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Benchtop plasma treatment of titanium surfaces enhances cell response

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ABSTRACT

Objective. Modifications to implant surface properties, including topography, chemistry, and wettability, alter immune response, osteoblast differentiation of bone marrow stromal cells (MSCs), and implant integration *in vivo*. Dielectric barrier discharge (DBD) plasma treatment has been used to sterilize surfaces and remove adsorbed carbon, improving wettability. However, unless it is used immediately prior to placement, ambient atmospheric hydrocarbons rapidly adhere to the surface, thereby reducing its hydrophilicity. Moreover, this method is not practical in many clinical settings. The aim of this study was to evaluate the effectiveness of an on-site benchtop modification technique for implants at time of placement, consisting of a DBD plasma that is used to sterilize implants that are pre-packaged in a vacuum. Effects of the plasma-treatment on implant surface properties and cellular response of MSCs and osteoblasts were assessed *in vitro*.

Methods. Titanium-aluminum-vanadium implant surfaces were grit-blasted (GB) or grit-blasted and acid-etched (AE), and packaged under vacuum. AE surfaces were also plasma-treated using the benchtop device (GB + AE) and then removed from the vacuum. GB surface morphology was altered with AE but AE microroughness was not changed with the plasma-treatment. Plasma-treatment increased the surface wettability, but did not alter surface atomic concentrations of titanium, oxygen, or carbon.

Results. MSCs and osteoblast-like cells (MG63 s) produced increased concentrations of osteocalcin, osteopontin, and osteoprotegerin after plasma-treatment of AE surfaces compared to non-plasma-treated AE surfaces; production of IL6 was reduced and IL10 was. Aging GB + AE surfaces for 7 days after plasma-treatment but still in the vacuum environment reduced the effectiveness of plasma on cellular response.

Significance. Overall, these data suggest that application of benchtop plasma at the time of implant placement can alter the surface free energy of an implant surface without modifying surface chemical composition and enhance the differentiation and activity of MSCs and osteoblasts that are in contact with these implant surfaces.

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1. Introduction

Dental implants can have a major impact on the quality of life in patients suffering from tooth and maxillofacial bone loss. Many of these implants are placed in older or compromised patients with reduced implant retention rates. To improve treatment for these patients, implants need to possess increased retention longevity and reduced secondary complications such as loosening of the implant through mechanical overloading, insufficient osseointegration, or peri-implantitis [1]. Peri-implantitis incidences are consistently around 15–30% within 10 years of implant placement [2–4]. Therefore, there is a growing need to develop technologies that can be used in the clinic to create implant surface properties that positively affect osseointegration and implant longevity.

Surface modifications that increase surface microroughness and alter surface chemistry have been used as a way to increase implant retention and enhance osseointegration [5,6]. Altering the surface roughness through grit-blasting (GB) and acid-etching (AE) is now common practice and has been shown to increase bone to implant contact *in vivo* [7] and stimulate bone marrow stromal cell (MSC) differentiation into osteoblasts *in vitro* [8], without the use of osteogenic media supplements [9].

Our lab and others have shown titanium implants possessing micron-scale and meso-scale surface roughness created by GB + AE, work by altering the cytoskeletal organization and activating transcription factors to induce the osteoblastic differentiation of MSCs [10]. As a result, the MSCs produce osteocalcin (OCN), osteopontin (OPN), and osteoprotegerin (OPG) as well as paracrine signaling factors to stimulate bone formation [11–13]. MSCs decrease production of proinflammatory cytokines such as interleukin 6 (IL6) while pro-regenerative cytokines like IL10 are increased compared to machined hydrophobic implant surfaces [14].

Conducting these modifications in a nitrogen environment, followed by storage in saline results in super hydrophilic surfaces with reduced carbon content compared to storage in ambient air. These surface properties further improve cellular response to the implant *in vitro* and osseointegration *in vivo* [7,15].

Superhydrophilic surfaces can also be achieved using conventional dielectric barrier discharge (DBD) plasma cleaning prior to use. DBD gaseous plasma treatment is a post-manufacturing modification technology that can modify surface atomic composition and crystallinity depending on the voltage, frequency, and time of exposure to the plasma and avoids the external addition of metallic ions of other plasma treatment methods by using high frequency radio frequency to initiate the plasma discharge [16]. Titanium implants treated with oxygen plasma have altered surface wettability due to the reduction in the carbon content on the surface [7,17–20]. This can assist in cell attachment and spreading [21], as well as improved osteoblast differentiation [22,23] and osseointegration [24].

Plasma treatments on implant surfaces are subject to aging and possible fouling. Studies on polymeric and metallic surfaces have shown that the materials eventually returned to their normal hydrophobic state regardless of plasma used [25]. Once implants are removed from an oxygen plasma, atmospheric hydrocarbons rapidly adsorb to the surface, restoring hydrophobicity and reducing the enhanced cell response achieved with the hydrophilic surface chemistry [8].

To be clinically useful, the plasma treatment must yield a surface that is stable enough to last until the implant is surgically placed. Therefore, there is room for a patient-side plasma treatment method that can alter implant surface properties immediately before placement in bone. Conventional oxygen plasma treatments have been proposed for this purpose [26], but this technology is not practical for many clinical practices. An alternative method is to use a benchtop DBD method to treat implant surfaces with nitrogen plasma at the time of placement.

