bacterial biofilm [6]. Conflicting results were presented with regards the effect of dental lasers [7] [8]. ~~Inconsistent results were presented by lasers or photodynamic therapy~~. Interestingly, implantoplasty appears the best way to remove infected contaminants [9] [10]. ~~When re-osseointegration of these treated contaminated implant surfaces was assessed, the quality of the implant surface after decontamination dictates the outcome~~

Plasma treatment was described to effectively decontaminate surfaces not impacting on their complex micro-topography [11]. In fact, although this technology was originally developed to decontaminate flat surfaces in the microelectronics industry, plasma cleaning was recently introduced into implant dentistry to clean implant abutments [12] and activate implant surfaces for cell attachment [13]. Indeed, non-thermal plasma, which may be produced either at atmospheric pressure or under vacuum [14], preserve the integrity of materials [15] while removing any possible organic debris [16]. This technology, once appropriate parameters (pressure, energy, type of gas) are set, is able to increase surface energy.[14] From a physico-chemical point of view, the accelerated electrons and ions within plasma may render surfaces hydrophilic, increasing the capability of the titanium oxide layer to interact with proteins and cells. Additionally, this treatment was proven capable to enhance tissue adhesion [17] and to further support osteoblast spreading [18].

Non-thermal plasma technology is available also for intraoral use producing plasma under atmospheric pressure. In this form, plasma represents an electrically neutral, ionized cold gas composed of ions, electrons, neutral particles, free radicals and chemically reactive neutral particles. At the same time, when oxygen was used as gas, in combination with brushing, it could dest roy and eliminate bacterial biofilms [19]. This might be very promising for treating peri-implantitis and improving the ability of re-osseointegration.

Hence, the purpose of this *in vitro* study were to text the effects of Argon atmospheric-pressure

dielectric-barrier-discharge (APDBD) on 3 different implant surfaces with regards to bacterial decontamination and osteoblast adhesion. ~~the purpose of the present study was to test the effect of a novel Argon atmospheric pressure dielectric barrier discharge (APDBD) plasma on osteoblasts grown on different commercially available implant surfaces. Bacterial decontamination was also assessed in a preliminary test.~~

Materials and methods

A power analysis was estimated from to a similar clinical study [12], mean fibroblast adhesion values of 181±37 and 135±26 at 2 hours (P =0.0039) was projected by setting effect size dz = 1.438, error probability a = 0.05, and power = 0.95 (1-b error probability), resulting in 12 sample from each sub-group (G* Power 3.1.7 for Mac OS X Yosemite, version 10.10.3).

Seven hundred twenty serially numbered, sterile 4 mm diameter disks (Sweden & Martina, Italy), made of grade 4 titanium, with ~~three~~ 3 different surface topographies: machined (MAC), plasma sprayed (TPS) and zirconia-blasted and acid etched (ZRT), were used in the present study. Surface topography analyses ~~of the different surfaces~~ were reported in the previously ~~reported~~ study [20]. ~~The titanium disks were divided into three sub-groups of 144 samples each according to the surface topography~~. Three computer-generated randomization lists (Random Number Generator Pro 2.08 for Windows, Segobit Software, http://www.segobit.com/) were used to randomly allocate the titanium disks into three additional sub-groups, consisting in an equal number of 24 titanium disks, to be used for the ~~six~~ 6 different ~~testing treatments~~ experimental procedures. In particular, contact angle measurement, bacterial adhesion, protein adsorption, cell adhesion, cells morphology and cell viability were tested. For some experimental procedures, several time points were taken in account (Figure 1).

For each treatment, ~~twelve~~ 12 titanium disks for each sub-group were randomly allocated as test group and underwent APDBD treatment (8 W at atmospheric pressure for 2 minutes) using a non-thermal Dielectric-Barrier-Discharge (Plasma Beam Mini, Diener Electronic GmbH, Ebhausen, Germany). Specimens were treated in a meander-like scanning mode, under ambient pressure and with a working distance of 2 mm between the nozzle of the plasma jet and the surface of the sample. The remaining 12 non-treated titanium disks of each sub-group were used as controls (Figure 1).

~~All the computer-generated randomization lists were prepared in advance by an external investigator not involved in the study and an independent consultant prepared all of the envelopes/containing numbers for randomization, which were opened immediately before the testing procedures.~~

For each experiment, altogether, 72 discs were treated (3 surfaces x 2 groups x ~~12 repetitions~~ 3 repetitions x 4 technical replicates). A flow diagram of the randomization sequence is reported in Figure 1.

