Plasma Medicine, 6(2): 193–207 (2016)

Osteogenic Induction of Human Mesenchymal Stem Cells by Cold Atmospheric Argon Plasma

Adil Shahzad Ahmed,[a,b,*] Joseph Chavarria,[b] Tyler Brenneman,[b] Kurt Johnson,[b] Erik Antonsen,[c] & Scott Rosenfeld[b]

[a]Department of Orthopaedic Surgery, University of South Florida Morsani College of Medicine, Tampa, FL; [b]Department of Orthopaedic Surgery, Baylor College of Medicine, Houston, TX; [c]Department of Medicine, Section of Space Medicine and Emergency Medicine, Baylor College of Medicine, Houston, TX

*Address all correspondence to: Adil Shahzad Ahmed, Morsani College of Medicine, University of South Florida, 808 North Franklin Street, Apartment 2507, Tampa, FL 33602; Tel.: (972) 742-0561; Fax: (888) 980-6123, E-mail: adilahmed0000@gmail.com

ABSTRACT: Cold plasmas induce angiogenesis, enhance coagulation and wound healing, and selectively ablate microbes without harming eukaryotes. Work on bone tissue has been minimal; initial studies show enhanced osseointegration, increased gene transcription, and mesenchymal stem cell growth. Mesenchymal stem cell osteoblastic differentiation is required for bone formation and healing. The present study employs a novel device to assess whether cold argon plasma induces osteogenic differentiation of human mesenchymal stem cells. Human mesenchymal stem cells were exposed to five conditions: growth, osteogenic stimulation, nonionized argon gas, argon plasma, and argon plasma with osteogenic stimulation. Known osteoblastic differentiation markers (alkaline phosphatase, osteocalcin, RANKL) were assessed on days 1, 10, and 28. Cellular DNA production was measured for normalization. Novel dielectric barrier discharge plasma device parameters were as follows: energy 5J, flow rate 30 psig/min, distance 22 mm, and duration 30 sec. Alkaline phosphatase level was decreased compared to other treatments, with varying significance. Nonionized argon gas significantly increased alkaline phosphatase ($p < 0.0014$) compared to other groups. Osteogenic stimulation did not result in difference from growth. Changes in osteocalcin or receptor activator of nuclear factor kappa-B ligand (RANKL) were not observed. A definitive claim regarding the ability of cold argon plasma to induce osteoblastic differentiation cannot be made. Lack of β-glycerophosphate addition on day 14 prevented osteogenic media from responding as expected. Interestingly, nonionized argon gas significantly increased alkaline phosphatase production. This novel finding, possibly due to argon shielding or shear force production, merits further study.

KEY WORDS: cold plasma, nonthermal plasma, osteogenic differentiation

I. INTRODUCTION

Plasma medicine is an emerging field bridging techniques and knowledge from the disciplines of plasma physics, engineering, and biology to exert effects on biological tissue. "Plasma" refers to the application of plasma as the fourth state of matter—a cloud of electrified gas composed of electrons, ions, radicals, UV photons, and transient electrical fields.[1,2] There are many different types of plasmas and different methods to create

1947-5764/16/$35.00

them, but the most appropriate for living tissue use are called nonthermal atmospheric pressure plasma (NTAPP) or cold atmospheric plasma (CAP).[1] They are so named because they function at much lower temperatures and pressures than other more familiar forms of plasma, such as stars.

The effects of CAP on living tissue are far-reaching, affecting a myriad of different cell populations and biological processes. CAP exposure has demonstrated the following broad effects: (1) inducing angiogenesis by increasing endothelial cell migration and proliferation[1,3,4]; (2) altering local nitric oxide concentration and cytokine milieu[1,5]; (3) increasing fibroblast migration and collagen deposition[1,5]; (4) modifying cell membrane properties and polarization[6]; (5) killing microbes without damaging eukaryotic cells[1,7–18]; (6) penetrating microbial biofilms[19]; (7) creating reactive oxygen and nitrogen species that penetrate deep into tissues[1,20]; (8) inducing apoptosis in malignant cells[9]; (9) accelerating blood coagulation by effects on coagulation proteins, platelet aggregation, and fibrin filament formation without altering pH, temperature, or calcium levels[1,9,21]; (10) accelerating wound healing[1,22,23]; (11) inducing changes in cell surface receptors[24,25]; and (12) displaying various effects on lymphocyte subpopulations.[26]

To date, minimal work has been done investigating the effects of CAP on bone tissue. Previous studies in animal dental and tibial implants,[27–31] biomaterial modification,[32–37] and cell culture[38–40] show initial promise. These experiments demonstrated enhanced implant osseointegration[27–31,33,34,36,37,41] (measured by surface hydrophilicity, removal torque, surface energy, biomechanical fixation, roughness, and wettability), and increased osteogenic gene transcripts and mesenchymal stem cell growth.[2,36,38–40] One possible explanation for these phenomena is that CAP induces mesenchymal stem cell (MSC) differentiation into osteoblasts, resulting in the previously mentioned enhanced osseointegration and increased gene transcription. No current studies in the literature have specifically assessed the potential for nonthermal plasma to convert MSCs into osteoblasts.

The potential clinical implications for the field of orthopedic surgery are significant. It is known that increased MSC osteoblastic differentiation results in accelerated and enhanced fracture healing.[42,43] Therapies such as bone morphogenetic protein[44] are employed specifically for this purpose, albeit with known adverse effects, such as vertebral osteolysis and postoperative radiculitis, and great expense.[45] Nonthermal plasma has been shown to be safe when applied to eukaryotic cells.[1,7–17] If osteoblastic differentiation of MSCs can be demonstrated, it holds future promise as an orthopedic surgical adjunct in situations such as the treatment of nonunion fractures and spinal fusion. The present study employs a novel dielectric barrier discharge device for nonthermal atmospheric pressure plasma creation, aiming to ascertain if cold atmospheric argon plasma induces human MSCs to differentiate into the osteoblastic lineage.

II. MATERIALS & METHODS

A. Sample Selection

The human MSCs were acquired from three human donors from the laboratory of James

Dennis, Ph.D., at Baylor College of Medicine in Houston, Texas. The cells were initially collected from the femoral necks from each of the donors.

