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Marla J Steinbeck  
*Drexel University*  

Natalie Chernets  
*Drexel University*  

Jun Zhang  
*Thomas Jefferson University*  

Deepa S Kurpad  
*Thomas Jefferson University*  

Gregory Fridman  
*Drexel University*  

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Authors
Marla J Steinbeck, Natalie Chernets, Jun Zhang, Deepa S Kurpad, Gregory Fridman, Alexander Fridman, and Theresa A. Freeman
Skeletal Cell Differentiation Is Enhanced by Atmospheric Dielectric Barrier Discharge Plasma Treatment

Marla J. Steinbeck¹*, Natalie Chernets²*, Jun Zhang³*, Deepa S. Kurpad⁴, Gregory Fridman¹, Alexander Fridman⁵, Theresa A. Freeman⁴*

¹ Department of Biomedical Engineering, Drexel University, Philadelphia, Pennsylvania, United States of America, ² Department of Electrical Engineering, Drexel University, Philadelphia, Pennsylvania, United States of America, ³ Department of Orthopaedics, The Second Hospital of Jilin University, Chang Chun, Jilin, China, ⁴ Department of Orthopaedic Surgery, Thomas Jefferson University, Philadelphia, Pennsylvania, United States of America, ⁵ Department of Mechanical Engineering and Mechanics, Drexel University, Philadelphia, Pennsylvania, United States of America

Abstract

Enhancing chondrogenic and osteogenic differentiation is of paramount importance in providing effective regenerative therapies and improving the rate of fracture healing. This study investigated the potential of non-thermal atmospheric dielectric barrier discharge plasma (NT-plasma) to enhance chondrocyte and osteoblast proliferation and differentiation. Although the exact mechanism by which NT-plasma interacts with cells is undefined, it is known that during treatment the atmosphere is ionized generating extracellular reactive oxygen and nitrogen species (ROS and RNS) and an electric field. Appropriate NT-plasma conditions were determined using lactate-dehydrogenase release, flow cytometric live/dead assay, flow cytometric cell cycle analysis, and Western blots to evaluate DNA damage and mitochondrial integrity. We observed that specific NT-plasma conditions were required to prevent cell death, and that loss of pre-osteoblastic cell viability was dependent on intracellular ROS and RNS production. To further investigate the involvement of intracellular ROS, fluorescent intracellular dyes Mitosox (superoxide) and dihydrorhodamine (peroxide) were used to assess onset and duration after NT-plasma treatment. Both intracellular superoxide and peroxide were found to increase immediately post NT-plasma treatment. These increases were sustained for one hour but returned to control levels by 24 hr. Using the same treatment conditions, osteogenic differentiation by NT-plasma was assessed and compared to peroxide or osteogenic media containing β-glycerophosphate. Although both NT-plasma and peroxide induced differentiation-specific gene expression, neither was as effective as the osteogenic media. However, treatment of cells with NT-plasma after 24 hr in osteogenic or chondrogenic media significantly enhanced differentiation as compared to differentiation media alone. The results of this study show that NT-plasma can selectively initiate and amplify ROS signaling to enhance differentiation, and suggest this technology could be used to enhance bone fusion and improve healing after skeletal injury.


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* E-mail: Theresa.freeman@jefferson.edu

These authors contributed equally to this work.

Introduction

The goal of this investigation was to examine the effect of non-thermal (NT)-plasma on skeletal cell differentiation. Medical use of plasma technology is most commonly associated with thermal plasmas, such as the plasma knife used in surgery to cut and simultaneously cauterize vessels as a result of the high temperature generated by plasma [1]. Advancements in atmospheric pressure plasma systems led to the development of a novel NT dielectric barrier discharge plasma with a discharge sufficiently uniform and cold to safely apply to living cells and tissues [2–6]. The NT-plasma discharge is generated by applying a high voltage, time-varying waveform between a dielectric covered electrode and the biological target [7,8]. To prevent high temperature build-up and transition to arc, high voltage current is alternated between the two electrodes, one of which is covered with a dielectric. Within the NT-plasma discharge, the molecules present in air (O₂, N₂, H₂O, CO₂, etc) are ionized resulting in the direct formation of numerous reactive oxygen species (ROS) and reactive nitrogen species (RNS) [8–10].

