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Hydrophilic implants generated using a low-cost dielectric barrier discharge plasma device at the time of placement exhibit increased osseointegration in an animal pre-clinical study: An effect that is sex-dependent

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Objectives: Increased wettability of titanium and titanium alloy surfaces due to processing and storage methods increases osteoprogenitor cell differentiation and osseointegration compared to microroughness alone. Implants that are exposed to air have a hydrophobic surface due to adsorption of atmospheric hydrocarbons, which can limit overall implant success. Dielectric barrier discharge plasma (DBD) is one method to increase surface hydrophilicity. Although current DBD methods yield a hydrophilic surface, adsorbed hydrocarbons rapidly restore hydrophobicity. We demonstrated that application of DBD to implants previously packaged in a vacuum, generates a hydrophilic surface that supports osteoblastic differentiation *in vitro* and this can be done immediately prior to use. In the present study, we tested the hypothesis that DBD treatment to alter surface wettability at the time of implant placement will improve osseointegration in vivo.

Materials and methods: Twenty male and sixteen female rabbits were used in a preclinical trans-axial femur model of osseointegration. Control and DBD treatment implants were inserted randomized per hind limb in each rabbit (1 implant/hind-limb). At 6 weeks post-surgery, bone-to-implant contact, adjacent bone volume, and torque to failure were assessed by micro-CT, calcified histology, and mechanical testing.

Results: DBD plasma treatment of vacuum-sealed implants increased surface wettability and did not change surface chemistry or roughness. Peak torque and torsional energy, and bone-to-implant contact increased with DBD treatment in males. In contrast, female

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rabbits showed increased osseointegration equal to DBD treated male implants regardless of DBD plasma treatment.

Conclusion: DBD treatment is an effective method to enhance osseointegration by increasing surface wettability; however, this response is sex dependent. In healthy female patients, DBD treatment may not be necessary but in older patients or patients with compromised bone, this treatment could be an effective measure to ensure implant success.

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1. Introduction

Titanium dental implants are one of the best methods to replace missing teeth due to their high success rate in healthy patients, restoration of functionality, and aesthetics [1]. However, implants have reduced long-term effectiveness in patients with comorbidities such as diabetes, osteoporosis, and many other systemic diseases [2]. One method to improve implant success is to modify the surface characteristics to enhance osteogenesis and ultimately osseointegration. In order to increase the quality of care for patients in underserved communities and in places lacking expertize in oral care, there is a need for low cost approaches to achieve the desirable surface properties.

Bone marrow stromal cells (MSCs) are one of the key regulators of implant integration with bone. These cells regulate key signaling pathways including angiogenesis, osteogenesis, and immune response [3]. There is strong *in vitro* and *in vivo* evidence demonstrating that titanium and titanium alloy implants possessing surface properties similar to those of native bone remodeling regulate MSC response to create a pro-osteoid depositing environment by regulating paracrine signaling factors, immunomodulatory cytokines, and inducing surface mediated osteoblastic differentiation [4–6].

Previous studies by our lab and others have investigated the effect of sex differences on the response of osteoprogenitor cells, including MSCs, to implant surface properties. These studies have demonstrated that cells from males and females respond differently to implant surfaces possessing microroughened surface topographies and varying degrees of wettability [7–9]. Interestingly, these studies have concluded that both sexes increase osteogenic factor production in response to surface properties; however, cells from males increase the production of osteoprotective paracrine signaling factors like osteoprotegerin, while female cells increase the production of bone formation factors like bone morphogenetic protein 2 (BMP2) and transforming growth factor beta 1 (TGFβ1). Whether these differences noted *in vitro* impact implant success *in vivo* is not known. Therefore, it is important to understand how implant surface treatments affect both sexes to ensure these differences do not affect overall implant success.

In the clinic, the most common and clinically proven method to improve osseointegration is through a combination of physical grit-blasting and chemical acid-etching modification methods. There is extensive literature demonstrating that MSCs cultured on surfaces treated by grit-blasting and acid-etching undergo surface mediated osteoblastic differentiation, and furthermore, surfaces that are modified and maintained in a hydrophilic environment further increase this phenotypic response of osteoblast precursors [10,11]. One method to increase osseointegration for all implants, independent of manufacturing processes, is to increase the wettability of the implant close to or at the time of placement. Dielectric barrier discharge (DBD) can be used to generate a hydrophilic surface and increase the overall success of an endosseous implant. This is achieved by production of an ambient temperature, cold plasma that spontaneously occurs when an insulating material interrupts the discharge path between two electrodes [2]. This technology was originally used to generate ozone, and in recent years has been a part of surface treatment methods to improve adhesion and wettability properties by increasing hydrophilicity [2], and has also been part of numerous medical applications for biological decontamination [12]. However, unless implants are used immediately or are stored in an aqueous environment, atmospheric hydrocarbons adsorb onto the surface, resulting in a hydrophobic surface chemistry.

Cleaning the implant prior to its use can temporarily restore hydrophilicity, but the technology to achieve this is not readily available in most clinical settings. A bench-top device that generates DBD to sterilize and improve surface characteristics at the time of implant placement could result in a better osseointegration outcome, with no need for special storage conditions to retain wettability on the implant surface. We assessed the effectiveness of a bench top DBD device to clean grit blasted and acid etched titanium-aluminum-vanadium (Ti6Al4V) disks that have been sealed in vacuum tubes [13]. The resulting surfaces were hydrophilic but the carbon content on the surfaces was unchanged. MSCs cultured on the surfaces exhibited enhanced osteogenic phenotypic markers compared to MSCs on untreated surfaces, suggesting that implants treated using the device might promote greater osseointegration in vivo. Therefore, in the present study, we evaluated the surface effects of DBD plasma on Ti6Al4V implant osseointegration and assessed the effectiveness of the treatment in an *in vivo* rabbit model. Moreover, to test if the effect is sex-dependent, the treated implants were placed in both male and female rabbits.

2. Methods

2.1. Substrate manufacturing

Titanium-aluminum-vanadium (Ti6Al4V) surfaces were produced as described previously [13]. Briefly, grade 4 Ti6Al4V rods were milled into 10.5 mm by 5.25 mm rectangular

substrates and surfaces treated by grit-blasting and acid-etching (GB+AE) in the same manner as the implants described below.

Implants were manufactured by computer numerical control (CNC) milling. Dual pitch screw implants were machined to 3.75 mm diameter and 8 mm in length. Surfaces and implants were calcium phosphate grit-blasted using proprietary technology (AB Dental, Ashdod, Israel). All surfaces were degreased and treated by HNO_3 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 H_2O for 10 min.

All surfaces and implants were blotted, air-dried, and half of them were packaged in glass vials fitted with implant mounts made from stainless steel. Storage vials were vacuum-sealed, maintained by rubber gaskets and sterilized by gamma radiation. Control implants were packed in clinically available plastic boxes with implant mounts made from stainless steel because vacuum storage of control substrates did not alter surface properties [13].

2.2. Dielectric barrier discharge plasma

Vacuum-sealed vials containing the implants 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 [13].

2.3. Surface characterization

2.3.1. 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 at a 5 mm working distance.

2.3.2. 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, 201.3 × 201.3 μm image size, and step size of 1 μm. Data were determined by the topographical software package addition (Carl Zeiss).

2.3.3. Surface wettability analysis

Surface wettability was analyzed by sessile drop test using a goniometer (CAM 250, Ramé-Hart). Samples were measured in 6 different locations and dried with nitrogen gas between measurements. 3 μL of ultrapure distilled water was used per measurement.

2.3.4. 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-Watt, 15 kV x-ray gun with a spot size of 200 μm, 20 ms dwelling time and 1 eV step size.

2.4. Surgical implantation

2.4.1. Ethics approval

All animal procedures were conducted in compliance with the US National Research Council's Guide for the Care and Use of Laboratory Animals, the US Public Health Service's Policy on Humane Care and Use of Laboratory Animals, and Guide for the Care and Use of Laboratory Animals. Approval was obtained by Virginia Commonwealth University's Institutional Animal Care and Use Committee (IACUC) under protocol number AD10000675. *In vivo* studies were only completed after validation *in vitro* [13]. ARRIVE guidelines and additional recommendations were used in the development, implementation, and analysis of this study and is available for review under supplemental materials.

