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ORIGINAL ARTICLE

Air atmospheric-pressure plasma-jet treatment enhances the attachment of human gingival fibroblasts for early peri-implant soft tissue seals on titanium dental implant abutments

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Abstract

Objective. Although dental implants are commonly used for tooth restoration, there is a lack of studies of treatment regimens for preventing extra-oral infection and decreasing osseointegration failures by establishing early peri-implant soft tissue seals on titanium dental implant abutments. In this study, air atmospheric-pressure plasma-jet (AAPPJ) treatment was applied to titanium disks to assay the potential for early peri-implant soft tissue seals on titanium dental implant abutment. ***Materials and methods.*** After titanium disks were treated with AAPPJ for 10 s at 250, 500, 1000 and 1500 sccm, surface analysis was performed; the control group received air only or no treatment. Human gingival fibroblasts (HGF) were seeded onto the specimens for evaluating cell attachment and proliferation and adherent-cell morphology was visualized via confocal microscopy. ***Results.*** In AAPPJ-treated specimens, the water contact angle decreased according to increased flow rate. Oxygen composition increased in XPS, but no topographical changes were detected. The effect of AAPPJ treatment at 1000 sccm was apparent 2 mm from the treated spot, with a 20% increase in early cell attachment and proliferation. Adherent HGF on AAPPJ-treated specimens displayed a stretched phenotype with more vinculin formation than the control group. ***Conclusions.*** Within the limitations of this study, the results indicate that AAPPJ treatment may enhance the early attachment and proliferation of HGF for establishing early peri-implant soft tissue seals on titanium dental implant abutments with possible favorable effects of osseointegration of dental implant.

Key Words: *air atmospheric pressure plasma jet, human gingival fibroblast, peri-implant soft tissue seals, titanium dental implant abutment*

Introduction

The replacement of missing teeth by means of titanium dental implants is a promising method [1]. Despite relatively high success rates of over 95% in regular surgeries of healed alveolar bone sites [2], ongoing research addresses the prevention of higher rates of dental implant failure after immediate surgeries in extraction sockets for better esthetic outcomes [3,4].

Since dental implants penetrate into the oral cavity, the interface between dental implants and oral soft tissues is considered to be a protective barrier that prevents communication between the oral environment and the alveolar bone [5]. Soft tissue around titanium dental implant abutments (DIAs) forms a soft-tissue seal that acts as an important barrier against infection from the oral environment [6]. In contrast to the soft-tissue seals consisting of connective-tissue fibers around natural teeth, the seals around titanium DIAs do not penetrate the implant, leading to a relatively weaker barrier [7]. Therefore, early establishment of peri-implant soft tissue seals around DIAs has been suggested as a solution to overcome the limitations of weaker soft-tissue barriers in early soft-tissue healing.

Anatomically, the soft-tissue seal consists of two layers: a well-keratinized oral epithelium that is

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composed of keratinocytes and a fiber-rich connective tissue that contains many gingival fibroblasts [8]. Therefore, 2-mm-wide periodontal soft tissues may be required to enable proper epithelial and connective tissue attachment; this width is referred to as the 'biological width' [9].

The effectiveness of DIA designs and materials has been largely investigated in terms of soft-tissue response and marginal bone loss [10]. Theoretically, titanium DIA surface modified by chemical treatment without topographical changes, a process that is currently hindered by technical limitations, may enhance the attachment of epithelial cells or fibroblasts, promoting the formation of peri-implant soft-tissue seals [11]. Increase of gingival fibroblasts attachment is attractive for enhancing peri-implant soft tissue seals because they secrete all of the extracellular matrix components, such as collagen fiber precursor, which act as a protective barrier [12].

Atmospheric-pressure plasma treatment, which can be applied in the dental chair, can be used to treat the DIA surface without causing topographical changes, resulting in improved early attachment of gingival fibroblasts for peri-implant soft-tissue seals [13]. The term 'plasma' refers to the partially ionized state of gas that has been widely used for various industrial applications [14]. Atmospheric-pressure plasma-jet technology has been extensively used on various dental implant surfaces to enhance osseointegration without topographical change and to carry out caries treatment, endodontic treatment and tooth bleaching [13,15–17]. However, no study has addressed the effects of this treatment on early peri-implant soft tissue seals on titanium DIAs with human gingival fibroblasts (HGFs).