Most biomedical studies on the effect of NT-plasma have focused on the bacteriostatic and bactericidal properties of this new technology [11–13]. Recently, it was reported that NT-plasma exposure promoted endothelial cell proliferation, which was abrogated by fibroblast growth factor (FGF)-2 neutralizing antibody [5]. Proliferation and FGF-2 release were blocked by N-acetyl-cysteine (NAC), which prevented changes in intracellular redox. Mechanistically, these studies directly link NT-plasma effects to ROS or RNS generation.

ROS and RNS are known to directly activate multiple proteins involved in signaling pathways that regulate cell function. ROS-responsive MAP kinases are known to control a wide range of cellular processes including: cellular differentiation, cell cycle control, cytokine and growth factor signaling, survival, hypotrophy and/or apoptosis [14–17]. For example, the Map5kinase
Apoptosis signal-regulating kinase 1 (ASK1), is particularly sensitive to ROS as its activity is tightly regulated by four ROS sensitive proteins thioredoxin, glutaredoxin, Akt and 14-3-3 [17–21]. ROS activated ASK1 phosphorylates and activates both p38 and jnk kinases, which play key roles in cellular differentiation [22,23] as well as the regulation of apoptosis [24]. Activation of ASK1, p38 and/or jnk promotes the differentiation of several cell lineages including chondrocytes [25–27], osteoblasts, neuronal [21], myoblasts [29] and keratinocytes [14,23,27].

In the case of mesenchymal cell differentiation into osteoblast lineages there is precedence for ROS stimulation to both direct and enhance this process [30–32]. Similarly, enhanced chondrogenesis has also been associated with ROS stimulation [27] and the occurrence of oxidative spikes as a driving force for transitional stages throughout developmental and regenerative processes is a commonly observed phenomena. The possibility of using NT-plasma technology to artificially trigger these transitions and direct these processes is intriguing. Herein, we show that specific exposure conditions to NT-plasma promote intracellular ROS production and skeletal cell differentiation.

**Materials and Methods**

**Cell Culture**

The pre-osteocytic cell line MLO-A5 was obtained from Dr. Linda Bonewald and cultured using the method described previously [33]. To induce maturation for qRT-PCR experiments MLO-A5, cells were placed in osteogenic differentiation media (Dulbecco’s Modified Eagle’s Medium (DMEM) (Cellgro, Fisher Scientific, USA), with 10% Fetal Bovine Serum (FBS) (Invitrogen, Life Science Technologies, USA), and supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin (Cellgro, Fisher Scientific, USA), 0.5 mM beta glycerophosphate and 100 μg/ml ascorbic acid (Sigma-Aldrich, St. Louis, MO) and incubated in 5% CO2 at 37°C with media changes every 2 days. N1511 mouse chondrocytes were obtained from Dr. Motomi Enomoto-Iwamoto. The cells were maintained in culture using the method described previously [34,35]. Briefly, the cells were plated at a concentration of 50,000 cells/ml in—MEM containing 10% FBS, 0.2% L-glutamine, and penicillin/streptomycin. To induce maturation, after 24 h, the adherent cells were treated with 200 ng/ml recombinant BMP2 (Alpha Diagnostic Intl., San Antonio, TX). Assessment of the maturation was performed at day 5 by measuring alkaline phosphatase activity and the expression of chondrogenic markers.

**Power Measurement for Calibration of Plasma Device**

Tektronix DPO4104B (1 GHz bandwidth, 5 GS/sec) digital oscilloscope with Tektronix P6015A high voltage divider (1000:1) and Ion Physics Corp CM-10-L current probe (1 V/V/1 A, 5 kA max) was used to record voltage and current waves at maximum sampling rate. Instant values for both were multiplied and resulting instant power was then integrated over the entire cycle. Ten measurements were taken for statistics analysis.

**Non-Thermal Plasma Treatment**

NT-plasma was generated by applying alternating polarity pulsed voltage of 20 kV magnitude (peak to peak), a 10 μs pulse width, at a frequency between 50 to 3500 Hertz (Hz), with a rise time of 5 V/ns between the quartz-insulated high voltage electrode and the sample undergoing treatment 2 mm from the electrode. General electrical schematics and description of the power supply (Plasma Power, LLC, Philadelphia USA) were as reported by the authors [4]. A 32 mm (6-well plate) electrode was used, which was composed of an inner core of copper surrounded by an outer insulating shell of acrylic, and a 0.5 mm quartz disc plasma-generating surface covered with a 0.01inch sheet of polyether ether ketone. Just before treatment, culture media was removed by pipet from the dish and replaced immediately following treatment.