2.4.2. Study design

Male and female New Zealand white rabbits, weighing between 3.2 and 3.5 kg, were single-housed and given *ad libitum* access to standard pellet and water, receiving fresh food daily. The entire cage was changed and sanitized biweekly, with waste control pans changed twice a week. The racks were situated to allow visual, auditory, and olfactory stimulation for all rabbits in the room, and all rabbits received toys as part of their environmental enrichment. Twenty male rabbits and sixteen female rabbits were used for all studies. These groups were divided into two cohorts. Cohort 1 underwent destructive mechanical testing (18 animals, $n = 10$ males, 20 implants, 10 from each treatment group and n = 8 females, 16 implants, 8 from each treatment group). Cohort 2 were designated for micro-CT and calcified histology (18 animals, $n = 10$ males, 20 implants, 10 from each treatment group and n = 8 females, 16 implants, 8 from each treatment group). One female from cohort 1 and one male rabbit from cohort 2 were withdrawn from the study because humane endpoints were met. The animal number per group was chosen based on a power analysis using an alpha of 0.05 and a power of 80% (delta = 5, sigma = 3, $m = 1$) to reveal a minimum of $n = 7$ per group for the study to yield statistical significance. Extra rabbits were used to ensure that experimental exclusions did not reduce power below the threshold.

2.4.3. Bilateral implant surgery

Rabbits were sedated with 20 mg/kg ketamine in combination with 3 mg/kg Xylazine by intramuscular injection into the quadriceps. Rabbits were intubated with a R3 or R3.5 intubation tube, and anesthesia was maintained using 0.5–4% inhaled isoflurane in O_2. Rabbits were randomly divided into the experimental cohorts, and control or plasma implant placement was randomized per hind-limb, contralateral hind-limb possessed the non-selected implant. The surgeon was blinded to the implant treatment. Implant placement was achieved using previously described surgical techniques [14].

Briefly, the greater trochanter of the femur was palpated, and a 5-cm incision was made overlying this anatomic

landmark. The muscles were separated, and the posterior surface of the both left and right proximal femur was localized. A physio dispenser connected to a dental hand piece with a maximum torque of 35 Nm was used to prepare the implant cavity. All parts of the surgery used a foot controller with irrigation to avoid undue heating of the bone. Implants were placed trans-axially into the underlying bone using a stepwise drilling process starting with a shallow pilot drill bit and ending with a maximum diameter of 3.65 mm dental drill bit (AB Dental, Ashdod, Israel). For plasma treatment groups, the implants were treated for as described above for 30 s during the stepwise drilling into the bone. Implant were secured using a dental torque wrench (AB Dental) until implants were flush with the cortical surface and primary fixation was achieved. A cover screw was then screwed into the implant, the muscles were re-approximated and secured with 5–0 vicryl suture, and the skin was closed with 5–0 nylon suture in a running technique.

2.4.4. *Sample harvest*

Animals were euthanized using an ear vein injection of sodium pentobarbital at post-surgical day 42. Femurs processed for micro-CT and calcified histology were fixed in 10% neutral buffered formalin for a minimum of 48 hr. Femurs processed for mechanical testing were kept moist, cut to size, with torque removal testing conducted the same day as harvest.

2.5. *Micro-CT analysis*

Micro-CT (SkyScan 1173, Bruker) was used to evaluate bone-to-implant contact (BIC) in the distal femur. Femurs used for micro-CT were fixed and stored in 10% neutral buffered formalin for at least 48 h prior to imaging. The proximal femur of fixed samples was scanned at a resolution of 1,7920 × 1520 pixels (image pixel size of 6.83 μm) over 360° using a 0.25 mm brass filter, scanning energies of 130 kV and 61 μA, 1350 ms exposure time, and 5 x-ray projections acquired every 0.3° averaged. A standard Feldkamp reconstruction was done using NRecon Software (Kontich, Belgium) with a beam hardening correction of 30% and a Gaussian-smoothing kernel of zero. Samples were analyzed using CTAn version 1.17.7.2. The implant was dilated by 4 pixels to ensure a conservative determination of bone-to-implant contact (BIC) and bone formation. BIC was then calculated by isolating all bone within 3 pixels (20.4 μm) of the outer three-dimensional surface of the dilated implant region of interest, and BIC reflects this value divided by the outer three-dimensional surface of the implant volume. Adjacent bone volume was calculated as the bone volume within 100 pixels adjacent to the dilated implant surface (680 μm).

2.6. *Histological analysis*

2.6.1. *Calcified histology*

Following micro-CT, samples were commercially processed (Histion, Everett, WA). Briefly, samples were embedded in methyl methacrylate and one section was taken from each specimen. Sections were stained with Stevenel's Blue/Van Gieson and imaged using Zen 2012 Blue Edition with an AxioCam MRc5 camera and Axio Observer Z.1 microscope (Carl Zeiss Microscopy).

2.6.2. *Histological bone-to-implant contact*

BIC was evaluated for male and female rabbits through ImageJ (NIH) [15]. Bone contact in the cortical and marrow regions of the femur was traced and measured in ImageJ with the scale calibrated to 1000 μm. Bone contact was defined as the purple stained sections touching the implant, and the sum for each region was divided by the total perimeter of that respective region to create a ratio of BIC/regional perimeter. The cortical region was classified as the striated portions of bone around the top and bottom of the implant, and the marrow region was the space in between the cortical bone.

2.6.3. *Adjacent bone area*

Adjacent bone area was quantified for the marrow, with a rectangular region standardized to width (2208.74 μm) and a specific height for each sample, as the height of the cortical regions would differ between samples. The bone region was isolated by using the shade of the stain as a threshold; its area was quantified and normalized to the total area of the rectangular region to achieve a ratio of bone area divided by tissue area.

2.7. *Mechanical analysis*

Destructive mechanical testing was performed on cohort 1 rabbits. Mechanical torque to failure was used to determine overall implant mechanical integrity using a Bose ElectroForce 3200 Series III Axial-Torsion mechanical testing system equipped with a 445 N/5.7 N-m load/torque transducer. The load cell was zeroed and a 0.1 Hz filter was applied to reduce noise. Samples were minimally shaved with a Dremel-300 rotary sander to flatten the sides of the femur for better gripping within the mechanical testing platens. Implants were first attached to a custom-made implant mount, using one stainless-steel implant mount welded into custom machined block with thread holes that match the fixation mounts on the mechanical testing device. Once the implant was secured tightly to the mount and was perpendicular to the axis of rotation, the sides of the cortical bone were then secured between two flat specimen clamps, ensuring not to induce significant preload (> ±0.15 N load) or torque (< ±0.1 N cm). The femurs were then rotated at 1 RPM (ASTM standard F543–00) to remove the implant from the surround bone. At the same time the mechanical testing frame raised the mount at a rate of 0.013 mm per second to ensure the threads of the mount would not catch on the implant threaded grooves and create compressive loads. Data collection was achieved at 100 Hz to 60 degrees of rotation. Mechanical evaluation was determined by generating torque *vs* degree graphs after the torque and rotation were all normalized to the same starting conditions (0 N cm and 0 degrees). Curve were fit to a bilinear model to distinguish the linear region from the toe region (SLM-Shape Language Modeling version 1.14, MATLAB) and were evaluated for the maximum torque at failure (N m), torsional stiffness (linear region slope, N m/radians), and torsional energy (area under the curve, millijoules).