2. Materials and methods

2.1. Titanium alloy surface production

Titanium-aluminum-vanadium (Ti6Al4V) rectangular surfaces 10.50 mm long, 5.25 mm wide and 1 mm in height were CNC-milled from Ti6Al4V (grade 4) rods. Surfaces were calcium phosphate grit-blasted using proprietary technology (AB Dental, Ashdod, Israel). Following degreasing and HNO_3 treatment for 5 min, surfaces were further modified by acid-etching *via* a series of proprietary acid washes using H_2SO_4 and HCl. All treatment groups were rinsed 3X in ultrapure distilled H2O for 10 min. Coupon surfaces were blotted, air dried, and packaged in glass vials fitted with implant mounts made out of stainless steel. Vials had rubber gaskets to vacuum seal each container after purging air with a gas mixture of primarily nitrogen. Packaged coupons were sterilized by gamma radiation.

2.2. Dieletric barrier discharge plasma (DBD) treatment

Glass vials containing sterile GB + AE coupons in a sub-atmospheric pressure nitrogen gas were placed in the tabletop plasma applicator (Nova Plasma Ltd., Hevel Megiddo, Israel). Immediately prior to use, plasma initiation was achieved by a 13 kV ignition voltage at 700 kHz and lasted for 30 s(P). Vials were then opened and the coupons used for cell culture or surface characterization. Plasma aging studies were conducted using GB + AE + P coupons that were treated and stored in unopened vials, in order to age for 1d, 4d, and 7d before surface analysis and for 7d for assessing cellular response.

2.3. Scanning Electron microscopy

Surface topography was qualitatively assessed using scanning electron microscopy (SEM; Hitachi SU-70, Tokyo, Japan). Surfaces were secured on SEM imaging mounts by carbon tape and imaged with 56 μA ion current, 5 kV accelerating voltage and 5 mm working distance.

2.4. Optical profilometry

Surface roughness was qualitatively assessed by laser confocal microscopy (LCM; Zeiss LSM 710). Z-stacks were obtained

with a Plan Apochromat 20x/0.8 M27 objective with a 5x optical zoom, using a 405 nm laser in reflection mode at 50% power. Scan parameters were 0.39 μs pixel dwell, and 25 μm pinhole, 402.1 × 402.1 μm image size, and step size of 1 μm.

2.5. Surface wettability analysis

Surface wettability was analyzed by sessile drop test using a goniometer (CAM 250, Ramé-Hart). Samples were measured in 5 different locations and dried with nitrogen between measurements. 5 μL of ultrapure distilled water was used per measurement.

2.6. Surface elemental composition

Surface chemistry was analyzed by X-ray photoelectron spectroscopy (XPS) (PHI VersaProbe III Scanning XPS, Physical Electronics Inc., Chanhassen, MN). Copper clips and instrument mount were sonicated in ethanol for 10 min prior to securing samples. Analysis was conducted using a 50 W, 15 kV x-ray gun with a spot size of 200 μm, 20 ms dwelling time and 1 eV step size. Peaks were aligned to 284.8 eV of the C1 peak. Carbon bonds on the surfaces before and after DBD treatments were determined using the curve fitting tool in the MultiPak analysis software: 284.8 eV (C—C or C–H bonds), 286 eV (CO— bonds), and 288 eV (C═O bonds.

2.7. Cell culture

Human female bone marrow stromal cells (MSCs) (Donor #8011 L, Texas A&M Institute for Regenerative Medicine, College Station, Texas) were cultured in MSC growth medium (GM) comprised of αMEM with 4 μM L-glutamine and 16.5% fetal bovine serum (FBS) at 37 °C in 5% CO2 and 100% humidity. At 80% confluence in T75 flasks (Corning Inc., Oneonta, NY), cells were transferred to test surfaces. MG63 cells (male human pre-osteoblast cell line, ATCC, Manassas, Virginia) were grown in Dulbecco's modified Eagle medium supplemented with 10% FBS (DMEM-FM) at 37 °C in 5% CO2 and 100% humidity and cultured to 80% confluence in T75 flasks (Corning Inc., Oneonta, NY) before plating on the surface.

Two surfaces of the same group were placed side-by-side per well in 24-well plates. Six wells per group were plated for each experiment. Cells were plated at a density of 20,000 cells/mL at 0.5 mL per well. MSCs or MG63 s cultured on tissue culture polystyrene (TCPS) served as experimental controls. 24 h after plating, coupons were moved into new 24-well plates to ensure that only cells in contact with the implant surfaces were assessed. Subsequently, GM were changed with media changes every 48 h. At day 7, cells were incubated for 24 h with fresh GM before harvest. Conditioned media were collected and stored at −80 °C. Cell layer lysates were rinsed twice with 1x PBS and lysed in 0.5 mL Triton-X100 and stored at −80 °C for biological assays.

2.8. Cellular response

Cell layer lysates were dispersed by ultrasonication at 40 V for 15 s/well (VCX 130; Vibra-Cell, Newtown, CT). The QuantiFluor* dsDNA system (Promega, Madison, WI) was used to determine total DNA content by fluorescence. Enzyme-linked immunosorbent assays were used to determine the levels of osteogenic factors in the conditioned media. OCN (ThermoFisher Scientific), OPN, OPG, VEGF-A, BMP2, IL6, and IL10 (R&D Systems, Inc.) were quantified according to the manufacturer's protocol. Data were normalized to total DNA content.