Here we investigated the effects of air atmospheric-pressure plasma-jet (AAPPJ) treatment on HGF immobilization of titanium DIAs as a possible method for promoting peri-implant soft tissue seals on DIAs (Figure 1A). The null hypothesis was that AAPPJ treatment does not affect early HGF attachment and proliferation on titanium DIAs.

Materials and methods

AAPPJ device

AAPPJ was developed and provided by Kwangwoon University (Figure 1A) [18]. Based on the results of a preliminary study and conventional use of air in dental clinic (data not shown), air was preferred for further study over nitrogen and helium. The plasma was formed by passing 250, 500, 1000 or 1500 standard cubic centimeters per minute (sccm) of compressed air through the plasma-jet device while applying a maximum discharge voltage of 2.24 kV and a discharge current of 1.08 mA, which yielded 2.4 W of discharge power for plasma generation [19] (Figure 1B). During plasma generation, discharge lasted 0.16 ms during a single period (16 ms) of discharge voltage, with ~100 discharges per period. Discharge voltage and current measurements were performed using a high-voltage probe (P6015A, Tektronix, Beaverton, OR) and a current probe (A6303, Tektronix) with a digital oscilloscope (MSO4032, Tektronix). Each sample underwent AAPPJ treatment for 10 s, with a 3-mm distance between the jet tip and the titanium disk. Control samples were not exposed to AAPPJ or were exposed to air only.

Surface characteristics

To study the effects of AAPPJ treatment on titanium DIAs, as-received machined titanium disks (grade 4, diameter 10 mm, thickness 2 mm) were used as models for DIAs. These disks were kindly provided by Ostem Implant (Pusan, Korea). The samples were decontaminated and sterilized as described elsewhere [20]. In brief, as-received specimens were rinsed ultrasonically in acetone, ethanol and distilled water for 10 min each. After drying under vacuum at 25°C for 24 h, the specimens were sterilized with ethylene oxide gas, then exposed to air for 48 h to eliminate any remaining gas.

Machined titanium disk was chosen for analysis, because the titanium composition was the same in the titanium dental implant fixture and the abutment, with the exception of structural differences (Figure 1A). Contact angle analysis was carried out before and after AAPPJ treatment with surface electro optics (Phoenix-300, SEO, Korea) and a standard jig for contact angle analysis [21]. Then, 7 µL of distilled water was pipetted onto each specimen and left for 10 s to reach equilibrium. Live images of the water and the specimen were captured and analyzed using a contact angle goniometer (DSA10, Kruss, Charlotte, NC).

X-ray photoelectron spectra (XPS; K-alpha, Thermo Scientific, Loughborough, UK) was obtained using a monochromated Al K-alpha source. The analysis was carried out at the treated spot and a line scan was performed in the 1000 sccm group to measure the effect distance. XPS was measured on two additional occasions to confirm the validity of the results. C1s at 284.8 eV was used as a reference and the atomic compositions of C, O, N and Ti were detected in detail.

Surface analysis was performed with an optical three-dimensional surface profilometer (Contour GT-X3 base, Bruker, Germany) to visualize topographical differences caused by AAPPJ treatment. For the standard camera, the objective magnification was 50×, the zoom was 1.0× and the visualized area was 0.095 mm × 0.127 mm. The back scan and scan length were both 15 µm.

All of the above experiments were performed in five replicates except for XPS analysis, which was