B. Experimental Design

The study employed five treatment groups (detailed later) of human mesenchymal stem cells (hMSCs) from three different human donor cell lines. The five experimental groups were as follows:

- Group 1: negative control (growth media only: Dulbecco's modified Eagle's medium with low glucose (DMEM-LG); fetal bovine serum (FBS); antibiotic/antimycotic
- Group 2: positive control (growth media + osteogenic media: dexamethasone, ascorbic acid, β-glycerophosphate)
- Group 3: argon gas control (growth media + nonionized argon gas flow)
- Group 4: argon plasma (growth media + ionized argon plasma)
- Group 5: combination (growth media + ionized argon plasma + osteogenic media)

Day 0 marked the day of exposure to the five different treatment conditions, with a single exposure to the respective treatments for each group. The hMSCs in all five groups were assessed with measurement of alkaline phosphatase, receptor activator of nuclear factor kappa-B ligand (RANKL), osteocalcin, and DNA assays at three time points (day 1, day 10, and day 28). Each of the three cell lines for each of the five groups was assessed in triplicate for all four assays at all three time points. All assays were assessed at each time point except for RANKL and osteocalcin, which were not assessed at time point 1 (day 1) because of resource limitations. The specific exposure parameters for argon gas (group 3) and argon plasma (groups 4 and 5) are detailed next.

C. Nonthermal Argon Plasma Device and Exposure Parameters

This experiment employed a novel device (Figs. 1 and 2) for nonthermal atmospheric pressure plasma creation, designed and manufactured by Starfire Industries, LLC (Champaign, IL). Several unique characteristics allow enhanced nonthermal plasma delivery. This device is a hybrid combination of dielectric barrier discharge (DBD) and jet-type emission, allowing downstream metastable argon gas flow to carry nonthermal plasma several centimeters from the device tip. This permits conformal nonthermal plasma coverage over surfaces, penetration into wounds or cavities, and placement of sheaths on surfaces to increase ion energy at atmospheric pressures. Frequency can also be titrated from low-frequency AC/pulsed (kHz) to high-frequency (MHz/GHz). The point-like emitter has a high electric field that breaks down easily with low power lev-

FIG. 1: CAP device setup.

FIG. 2: Argon plasma beam.

els. The dielectric surface itself can be either alumina or glass, and the dielectric barrier limits transferrable charge, minimizing streamer transition and direct-metal arcs. These features allow simultaneous creation of both direct and indirect nonthermal plasma, and additionally allow a DBD functional distance of 22 mm, a significant increase in working distance compared to other DBD devices.

The argon plasma parameters for the present study were as follows: energy level of 5 J, argon gas flow rate of 30 psig/min, exposure for 30 sec, and exposure distance of 22 mm, measured from the tip of the device orifice to the fluid layer surface in the 6-well (35 mm) plates. Parameters were selected based on a literature review of operating parameters for nonthermal atmospheric pressure plasma devices. Argon gas for the experiment was purchased in a T type cylinder (330 cu ft) from Matheson Tri-Gas (Houston, TX).

D. *In vitro* Osteogenic Induction of Human Mesenchymal Stem Cells

Human mesenchymal stem cell differentiation was accomplished through exposure to dexamethasone and ascorbic acid. The cells were initially thawed at room temperature. The osteogenic-supplemented (OS) culture media consisted of FBS containing DMEM-LG, with final concentrations of 10^{-7} M dexamethasone and 120 nM ascorbic acid 2-phosphate. Cells were counted and seeded at 5×10^3 per cm^2 in serum containing medium on 6-well (35 mm) plates and grown to approximately 70%–80% con-

fluence; 3 mL of culture media was exchanged every 3 days throughout the 28-day experimental process.

E. Alkaline Phosphatase Assay

The cells were assessed using a quantitative alkaline phosphatase activity assay. Cellular production of alkaline phosphatase typically reaches peak levels between 9 and 12 days after plating. For the quantitative biochemical assay, alkaline phosphatase cleaves a phosphate ion from *p*-nitrophenyl phosphate. The product, *p*-nitrophenol, is then added to a solution containing sodium hydroxide and the absorbance of the resulting solution is read at 405 nm with a Tecan microplate reader. The measured results were then compared to a standard curve that was also generated.

F. Osteocalcin and RANKL Enzyme-Linked Immunosorbent Assay (ELISA)

The human osteocalcin and RANKL levels were measured in cell culture supernatants using enzyme-linked immunosorbent assay (ELISA) kits, purchased from R&D Systems, Inc. (Minneapolis, Minnesota). The measurements were completed in triplicate, and the optical density was determined at 540 nm using a Tecan plate reader. Sensitivity of the osteocalcin assay was 0.402 ng/mL. The intra-assay and inter-assay precision coefficients of variation were 2.3% and 6.2%, respectively. Data were assessed on a log/log graph by plotting the mean absorbance for each standard against the concentration. Concentration of RANKL in the plates was determined by plotting the mean absorbance for each standard against the concentration.

G. DNA Assay

The Hoechst dye method was used to assess for cellular DNA production, permitting normalization of data per cell number. The samples from the 6-well (35 mm) plates were collected and 50 µl was placed in wells on 96-well black plates; 100 µl of Hoechst dye was added to each well, and the plates were read at an excitation of 365 nm and emission at 460 nm under a low intensity setting (1,100 V, gain of 1, lamp brightness of 1) on a fusion plate reader. The data were assessed using a standard curve generated through the same protocol with a calf thymus DNA standard.

H. Statistical Analysis

Statistical analysis was performed with the assistance of Wei Zhang, Ph.D. at the Outcomes & Impact Service at Texas Children's Hospital in Houston, Texas. A generalized linear model was applied to assess group and donor effects. Analysis was performed for overall group and donor effects, pairwise group comparisons, and pairwise group comparisons with each of the three human donors.