2.9. Statistical analysis

Data are means ± standard error mean of six independent cultures/variable. All experiments were repeated to ensure the validity of observations, with results from individual experiments shown. Statistical analysis among groups was performed by one-way analysis of variance (ANOVA) and multiple comparisons between the groups were conducted with a two-tailed Tukey correction. A p-value of less than 0.05 was considered statistically significant. All statistical analysis was performed with GraphPad Prism v5.04.

3. Results

SEMs demonstrated physical differences in surface topography due to the surface processing method. GB alone created a jagged rough surface morphology at 20KX magnification (Fig.1A) with microscale peaks evident at 100KX magnification (Fig.1B). AE further modified the Ti6Al4V surface, creating pit-like structures and microscale peaks on top of and within the pits (Fig.1C-D); however, further modification by DBD treatment was not evident at either low or high magnification (Fig.1E-F).

All three surfaces possessed similar microroughness using optical profilometry (Fig.2A). Peak to valley distance was unchanged with AE treatment and DBD treatment (Fig.2B). XPS showed that the atomic concentration on the surface of these treated Ti6Al4V surfaces was unchanged with no differences in oxygen, titanium, or carbon content (Fig.2C). Contact angle measurements showed that AE increased surface wettability compared to GB only, and DBD treatment created a super hydrophilic surface (Fig.2D). XPS and contact angle measurement of aged plasma surfaces after 7 days of sterile environmental exposure showed no changes in atomic composition (Fig.2E) but a loss of surface wettability (Fig.2F). Curve fitting analysis of the high-resolution scans in the carbon region showed that the types of carbon bonds were altered after DBD treatment with a robust increase in C—C or C–H bonds at 284.8 eV and a decrease in both C—O (286 eV) and CO═ (288 eV). Aging of the surfaces in their vacuum containers for up to 7 days after DBD treatment shows that the amount of C—O bonds did not change but the degree of C═O increased with surface aging (Fig.2G).

Cell response varied with surface treatment. MSCs on GB surfaces had decreased total DNA content compared to TCPS and AE further reduced DNA; however, DBD treatment did not change DNA content compared to AE without DBD treatment (Fig.3A). OCN, OPN, and OPG were increased on all Ti6Al4V surfaces compared to TCPS (Fig.3B-D). AE increased OCN production compared to GB, and DBD treatment increased OCN compared to both GB and AE treatment groups (Fig.3B). OPN