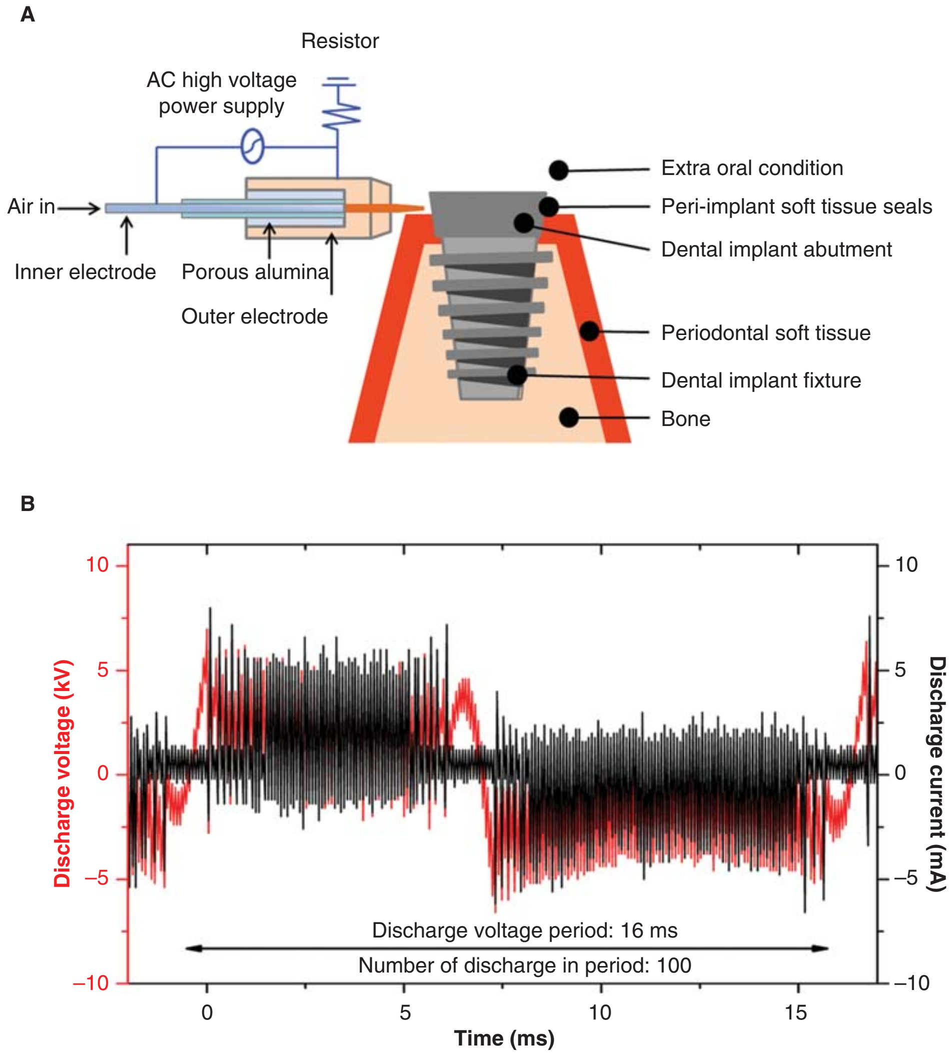

Figure 1. (A) Schematic diagram of the AAPPJ device (provided by the Plasma Bioscience Research Center at Kwangwoon University, Korea) and a graphical illustration of AAPPJ treatment of titanium DIA. (B) Voltage and current during AAPPJ. (The Japan Society of Applied Physics about voltage and current, for interpretation of the references to color in this figure legend, the reader is referred to the web version of this article [© 2013]).

performed in triplicate. Data averages with standard deviations and representative images are shown.

HGFs and cell culture

A primary HGF cell line (CRL-2014, American Type Culture Collection, Manassas, VA) was used at 5–10 passages. This cell line was established from biopsied human gingival connective tissue [22]. Cells (1×10^4 cells/100 μL) were prepared from 90% confluent cells for further experiments. All investigations employed Dulbecco's Modified Eagle Medium (Welgene, Daegu, Korea) with 10% fetal bovine serum (Gibco, NY) and 1% antibiotics (penicillin/streptomycin, Gibco).

Cell attachment

Machined titanium disks were exposed to AAPPJ treatment at 250, 500 or 1000 sccm for 10 s. HGFs were allowed to attach to the treated disks for 4 h, then unattached cells were washed away through several rounds of washing with phosphate-buffered saline. PrestoBlue reagent (10%; Molecular Probes, Grand Island, NY) was added to the culture medium according to the manufacturer's protocol. Optical absorbance at 570 nm using a microplate reader (Epoch, BioTek, Winooski, VT) yielded the number of attached cells after subtracting the absorbance at 600 nm [23]. Results are expressed as the percentage of optical density in each experimental specimen compared to untreated specimens. Experiments were performed in five replicates. Data averages with standard deviations are shown.