III. RESULTS

Alkaline phosphatase, osteocalcin, and RANKL were assessed for all five experimental groups at each of the three time points (days 1, 10, and 28). Cellular production of DNA was assessed to allow normalization of these three metrics per cell number. For normalization, two wells per donor per group per time point were considered for alkaline phosphatase, and one well per donor per group per time point was considered for osteocalcin and RANKL.

Figures 3, 4, and 5 demonstrate normalized measurements of alkaline phosphatase, osteocalcin, and RANKL.

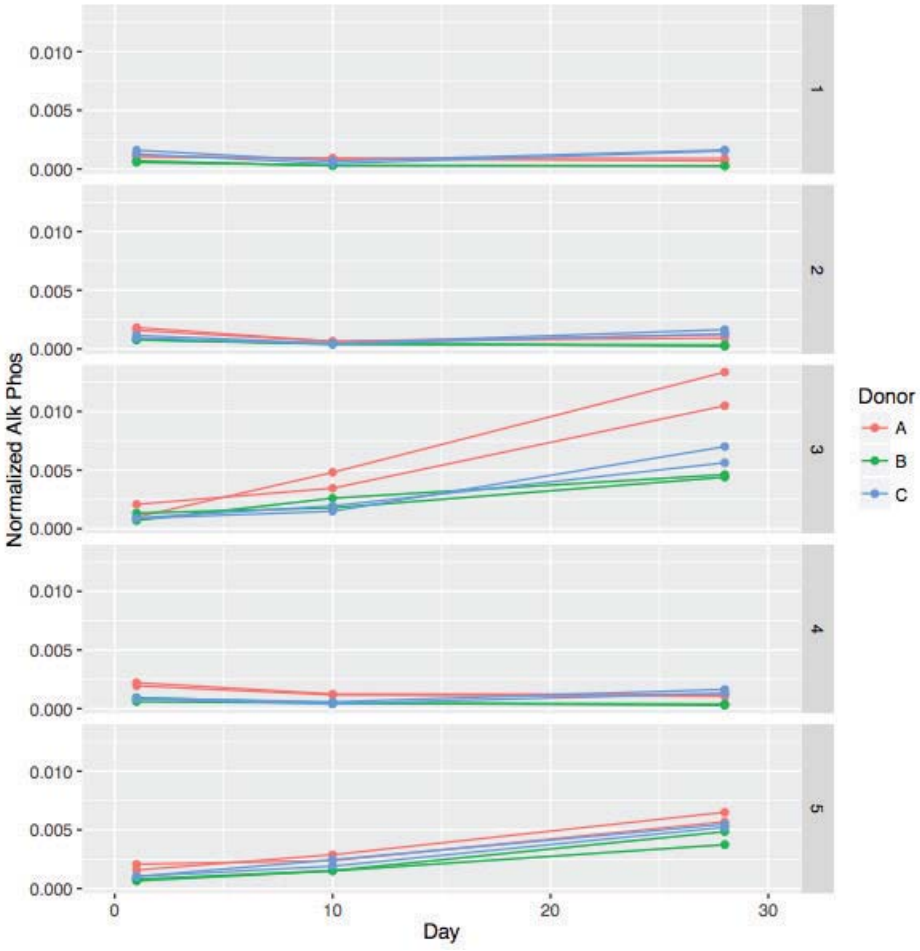

FIG. 3: Normalized alkaline phosphatase levels for groups 1–5 at days 1, 10, and 28. The *x*-axis represents duration of the experiment, from day 0 to day 28. The *y*-axis represents nmol/mL/min of alkaline phosphatase per ng/mL of DNA