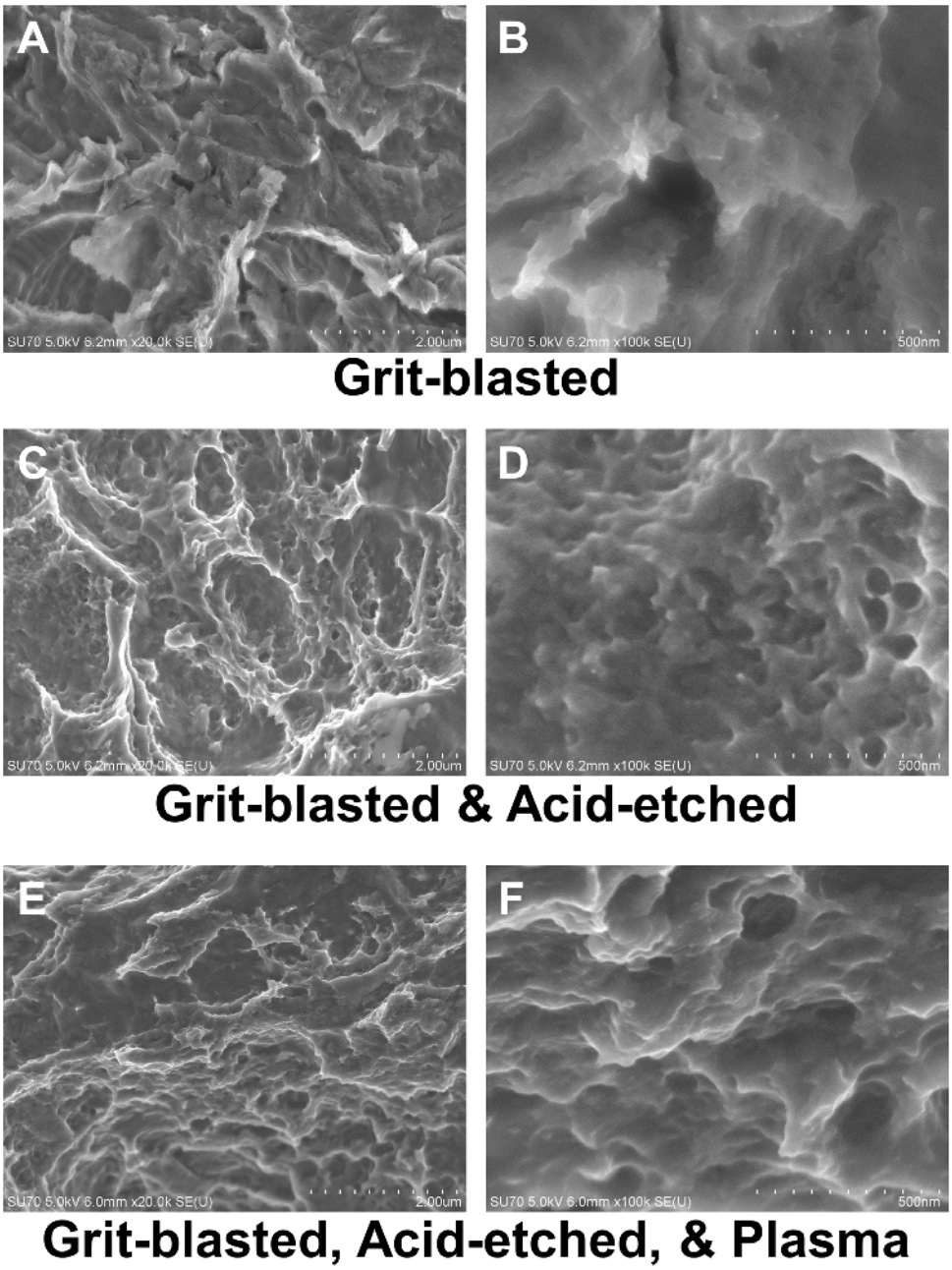

Fig. 1 – Scanning electron images of titanium surfaces. Scanning electron microscopy was used to image grit-blasted (GB; A,B), grit-blasted and acid-etched (GB + AE; C,D), and GB + AE that were plasma treated (E,F) at both 20,000X and 100,000 × . Representative images are shown.

was increased compared to GB only and DBD treatment did not affect production (Fig.3C). OPG was maximally produced after DBD treatment and AE alone did not increase the production of OPG compared to GB (Fig.3D). Paracrine signaling factors BMP2 and VEGF-A were increased on Ti6Al4V compared to TCPS, but DBD treatment increased BMP2 compared to GB, and DBD treatment or AE did not alter VEGF production (Fig.3E-F).

Immunomodulatory cytokines IL6 and IL10 were also assessed in conditioned media. Pro-inflammatory IL6 was increased on GB treated surfaces compared to TCPS and AE reduced production to similar levels. Treatment by plasma further reduced IL6 production (Fig.3G). Anti-inflammatory IL10 was increase in an AE and DBD treatment manner compared to GB and TCPS groups (Fig.3H).

Studies using MG63 cells showed that total DNA content decreased on Ti6Al4V surfaces compared to TCPS and was affected by AE but not by DBD treatment (Fig.4A). OCN was increased on both AE and GB + AE + P groups compared to GB and TCPS (Fig.4B). OPN was only significantly increased on plasma treated surfaces (Fig.4C), while OPG was increased on plasma treated surface but not different from untreated plasma (Fig.4D). Mean BMP2 production increased on plasma treated Ti6Al4V surfaces, but cultures grown on GB or GB +