**Cell Viability and Cell Cycle Analysis**

Cell viability was measured by live/dead flow cytometry in cells stained with propidium iodide (PI; Sigma), an indicator of cell death. PI was added immediately before analysis on the MACSQuant VYB flow cytometer (Miltenyi Biotec, Auburn, CA). Ten measurements were taken for statistics analysis. Ten measurements were taken for statistics analysis. The medium was replaced after 3 hr without H2O2 or TEMPO.

To determine cell cycle kinetics, 24 hr after NT-plasma treatment cells were prepared for flow cytometric cell cycle analysis on the MACSQuant VYB (Miltenyi Biotec, Auburn, CA), following the Miltenyi cell cycle analysis protocol. Briefly, cells were collected in sample buffer (Ca2+ and Mg2+ free PBS, with 1 g/L glucose), washed and placed in 70% ethanol overnight at 4°C. After re-suspension in sample buffer, the propidium iodide (PI (1 ug/ml); Sigma, St. Louis, MO) was added and the stained cells were analyzed with MACSQuant software (Miltenyi Biotec, Auburn, CA). This procedure was repeated in quadruplicate on 3 separate days.

**Western Blot Analysis for DNA and Mitochondrial Damage**

Protein from NT-plasma treated cells was collected 24 hr after treatment. Cells were isolated with MPER®, 5 M NaCl, complete protease inhibitor cocktail, DTT, 100 μM NaF, and 100 μM Na3VO4 and quantified using the Bradford assay (Pierce Biotechnology Thermal Scientific, Rockford IL). Protein (40 μg) was then loaded onto 10% Tris polyacrylamide gels. Samples were transferred to PVDF membranes and subsequently blocked with ECL Blocking agent (RPN 2125, GE Health Life Sciences, Cleveland, OH) (2.0% TBS-T +2.0 g dried milk). Blots were treated overnight with antibodies to either H2Axl (Millipore, Billerica, MA) or Cytochrome C (BD Biosciences, Franklin Lakes, NJ) at a 1:500 dilution in 2% blocking reagent. The blots were washed three times in TBST and species specific HRP conjugated secondary antibodies (Santa Cruz Biotechnology, Dallas, TX) were applied for 1 hr at room temperature. After three washes in TBST, the membrane was detected with Western Bright Quantum kit (Advanta, Menlo Park, CA) for 5 min at 25°C. Photographs of the membranes were taken in a GE Image quant LAS 4000 dark box with a GE Luminescent Image Analyzer LAS-4000 (GE Health Life Sciences, Cleveland, OH) and digital images were collected using the Image quant TL 1D gel analysis software (GE Health Life Sciences, Cleveland, OH).
ROS Measurements
Mitsox and dihydrothreomethine (DHR) dyes (Invitrogen, Carlsbad, CA) were used to detect the presence of intracellular superoxide anion and hydrogen peroxide, respectively. NT-plasma or sham treatments were performed on MLO-A5 cells plated overnight in 6 well plates and fluorescent intensity was measured using a Tercan Infinite 1000 plate reader (Tecan, Research Triangle, NC). Inhibitors (NAC, 200 μM and TEMPO, 6 mM (Sigma, St. Louis, MO) were added to the medium for one hour before NT-plasma treatment and replaced 3 hr after with regular media.

Real Time-PCR for Differentiation Markers and Comparative Analyses
Confluent cells in 6 well dishes were either treated with differentiation media (β-GP), H2O2 (100 μM) or treated with 1000 Hz NT-plasma for 10 sec and harvested after 24 hr. Alternatively, confluent cells from 6 well dishes were placed in differentiation media (MLO-A5 with β-GP or N1511 with BMP) 24 hr before NT-plasma or sham-plasma control treatment and harvested after 24 hr or 56 hr. All harvested cells were washed with DEPC water before total RNA was isolated using the QiagenRNeasy® Mini kit (Qiagen, Valencia, CA). RNA yield was quantified using the MyIQ Real-Time PCR System (BioRad, Hercules, CA). The expression level of the housekeeping gene, β-actin was used to normalize the data presented. Melting curves were analyzed to verify the specificity of the RT-PCR reaction and the absence of primer dimer formation. Each sample is analyzed in duplicate and included a template-free control. All the primers (Table 1) used were synthesized by Integrated DNA Technologies, Inc. (Corvalle, IN).