Cell proliferation

A BrdU test kit (Cell Proliferation ELISA BrdU, Roche Applied Science, Penzberg, Germany) was used to analyse DNA synthesis in cells as an assay of cell proliferation. HGFs were seeded onto the specimens and cultured for 4 h. The BrdU procedure was then performed according to the manufacturer's protocol. Absorbance of the final solution was measured at 370 nm (reference wavelength = 492 nm). Experiments were performed in five replicates. Means and standard deviations are shown.

Confocal laser microscopy

Confocal microscopy (LSM 700, Carl Zeiss, Jena, Germany) was used to detect vinculin and actin filaments after cells were stained with the Actin Cytoskeleton and Focal Adhesion Staining Kit (FAK100, Millipore, Bedford, MA) according to the manufacturer's protocol. Experiments were performed in triplicate and representative images are shown.

Statistics

The resulting data, except surface roughness data, were analysed using one-way ANOVA. Surface roughness before and after AAPPJ treatment was analysed via paired Student's *t*-test. Statistical significance was accepted at a confidence level of 95% ($p < 0.05$) according to Tukey's test for multiple comparisons. Statistical analysis was performed with IBM SPSS Statistics 20 (IBM, New York, NY).

Results and discussion

Surface characteristics

We characterized the surfaces of AAPPJ-treated titanium disks, which mimic AAPPJ-treated titanium DIAs before implantation in the dental clinic. Specimen wettability was evaluated through contact angle analysis. AAPPJ treatment decreased the contact angle to 35° as the flow rate increased (Figure 2). No treatment and air-only treatment (1000 sccm) led to less hydrophilic characteristics, with a contact angle of 75° (Figure 2). Since there were no statistical differences between treatment at 1000 sccm and treatment at 1500 sccm (Figure 2), we chose 1000 sccm for further investigation. No treatment and air-only treatment remained the control conditions.

XPS was assessed to characterize specific changes in chemical composition following AAPPJ treatment. Compared to samples that received no treatment or treatment with air alone, the carbon composition (C-H) of AAPPJ-treated disks decreased due to cleansing of carbon contamination on the titanium disks (Figure 3A). Oxygen bonding increased in AAPPJ-treated disks, for example in moieties such as C=O, O-H and Ti-O (Figures 3B and E). According to the N1s analysis, AAPPJ treatment at 1000 sccm led to an increase in the N-Ti peak and increased nitrogen content (~4%) compared to no treatment and air-only treatment (~3%; Figures 3D and E). Consequently, an increase in the TiO_2 peak was observed following AAPPJ treatment, as was an increase in the titanium content of the sample (Figures 3C and E). The observed titanium-related changes are known to favor hydrophilic surfaces and to increase cell attachment [24–26]. The effect of AAPPJ treatment at 1000 sccm persisted ~2 mm from the treated spot (Figure 3F). Previously, the range of the AAPPJ effect on the substrate was measured inaccurately, using a metal mold to calculate the effect range in the presence of cells adhering to the substrate [27]. In the present investigation, XPS

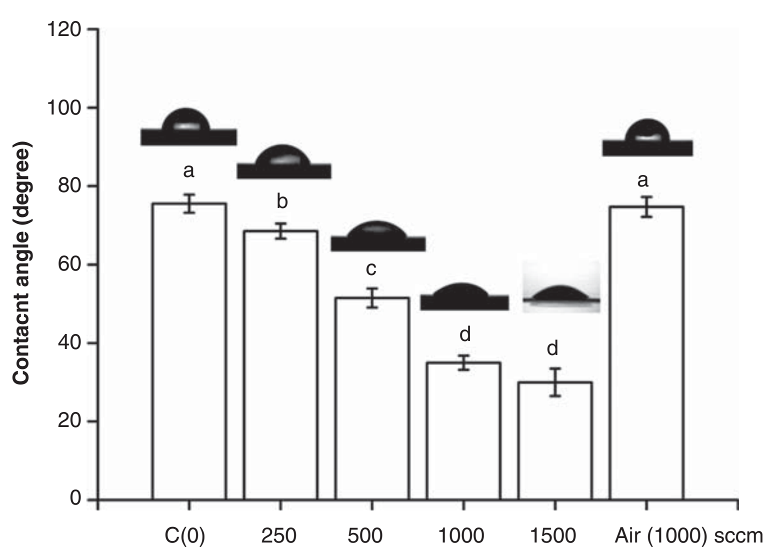

Figure 2. Contact angle analysis of titanium disks exposed to AAPPJ treatment for 10 s at 0, 250, 500, 1000 and 1500 sccm. Contact angle was determined with distilled water at 20°C. Different letters represent statistical differences between groups ($n = 5$). Control titanium disks were exposed to air only for 10 s at 1000 sccm.