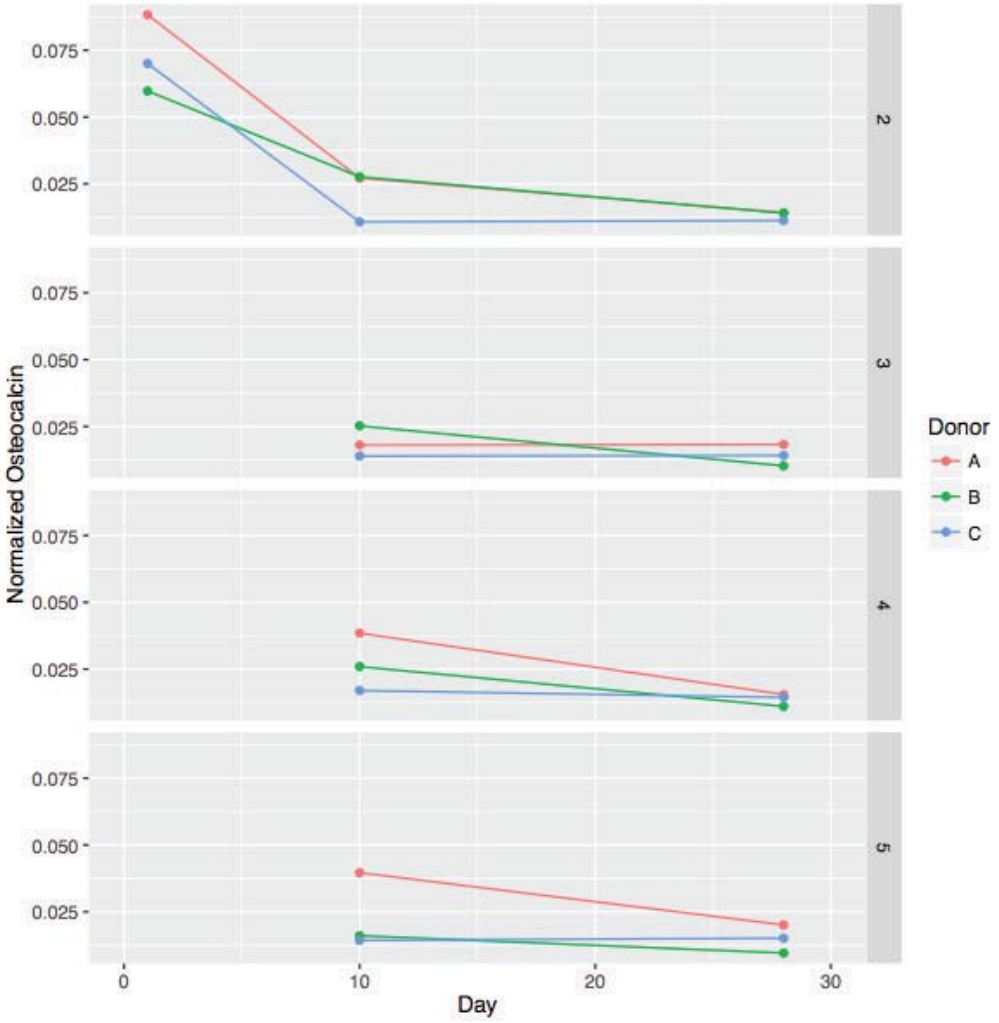

FIG. 4: Normalized osteocalcin levels for group 2 at days 1, 10, and 28, and for groups 3–5 at days 10 and 28. The *x*-axis represents duration of the experiment, from day 0 to day 28. The *y*-axis represents ng/mL of osteocalcin per ng/mL of DNA

Of note, group 1 (growth media only) was not assessed for osteocalcin and RANKL. Time point 1 (day 1) for groups 3–5 was also not assessed for osteocalcin and RANKL.

The results for pairwise comparison for group and donor are described in Tables 1 to 3. Pairwise comparisons of osteocalcin and RANKL with respect to each donor were not feasible because of insufficient data. In the tables, "Baseline" refers to initial estimated value (day 1) and "Growth Rate" refers to the speed of growth, taking into account all three time points to estimate the slope of the line in the plots.

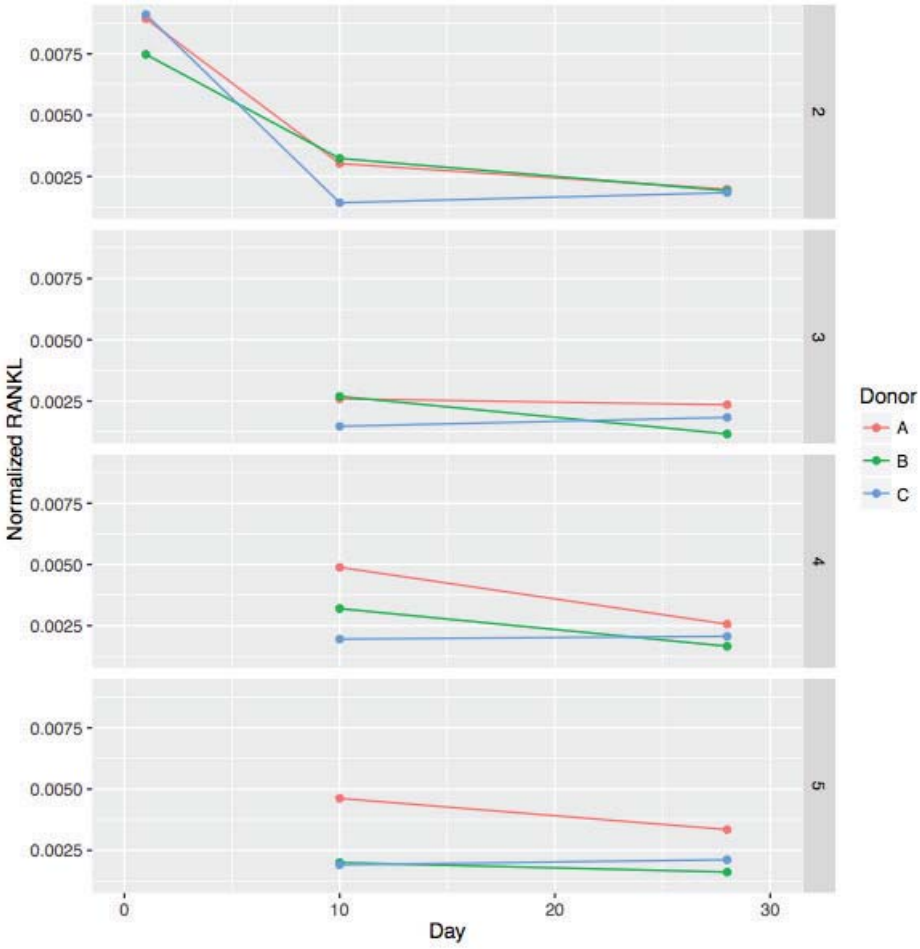

FIG. 5: Normalized RANKL levels for group 2 at days 1, 10, and 28, and for groups 3–5 at days 10 and 28. The *x*-axis represents duration of the experiment, from day 0 to day 28. The *y*-axis represents pg/mL of RANKL per ng/mL of DNA.

TABLE 1: *P*-values of overall group and donor effects

	Alkaline Phosphatase	RANKL	Osteocalcin
Baseline			
Group Effect	<0.0001	0.3660	0.7220
Donor Effect	0.0002	0.0294	0.0098
Growth Rate			
Group Effect	<0.0001	0.3777	0.5028
Donor Effect	0.0425	0.0153	0.0330

Effects of p-value < 0.05 were considered significant.

TABLE 2: Pairwise group effect comparison

	Alkaline Phosphatase		RANKL		Osteocalcin	
	Effect	**P-Value**	**Effect**	**P-Value**	**Effect**	**P-Value**
Baseline						
2 vs. 1	0.000047	0.9376	0.003999	0.0579	0.03336	0.0524
3 vs. 1	–0.00023	0.6990	–0.00060	0.8033	–0.00622	0.7477
4 vs. 1	0.000178	0.7660	0.000923	0.7034	0.006643	0.7313
5 vs. 1	–0.00003	0.9614	NA	NA	NA	NA
3 vs. 2	–0.00028	0.6423	–0.00460	0.0344	–0.03958	0.0271
4 vs. 2	0.000131	0.8264	–0.00308	0.1273	–0.02672	0.1058
5 vs. 2	–0.00008	0.8992	–0.00400	0.0579	–0.03336	0.0524
4 vs. 3	0.000410	0.4954	0.001525	0.5326	0.01286	0.5105
5 vs. 3	0.000203	0.7350	0.000602	0.8033	0.006221	0.7477
5 vs. 4	–0.00021	0.7295	–0.00092	0.7034	–0.00664	0.7313
Growth Rate						
2 vs. 1	0	0.9657	–0.00019	0.0870	–0.00149	0.0819
3 vs. 1	0.000246	<0.0001	7.963E-7	0.9947	0.000198	0.8339
4 vs. 1	0	0.9164	–0.00004	0.7269	–0.00028	0.7651
5 vs. 1	0.000157	<0.0001	NA	NA	NA	NA
3 vs. 2	0.000247	<0.0001	0.000188	0.0858	0.001689	0.0529
4 vs. 2	–1.64E-6	0.9505	0.000145	0.1736	0.001209	0.1489
5 vs. 2	0.000158	<0.0001	0.000188	0.0870	0.001491	0.0819
4 vs. 3	–0.00025	<0.0001	–0.00004	0.7220	–0.00048	0.6127
5 vs. 3	–0.00009	0.0013	–7.96E-7	0.9947	–0.00020	0.8339
5 vs. 4	0.000159	<0.0001	0.000042	0.7269	0.000282	0.7651