Contact angle characterization

The surface wettability was estimated according to Duske et al. (2012)[18] ~~by the measurement of~~ measuring the water contact angle in control (no treatment) and test (120" of treatment) samples. ~~Briefly, a~~ A drop of distilled water with a volume of 0.5 µl was poured onto the tested surfaces ~~recurring to~~ by using a dynamic contact angle tester Data Physics OCAH 200 (Data Physics Corp., USA, California, San Jose). A picture of the drop was acquired by using the high-resolution camera of the contact angle tester immediately after and 1 minute thereafter. The contact angle was determined using the data analysis software provided along with the instrumentation by fitting the sessile drop profile. ~~For each sample, the contact angle measurement was repeated seven times.~~

Bacterial Adhesion

Bacterial adhesion test was performed as follows. The samples were washed in 70% ethanol and subsequently in sterile water. Finally the specimens were dried under a sterile hood. *Streptococcus mitis* was purchased by ATCC and suspended following these indications: 100 µl were seeded on Luria Agar (LA) and incubated for 12h (at 37 °C). Three colonies were transferred into 20 ml of Luria Broth (LB), incubated for 24h (at 37 °C, 5% CO_2) and then 100 µl of bacterial suspension were refreshed in new 500 ml of new LB for 24h. ~~Meanwhile,~~ Afterwards, the samples were placed into 120ml of bacterial suspension under constant stirring (650–700 rpm), at 37 °C for 2 h. After rinsed with Phosphate Buffer Saline (PBS), samples were: a) treated (except the control condition, left untreated) with APDBD for 30, 60 and 120 seconds, using 36 titanium disk for each time-point; b) washed with PBS; c) fixed by using ~~in~~ 2.5% glutaraldehyde in PBS for 30 min at 4 °C; d) washed with pure water and e) dehydrated with immersion in increasingly high ethanol-water solution till 100% ethanol. Steps from b) to e), was necessary to prepare the samples for scanning electron microcopy (SEM) by fixing a dehydrating

bacteria. Samples were finally metalized with chromium. Images were acquired recurring to a SEM (Zeiss EVO 50, Carl Zeiss AG, Oberkochen, Germany) at 10000 and 35000 magnifications. Sterile forceps were used to avoid contamination. At least 12 different fields were acquired for each sample. Three different samples were used for each condition tested and the experiments were replicated three times (n = 3).

Protein adsorption

To determine the quantity of protein adsorbed onto the titanium disks, a 1% solution of Fetal Bovine Serum (FBS) in PBS was used to incubate the titanium disks (36 disks, control group and 36 disks, APDBD treatment for 120'') at 37°C for 30 minutes. Then the samples were washed twice with PBS and the adsorbed protein was eluted from the disks using Tris Triton buffer (10mM Tris (pH 7.4), 100mM NaCl, 1mM EDTA, 1mM EGTA, 1% Triton X-100, 10% Glycerol and 0.1% SDS) for 10 minutes. Total protein amount eluted from the disks was quantified using Pierce™ BCA Protein Assay Kit (Life Technologies) according to the manufacturer's protocol measuring the absorbance at 562nm.

Cell culture

To characterize in vitro the biological response, a pre-osteoblastic murine cell line MC3T3-E1 (ECACC) was used [21]. Cells were maintained in Alpha MEM supplemented with 10% fetal bovine serum (Gibco Life Technologies, Milan, Italy), 100 U/ml penicillin, 100 µg/ml streptomycin, were passaged at subconfluency to prevent contact inhibition and were kept under a humidified atmosphere of 5% CO_2 in air, at 37°C.

Cell adhesion assay

Cell adhesion was evaluated on titanium disks using a 96 well plate as support. Cells were detached using trypsin for 3 minutes, carefully counted and seeded at 3 x 10^3 cells/disk in 100µl of growth medium on 36 disks for control group (left untreated) and 36 for APDBD treatment (120'')~~disks with different roughness~~. The 96-well plates containing the titanium disks were kept at 37°C, 0,5% CO_2 for 12 min. After 12min of adhesion, the cells seeded on titanium disk were carefully washed with PBS and then fixed using 4% Paraformaldehyde. Cells were washed twice

with PBS and then they were stained with 1μM DAPI for 15 min at 37°C as to visualize cell nucleus. Images were acquired using a Nikon Eclipse T-E microscope with a 4× objective. The nuclei were counted using the automated cell count tool of ImageJ software (U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/).