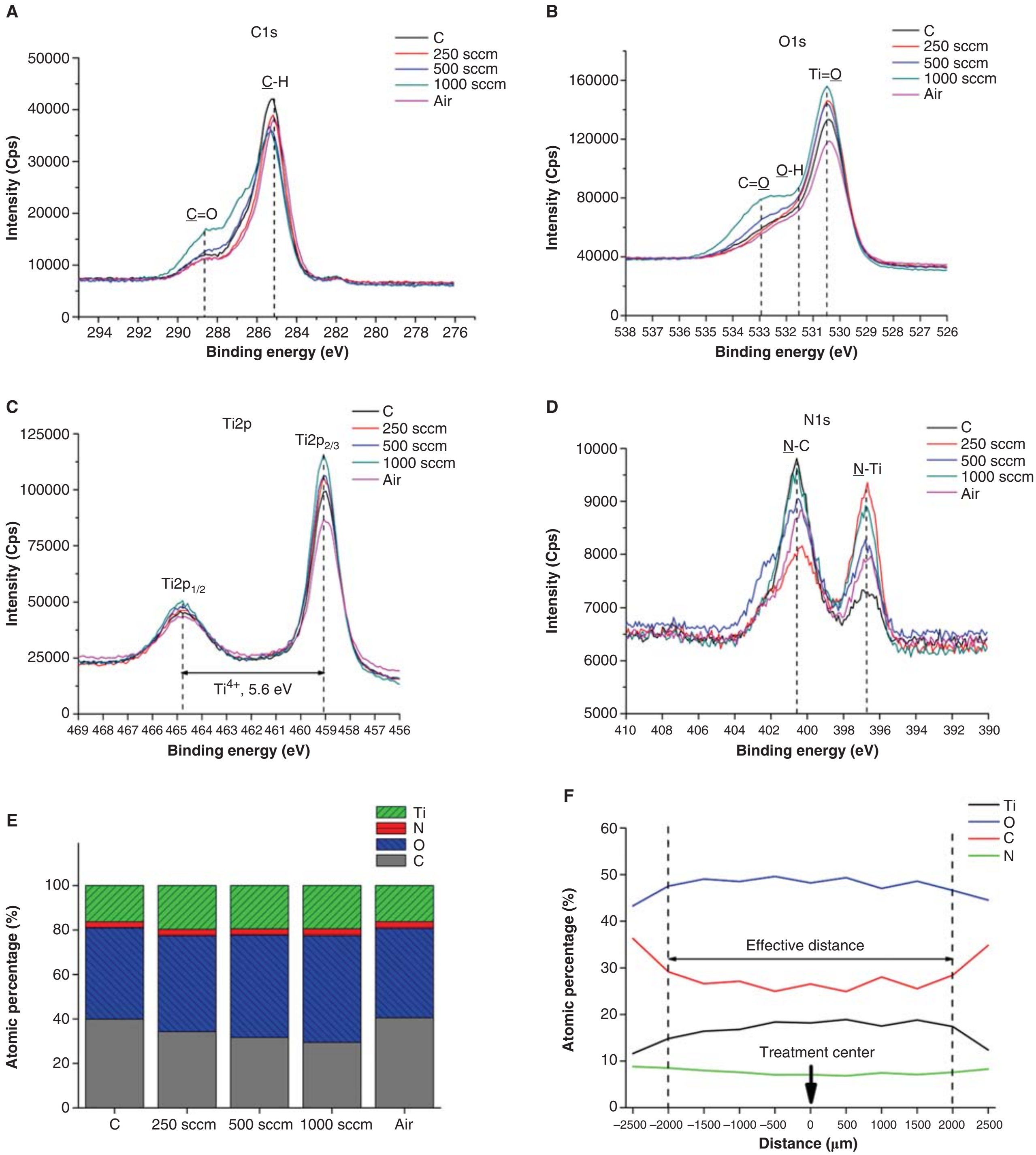

Figure 3. High-resolution XPS of untreated (C) titanium disks, disks exposed to AAPPJ treatment at 250, 500 or 1000 sccm and disks treated with air only (Air). XPS were determined for (A) C1s, (B) O1s, (C) Ti2p and (D) N1s. (E) Atomic composition percentage of all specimens and (F) that of an AAPPJ-treated disk at 1000 sccm according to the distance from the treated center spot. Data are representative of triplicate analysis. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