G1: growth media; G2: osteogenic media + growth media; G3: nonionized argon gas + growth media; G4: argon plasma + growth media; G5: argon plasma + osteogenic media + growth media

As demonstrated in Tables 1–3, no significant differences in alkaline phosphatase production were observed at baseline. During the growth period, nonionized argon gas (group 3) exhibited significant increases in hMSC alkaline phosphatase production compared to group 1 (0.000246, $p < 0.0001$), group 2 (0.000247, $p < 0.0001$), group 4 (0.00025, $p < 0.0001$), and group 5 (0.00009, $p = 0.0013$). Argon plasma (group 4) decreased alkaline phosphatase levels compared to all other groups, with varying levels of significance. The osteogenic group (group 2) did not demonstrate significant changes compared to the control group (group 1: growth media only). There were no significant changes in any of the experimental groups in terms of levels of osteocalcin and RANKL at the three time points.

TABLE 3: Pairwise group effect comparison of alkaline phosphatase with respect to donor

Alkaline Phosphatase	Donor A		Donor B		Donor C	
	Effect	***P*-Value**	**Effect**	***P*-Value**	**Effect**	***P*-Value**
Baseline						
2 vs. 1	0.000302	0.6271	0.000173	0.5094	–0.00033	0.4508
3 vs. 1	–0.00027	0.6594	0.000343	0.2180	–0.00077	0.1200
4 vs. 1	0.000824	0.2170	0.000168	0.5212	–0.00046	0.3142
5 vs. 1	0.000347	0.5782	–0.00014	0.5922	–0.00029	0.5033
3 vs. 2	–0.00058	0.3697	0.000170	0.5161	–0.00043	0.3396
4 vs. 2	0.000522	0.4119	–5.02E-6	0.9843	–0.00012	0.7755
5 vs. 2	0.000045	0.9416	–0.00031	0.2561	0.000039	0.9268
4 vs. 3	0.001097	0.1189	–0.00018	0.5043	0.000308	0.4847
5 vs. 3	0.000620	0.3367	–0.00048	0.1045	0.000471	0.3015
5 vs. 4	–0.00048	0.4507	–0.00031	0.2629	0.000162	0.7073
Growth Rate						
2 vs. 1	–6.81E-6	0.8520	–5.43E-6	0.7243	8.833E-6	0.7619
3 vs. 1	0.000401	<0.0001	0.000141	<0.0001	0.000196	<0.0001
4 vs. 1	–0.00002	0.5719	–2.78E-6	0.8566	0.000015	0.6034
5 vs. 1	0.000173	0.0002	0.000147	<0.0001	0.000150	0.0001
3 vs. 2	0.000408	<0.0001	0.000146	<0.0001	0.000187	<0.0001
4 vs. 2	–0.00001	0.7033	2.654E-6	0.8630	6.355E-6	0.8273
5 vs. 2	0.000179	0.0002	0.000153	<0.0001	0.000141	0.0002
4 vs. 3	–0.00042	<0.0001	–0.00014	<0.0001	–0.00018	<0.0001
5 vs. 3	–0.00023	<0.0001	6.541E-6	0.6714	–0.00005	0.1295
5 vs. 4	0.000193	<0.0001	0.000150	<0.0001	0.000135	0.0003

G1: growth media; G2: osteogenic media + growth media; G3: nonionized argon gas + growth media; G4: argon plasma + growth media; G5: argon plasma + osteogenic media + growth media

IV. DISCUSSION

Nonthermal plasmas have shown remarkable effects in many biological applications, such as accelerated coagulation,[1,9,13] increased angiogenesis,[1,3,4] accelerated wound healing,[1,6,22,23] and selective ablation of microbes without harming eukaryotic cells.[1,7,8,11,13,15–17,46] Furthermore, experiments on bone tissue have demonstrated enhanced implant osseointegration[28–31,33,34,36,37,41,47] and increased production of osteogenic gene transcripts and mesenchymal stem cell growth.[2,36,38–40] These initial experiments are promising in the study of bone formation and clinical applications in orthopedic surgery, because MSC differentiation into osteoblasts is a necessary step in bone healing.[43,44] If

nonthermal plasma can indeed induce human mesenchymal stem cells to differentiate into the osteoblastic lineage, it has the potential to positively impact bone healing and biomechanical implant fixation, without the risks and financial costs associated with current therapeutics such as bone morphogenetic proteins.[45]

In the present study, a definitive claim cannot be made about the ability of cold atmospheric argon plasma to induce hMSC differentiation into osteoblasts. The osteogenic group (group 2) was meant to serve as a positive control for hMSC differentiation into the osteoblastic lineage. However, a lapse occurred in the addition of β-glycerophosphate (BGP) on day 14 of the experiment. Along with dexamethasone and ascorbic acid, BGP is necessary for *in vitro* osteogenic differentiation of MSCs.[48] BGP addition on day 14 allows for mineralization of preosteoblastic cells; without it, a necessary step in MSC osteogenic differentiation cannot occur. Lacking this known control, we are unable to definitively state whether the apparent lack of osteogenic induction was due to CAP's inherent inability to trigger such a response, or rather a consequence of nonreactive donor cells. Furthermore, this limited our study of the combination group (group 5), because the osteogenic media was incomplete. The fact that BGP must be added on day 14, two weeks after dexamethasone and ascorbic acid, increases the probability of error. It is imperative that future researchers enact stringent methodologies to prevent similar lapses.