Cell morphology

Cells were seeded on titanium disks in both control and plasma APDBD treatment condition (120'') at a concentration of 10^4 cells/well in a 24-well plate (BD, Milan Italy) and then kept in growth condition. After 30 minutes and 8 hours (36 disks for control group and 36 for APDBD treatment for each time-point) the titanium specimens were washed in PBS and then the cells were fixed with 4% paraformaldehyde (PFA) in PBS for 15min. After two washes with PBS, cells were permeabilized with 0,1% Triton X-100 (Sigma-Aldrich) in PBS. Following the manufacturer's protocol, cells were stained with Rodhamine-Phalloidin (Life Technologies) and 1uM Dapi (Life Technologies) to respectively detect the cytoskeleton and the nuclei. Image acquisition was made recurring to a Nikon Eclipse Ti-E microscope with a 20X and 40X objective (Plan Fluor Nikon). Image analysis was performed by means of ImageJ software (ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/).

Viability Assay

To test the cell viability post-treatment, cells were plated at density of 3 x 10^3 cells/disk and the viability was assessed as reported elsewhere [22] by CellTiter-Glo® Luminescent Cell Viability Assay (Promega Corporation, Madison, WI, USA), according to the manufacturer's protocol at 1, 2 and 3 days using 36 disks for control group and 36 for APDBD treatment for each time-point.

Statistical Analysis

Due to the nonparametric nature of the data ~~collected~~, differences between groups were analyzed using the Mann–Whitney test, by means of GraphPad Prism 6 software (GraphPad Software, Inc., La Jolla, CA, USA). All of the statistical comparisons were conducted with a 0.05 level of significance.

Results

Contact angle characterization

The contact angle measurements are reported in table 1. On all treated samples, values were lower than 10°, which is the minimum angle measurable by the instrument both immediately after treatment and 1 minute thereafter. The untreated samples showed values of around contact angle of 80°, 100° and 110°, respectively for MAC, TPS and ZRT. Values remained stable after 1 minute. The statistical analysis, ~~carried out considering a value of 10° for the contact angle of all treated samples~~, showed a significant difference ($p<0.01$) between the contact angles measured on treated and untreated samples for all the surface modifications.

Protein adsorption

Outcomes of the protein adsorption at 1% concentration are summarized in Table 2~~ and Figure 2~~. The use of plasma of Argon was able to increase the quantity of proteins adsorbed on the rough surfaces (TPS and ZRT), in a statistically significant way. However, no significant effect was noted on the MAC disks.

Bacterial adhesion

A qualitative representation of MAC, ZRT and TPS surfaces after bacterial incubation and plasma argon decontamination is depicted in Table 3 and Figure 2 ~~3~~. Untreated controls show clearly bacteria on their surfaces, while little or even no microorganisms can be seen at increasing plasma treatment times (30, 60, 120 seconds). Quantitative analysis of a convenient number of fields (n=12) is reported in Figures 3A and 3B ~~4A and 4B.~~

Cell adhesion, morphology and viability

The treatment with plasma of argon significantly improved ~~in a statistically significant way~~ the number of osteoblasts adherent at 12 minutes in all tested surfaces (Table 4 ~~and Figure 5~~). As it can be seen in Figure ~~6~~ 4, MC3T3 cells seeded on plasma treated titanium disks appeared more spread than the cells seeded on untreated control disks. Indeed, as shown in Table 5, the APDBD treated samples showed a statistically significant increase of osteoblast cell spreading.

~~Among the morphometric parameters investigated, cell surface areas differed in a statistically significant way when plasma-treated samples were compared to the untreated ones (Table 5). Particularly, , as portrayed in Figure 7. Also, non-treated samples promoted a less spindle shaped morphology as numerically assessed by the major to minor cell axis ratio (Table 4). Thus a clear and significant trend towards decreasing cell elongation was detected.~~

On the other hand, data showed that the treatment with plasma of argon will not affect osteoblast proliferation and viability at 1,2 and 3 days (Figure ~~8~~ 5).

Discussion

Non-thermal plasmas (NTPs) were shown to clean and decontaminate surfaces at atmospheric pressure [23]. Positive effects of NTP ~~lasma of Argon~~ have been ~~already reported, as~~ documented for cell spreading, wettability [18] and early osseointegration in animal models [24].

~~Both above~~ These studies ~~reported the~~ used a plasma device (KinPen, INP- Greifswald, Germany) with 1% O_2/Ar gas for a period of 60 or 120 seconds. Although this approach was shown to increase ~~While it enhances the performance of the plasma by increasing~~ the sample surface reactivity ~~also at low power~~, however, the use of oxygen might possess potential ~~danger~~ concern in a clinical environment. The novelty of the present paper is due to the use of ~~Argon Atmospheric Pressure Dielectric Barrier Discharge (~~APDBD, as a possible alternate approach to obtain similar outcomes. Although only argon was used, surface wettability, increase osteoblast adhesion and protein adsorption were clearly demonstrated. ~~This result~~ The same effect was accomplished by modifying the power (8W instead of 2-3W), changing the time of exposure (120 sec instead of 60 sec) and ~~the~~ increasing microwave power ~~supply~~ (2.45 GHz instead of 1,82 MHz).