analysis was used to calculate the exact range of the effect of AAPPJ treatment on atomic composition (Figure 3F).

Surface roughness may change due to AAPPJ treatment. Therefore, surface roughness profile (Ra) and area (Sa) were measured before and after AAPPJ treatment at 1000 sccm, which could most powerfully induce surface chemical changes. We used a three-dimensional optical profilometer to evaluate sub-nanoscale surface roughness because the resolution of this machine spans the sub-angstrom to millimeter scales at any magnification. The air-only group was excluded due to no change of surface roughness (data not shown). The machine-cut surface was only visualized in the control and 1000-sccm groups, which did not display changes in surface

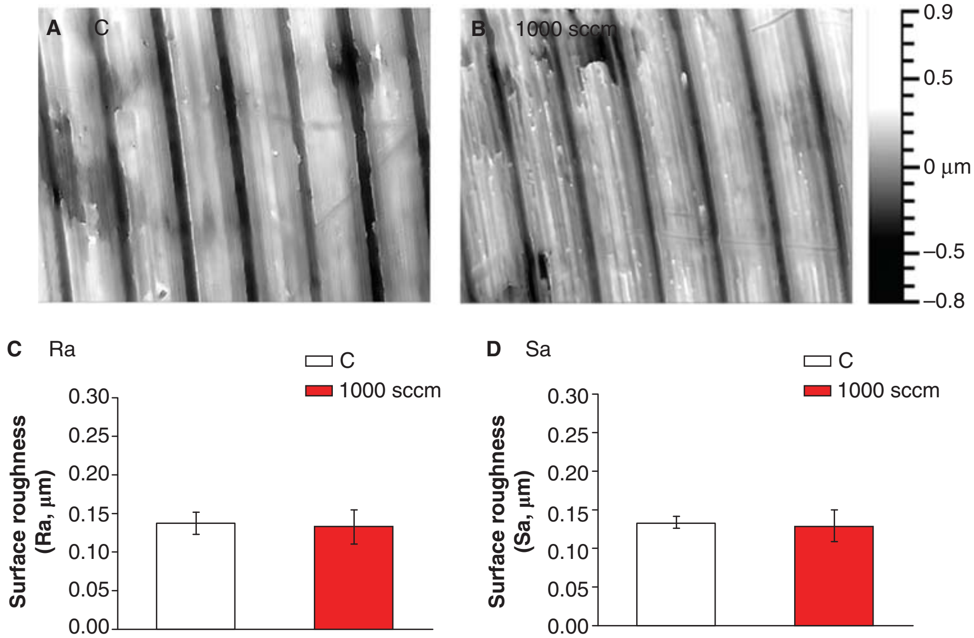

Figure 4. Representative topographical features of titanium disks before (A) and after (B) AAPPJ treatment with 1000 sccm. Surface roughness profiles and areas appear in (C) and (D), respectively. Statistical differences were not detected ($n = 5$, $p > 0.05$).

topography (Figures 4A and B). Surface roughness did not significantly differ before and after AAPPJ treatment ($p > 0.05$, Figures 4C and D). The mean surface roughness profile (Ra) with standard deviation before and after AAPPJ treatment was 0.137 ± 0.014 µm and 0.132 ± 0.023 µm, respectively. The surface area (Sa) before and after AAPPJ treatment was 0.134 ± 0.007 µm and 0.129 ± 0.020 µm, respectively. Thus, surface topographical changes were not detected after AAPPJ treatment. The hydrophilic surface properties of AAPPJ-treated specimens did not result from changes in surface roughness, but rather in changes in chemical composition, as described by a previous investigation of nitrogen atmospheric-pressure plasma-jet-treated titanium as well [13].

