Atmospheric Pressure Plasma Treatment with Nitrogen Induces Osteoblast Differentiation and Reduces iNOS and COX-2 Expressions

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Abstract: In recent years, atmospheric pressure plasma jet (APPJ) has been widely developed for various medical applications, such as medical equipment sterilization, gene transfection, cell proliferation, and wound healing. In particular, non-thermal APPJ enables direct treatment of the biological system without any thermal-associated damage. The effect of cells on APPJ depends upon the gas species used in the treatment. However, the mechanisms underlying osteoblast differentiation mediated by APPJ with nitrogen are yet to be studied. This study investigated the effects of nitrogen-APPJ on osteoblast differentiation by assessing the transcription factors, extracellular matrix proteins (ECM proteins), alkaline phosphatase (ALP), activity, and the mRNA and protein expressions of ALP, inducible nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX-2), in an osteoblast mouse cell line. We found that nitrogen-APPJ induced osteoblast differentiation-related transcription factors (runt-related transcription factor (Runx2) and osterix), osteocalcin (OCN), and ALP activity, as well as reduced the mRNA and protein expressions of iNOS and COX-2. Thus, we concluded that APPJ affects differentiation of the osteoblast cells.

Key words: Atmospheric pressure plasma jet, Osteoblast differentiation, iNOS

Introduction
Concerning for to the terminologies used in physics, plasma is referred to as a partially or completely ionized gas. Ionized gas differs from non-ionized gas in terms of its physical properties. Plasma is known as the fourth state of matter1 and can be classified based on pressure as low pressure2, atmospheric pressure, or high-pressure plasmas; furthermore, it can be classified based on temperature as cold and hot plasmas3. Apart from the natural occurrence, plasma is also generated artificially4. However, the exact composition and the ratio of plasma among the different components, depending on the operating pressure, employed process gas or gas mixture, and the origin of plasma. Non-noble gas plasmas, such as oxygen or nitrogen plasmas generate less intense ultraviolet radiation and high amounts of reactive species5.

Previous studies have investigated the gene and protein expressions, as well as the impact of cold atmospheric pressure plasma jet (APPJ) in vitro5. Kaneko et al. reported that indirect APPJ facilitates cell-membrane permeabilization via plasma-produced hydroxyl ions [OH]-. Atmospheric pressure plasma and nitrogen plasma jets from a micronozzle array induce apoptosis through the reactive oxygen/nitrogen species (ROS/RNS). The ROS/RNS generated by the plasma-triggered signaling pathways involving Jun N-terminal kinase (JNK) and p38, promotes mitochondrial perturbation, which further leads to cancer cell apoptosis6. Although majority of the previous studies have suggested that direct or indirect APPJ induces cell death or apoptosis in vitro by ROS/RNS-mediated toxicity, the biological efficacy of APPJ as a medical treatment alternative without causing cell death or apoptosis has not been widely studied.

Bone turnover or remodeling maintains a state of dynamic equilibrium by bone formation and resorption. Osteoblasts regulate bone formation and osteoblast differentiation by expressing various transcription factors and extracellular matrix proteins (ECM proteins) associated with osteoblast differentiation, which promote bone formation. Examples of such transcription factors are runt-related transcription factor 2 (Runx2), a master regulator of osteoblast differentiation and the transcription factor osterix that acts downstream of Runx2. In osteoblasts, both the transcription factors play an important role in bone formation. Osteocalcin (OCN) is a crucial ECM associated with osteoblast differentiation. This study investigated the effects of nitrogen-APPJ on osteoblast differentiation by assessing the transcription factors, ECM proteins, alkaline phosphatase (ALP) activity, and the mRNA and protein expressions of ALP, inducible nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX-2) in an osteoblast mouse cell line.

Materials and Methods
Cell culture
Mouse osteoblastic cell line, MC3T3-E1 was purchased from Riken...
BioResource Research Center (Tsukuba, Japan). Cells were cultured for up to 14 days, as previously described6.

**APPJ stimulation**

The 20 ml culture medium was irradiated directly for up to 180 sec, by APPJ (Damage-Free-Multi-Gas Plasma Jet, PCT-DFJM-02; Plasma Concept Tokyo Co., Tokyo, Japan) with nitrogen (outflow 6 l/min), and this medium was then used to treat the cells. The distance between the APPJ and medium was approximately 5-10 mm. Untreated control cells were not stimulated with APPJ.