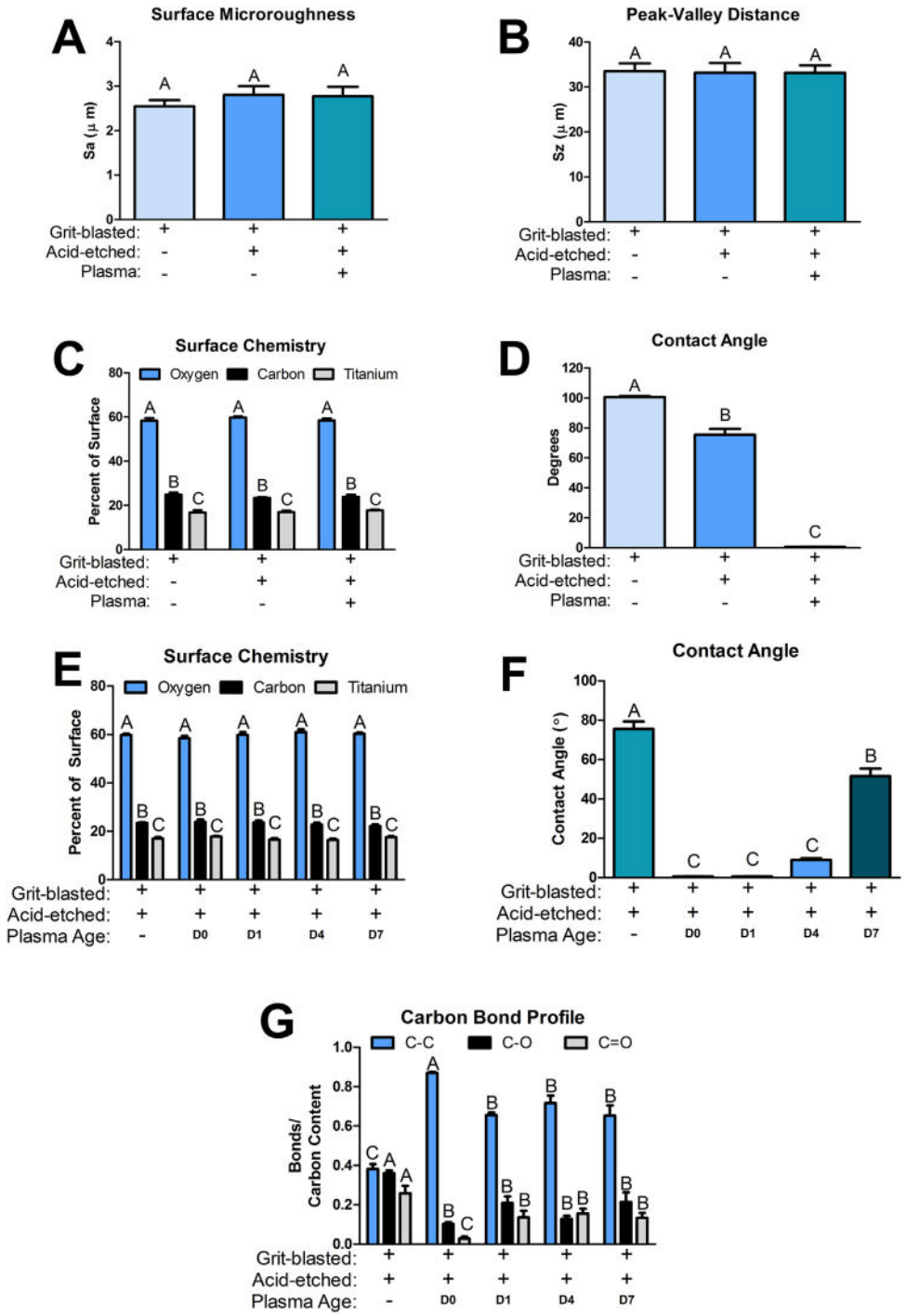

Fig. 2 – Surface characterization of titanium surfaces after DBD treatment. Laser confocal microscopy was used to quantitatively assess surface roughness and morphology. Average microroughness (A), average peak-to-valley distance (B), surface chemistry (C), and surface wettability (D) were determined for grit-blasted (GB), grit-blasted and acid-etched (GB + AE), and GB + AE plus DBD treatment (GB + AE + P). Aging of the DBD treatment was evaluated at 1 day, 4 days, and 7 days after DBD treatment and surface chemistry (E) and wettability (F) were assessed. Carbon bond percentages (G) of the total carbon composition was determined with curve fitting software at 284.8 eV (C—C or CH), 286 eV (CO), and 288 eV (CO——=). Groups were assessed by ANOVA with tukey posttest. Groups not sharing letters are significant at a $p < 0.05$.

AE coupons did not differ from TCPS (Fig.4E). VEGF-A was decreased on all Ti6Al4V surfaces compared to TCPS; this reduction was less in cultures grown on GB + AE and GB + AE + P compared to GB only (Fig.4F). IL6 was decreased on all Ti6Al4V groups compared to TCPS; there was a further reduction in IL6 production in cultures grown on GB + AE, with the lowest concentration of IL6 produced on plasma treated surfaces (Fig.4G). IL10 was increased on GB and GB + AE surfaces but