2.8. *In vitro analyses*

2.8.1. *Osteoblast isolation*

Based on the surprising results of the *in vivo* study, *in vitro* analysis of calvarial osteoblasts isolated from male and female rabbits were conducted to examine cellular response to implant surface properties. Rabbit male and female (2.9–3.2 kg) osteoblasts were isolated from the calvarial bones of 3 donors per sex. To avoid using more animals for calvarial bone isolation, rabbit calvarial bones were obtained at an experimental endpoint in other IACUC approved studies from collaborations at Virginia Commonwealth University. Rabbits were sedated and euthanized according to approved IACUC protocols. The heads were sanitized with 70% ethanol before the dermal layers were separated by scalpel and parietal bones were removed by dental rongeurs tool. Bones were cleaned of soft tissue and periosteum by scalpel and periosteal elevator. Bones were then placed in Dulbecco's modified Eagle medium (DMEM) containing 150 units/mL of penicillin and streptomycin on ice and transported to the lab.

Bones were then placed into Petri discs and further cleaned of soft tissue by surgical scissors and scraping with a periosteal elevator. Bones were subsequently minced into 1 mm bone pieces and rinsed in sterile PBS until the PBS washes were clear. The bone chips were then placed in a tissue culture 6-well plate (75,000 cell/cm^2) spacing the bone chips by roughly 2 mm apart. Osteoblasts were allowed to grow out of the bone chips for 1 week. After 1 week, the cells that had grown out of the bone chips were passaged using 0.25% trypsin for 5 min. The trypsin was inactivated using DMEM full media (DMEM-FM) containing 10% FBS and 50 units/mL of penicillin/streptomycin. Subsequently, cells were from donors of the same sex were pooled and passaged into new 6-well culture plates by collecting the supernatant and spinning the cells down at 400 g for 10 min and re-suspending in 5 mLs of DMEM-FM, cells were counted and plated at a density of (75,000 cells/cm^2).

Characterization to confirm the isolated cells were osteoblasts was conducted by culturing cells in a 24-well plate at a density of 10,000 cells/cm^2 till confluence and then subsequently treating with 10^{-8} M 1α,25-dihydroxyvitamin D_3 (1α,25$(OH)_2D_3$) for 24 h. 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-X 100 and stored at − 80 °C for biological assays. Osteoblastic response of these isolated cells was determined by alkaline phosphatase activity of cell layer lysates and osteocalcin content of the conditioned media (R&D Systems, Inc.) normalized to total DNA content (Promega). These cells were confirmed as osteoblasts as they presented high levels of alkaline phosphatase and osteocalcin, and also responded to treatment with 1α,25$(OH)_2D_3$ by increasing osteocalcin production.

2.8.2. *Cellular response and sex differences*

Two control, non-DBD plasma treated rectangular implant surfaces were placed side-by-side per well in 24-well plates (n = 6wells/group) to determine the response of rabbit osteoblasts to the surface microroughness. Osteoblasts were plated at a density of 10,000 cells/cm^2, with osteoblasts cultured for each experiment on tissue culture polystyrene (TCPS) serving as controls. Surfaces were moved into new 24-well plates after 24 h to ensure that only cells in contact with the implant surfaces were assessed. Subsequently, media was changed every 48 h. At day 7, cells were incubated for 24 h with fresh DMEM-FM 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.

2.8.3. *Biological assays*

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. Osteocalcin (OCN), osteoprotegerin (OPG), and BMP2 (R&D Systems, Inc.) were quantified according to the manufacturer's protocol. Data were normalized to total DNA content (Promega).

2.9. *Statistical analysis*

For the *in vivo* study, mechanical, histological, and microCT analyses comparing treatment to non-treated implants used a paired T-test, and comparisons across all experimental groups used ANOVA with two-tailed Tukey *post hoc* analysis with p-values equal or less than < 0.05 considered statistically significant. Data are reported as means ± standard error mean (S.E.M.). In the supplemental histological analysis; cortical, marrow, and combined bone BIC/regional perimeter were averaged separately and Wilcoxon t-test was used to compare treatment over control with p-values equal or less than < 0.05 considered statistically significant.

In vitro data are means ± S.E.M. of six independent cultures per variable (n = 6 wells/group). Experiments were repeated to ensure the validity of observations (N = 2), with results from an individual experiment shown in the figures. Statistical analysis among cell culture groups was performed by one-way analysis of variance (ANOVA) and multiple comparisons between the groups were conducted with a two-tailed Tukey *post hoc*-test with p-values equal or less than < 0.05 considered statistically significant. Unpaired Student's t-test was used to compare each sex of osteoblasts on GB+AE and TCPS. Graph Pad Prism 5.04 was the statistical software used to evaluate the results. Data are available upon reasonable request to the corresponding author.

All animal procedures were conducted in compliance with the US National Research Council's Guide for the Care and Use of Laboratory Animals, the US Public Health Service's Policy on Humane Care and Use of Laboratory Animals, and Guide for the Care and Use of Laboratory Animals. Approval was obtained by Virginia Commonwealth University's Institutional Animal Care and Use Committee (IACUC) under protocol number AD10000675. *In vivo* studies were only completed after validation *in vitro* [13]. ARRIVE guidelines and additional recommendations were used in the development, implementation, and analysis of this study and is available for review under supplemental materials.

3. Results

3.1. *Effect of dielectric barrier discharge plasma on implant surface properties*

The physical properties of the Ti6Al4V screw implants were not changed after the 30-second DBD plasma treatment. Scanning electron micrographs of the untreated control implant surface show pit like morphologies at microscale (Fig. 1 A & C) with ridgelines similar to that of native bone at the nanoscale (Fig. 1E). Furthermore, DBD plasma treatment did not alter surface morphology (Fig. 1B, D and F). Optical profilometry showed GB+AE created surface microroughness of roughly 2.9 µm (Sa) and DBD plasma treatment did not alter this roughness (Fig. 1 G) or average peak-to-valley height (Fig. 1H).

Plasma treatment did alter surface wettability as shown by the sessile drop contact angle measurement. DBD treatment created a super hydrophilic surface with a contact angle of 0 degrees compared to roughly 80 degrees for the untreated controls (Fig. 1I). X-ray photoelectron analysis showed these Ti6Al4V surface readily oxidize with high concentrations of oxygen, followed by carbon, and then titanium respectively. There were no differences between aluminum, vanadium, and nitrogen. DBD treatment did not alter surface chemical composition, as treated implants possess the same concentrations of each element compared to the untreated implant surface (Fig. 1J).

3.2. *Effect of plasma on implant osseointegration at 6 weeks*

Micro-CT assessment of implant osseointegration at 6 weeks post implantation showed that DBD plasma treatment enhanced osseointegration in male rabbits. MicroCT images of untreated and DBD plasma treated implant in the rabbit bone showed bone growth creeping in the marrow cavity from the cortical bone (Fig. 2A-D). The female groups qualitatively showed higher quantities of trabecular bone-like structures within the marrow cavity (Fig. 2C-D).

Analysis of the microCT reconstructions revealed that plasma treatment increased bone-to-implant contact in male rabbits (Fig. 3A, & C). Interestingly, female rabbits did not increase bone-to-implant contact in response to the DBD plasma treatment (Fig. 3B, D). However, both female implant groups had increased bone-to-implant contact, similar to the plasma treated male implants (Fig. 3C). Moreover, assessment of adjacent bone volume showed a response to DBD treatment in males and an increase in both female implant groups to similar volumes as the plasma treated males (Fig. 3D).

Qualitative calcified histology showed similar results to micro-CT analysis. The calcified histology showed increased bone formation with the application of DBD treatment in males. The female groups had marked increase in bone formation compared to untreated male implant groups, which was similar to the DBD treated male (Fig. 4A-D). Histomorphmetric analysis revealed increased total bone-to-implant contact in response to DBD plasma treatment in males, with no differences between treatments in females (Fig. 4E-G).

Adjacent bone area was quantified in 2-dimensions by calcified histology (Fig. S1). However, the effect of DBD plasma treatment in males as seen in the micro-CT 3-dimension analysis was not seen in adjacent bone area by calcified histology (Fig. S1). Furthermore, adjacent bone area was not different between implant treatment or sex as shown by the quantification of the calcified histology (Fig. S1).