~~During bone healing, the first interaction between implants and bone is the contact of water molecules and salt ions, immediately followed by blood proteins~~. In the present study a protein adsorption assay was implemented ~~in order~~ to simulate the protein adsorption occurring in vivo. ~~At a proper FBS concentration (1%),~~ Argon based APDBD could significantly increase ~~in a statistically significant way,~~ the amount of adsorbed protein. This is in agreement with the finding reported report in literature [25] ~~However, the same effect could not be detectable at a higher concentration~~. Furthermore, our finding is in line with the studies that showed the removal of the adsorbed carbon from the implant surface can be accomplished by ~~This is consistent with the substantial increase in surface energy (in both polar and disperse components) elicited by~~ using plasma of argon ~~owing to the removal of the adsorbed Carbon species from the surface~~ [26–28].

Studies have also shown that increased hydrophilicity induces osteoblast differentiation, growth factor production, angiogenesis and osteogenic gene expression [29–31]. ~~On the contrary~~ As illustrated, highly hydrophobic surfaces hinders cell adhesion by the adsorption of proteins in a denatured and rigid state. Our ~~Accordingly, as per the~~ adhesion assays showed, a higher number of osteoblasts adhered onto the treated (i.e. more hydrophilic) surfaces than onto the untreated ones. In addition ~~It is very interesting to correlate the effect observed on cell adhesion to cell morphology observed in Figure 4, indeed~~ cells seeded on argon plasma treated disks display more cell spreading activity. In conclusion, data obtained from the present study are consistent with previous reports showing that plasma treatment of titanium implant surfaces can positively affect osteoblast adhesion [34,35]. However, cell proliferation ~~was instead~~

~~not different among~~ did not differ from the conditions tested, this is probably due to ~~the~~ powerful, yet transient effect of plasma treatment.

One of the limitations of the current study is *in vitro* study design, however, as reported by Coelho et al. [38], who supported the reliability of these *in vitro* findings when compared with a sophisticated histometric approach in beagle dogs. Other limitation is murine MC3T3-E1 cell line was used to test our hypothesis. As demonstrated in the literature, most of the studies used the same cell line to conduct research and supported the notion of using this murine cell line as a pre-osteoblastic phenotype [32] and a reliable approach for these types of interface research [21,33].

~~The MC3T3-E1 cells are the most used osteoblast cell line endowed with a pre-osteoblastic phenotype [32] and, in spite of their murine origin, represent a reliable in vitro model for interface research~~

~~Data from the present study are consistent with previous reports showing that Plasma treatment of titanium implant surfaces can positively affect osteoblast adhesion. A recently published study using non-thermal Argon plasma reactor [12] reported better outcomes in terms of protein adsorption and cell adhesion. This could be possibly due to the effect of vacuum. However, it must be highlighted that the mentioned device has a completely different clinical use.~~

As for the capability to remove bacterial contamination illustrated ~~portrayed~~ in figure 3, it highlights ~~that the present study supported the potential usage of~~ the benefit of using APDBD to disinfect implant surface. Although complete elimination of microbiota was observed only after 120 sec but the bacterial load was significantly reduced even after 30 sec. ~~In fact, from a clinical point of view, the efficacy of APDBD becomes very interesting in light of the possible use as a means of decontamination in implant surface. This technology is positively versatile and can be successfully used intraorally easily reaching the infected sites.~~

~~The reported~~ This data are ~~aligned~~ in agreement with ~~another study by~~ Duske et al. (2012) [18], who demonstrated similar results ~~recurring to~~ by using the combination of established brushing technique and O_2/Ar plasma application. However, in contrast to Duske et al. (2015) [19] where a complex oral biofilm was used, we only used *Streptococcus mitis* to test the concept. ~~limitation of the present study could be represented by the simplified in vitro settings of the bacterial adhesion test that aimed just at performing a preliminary assay. In fact, dynamics of plaque formation are very complex and require more accurate biological evaluation.~~ For this reason, additional studies on different bacterial species ~~based more verisimilar bacteria biofilm formation conditions~~ are required to fully confirm the reliability of this device in plaque removal. Interestingly, from a clinical point of view, the efficacy of APDBD might become useful tool in assisting clinician to decontamination implant surface especially in the field of implant complication management.

Conclusion

Within the limitations of this *in vitro* study, argon atmospheric-pressure dielectric-barrier-discharge (APDBD) showed ~~a promising armamentarium to promote~~ ability in enhancing osteoblasts attachment and spreading as well as ~~to decontaminate~~ bacterial decontamination. ~~adhesion~~.

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