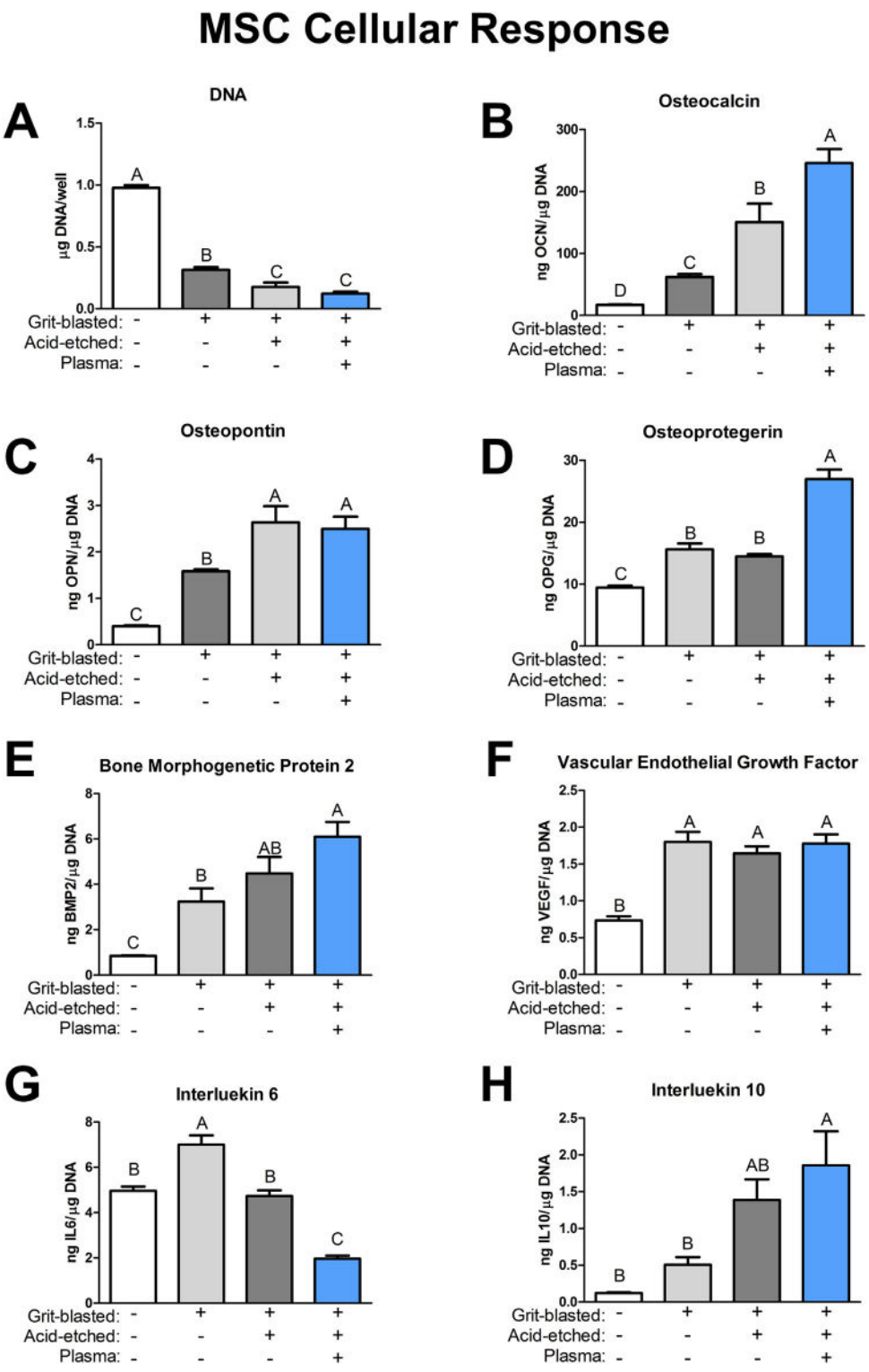

Fig. 3 – Cellular response of mesenchymal stromal cells to titanium surface properties. MSCs were cultured on grit-blasted (GB), grit-blasted and acid-etched (GB + AE), and GB + AE plus DBD treatment (GB + AE + P) titanium surfaces. Total DNA content (A) was determined in the cell layer lysate. Osteocalcin (B), osteopontin (C), osteoprotegerin (D), bone morphogenetic protein 2 (E), vascular endothelial growth factor A (F), interleukin 6 (G), and interleukin 10 (H) were determined in the conditioned media and normalized to Total DNA. Groups are 6 cultures/variable and stats were determined by ANOVA with tukey posttest. Groups not sharing letters are significant at a $p < 0.05$.

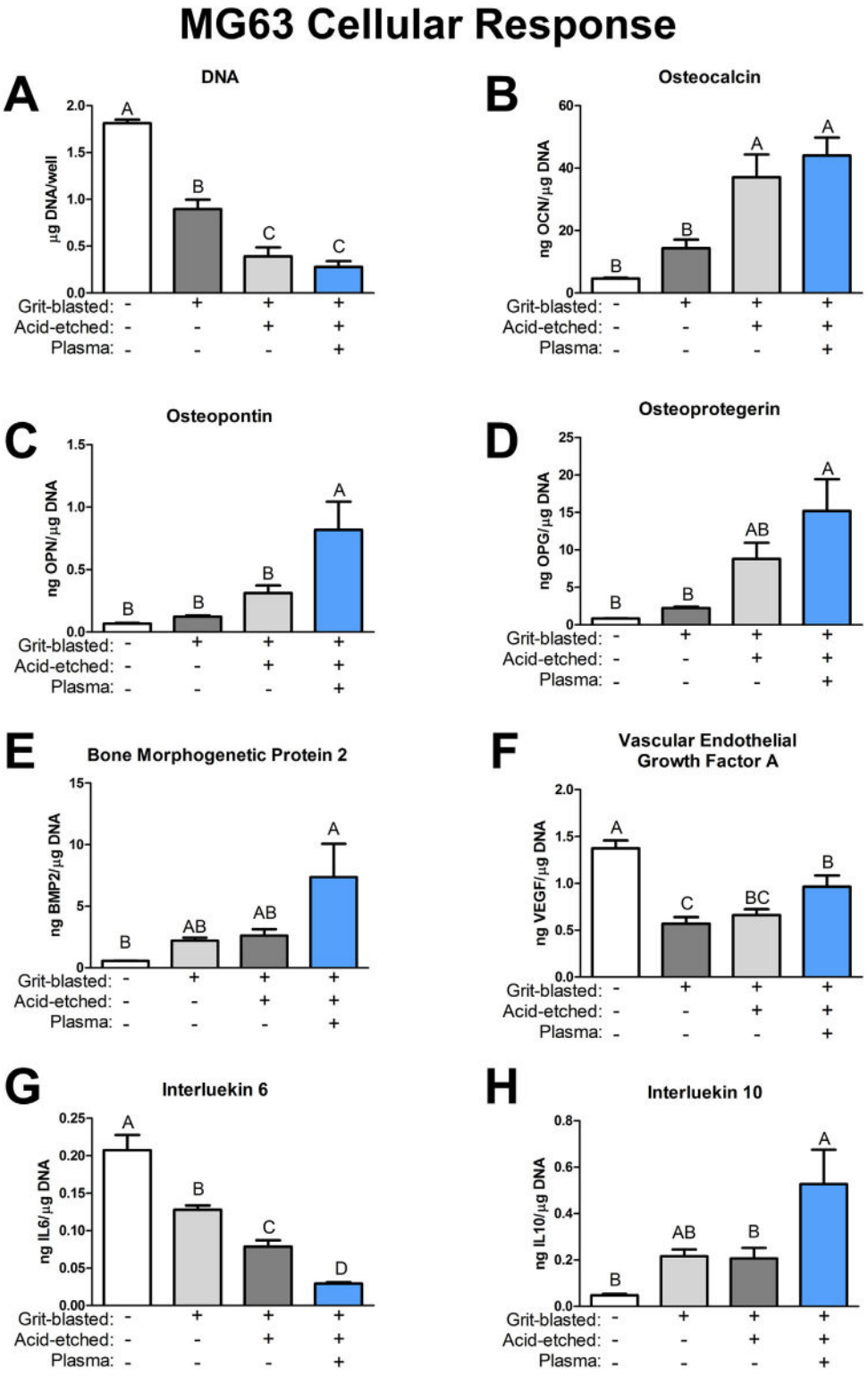

Fig. 4 – Cellular response of osteoblasts to titanium surface properties. MG63 s were cultured on grit-blasted (GB), grit-blasted and acid-etched (GB + AE), and GB + AE plus DBD treatment (GB + AE + P) titanium surfaces. Total DNA content (A) was determined in the cell layer lysate. Osteocalcin (B), osteopontin (C), osteoprotegerin (D), bone morphogenetic protein 2 (E), vascular endothelial growth factor A (F), interleukin 6 (G), and interleukin 10 (H) were determined in the conditioned media and normalized to Total DNA. Groups are 6 cultures/variable and stats were determined by ANOVA with Tukey post-test. Groups not sharing letters are significant at a $p < 0.05$.

was not different from TCPS at 7 days; DBD treatment further increased IL10 compared to TCPS and AE (Fig.4H).