Cell attachment and proliferation

Soft-tissue seals around titanium DIAs are important protective barriers against infection from the oral environment [6]. Here, we incubated HGFs on AAPPJ-treated titanium disks to mimic peri-implant soft tissue seals by AAPPJ treatment around titanium DIAs. Even though ability of AAPPJ was not evaluated as an *in vivo* study, titanium disks and HGF were rationally chosen due to their material composition and representative cell type among peri-implant soft tissue, respectively, to mimic the clinical situation. The sealing ability on titanium disk by AAPPJ was evaluated as cell attachment and proliferation, which play a pivotal role to achieve initial interaction between cell and material surface and consequently enhance sealing ability [28].

Compared to the no-treatment group, HGF attachment increased on specimens treated with AAPPJ at 500 and 1000 sccm ($p < 0.05$; Figure 5A). Generally, hydrophilic surface results in increased initial cell attachment [13,25]. Along with the above reports, the air-only treatment group had similar cell attachment compared to the no-treatment group, which was considered due to similar low hydrophilic characteristics (75°) and no change of chemical composition on the specimen (Figure 3). It was previously reported that increase of surface roughness induces HGF attachment on titanium [29]. On the other hand, in this study, chemical modification on the specimen, not surface roughness change, contributed to HGF attachment on titanium (Figures 3 and 4). The formation of oxygen-bonded functional groups on the treated specimen was observed in the AAPPJ treated specimen, which may attract cells to the treated area (Figure 3E). Increases in oxygen-involved functional groups on a substrate previously accelerated cellular activity [30]. However, our BrdU assay failed to detect significant increases in DNA replication ($p > 0.05$; Figure 5B), although a trend in proliferation was observed and a 15% increase was shown in 1000 sccm. This increased tendency was likely due to increased cell attachment, which consequently increased the amount of BrdU-labeled DNA replication during the 4-h incubation relative to the amount of DNA replication in the less-adherent cells in the control group.

Confocal microscopy images of adherent HGFs 2 mm from the treated spot confirmed that AAPPJ treatment increased the actin cytoskeleton and vinculin localization (arrow in Figure 6) as flow