3.3. *Effect of plasma on implant mechanical anchorage at 6 weeks*

In males, mechanical torque to failure revealed that plasma treatment increased peak torque (Fig. 5A) and torsional energy (Fig. 5E) compared to untreated contralateral implants. Interestingly there were no differences for torsional stiffness (Fig. 5C) in the male rabbits. In the female rabbits' peak torque at failure (Fig. 5B) and energy (Fig. 5F) were not increased with plasma treatment; however, plasma treatment did increase the torsional stiffness (Fig. 5D). Furthermore, peak torque at failure and torsional energy for female rabbits of both implant types was similar to that of the plasma treated implants in males, and peak torque for untreated implants in males was lower than all other groups (Fig. 5G & I). The only difference in torsional stiffness was between plasma treated and non-treated implants in females; males with implants from both groups were not different from either female implant group (Fig. 5H).

3.4. *Sex differences between male and female osteoblasts in response to surface topography*

In vitro assessment of male and female osteoblasts isolated from rabbit calvaria bones showed these cells produced high levels of osteocalcin and responded to 10^{-8} M $1\alpha,25(OH)_2D_3$ treatment for 24 h (Fig. S2). Osteoblasts were cultured on non-DBD plasma treated Ti6Al4V substrates for 7 days to determine sex differences in response surface microroughness to determine if the *in vivo* response was species specific and for comparison to our previous pre-clinical evaluations of sex differences in both rats and humans. After culture for 7 days on titanium surfaces female cells had much higher concentrations of total DNA on either TCPS or the GB+AE Ti6Al4V implant surface compared to their male counter parts (Fig. 6A). Total DNA content at day 7 for female cells on the TCPS was greater than that of the male cells; DNA content in female osteoblast cultures on GB+AE surface was similar to that of male cells on TCPS, and male cells on the GB+AE had the least amount of DNA (Fig. 6A). Males produced higher quantities of OCN, BMP2, and OPG on the GB+AE surfaces, which was significant compared to cells on TCPS. Females produced similar quantities of OCN, BMP2, and OPG on the GB+AE surfaces compared to TCPS (Fig. 6B-D). Male cells produced significantly higher levels of OCN, BMP2, and OPG compared to their female counterparts when grown on the titanium surface (Fig. 6B-D).

4. Discussion

The present study shows that DBD plasma treatment increases osseointegration in a preclinical model. Micro-CT and

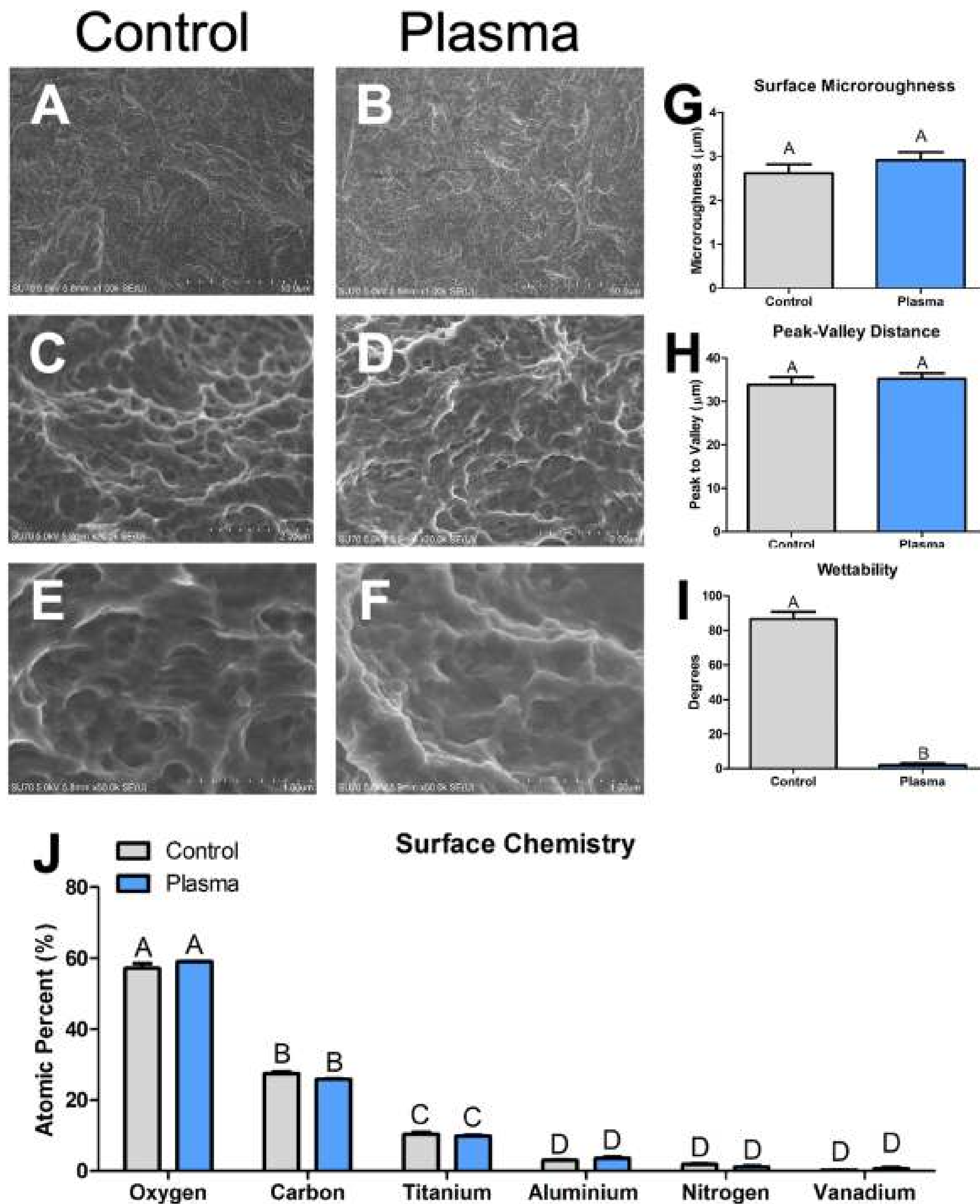

Fig. 1 – Implant surface characterization shows increases in surface wettability without altering surface topography and chemistry. Scanning electron micrographs at 1kX (A,B), 20kX (C,D), and 50kX (E,F) magnification. Optical profilometry measurements of surface microroughness (G), peak to valley height (H). Contact angle measurement to assess wettability (I). X-ray photoelectron spectroscopy to assess atomic concentration of elements on the surface (J). Groups not sharing letters are significant at p-values equal or less than < 0.05.

calcified histology showed significant increases in bone-to-implant contact, which was correlated to improved mechanical response. Collectively, this preclinical study and prior *in vitro* studies using DBD plasma treatment have demonstrated the potential to improve osteogenesis by increasing local factor production necessary for osteoblastic differentiation, and ultimately stimulate bone formation [13,16,17].

While there are other methods to treat surfaces using cold plasma, they rely on the clinician or technician to clean the entire surface in an open air-environment. Moreover, the instruments are often bulky and do not maintain the implant in