Another point of consideration for future study is single versus successive nonthermal plasma exposure. Human MSCs in our study were exposed to a single treatment of CAP, for the purpose of modeling the device as a potential adjunct during orthopedic surgeries. In a surgical setting, the increased morbidity from repeatedly opening a surgical wound for successive nonthermal plasma treatments would negate possible benefits achieved through the adjunct. Other *in vitro* nonthermal plasma studies have employed multiple plasma treatments over the duration of experiments.[8,19,22] This repeat exposure to nonthermal plasma may play a factor as mesenchymal stem cells progress through the three main stages of osteoblastic differentiation.[49] Future studies employing multiple CAP treatments may exhibit yet unseen findings as MSCs progress through these differentiation stages to fully commit to the osteoblastic lineage.

We additionally report a novel finding that has not previously been documented in the literature. A single treatment of nonionized argon gas significantly increased human mesenchymal stem cell production of alkaline phosphatase. A prior study by Shuvalova et al.[50] demonstrated that mesenchymal stem cells exposed to a gas mixture containing high concentrations of argon gas (93%) led to enhanced MSC proliferation. However, increased alkaline phosphatase production by human MSCs exposed to argon gas has not been reported. This finding could possibly be explained by the creation of a relative hypoxic environment above the MSCs, in a process called argon shielding (also referred to as argon blanketing).[51,52] This refers to the use of inert gases, such as argon, in steel welding to shield a site of interest from oxygen and water vapor. In biological application, argon shielding will naturally create a local hypoxic climate due to its higher molecular weight and density compared to atmospheric air. Additionally, the production of shear

forces by the argon gas stream may also be a contributing factor. The anaerobic environment from argon shielding and the shear force from the gas stream may trigger a stress response in human MSCs, resulting in increased metabolic activity and enhanced production of cellular factors such as alkaline phosphatase. The fact that groups 4 and 5 did not exhibit increased hMSC alkaline phosphatase production may indicate that ionization of argon into plasma prevents argon shielding and/or shear force production. The creation of smaller molecules and charged species within the plasma[1,2] may somehow interfere with shielding and shearing effects. Future studies assessing MSC response to nonionized argon gas can shed light on the effects of differing flow rate, percentage of argon in the gas mixture, and MSC production of other molecules in addition to alkaline phosphatase.

In Figures 4 and 5, one will notice that several data points are missing. A final limitation of our study was insufficient funding to assess all wells in the experiment. Measurements of osteocalcin and RANKL had to be eliminated for group 1 (growth media only) and for time point 1 (day 1) for groups 3 to 5, because of the cost associated with ELISAs.

Ultimately, the present study is inconclusive in demonstrating whether cold atmospheric argon plasma causes osteogenic induction of human mesenchymal stem cells. The lapse in BGP addition invalidated the osteogenic positive control. Thus, we are unable to definitively state whether the lack of osteogenic induction was due to nonreactive cells or the inability of CAP to trigger such a response. Future studies with proper controls should reveal the effects of single and repeat nonthermal plasma application on hMSC osteoblastic differentiation. Finally, a single exposure to nonionized argon gas significantly increased hMSC production of alkaline phosphatase. This novel finding, possibly resulting from cellular response to argon shielding and/or shear forces, merits further exploration.

ACKNOWLEDGMENTS

We wish to thank Jeffrey P. Sutton, M.D., Ph.D., and the Center for Space Medicine at Baylor College of Medicine in Houston, Texas, for providing financial support. Additionally, special thanks goes to Danielle MacKay, Ph.D., Thomas Kean, Ph.D., and James Dennis, Ph.D., in the Department of Orthopedic Surgery at Baylor College of Medicine for their incredible assistance.

REFERENCES

1. Fridman G, Friedman G, Gutsol A, Shekhter AB, Vasilets VN, Fridman A. Applied plasma medicine. Plasma Process Polym. 2008;5(6):503–33.
2. Wang M, Holmes B, Cheng X, Zhu W, Keidar M, Zhang LG. Cold atmospheric plasma for selectively ablating metastatic breast cancer cells. PLoS One. 2013;8:e73741.
3. Arjunan KP, Clyne AM. Non-thermal dielectric barrier discharge plasma induces angiogenesis through reactive oxygen species. Conf Proc IEEE Eng Med Biol Soc. 2011;2011:2447–50.
4. Kalghatgi S, Friedman G, Fridman A, Clyne AM. Endothelial cell proliferation is enhanced by low dose non-thermal plasma through fibroblast growth factor-2 release. Ann Biomed Eng. 2010;38(3):748–57.
5. Arndt, S, Unger, P, Wacker, E, Shimizu, T, Heinlin, J, Li, Y.-F, Thomas, HM, Morfill, GE, Zimmer-