Based on our data from the surface characterization after aging the plasma treated surfaces, the 7 day time-point was selected to determine cellular response in the worse-case scenario of not placing an implant until 1 week after it was plasma treated. Total DNA content of MSC cultures on aged surfaces was increased compared to DBD treatment immediately prior to culture (Fig.5A). Osteocalcin was not affected by aging of the surface (Fig.5B). However, OPN, OPG, BMP2, and VEGF-A were all decreased compared to DBD treatment immediately before cell culture (Fig.5C-F). Aged plasma surfaces were still capable of inducing surface mediated cellular differentiation of MSCs but not to the same extent as DBD treatment immediately before cell culture (Fig.5A-F). Immunomodulatory IL6 and IL10 were not affected by plasma aging. IL6 was still decreased compared to control on both plasma surfaces (Fig.5G) but IL10 was not different from the control (Fig.5H).

4. Discussion

Our results demonstrate that treatment using a DDB plasma in a sub-atmospheric pressure N2 environment does not alter the surface elemental composition shown to occur with previous long-duration conventional plasma treatments. The oxygen plasma eliminates adsorbed hydrocarbons from the implant surface by creating a backflow pressure that continuously moves fast moving ionized gas particles through the sample chamber, allowing impact forces and microcombustion to convert any contaminants into gas and remove them from the chamber [17,26]. In this study, although the wettability of the surface changed, the vacuum sealed implant packaging cannot allow gas to flow through the packing; thus, any volatized carbon is not removed from the tube and rapidly reabsorbs onto the surface.

The MSCs and osteoblasts responded robustly to the alterations in surface wettability even though surface carbon content was unaffected. DBD treatment immediately prior to culture increased MSC-to-osteoblast differentiation, increased osteoblast activity of immature osteoblasts, and reduced the production of pro-inflammatory cytokines. In contrast, coupons that were aged in the vacuum vials did not exhibit a change in elemental composition, but they did exhibit a reduction in wettability and a corresponding reduction in production of osteoblast differentiation markers. These observations indicate that the transient increase in hydrophilicity that was achieved using the benchtop approach was sufficient for improving cellular outcomes but stress the need to use devices treated in this manner soon after applying the DBD plasma.

The present study shows that surface morphology is not altered by the DBD treatment. However, this method is different from previous non-plasma methods to produce a hydrophilic surface on the GB + AE substrates that created additional nanoscale perturbations as a result of their storage in aqueous solutions [27]. These nanoscale alterations did not alter cellular response to the GB + AE surface topography [8], however, suggesting that the implant surface wettability generated in the present study and the underlying microscale roughness play a larger part in surface mediated differentiation [8].

In previous literature, examination of surface hydrocarbon concentration after conventional oxygen plasma treatment showed that the surfaces had increased titanium dioxide compared to their non-plasma treated controls due to robust removal of detectable carbon [17,18,22]. Additionally, surfaces that were acid-etched in a nitrogen environment generated hydrophilicity through a similar mechanism as conventional plasma treatment by reducing carbon concentration. Therefore, changes in cellular response to these surfaces was attributed to increased oxygen and decreased total carbon on the implant surfaces. Here we show that it is not a reduction in carbon content that was the determinate of increased osteoblast response, as this short duration DBD treatment method increased cellular response without altering surface carbon.

The impact of surface wettability *versus* hydrocarbon concentration on the cellular response has not been elucidated in the literature. Research into surface wettability shows that protein adsorption is highly regulated by surface hydrophilicity. Hydrophobic surfaces have increased concentrations of adsorbed proteins at the interface while hydrophilic surfaces have significantly lower protein concentrations [28]. Additionally, studies using fluorescently labeled probes show that hydrophilic surfaces have more homogenous spreading of adsorbed proteins and the concentration of fibronectin is increased with both microroughness and wettability [29,30]. Furthermore, *in vitro* studies examining microroughness have highlighted that surface wettability may be a contributing factor to clot formation and extension [31,32].

Our data show that it is not the carbon concentration per se but the wettability and type of carbon bonds on the implant surface that are more influential on overall cellular response. Increases in the number of C—C or C–H bonds is correlated with improved cellular response, even though the total carbon on the implant surface remained constant during this short-term DBD treatment. Aging the implant surfaces in their sterile packaging after DBD treatment, thereby preventing exposure to ambient aerosolized hydrocarbons, prevented complete loss of the DBD treatment effect, but the types of carbon bonds changed during the process as early as day 1 of aging with increasing percentages of C═O and remained constant throughout the 7 days of aging. This led to altered cellular response compared to the unaged plasma treated surfaces similar to that of untreated controls. This suggests that packaging and duration time of plasma treatment alter cellular response by only altering surface wettability and not surface elemental composition.