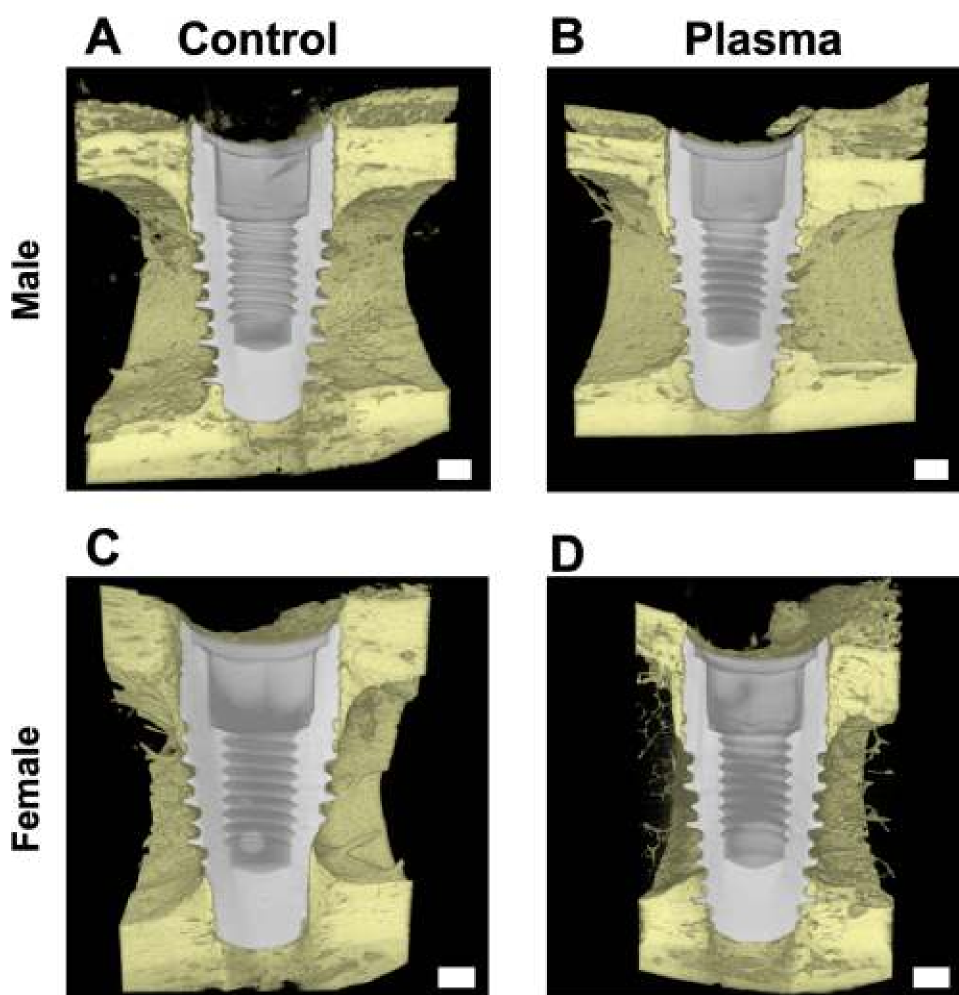

Fig. 2 – Micro-CT reconstructions of implanted screw implants after 6 weeks of implant placement. Representative images of male rabbits for both control (A) and DBD plasma treated implants (B). Representative images of female rabbits for control (C) and DBD plasma treated implants (D). Scale bar is 1 mm.

a sterile environment after treatment [16,18]. The method of vacuum packaging used in the present study ensures complete coverage of the implant surface by DBD plasma and the maintenance of sterility right up until the time of implantation.

Whereas implant osseointegration in the male animals was markedly improved by DBD treatment of the implants, we saw a high degree of implant integration in female animals regardless of implant treatment. Bone-to-implant contact, adjacent bone formation, and mechanical anchorage were increased compared to the untreated male implant group. These results suggest the environment surrounding the implant was already highly osteogenic in the females and this superior sex-dependent bone formation may have masked the effects of DBD plasma treatment.

One explanation for the sex difference in bone formation in response to plasma treatment is rabbit maturation and physiology during adolescence. Our rabbits were 2 months old at the time of the study; rabbit puberty can begin as early as 3 months of age, which would coincide with the middle of this study, and female rabbits are more likely to undergo puberty before male rabbits [19]. This potential earlier onset of puberty could overshadow the effect of the plasma treatment as puberty is one of the most important stages of bone mass accrual and bone mineral density almost double by the end of adolescence [20]. Prior *in vitro* studies evaluating DBD plasma used donor cells from both males and females and reported that DBD plasma can increase osteogenesis when systemic factors are removed [13,17].

In other studies, using implant surfaces with similar surface microroughness, a similar result was seen where osseointegration of super hydrophilic implants placed into young mice was not different from their hydrophobic counterparts. This further enforces the hypothesis that wettability is less important in healthy bone environments. This hypothesis is also backed up by compromised bone models where implant surface properties, primarily wettability, were able to increase bone-to-implant contact in ovariectomized, aged rats and improve bone formation parameters and neovascularization in aged mice [21].

These results demonstrate that DBD plasma treatment improves bone formation and implant integration and suggest

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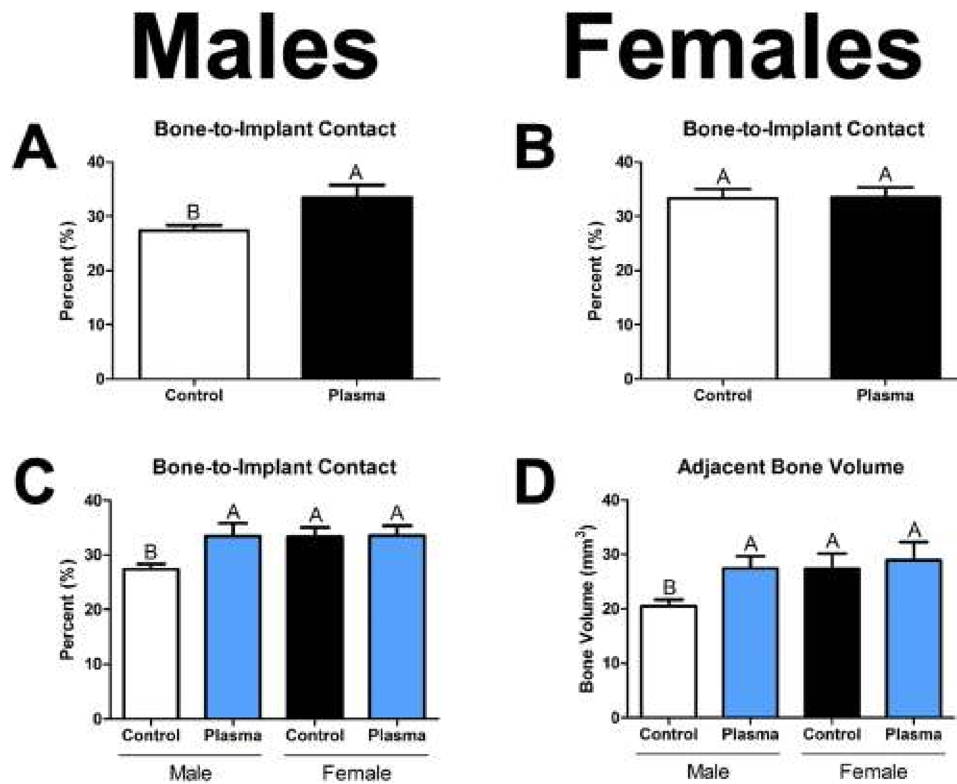

Fig. 3 – Quantified analysis of micro-CT shows increased bone-to-implant contact (BIC) with DBD plasma treatment in males. Male BIC is increased with DBD plasma treatment (A, C). BIC in female rabbits is not altered with DBD treatment (B,C). Adjacent bone volume was quantified within 100 pixels (680 micros) from the implant surface (D). Groups not sharing letters are significant at p-values equal or less than < 0.05.

that this effect may be dependent on bone quality. Importantly, we show that this treatment does not impede implant osseointegration in healthy populations, while providing surface properties that have been shown to enhance bone formation in compromised bone qualities. Therefore, we show that application of DBD to vacuum sealed implants could be a viable method to alter surface wettability in the clinic.

Previous studies evaluating the effect of sex differences on osseointegration showed that osteoprogenitor cells from both sexes respond to implant surface topographies to increase osteogenic soluble signaling proteins [8], but the extent of each individual protein varied. In the case of human osteoblasts from young patients, females produced higher levels of bone formation proteins, while males produced higher levels of anti-resorption proteins like osteoprotegerin [13]. This response in human female osteoblast *in vitro* could also correlate with a similar phenotype we see in the current preclinical study.