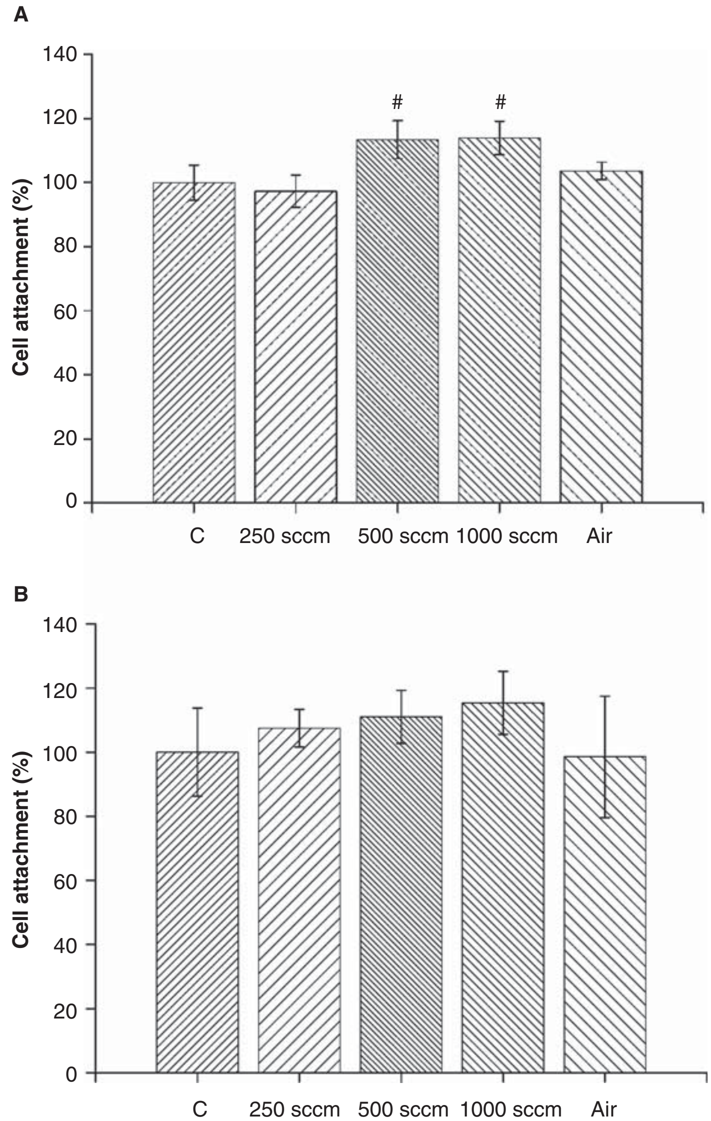

Figure 5. HGF activity on titanium disks after no treatment (C), treatment with 250 sccm, 500 sccm or 1000 sccm AAPPJ or air-only treatment at 1000 sccm. (A) Cell attachment according to the PrestoBlue assay. (B) Cell proliferation according to the BrdU assay. #Significant difference vs no treatment ($n = 5$, $p < 0.05$).

rate increased. Extensive vinculin development was evident in the 500 and 1000 sccm groups compared to groups that underwent no treatment or air-only treatment (Figure 6). Vinculin is known to be involved in the linkage between cell adhesion molecules and actin filaments and serves a key role in initiating and establishing cell adhesion, cell shape and cytoskeletal development [31]. Vinculin localization reflects establishing tight cell adhesion, inducing a stretched morphology, increasing cell invasion and promoting formation of the extracellular matrix three-dimensionally, which is very important to establish peri-implant soft tissue seals between titanium DIA and peri-implant soft tissue [32,33].

In conclusion, the null hypothesis was rejected. Short AAPPJ treatment (10 s) improved the attachment of HGFs to titanium disks, perhaps due to oxygen present in functional groups on the surface and/or decreased levels of carbon contamination, but not due to changes in surface roughness. The early formation of vinculin and actin filaments on AAPPJ-treated titanium disks after 4 h of incubation is established (Figure 6), which suggests the development of early peri-implant soft-tissue seals on AAPPJ-treated DIAs compared to untreated DIAs. Within the limitations of this study, AAPPJ treatment could be used to enhance peri-implant soft-tissue seals on titanium dental implant abutments, possibly preventing infection from the oral environment and decreasing implant failure. These results provide valuable information for the future application of AAPPJ treatment in dental implant surgery. However, further studies are warranted to investigate the safety and efficacy of AAPPJ application *in vivo* and in the clinic.

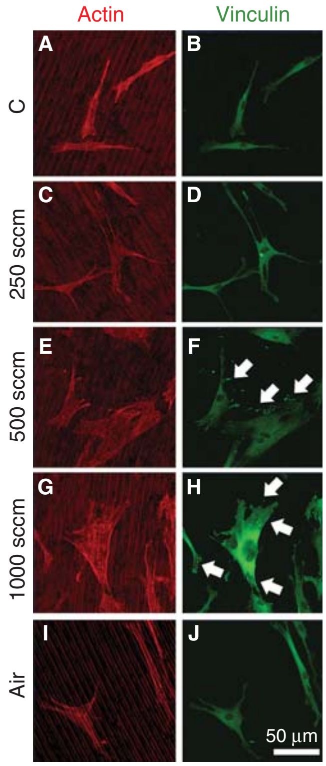

Figure 6. HGF morphology observed via confocal laser microscopy after 4 h of culture and staining with tetramethylrhodamine-conjugated phalloidin (against actin, red) and anti-vinculin (green). Titanium disks were exposed to (A, B) no AAPPJ treatment or to 10 s of AAPPJ treatment at (C, D) 250 sccm, (E, F) 500 sccm or (G, H) 1000 sccm. (I, J) Air-only treatment with 1000 sccm was also performed. Arrow, vinculin localization. These images are representative of triplicate experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

Summary

- Air atmospheric-pressure plasma-jet treatment (AAPPJ) enhances the early attachment and proliferation of human gingival fibroblasts on titanium dental implant abutment.
- AAPPJ's effects on the attachment and proliferation of human gingival fibroblasts on titanium dental implant abutment depended on the supplied air-flow rate.
- The application of AAPPJ to titanium dental implant abutment is likely to support early peri-implant soft tissue seals.

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