Statistical Analysis
Statistical analysis between groups was performed using a one-way ANOVA for normality and student’s t-test for continuous variables. A level of significance (α), or a p-value of less than 0.05, with a 95% confidence interval was determined. Representative data are presented as the mean ± SD of individual samples from 2 to 16 independent analyses, unless otherwise stated. In this work “n” indicates number of separate experimental trials, with repeats varying from 3 to 8, or more. For normally distributed data, differences between groups were determined by independent t-tests. For non-parametric data, Mann Whitney U tests were used to evaluate differences.

Results
Determining the Appropriate NT-Plasma Dose
To investigate the appropriate NT-plasma dose, MLO-A5 osteoblastic cells in 6 well plates were treated with NT-plasma for 10 sec at a voltage of 20 kV and pulse width of 10 μs), varying only the frequency at either 50, 1000, or 3500 Hertz (Hz). Controls included the NT-plasma electrode without power as a sham treatment, and H2O2 (100 μM) as a positive control for ROS. Cell death was determined 24 hr after NT-plasma treatment using a fluorescent flow cytometric live/dead assay. Less than 4% of the cells underwent cell death in response to NT-plasma treatment, although the 50 Hz (p<0.05) and 3500 Hz (p<0.05) treatments were significantly above control (Fig. 1A). H2O2 induced a slightly higher 5.6% increase in cell death (p<0.01). Results for the 3500 Hz treatment, were an under estimate of cell damage, as large areas of the culture plate were devoid of cells immediately after treatment (Fig. 1B). Due to cell detachment this setting was not used in subsequent assays.

To further assess the 50 and 1000 Hz dose rates, MLO-A5 cell cytotoxicity was measured using lactate dehydrogenase (LDH) (Fig. 1C). At 24 h LDH release in response to 50 Hz was 5.1% (not significant; ns) and 1000 Hz was 7.7% (p<0.01) above NT-plasma sham control. Both treatments were less than H2O2, which induced an 11.2% increase in cell death (p<0.01). To prevent changes in intracellular oxidant levels, TEMPOL (6 mM) a membrane permeable scavenger of both ROS and RNS, was added 1 hr before NT-plasma or H2O2 treatment [36,37]. TEMPOL reduced the release of LDH in response to 1000 Hz NT-plasma (ns) and significantly decreased LDH release in response to H2O2 (p<0.01), indicating ROS/RNS are being generated in response to NT-plasma and cause cell damage.

To determine whether NT-plasma treatment affected cell proliferation (increased S phase) or caused DNA damage (blockage in G2), flow cytometric analysis of cell cycle phase was determined 24 hours after treatment of MLO-A5 cells with 50 and 1000 Hz NT-plasma or H2O2 (Fig. 1D). No significant differences were observed in the G1, S or G2/M phase after any treatment, indicating no increase in cell proliferation or blockage of the cell cycle due to DNA damage.

Additionally, Western blot analysis was performed to assess damage to DNA or mitochondrial membranes using antibodies to the histone2A variant (H2AX) and cytoplasmic cytochrome c, respectively (Fig. 1E). The 1000 Hz NT-plasma treatment dose was achieved by varying the time to include 10 sec (4.08 Joules J)/

Table 1. PCR primer information.

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cm²), 30 sec (12.24 J/cm²) and 60 sec (24.48 J/cm²) dose. The 10 sec dose did not induce DNA or mitochondrial damage within 1 hr after treatment, but damage after both the 30 and 60 sec treatments was observed.

Taken together the results show that NT-plasma treatment at 1000 Hz for 10 sec did not significantly reduce cell viability, alter the cell cycle or cause DNA or mitochondrial damage in MLO-A5 cells. Based on these findings the 1000 Hz, 10 sec NT-plasma treatment was chosen as the NT-plasma optimal treatment.

Production of Intracellular ROS in Response to NT-Plasma
To evaluate intracellular reactive species levels in response to NT-plasma at 1000 Hz, 10 sec did not significantly reduce cell viability, alter the cell cycle or cause DNA or mitochondrial damage in MLO-A5 cells. Based on these findings the 1000 Hz, 10 sec NT-plasma treatment was chosen as the NT-plasma optimal treatment.