The results from our *in vitro* analysis of isolated rabbit osteoblasts from both males and females also shows that cell maturation and environment may be contributing factors in differences in cellular response to DBD treatment of GB+AE implant surfaces. Osteoblasts from female calvarial bones grew much more quickly than their male counterparts suggesting systemic environmental factors affect proliferation and cell number peri-implant. However, the male osteoblasts produced higher levels of osteocalcin, BMP2, and osteoprotegerin. This suggests that female osteoblasts in culture may be responding to decreases in soluble estrogen available *in vitro* (compared to circulating estrogen *in vivo*) and may be less mature when removed from a robustly positive bone-forming environment. Previous studies evaluating the response of anti-resorption proteins show that when cells are removed from a compromised bone environment, they produce higher levels of osteogenic signaling proteins *in vitro* as a potential feedback mechanism to compensate for diminished bone quality [22–24]. These results suggest that cells maintain a memory of the condition of bone quality from which they were harvested. In this case, we might see higher degrees of osteogenic signaling by male cells because the systemic environment in our male animals was not as osteogenic as in female rabbits.

In males the decreased growth rate seen in our osteoblast population suggests that the induction of more osteogenic factors by the plasma treatment may be more potent because the cells are more sensitive to pro-osteogenic surface properties because they are not receiving systemic high levels of endocrine signaling proteins to induce osteogenesis and therefore produce higher quantities of osteogenic signaling proteins to compensate. These differences in growth rates *in vitro* and bone formation *in vivo* suggest other signaling pathways are involved, and it is likely systemic.

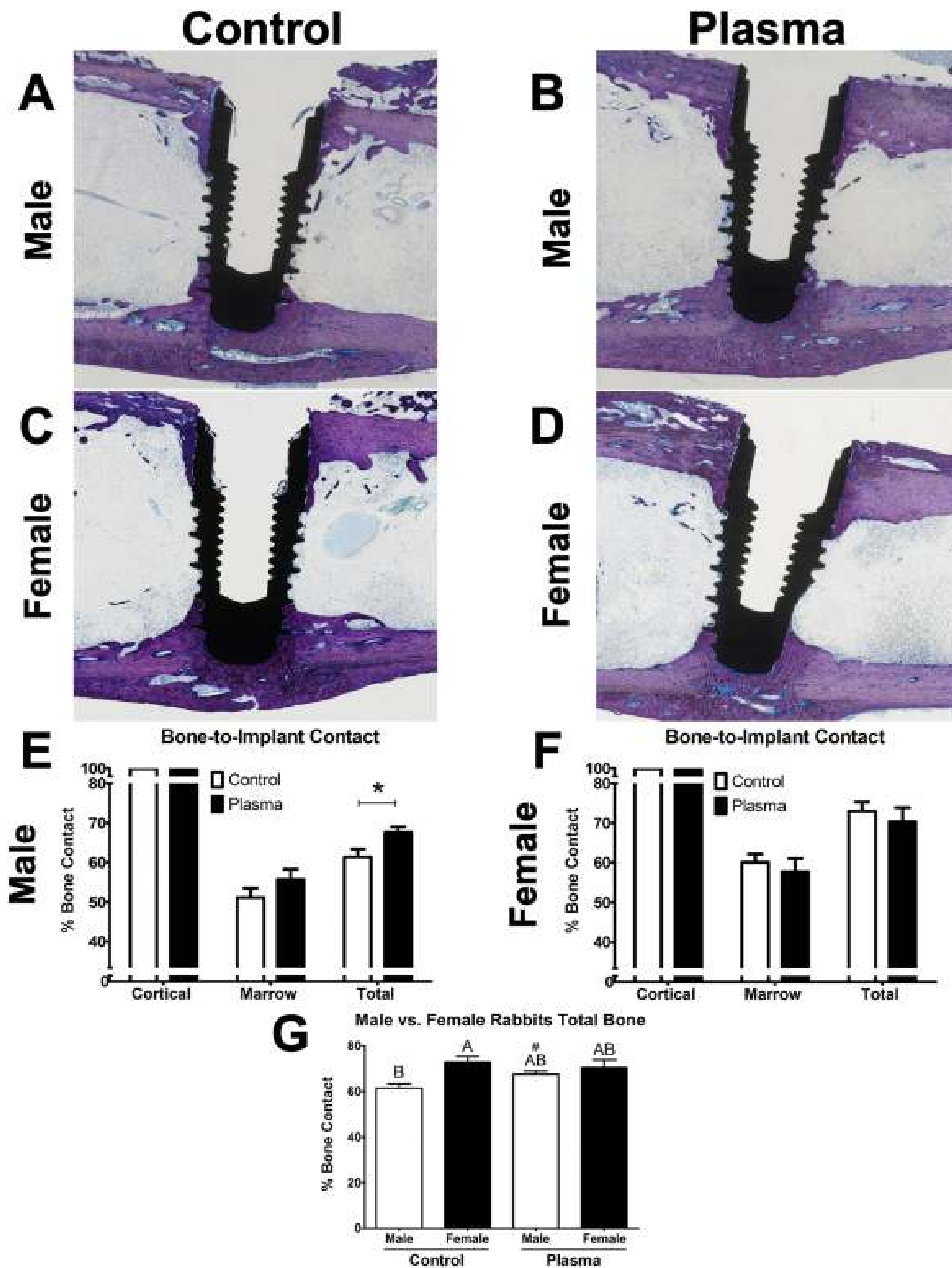

Fig. 4 – Representative calcified histology images of implanted screw implants after 6 weeks of implant placement. Representative images of male rabbits for both control (A) and DBD plasma treated implants (B). Representative images of female rabbits for control (C) and DBD plasma treated implants (D). Quantification of bone-to-implant contact in cortical and marrow area, and total BIC in males (E) and females (F). Comparison of all groups for total BIC (G). Groups not sharing letters are significant at p-values equal or less than < 0.05.

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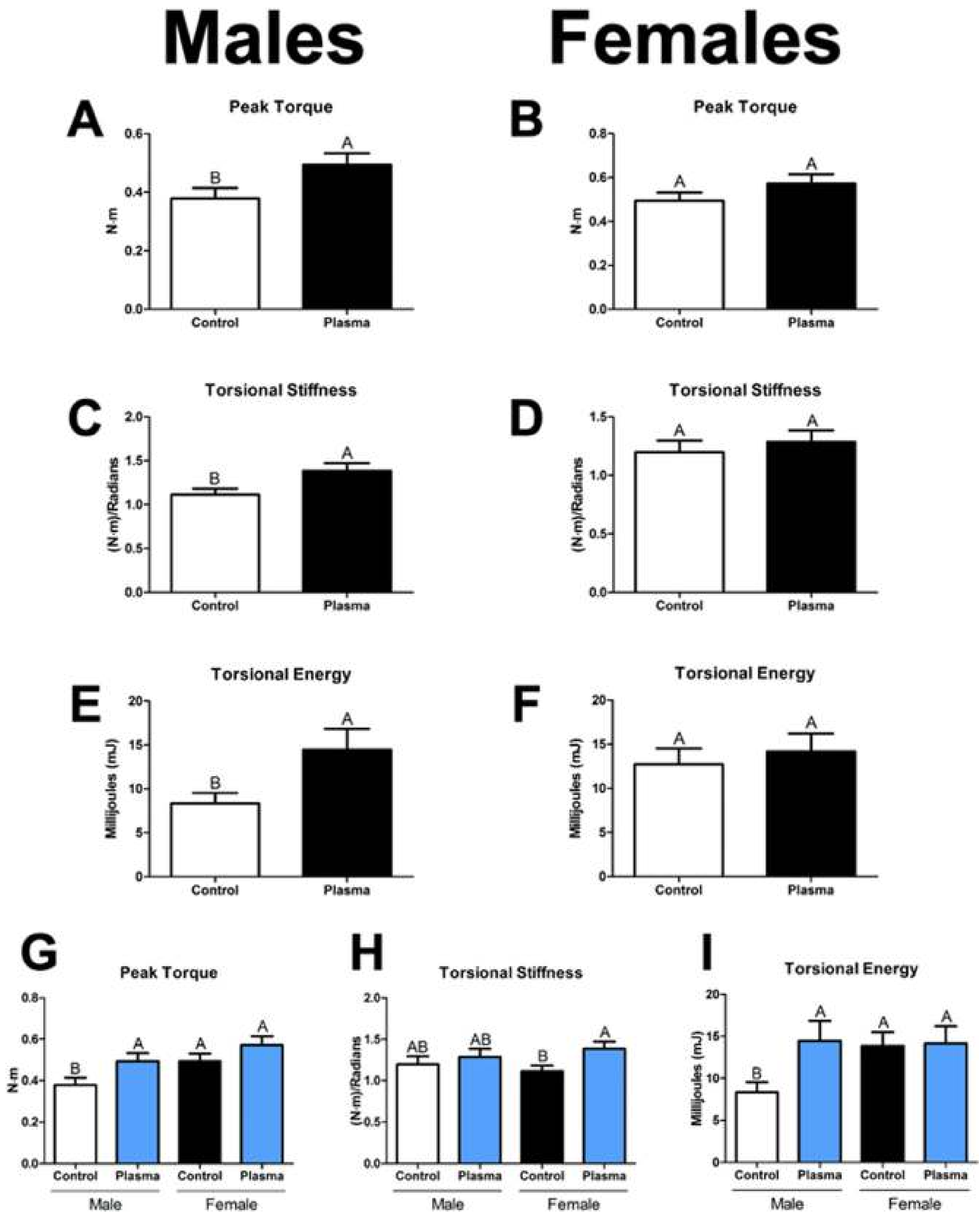