mann, JL, Bosserhoff, A-K, Karrer, S. Cold atmospheric plasma (CAP) changes gene expression of key molecules of the wound healing machinery and improves wound healing *in vitro* and *in vivo*. PLoS One. 2013;8(11):e79325.
6. Babaeva NY, Ning N, Graves DB, Kushner MJ. Ion activation energy delivered to wounds by atmospheric pressure dielectric-barrier discharges: sputtering of lipid-like surfaces. J Phys D: Appl Phys. 2012;45(11):115203.
7. Blackert S, Haertel B, Wende K, von Woedtke T, Lindequist U. Influence of non-thermal atmospheric pressure plasma on cellular structures and processes in human keratinocytes (HaCaT). J Dermatol Sci. 2013;70(3):173–81.
8. Daeschlein G, Scholz S, Ahmed R, von Woedtke T, Haase H, Niggemeier M, Kindel E, Brandenburg R, Weltmann K-D, Juenger M. Skin decontamination by low-temperature atmospheric pressure plasma jet and dielectric barrier discharge plasma. J Hosp Infect. 2012;81(3):177–83.
9. Dobrynin D, Fridman G, Friedman G, Fridman A. Physical and biological mechanisms of direct plasma interaction with living tissue. New J Phys. 2009;11(11):115020.
10. Wu AS, Kalghatgi S, Dobrynin D, Sensenig R, Cerchar E, Podolsky E, Dulaimi E, Paff M, Wasko K, Arjunan KP, Garcia K, Fridman G, Balasubramanian M, Ownbey R, Barbee K, Fridman A, Friedman G, Joshi SG, Brooks AD. Porcine intact and wounded skin responses to atmospheric nonthermal plasma. J Surg Res. 2013;179(1):e1–12.
11. Fridman G, Peddinghaus M, Balasubramanian M, Ayan H, Fridman A, Gutsol A, Brooks A. Blood coagulation and living tissue sterilization by floating-electrode dielectric barrier discharge in air. Plasma Chem Plasma Process. 2006;26(4):425–42.
12. Isbary G, Heinlin J, Shimizu T, Zimmermann JL, Morfill G, Schmidt H-U, Monetti R, Steffes B, Bunk W, Li Y, Klaempfl T, Karrer S, Landthaler M, Stolz W. Successful and safe use of 2 min cold atmospheric argon plasma in chronic wounds: results of a randomized controlled trial. Br J Dermatol. 2012;167(2):404–10.
13. Kalghatgi, S, Kelly, CM, Cerchar, E, Torabi, B, Alekseev, O, Fridman, A, Friedman, G, Azizkhan-Clifford, J. Effects of non-thermal plasma on mammalian cells. PLoS One. 2011;6(1):e16270.
14. Kong, MG, Kroesen, G, Morfill, G, Nosenko, T, Shimizu, T, van Dijk, J, Zimmermann, JL. Plasma medicine: an introductory review. New J Phys. 2009;11(11):115012.
15. Laroussi M, Paper I. Low-temperature plasmas for medicine? IEEE Trans Plasma Sci. 2009;37(6): 714–25.
16. Maisch T, Shimizu T, Li Y-F, Heinlin J, Karrer S, Morfill G, Zimmermann JL. Decolonisation of MRSA, *S. aureus* and *E. coli* by cold-atmospheric plasma using a porcine skin model *in vitro*. PLoS One. 2012;7(4):e34610.
17. Martines E, Zuin M, Cavazzana R, Gazza E, Serianni G, Spagnolo S, Spolaore M, Leonardi A, Deligianni V, Brun P, Aragona M, Castagliuolo I. A novel plasma source for sterilization of living tissues. New J Phys. 2009;11(11):115014.
18. Joshi SG, Cooper M, Yost A, Paff M, Ercan UK, Fridman G, Friedman G, Fridman A, Brooks AD. Nonthermal dielectric-barrier discharge plasma-induced inactivation involves oxidative DNA damage and membrane lipid peroxidation in *Escherichia coli*. Antimicrob Agents Chemother. 2011;55(3):1053–62.
19. Fricke K, Koban I, Tresp H, Jablonowski L, Schröder K, Kramer A, Weltmann K-D, von Woedtke T, Kocher T. Atmospheric pressure plasma: a high-performance tool for the efficient removal of biofilms. PLoS One. 2012;7(8):e42539.
20. Dobrynin D, Fridman G, Friedman G, Fridman A. Penetration deep into tissues of reactive oxygen species generated in floating-electrode dielectric barrier discharge (FE-DBD): an in vitro agarose gel model mimicking an open wound. Plasma Med. 2012;2(1–3):71–83.
21. Kalghatgi SU, Fridman G, Cooper M, Nagaraj G, Peddinghaus M, Balasubramanian M, Vasilets VN, Gutsol AF, Fridman A, Friedman G. Mechanism of blood coagulation by nonthermal atmospheric pressure dielectric barrier discharge plasma. IEEE Trans Plasma Sci. 2007;35(5):1559–66.