Levels of both oxidants returned to PRE-PL levels by 24 hr. The immediate increase in H₂O₂ may be due to ROS produced by NT-plasma but the extended detection indicates ROS are being actively produced in response to NT-plasma.

To confirm the production of ROS in response to NT-plasma treatment, MLO-A5 cells were pretreated for 1 hr with either NAC (200 µM), to maintain intracellular redox balance and interfere with ROS production [38] or TEMPOL (6 mM), a scavenger of ROS as well as RNS. After pre-incubation with the inhibitor, the cells were subjected to NT-plasma treatment at 1000 Hz for 10 sec. NAC significantly inhibited the POST-PL and 1 hr increase in O₂⁻ (Fig. 2A) and H₂O₂ (Fig. 2B). TEMPOL also significantly inhibited the POST-PL and 1 hr increase in O₂⁻ (Fig. 2C), and it inhibited the POST-PL increase in H₂O₂ (Fig. 2D). To lesser extent TEMPOL inhibited the 1 hr increase in H₂O₂ in response to NT-plasma, and at 24 hr H₂O₂ production was increased (p<0.01). One possible explanation for the decreased effectiveness of TEMPOL after 1 hr and 24 hr after NT-plasma/TEMPOL treatment is that removal of NO prevents its reaction with O₂⁻ and the production of peroxynitrite (ONOO⁻). Thus, by scavenging NO TEMPOL...
allows H$_2$O$_2$ to be generated from O$_2$ rather than ONOO$^-$, indicating some NO$^+$ is being generated although at significantly lower amounts compared to H$_2$O$_2$.

### NT-plasma Differentiation of Osteoblasts and Chondrocytes in vitro

To determine if NT-plasma treatment influenced the differentiation of MLO-A5 cells, alkaline phosphatase (ALKP), bone morphogenetic protein 2 (BMP2), bone sialoprotein (BSP) and fibroblast growth factor-2 (FGF-2) expression was evaluated by qPCR (Fig. 3). We compared differentiation media containing β-glycerophosphate (βGP) to either NT-plasma or H$_2$O$_2$ treatment; used to evaluate the effects of redox. Both NT-plasma and H$_2$O$_2$ increased the expression of osteogenic genes above controls, which were undifferentiated cells sham treated with NT-plasma (Fig. 3A). ALKP expression was upregulated 3 (ns) and 11 (p<0.01) fold, respectively, and BMP2 and BSP were increased 5 fold by 24 hr (p<0.05). However, compared to the 14–23 fold increases induced above control at 24 hr, presumably due to removal of baseline NO$^+$. The results are expressed as the mean ± standard deviation. Statistical significance was determined by the Mann-Whitney U test for non-parametric data; * or # (p<0.01) ** or ## (p<0.001).

![Figure 2. Direct effects of NT-plasma on intracellular ROS production. (A and B)](image)

(NT-Plasma Enhances Skeletal Cell Growth)

**Figure 2. Direct effects of NT-plasma on intracellular ROS production. (A and B)** Intracellular O$_2$ and H$_2$O$_2$ levels were measured using fluorescent indicators MitoSox (n = 3) and dihydrorhodamine (DHR), (n = 3) respectively. Immediately post-treatment (POST-PL) and at 1 hr, 1000 Hz NT-plasma treated cells generated significantly increased amounts of O$_2$ and H$_2$O$_2$ (p<0.001) as compared to sham control or pre-treatment levels (PRE-PL). Amounts of both ROS were decreased by 24 hr. NAC and TEMPOL quenched the ROS increase POST-PL and at 1 hr (p<0.01–0.001).

However, H$_2$O$_2$ levels in the TEMPOL (p<0.01) inhibitor group were significantly increased above control at 24 hr, presumably due to removal of baseline NO$^+$. The results are expressed as the mean ± standard deviation. Statistical significance was determined by the Mann-Whitney U test for non-parametric data; * or # (p<0.01) ** or ## (p<0.001).