Fig. 5 – Torque to failure of a separate cohort of screw implants treated by DBD plasma treatment after 6 weeks of integration. Peak torque at the time of failure in males (A) and females (B). Torsional stiffness is the slope of the linear region and a measure of the bone quality in the immediately adjacent bone for both males (C) and females (D). Torsional energy absorbed by the bone before failure for males (E) and females (F). Comparison of all groups for peak torque (G), torsional stiffness (H), and torsional energy (I). Groups not sharing letters are significant at p-values equal or less than < 0.05.

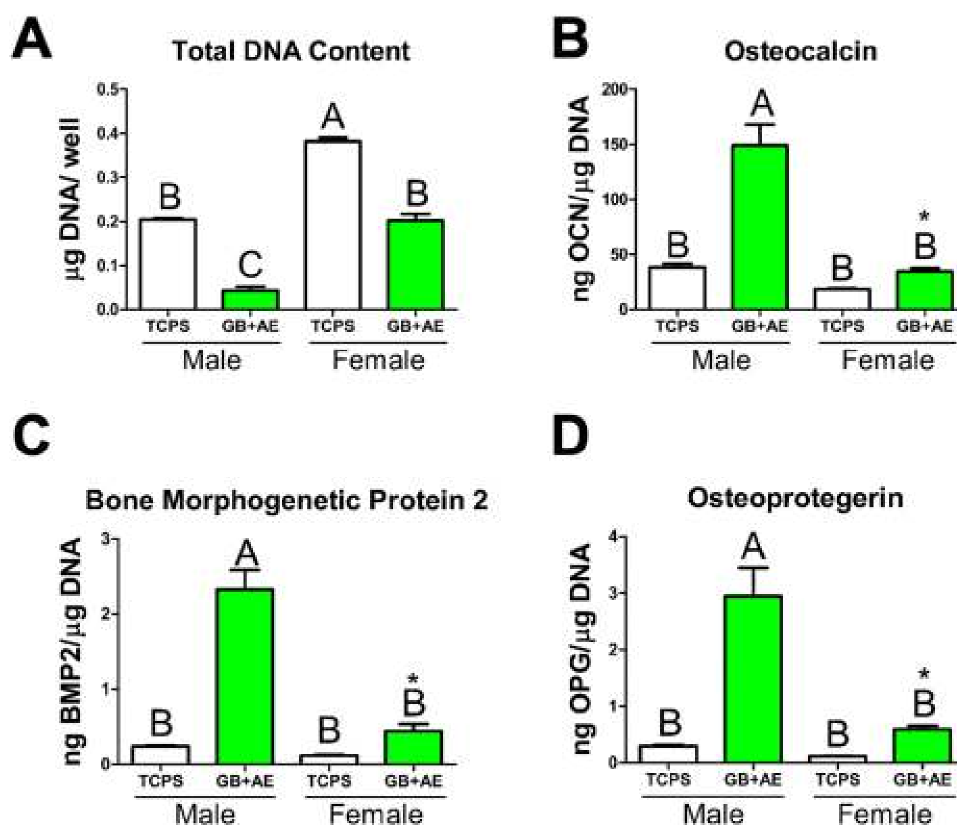

Fig. 6 – *In vitro* assessment of calvarial osteoblasts isolated from male and female rabbits. Total DNA content at 7 days of culture (A). Production of osteoblastic maturation marker osteocalcin (B), paracrine signaling factors bone morphogenetic protein (C), and osteoprotegerin (D) in response to surface microroughness for both male and female osteoblasts. Groups not sharing letters are significant at p-values equal or less than < 0.05. * denotes significance between GB+AE and TCPS within the same sex at p-values equal or less than < 0.05.

One possible mechanism of action for these differences is differential systemic signaling by estrogen. Male rabbits may be more sensitive to surface topography *in vitro* and plasma treatment *in vivo* due to lower responsiveness to systemic estrogen signaling. The literature has demonstrated that although both male and female osteoblasts possess receptors for estrogen and androgens, female cells are more responsive to circulating estrogen and concurrently, estrogen in the plasma is a much higher concentration than in males [25,26]. However, knockout studies have shown that males need estrogen receptors to form bone properly [27]. Additionally, any circulating estrogen that is internalized by male osteoblasts may be converted by aromatase leading to these cells being insensitive to estrogen [7]. These differences in circulating hormones may contribute to the overall differences in osteogenic behavior. Females possess a dual osteogenic effect as bone formation in the periosteum is stimulated by estrogen signaling and endocortical osteoclast mediated bone resorption is inhibited by estrogen signaling [15]. This could explain why bone formation is greater in the female rabbits regardless of implant treatment.

These data suggest that the wettability induced by DBD treatment increases the osteogenic potential of cells locally. However, osseointegration is regulated by a series of complex signaling cascades that are differentially regulated in both males and females. These differences in systemic bone formation can overshadow the effect of local plasma treatment in healthy bone. However, in compromised bone qualities these local effects may contribute more to the overall implant success. Therefore, this plasma effect should be evaluated in a compromised model of osseointegration.

5. Conclusions

These results show that DBD treatment in a sealed vacuum is an effective method to increase the osteogenic potential of an implanted dental implant. DBD plasma treatment improves osseointegration and this effect may be dependent on bone quality and systemic signaling cascades governing bone formation. Importantly, we show that this treatment does not impede implant osseointegration in healthy populations, while providing surface properties that have been shown to enhance bone formation in compromised bone qualities. Interestingly, we also show that males and females respond differently. Collectively, this benchtop plasma treatment is able to increase wettability on demand at the time of implant placement and creates another cost-effective method for clinicians to improve osseointegration by enhancing wettability. The device is also reusable and, therefore, could

ultimately provide a more cost-effective alternative to other more expensive surface treatments. Furthermore, many factory surface treatments have an expiration date and biological capability can degrade with time [15], thus DBD used at the time of implant placement could extend shelf life of implants.

CRediT authorship contribution statement

Michael B. Berger, D. Joshua Cohen: Contributed equally as first authors. **Michael B. Berger, D. Joshua Cohen, Barbara D. Boyan, Zvi Schwartz:** Designed experimental analyses. **Michael B. Berger, Michael M. Levit**: Conducted assays, assisted on the surgery, Analyzed data, Contributed to the interpretation. **Jennifer L. Puetzer**: Provided expertize on the mechanical testing and aided in the testing. **Michael B. Berger, Michael M. Levit**: Wrote the first manuscript draft. **D. Joshua Cohen**: Performed the surgery, Analyzed data. All authors contributed to the interpretation of the results and final manuscript.