22. Ermolaeva SA, Varfolomeev AF, Chernukha MY, Yurov DS, Vasiliev MM, Kaminskaya AA, Moisenovich MM, Romanova JM, Murashev AN, Selezneva II, Shimiz, T, Sysolyatina EV, Shaginyan IA, Petrov OF, Mayevsky EI, Fortov VE, Morfill GE, Naroditsky BS, Gintsburg AL. Bactericidal effects of non-thermal argon plasma in vitro, in biofilms and in the animal model of infected wounds. J Med Microbiol. 2011;60(Pt 1):75–83.
23. Yu Y, Tan M, Chen H, Wu Z, Xu L, Li J, Cao J, Yang Y, Xiao X, Lian X, Lu X, Tu Y. Non-thermal plasma suppresses bacterial colonization on skin wound and promotes wound healing in mice. J Huazhong Univ Sci Technolog Med Sci. 2011;31(3):390–4.
24. Haertel B, Hähnel M, Blackert S, Wende K, von Woedtke T, Lindequist U. Surface molecules on HaCaT keratinocytes after interaction with non-thermal atmospheric pressure plasma. Cell Biol Int. 2012;36(12):1217–22.
25. Haertel B, Straßenburg S, Oehmigen K, Wende K, von Woedtke T, Lindequist U. Differential influence of components resulting from atmospheric-pressure plasma on integrin expression of human HaCaT keratinocytes. Biomed Res Int. 2013;2013:761451.
26. Haertel B, Volkmann F, von Woedtke T, Lindequist U. Differential sensitivity of lymphocyte subpopulations to non-thermal atmospheric-pressure plasma. Immunobiology; 2012;217(6):628–33.
27. Coelho PG, Giro G, Teixeira HS, Marin C, Witek L, Thompson VP, Tovar N, Silva NRFA. Argon-based atmospheric pressure plasma enhances early bone response to rough titanium surfaces. J Biomed Mater Res A. 2012;100(7):1901–6.
28. Teixeira HS, Marin C, Witek L, Freitas A, Silva NRF, Lilin T, Tovar N, Janal MN, Coelho PG. Assessment of a chair-side argon-based non-thermal plasma treatment on the surface characteristics and integration of dental implants with textured surfaces. J Mech Behav Biomed Mater. 2012;9:45–9.
29. Cunha A, Renz RP, Blando E, de Oliveira RB, Hübler R. Osseointegration of atmospheric plasma-sprayed titanium implants: influence of the native oxide layer. J Biomed Mater Res A. 2014;102(1):30–6.
30. Guastaldi, FPS, Yoo, D, Marin, C, Jimbo, R, Tovar, N, Zanetta-Barbosa, D, Coelho, PG. Plasma treatment maintains surface energy of the implant surface and enhances osseointegration. Int J Biomater. 2013;2013:354125.
31. Shon WJ, Chung SH, Kim HK, Han GJ, Cho BH, Park YS. Peri-implant bone formation of non-thermal atmospheric pressure plasma-treated zirconia implants with different surface roughness in rabbit tibiae. Clin Oral Implants Res. 2014;25:573–9.
32. Ring A, Tilkorn DJ, Goertz O, Langer S, Schaffran A, Awakowicz P, Hauser J. Surface modification by glow discharge gasplasma treatment improves vascularization of allogenic bone implants. J Orthop Res. 2011;29:1237–44.
33. Wang M, Cheng W, Zhu W, Holmes B, Keidar M, Zhang LG. Design of biomimetic and bioactive cold plasma-modified nanostructured scaffolds for enhanced osteogenic. Tissue Eng Part A. 2014;20(5–6):1060–71.
34. Choi YR, Kwon JS, Song DH, Choi EH, Lee YK, Kim KN, Kim KM. Surface modification of biphasic calcium phosphate scaffolds by non-thermal atmospheric pressure nitrogen and air plasma treatment for improving osteoblast attachment and proliferation. Thin Solid Films. 2013;547:235–40.
35. Gugala Z, Gogolewski S. Attachment, growth, and activity of rat osteoblasts on polylactide membranes treated with various low-temperature radiofrequency plasmas. J Biomed Mater Res A. 2006;76:288–99.
36. Barradas AMC, Lachmann K, Hlawacek G, Frielink C, Truckenmoller R, Boerman OC, Van Gastel R, Garritsen H, Thomas M, Moroni L, Van Blitterswijk C, De Boer J. Surface modifications by gas plasma control osteogenic differentiation of MC3T3-E1 cells. Acta Biomater. 2012;8:2969–77.
37. Duske K, Koban I, Kindel E, Schröder K, Nebe B, Holtfreter B, Jablonowski L, Weltmann KD, Kocher T. Atmospheric plasma enhances wettability and cell spreading on dental implant metals. J Clin Periodontol. 2012;39:400–7.
38. Steinbeck MJ, Chernets N, Zhang J, Kurpad DS, Fridman G, Fridman A, Freeman T. Skeletal cell differentiation is enhanced by atmospheric dielectric barrier discharge plasma treatment. PLoS One. 2013;8(12):e82143.

39. Chernets N, Zhang J, Steinbeck MJ, Kurpad DS, Koyama E, Friedman G, Freeman TA. Nonthermal atmospheric pressure plasma enhances mouse limb bud survival, growth, and elongation. Tissue Eng Part A. 2015;21(1–2):300–9.
40. Kwon JS, Kim YH, Choi EH, Kim KN. The effects of non-thermal atmospheric pressure plasma jet on attachment of osteoblast. Curr Appl Phys. 2013;13(Suppl 1):S42–7.
41. Chen Z, Zhang M, Qiu J. Histomorphologic study of the bone repair materials by using the cold plasma technique. Zhonghua Kou Qiang Yi Xue Za Zhi. 1998;33:294–6.
42. Chen G, Deng C, Li Y-P. TGF-β and BMP signaling in osteoblast differentiation and bone formation. Int J Biol Sci. 2012;8:272–88.
43. Szpalski M, Gunzburg R. Recombinant human bone morphogenetic protein-2: a novel osteoinductive alternative to autogenous bone graft? Acta Orthop Belg. 2005;71(2):133–48.
44. Mulconrey DS, Bridwell KH, Flynn J, Cronen GA, Rose PS. Bone morphogenetic protein (RhBMP-2) as a substitute for iliac crest bone graft in multilevel adult spinal deformity surgery: minimum two-year evaluation of fusion. Spine (Phila Pa 1976). 2008;33(20):2153–9.
45. Tannoury CA, An HS. Complications with the use of bone morphogenetic protein 2 (BMP-2) in spine surgery. Spine J. 2014;14(3):552–9.
46. Dobrynin D, Wu A, Kalghatgi S, Park S, Shainsky N, Wasko K, Dumani E, Ownbey R, Joshi S, Sensenig R, Brooks AD. Live pig skin tissue and wound toxicity of cold plasma treatment. Plasma Med. 2011;1(1):93–108.
47. Coelho PG, Bonfante EA, Pessoa RS, Marin C, Granato R, Giro G, Witek L, Suzuki M. Characterization of five different implant surfaces and their effect on osseointegration: a study in dogs. J Periodontol. 2011;82(5):742–50.
48. Langenbach F, Handschel J. Effects of dexamethasone, ascorbic acid and β-glycerophosphate on the osteogenic differentiation of stem cells *in vitro*. Stem Cell Res Ther. 2013;4(5):117.
49. Birmingham E, Niebur GL, Mchugh PE, Shaw G, Barry FP, McNamara LM. Osteogenic differentiation of mesenchymal stem cells is regulated by osteocyte and osteoblast cells in a simplified bone niche. Eur Cell Mater. 2012;23:13–27.
50. Shuvalova NS, Kordium VA. Comparison of proliferative activity of Wharton jelly mesenchymal stem cells in cultures under various gas conditions. Biopolym Cell. 2015;31(3):233–9.
51. Takayama Y, Nomoto R, Nakajima H, Ohkubo C. Effects of argon gas flow rate on laser-welding. Dent Mater J. 2012;31(2):316–26.
52. Watanabe I, Topham DS. Laser welding of cast titanium and dental alloys using argon shielding. J Prosthodont. 2006;15(2):102–7.