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As NT-plasma treatment evoked only a weak induction of osteogenic gene expression, a potential synergism between βGP and NT-plasma was investigated. NT-plasma treatment applied 24 hr after incubation in βGP, induced a 2–7 fold increase in the expression levels of osteogenic markers BMP2 and ALKP above βGP alone (p<0.05) (Fig. 3B). The expression of osterix (ostx), a key transcription factor for osteogenesis, was increased 15 fold (p<0.01). At 56 hr after NT-plasma treatment, the late osteoblast markers, BSP and osteocalcin (OSTCN) were increased 17–24 fold above βGP-treated control (p<0.05).

To determine if the differentiation effect of NT-plasma was osteoblast specific, the N1511 chondrocyte cell line was subjected to the same NT-plasma treatment in the presence of BMP2 (200 ng/ml), a known inducer of chondrocyte differentiation [34]. 24 hr after incubation in βGP, induced a 2–7 fold increase in the expression levels of osteogenic markers BMP2 and ALKP above βGP alone (p<0.05) (Fig. 3B). The expression of osterix (ostx), a key transcription factor for osteogenesis, was increased 15 fold (p<0.01). At 56 hr after NT-plasma treatment, the late osteoblast markers, BSP and osteocalcin (OSTCN) were increased 17–24 fold above βGP-treated control (p<0.05).

To determine if the differentiation effect of NT-plasma was osteoblast specific, the N1511 chondrocyte cell line was subjected to the same NT-plasma treatment in the presence of BMP2 (200 ng/ml), a known inducer of chondrocyte differentiation [34]. 24 hr after treatment, chondrocyte differentiation markers Runx2, ALKP were increased 3–6-fold above BMP-treated controls (p<0.05) (Fig. 3C). By 56 hr, collagen type X (Col X) and another late marker, matrix metalloprotease 13 (MMP13) were both increased 20 (p<0.01) and 4-fold (p<0.05), respectively above BMP-treated control.

We conclude from these studies that NT-plasma alone was not sufficient to initiate significant changes in cell differentiation.
Figure 3. NT-plasma induced osteogenic differentiation using qPCR markers. (A) Fold increases in the expression of alkaline phosphatase (ALKP), bone morphogenetic protein 2 (BMP2), bone sialoprotein (BSP) and fibroblast growth factor-2 (FGF-2) in response to NT-plasma treatment, H2O2 or β-glycerol phosphate (βGP) normalized to sham treated control cells after 24 hr. ALKP was upregulated 3- (NS) and 11-fold (p<0.01), respectively, and BMP2 and BSP were increased 5 fold (p<0.05). βGP increased expression 14–23 fold (p<0.01). FGF-2 expression was not affected by NT-plasma, H2O2 or βGP. (B) After a 24 hr incubation in βGP NT-plasma was applied. 24 hr later there was a 2–7 fold increase in the induction of BMP2, ALKP (p<0.05) and Osterix was increased 15 fold (p<0.01) compared to βGP treatment alone. At 56 hr, BSP and osteocalcin (OSTCN) were increased 17–24 fold above βGP-treated control (p<0.05). The results are expressed as the mean ± standard deviation (n = 2). Statistical significance was determined by the Wilcoxin Mann-Whitney test for non-parametric data; * (p<0.05), ** (p<0.01).

doi:10.1371/journal.pone.0082143.g003
However, once differentiation had been initiated, NT-plasma enhanced osteogenesis and chondrogenesis at both early and late time points.

**Discussion**

This study investigated the potential of NT-plasma to enhance both chondrocyte and osteoblast differentiation. Initially, we determined the specific NT-plasma condition required to maintain cell viability and showed both an immediate and long-term increase in intracellular ROS and RNS in response to NT-plasma. Furthermore, we demonstrate that NT-plasma enhanced the expression of differentiation specific genes by MLO-A5 in media without differentiation factors and in differentiation media. In media alone, the levels of expression were equal to those induced by H2O2, but not as effective as differentiation medium. However, when NT-plasma was applied 24 hr after the cells were placed in differentiation media a synergistic enhancement of chondrogenic and osteogenic differentiation was observed.