Conflict of Interest

B.D.B. is an unpaid consultant for Institut Straumann AG (Basel, Switzerland) and a paid consultant for Medtronic Spine (Memphis, TN) and Spineology Inc. (St. Paul, MN). Z.S. is an unpaid consultant for AB Dental (Ashdod, Israel) and an unpaid consultant for Institut Straumann AG (Basel, Switzerland).

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.dental.2022.02.002.

REFERENCES

[1] Beschnidt SM, Cacaci C, Dedeoglu K, Hildebrand D, Hulla H, Iglhaut G, Krennmair G, Schlee M, Sipos P, Stricker A, Ackermann KL. Implant success and survival rates in daily dental practice: 5-year results of a non-interventional study using CAMLOG SCREW-LINE implants with or without platform-switching abutments. Int J Implant Dent 2018;4:33. https://doi.org/10.1186/s40729-018-0145-3
[2] Donos N, Calciolari E. Dental implants in patients affected by systemic diseases. Br Dent J 2014;217:425–30. https://doi.org/10.1038/sj.bdj.2014.911
[3] Terheyden H, Lang NP, Bierbaum S, Stadlinger B. Osseointegration - communication of cells. Clin Oral Implants Res 2012;23:1127–35. https://doi.org/10.1111/j.1600-0501.2011.02327.x
[4] Le Guéhennec L, Soueidan A, Layrolle P, Amouriq Y. Surface treatments of titanium dental implants for rapid osseointegration. Dent Mater 2007;23:844–54. https://doi.org/10.1016/j.dental.2006.06.025
[5] Boyan BD, Lotz EM, Schwartz Z. (*) Roughness and hydrophilicity as osteogenic biomimetic surface properties. Tissue Eng - Part A 2017;23:1479–89. https://doi.org/10.1089/ten.TEA.2017.0048
[6] Javed F, Ahmed HB, Crespi R, Romanos GE. Role of primary stability for successful osseointegration of dental implants: Factors of influence and evaluation. Inter Med Appl Sci 2013;5:162–7. https://doi.org/10.1556/IMAS.5.2013.4.3
[7] Berger MB, Cohen DJ, Olivares-navarrete R, Williams JK, Cochran DL, Boyan BD, Schwartz Z. Human osteoblasts exhibit sexual dimorphism in their response to estrogen on microstructured titanium surfaces. Biol Sex Differ 2018;9:1–10. https://doi.org/10.1186/s13293-018-0190-x
[8] Olivares-Navarrete R, Hyzy SL, Chaudhri RA, Zhao G, Boyan BD, Schwartz Z. Sex dependent regulation of osteoblast response to implant surface properties by systemic hormones. Biol Sex Differ 2010;1:4. https://doi.org/10.1186/2042-6410-1-4
[9] Olivares-Navarrete R, Hyzy SL, Boyan BD, Schwartz Z. Regulation of osteoblast differentiation by acid-etched and/or grit-blasted titanium substrate topography is enhanced by $1,25(OH)_2D_3$ in a sex-dependent manner. Biomed Res Int 2015;2015:365014https://doi.org/10.1155/2015/365014
[10] Zhao G, Zinger O, Schwartz Z, Wieland M, Landolt D, Boyan BD. Osteoblast-like cells are sensitive to submicron-scale surface structure. Clin Oral Implants Res 2006;17:258–64. https://doi.org/10.1111/j.1600-0501.2005.01195.x
[11] Kopf BS, Ruch S, Berner S, Spencer ND, Maniura-Weber K. The role of nanostructures and hydrophilicity in osseointegration: In-vitro protein-adsorption and blood-interaction studies. J Biomed Mater Res - Part A 2015;103:2661–72. https://doi.org/10.1002/jbm.a.35401
[12] Gupta TT, Matson JS, Ayan H. Antimicrobial effectiveness of regular dielectric-barrier discharge (DBD) and jet DBD on the viability of *Pseudomonas aeruginosa*. IEEE Trans Radiat Plasma. Med Sci 2018;2:68–76. https://doi.org/10.1109/TPS.2017.2748982
[13] Berger MB, Bosh KB, Cohen DJ, Boyan BD, Schwartz Z. Benchtop plasma treatment of titanium surfaces enhances cell response. Dent Mater 2021;37:690–700. https://doi.org/10.1016/j.dental.2021.01.026
[14] Cohen DJ, Scott KM, Kulkarni AN, Wayne JS, Boyan BD, Schwartz Z. Acellular mineralized allogenic block bone graft does not remodel during the 10 weeks following concurrent implant placement in a rabbit femoral model. Clin Oral Implants Res 2020;31:37–48. https://doi.org/10.1111/clr.13544
[15] Minamikawa H, Att W, Ikeda T, Hirota M, Ogawa T. Long-term progressive degradation of the biological capability of titanium. Mater 9. Switzerland: Basel,; 2016. p. 102. https://doi.org/10.3390/ma9020102
[16] Canullo L, Genova T, Wang H-L, Carossa S, Mussano F. Plasma of argon increases cell attachment and bacterial decontamination on different implant surfaces. Int J Oral Maxillofac Implants 2017;32:1315–23. https://doi.org/10.11607/jomi.5777
[17] Duske K, Koban I, Kindel E, Schröder K, Nebe B, Holtfreter B, et al. Atmospheric plasma enhances wettability and cell spreading on dental implant metals. J Clin Periodo 2012;39:400–7. https://doi.org/10.1111/j.1600-051X.2012.01853.x
[18] Yamamoto H, Shibata Y, Miyazaki T. Anode glow discharge plasma treatment of titanium plates facilitates adsorption of extracellular matrix proteins to the plates. J Dent Res 2005;84:668–71. https://doi.org/10.1177/154405910508400717

[19] Bays T.. Behavior of Small Mammals. In: Quesenberry K, Carpenter J, editors. Ferrets, Rabbit. Rodents Clin. Med. Surg. 3rd ed., St. Louis: Elsevier; 2012, p. 545–9.
[20] Saggese G, Baroncelli GI, Bertelloni S. Puberty and bone development. Best Pr Res Clin Endrocrinol Metab 2002;16:53–64. https://doi.org/10.1053/beem.2002.0180
[21] Olivares-Navarrete R, Raines AL, Hyzy SL, Park JH, Hutton DL, Cochran DL, et al. Osteoblast maturation and new bone formation in response to titanium implant surface features are reduced with age. J Bone Min Res 2012;27:1773–83. https://doi.org/10.1002/jbmr.1628
[22] Lotz EM, Cohen DJ, Ellis RA, Wayne JS, Schwartz Z, Boyan BD. Ibandronate treatment before and after implant insertion impairs osseointegration in aged rats with ovariectomy induced osteoporosis. JBMR 2019;3:e10184https://doi.org/10.1002/jbm4.10184
[23] Lotz EM, Lohmann CH, Boyan BD, Schwartz Z. Bisphosphonates inhibit surface-mediated osteogenesis. J Biomed Mater Res A 2020;108:1774–86. https://doi.org/10.1002/jbm.a.36944
[24] Lotz EM, Cohen DJ, Schwartz Z, Boyan BD. Titanium implant surface properties enhance osseointegration in ovariectomy induced osteoporotic rats without pharmacologic intervention. Clin Oral Implants Res 2020;31:374–87. https://doi.org/10.1111/clr.13575
[25] Nastzky E, Schwartz Z, Soskoline W, Brooks B, Dean D, Boyan B, et al. Evidence for receptors specific for 17 beta-estradiol and testosterone in chrondrocyte cultures. Connect Tissue Res 1994;30. 277–94.
[26] Bord S, Ireland DC, Beavan SR, Compston JE. The effects of estrogen on osteoprotegerin, RANKL, and estrogen receptor expression in human osteoblasts. Bone 2003;32:136–41. https://doi.org/10.1016/S8756-3282(02)00953-5
[27] Frank G. The role of estrogen in pubertal skeletal physiology: epiphyseal maturation and mineralization of the skeleton. Acta Paediatr 1995;84. 627–30.