Clinically, this dielectric discharge barrier plasma method for induction of hydrophilicity is advantageous because any brand of implant can be packaged and treated by this benchtop method to improve implant outcomes without the need for shipment back to the manufacturing facility. Additionally, the portability of this approach can improve periodontal treatment in developing areas the lack access to higher-cost implants. The increased access to implant treatment methods provided by this on-demand benchtop technology treatment partnered with the current and historical data showing increased wettability improved cellular response

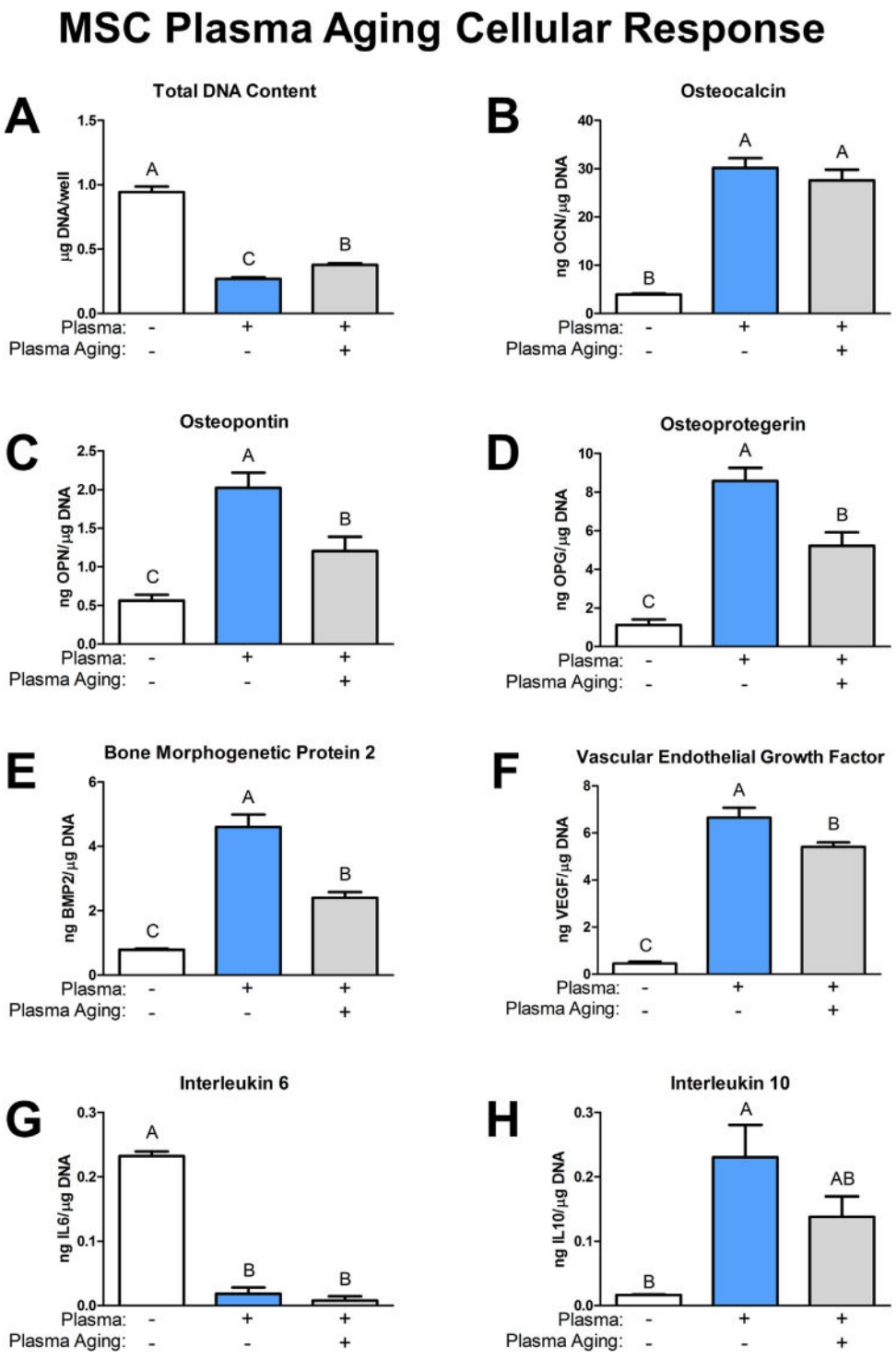

Fig. 5 – Cellular response of mesenchymal stem cells to plasma treated and aged plasma titanium surface properties. MSCs were cultured on tissue culture polystyrene (TCPS), and titanium surfaces that were grit-blasted and acid-etched (GB + AE) with DBD treatment or with DBD treatment and 7-day aging. Total DNA content (A) was determined in the cell layer lysate. Osteocalcin (B), osteopontin (C), osteoprotegerin (D), bone morphogenetic protein 2 (E), vascular endothelial growth factor A (F), interleukin 6 (G), and interleukin 10 (H) were determined in the conditioned media and normalized to Total DNA. Groups are 6 cultures/variable and stats were determined by ANOVA with Tukey post-test. Groups not sharing letters are significant at a $p < 0.05$.

in vitro and osseointegration *in vivo* creates great potential for this hydrophilicity application method. Future studies will evaluate the effectiveness of this novel DBD treatment *in vivo*.

Author contributions

MBB, ZS, DJC, and BDB contributed to conceptualization and methodology of these studies. ZS, and BDB were responsible for funding acquisition. MBB and KBB were responsible for investigation and formal analysis. MBB was responsible for validation and visualization of published work. MBB prepared the original draft of the manuscript. KBB, ZS, DJC, and BDB were involved with review and editing of the manuscript. All authors gave final approval before submission.

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