The NT-plasma generated ROS and RNS at the cell/environment interface initiates an immediate intracellular oxidative response (Fig. 2). It is interesting to speculate that this reaction may mimic naturally occurring ROS-associated cell signaling known to be associated with stem cell differentiation, cell fate decisions and regeneration. Within the stem cell pool, quiescence and pluripotency is maintained by the repression of ROS generation [39,40]. As such, mouse and human embryonic stem cells have immature mitochondria, reduced expression of OXPHOS enzymes, low metabolic activity, low oxygen consumption, decreased levels of ATP production [41], express modest levels of antioxidant enzymes [40] and have a high glycolytic flux [41]. In vivo, embryonic stem cells are maintained in hypoxic conditions (2 to 3%) before vascularization [42] and in vitro, low oxygen culture conditions promote maintenance of pluripotency [5]. Rapid changes in ROS production and oxidative status associated with vascularization, growth factor binding, or increased mitochondrial biogenesis and activity, all promote cellular differentiation and determine cell fate [30].

Our findings highlight the capability of NT-plasma to promote osteo and chondrogenesis. The mechanism(s) by which both proliferation and differentiation occur is dependent on both immediate and long-term signaling molecules. In the short term, O2-' and H2O2 are produced during the 10 sec NT-plasma exposure, however their sustained increases at 1 and 24 hr after treatment is due to intracellular ROS production. The most likely sources of the increased intracellular ROS are NADPH oxidase and mitochondrial production of O2-, H2O2 and NO- [42,43].

All three are signaling factors known to participate in cellular proliferation and differentiation by directly activating ROS or RNS-responsive proteins or indirectly by altering the redox status of the cell [44–57]. Specifically, MAP kinases are known to respond rapidly to ROS stimulation and these kinases control a wide range of cellular processes including: cellular differentiation, cell cycle control, cytokine and growth factor signaling, survival, hypertrophy and/or apoptosis [14–17].

ROS also promote downstream cellular responses by affecting the activity and expression of specific transcription factors [24,58,59]. In osteoblast progenitors, continuous production of low levels of H2O2 stimulates proliferation and augments their potential to differentiate into mature osteoblasts through up-regulation of Runx2 and osterix, [22]. In turn, these transcription factors promote ALKp and osteocalcin expression in differentiating osteoblasts. Similarly, in the current study, NT-plasma induced ROS production increased the expression of Runx2, osterix, ALKp and osteocalcin in pre-osteoblastic MLO-A5 cells.

Conflicting findings have been reported concerning the role of ROS in cell differentiation, but these discrepancies may be due to differing methodologies used for the generation of ROS and the delicate balance required for the appropriate response [57]. In a recent study comparing glucose oxidase continuous production of low levels of H2O2 to a single bolus of chemical H2O2, only glucose oxidase generated H2O2 promoted osteoblast differentiation [22]. This finding is not unexpected as the glucose oxidase system mimics cellular levels of H2O2 production. In the same way, a 10 sec exposure to NT-plasma initiates low-level intracellular production of ROS. This emphasizes that both timing and ROS concentration are critical in directing cell function. In the same way, chondrocyte differentiation in response to NT-plasma may be directly linked to ROS induced expression of SOX-9 and Runx2, which control collagen and aggrecan expression [60]. In a related study, laser irradiation induced intracellular ROS production and enhanced SOX-9 expression leading to chondrocyte differentiation and expression of collagen and aggrecan [61]. The roles of ROS in progenitor cell proliferation and differentiation described above may also be directly applicable to more complex tissue systems such as regenerating limbs or limb development.

While very little has been reported on the direct contribution of ROS in the developmental process, it is well established that a majority of the required signaling molecules (BMPs, WNTs and FGFs) either directly signal through ROS or are indirectly controlled by ROS [62–64].

Current studies investigating NT-plasma report multiple effects can be induced in living cells and tissues [3]; these include increased cell proliferation [3], enhanced cell transfection [65–67], selective killing of cancer cells [68] and improved wound healing [69,70]. The multiple effects can be attributed to the in vivo conditions selected during cellular treatment with NT-plasma. Results of the current study highlight the use of NT-plasma as a tool to initiate a non-lethal oxidative cellular burst that promotes osteoblast differentiation. Furthermore, these findings suggest that ROS and RNS produced in response to NT-plasma influence signaling pathways that are responsible for cellular proliferation and differentiation. Thus, it would not be unreasonable to assume that NT-plasma could be used to promote musculoskeletal cell differentiation and tissue regeneration.

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**Author Contributions**

Conceived and designed the experiments: TAF GF AF MJS. Performed the experiments: JZ NC DSK TAF. Analyzed the data: TAF MJS NC. The writer of the paper: TAF MJS NC.

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