**Cell viability and ALP activity**

Cell viability and ALP activity of the cultured cells were determined, as mentioned previously7. Briefly, MC3T3-E1 cells were placed in 96-well microplates at a density of 2.0 × 10^4 cells/cm². Control cells and cells subjected to APPJ treatment for 60, 120, and 180 sec were cultured for up to 14 days. At the time points indicated, the existing culture medium was replaced with a fresh medium containing 10% (vol/vol) cell-counting-kit-8 reagent (Dojindo Laboratories, Kumamoto, Japan), and was incubated for 1 h to measure cell viability. ALP activity was determined as described by Tanabe et al.7. Briefly, 100 μl of the enzyme assay solution (8 mM p-nitrophenyl phosphate, 12 mM magnesium chloride (MgCl₂), and 0.1 mM zinc chloride (ZnCl₂) in 0.1 M glycine-sodium hydroxide (NaOH) buffer, pH 10.5) was added to each well and the plate was incubated for several minutes at 37°C. The enzyme reaction was terminated by the addition of 50 μl of 0.2 M NaOH. The amount of p-nitrophenol released in the enzyme reaction was determined by measuring the absorbance at 405 nm using Benchmark Plus (Bio-Rad Laboratories, Inc., Hercules, CA, USA). One unit of ALP activity was defined as the amount of enzyme required to liberate 1.0 μmol p-nitrophenol per min. The enzyme activity was recorded as milliunits (mU)/10⁴ cells.

**Real-time polymerase chain reaction (real-time PCR)**

MC3T3-E1 cells were collected on days 3, 7, and 14 of culture. mRNA isolated from the samples was reverse transcribed to yield the complementary DNA (cDNA). Real-time PCR was performed to measure the target mRNA level, as previously mentioned6. PCR primer sequences used in real-time PCR are listed in Table 1. The target mRNA level was calculated using the 2ΔΔCt method. mRNA level of β-actin was used as the internal control.

**Western Blotting**

Total protein concentrations in the cell lysates were quantified using 40-80 μg of protein from each sample. As described previously8, the cell lysates per sample were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, the gels were transferred onto polyvinylidene fluoride (PVDF) membranes. The blots were then blocked and incubated with rabbit polyclonal immunoglobulin G (IgG) antibody or mouse monoclonal IgG antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), specific for Runx2, osterix, type I collagen (Col I), OCN, ALP, iNOS, COX-2, and β-actin (Santa Cruz Biotechnology). β-actin was used as an internal standard. Protein bands were visualized via ECL prime reagents (GE Healthcare, Chicago, IL, U.S.A.). Image J software was used to quantify the band intensity.

**Statistical analysis**

All data represent the results of three independent experiments. The normality of the primary data was confirmed by the Shapiro-Wilk test,

| Target | Primers |
|--------|---------|
| Runx2  | 5'-CACCTGGCTTTGGGAAGAG-3' |
|        | 5'-GCAGTTCCAACGATTCAT-3' |
| osterix| 5'-GGTAGGCTGTCCCATGTGTT-3' |
|        | 5'-AGACGGAGCAGCCAACCTAG-3' |
| Col I  | 5'-AGAAAGGTGTTGACGAGTTG-3' |
|        | 5'-ACACCACTGTTCAAGCTAGG-3' |
| OCN    | 5'-CAGACACCATGAGGACCA-3' |
|        | 5'-AAAGGCCTTGTAGTACACTAG-3' |
| ALP    | 5'-GTGGCGCAAGTTCTAGTTC-3' |
|        | 5'-AGCTCTGAGGCGTTCACAAACAT-3' |
| iNOS   | 5'-CAAAGCTGAACTTGAGGAGA-3' |
|        | 5'-TTTACTCAGTGGCAGAAGCTGA-3' |
| COX-2  | 5'-GCCACCGCTAATCCTGAAACA-3' |
|        | 5'-GCTCAAGGGCCACTGATACTA-3' |
| β-actin| 5'-CATCCGTAAAGACCTCTAGGCAAC-3' |
|        | 5'-ATGGAGGCCACCGATCCACA-3' |

Figure 1. Cell viability and ALP activity of N-APPJ. MC3T3-E1 cells were cultured with APPJ (0, 60, 120, and 180 sec) for 1, 3, 5, 7, 10, and 14 days (cell viability: a, ALP activity: b); *p <0.05, APPJ vs. untreated control.
while the F test or Bartlett test confirmed the homoscedasticity. Differences between the groups were analyzed using either the student t-test or one-way analysis of variance (ANOVA) with Tukey’s multiple comparisons test, or Mann-Whitney U test. The differences were considered statistically significant at \( P < 0.05 \). Statistical analysis was performed using the EZR software (EZR 1.23; Jichi Medical University Saitama Medical Center, Saitama, Japan). The experimental values are expressed the mean ± standard deviation (SD).

**Results**

**APPJ affects ALP activity and cell viability**

To confirm that APPJ affects cell toxicity, we determined the effects of APPJ on the viability of MC3T3-E1 cells and found that APPJ did not affect cell viability (Fig. 1a). Additionally, the ALP activity assay revealed that APPJ (120 sec) significantly increased ALP activity on day 14 of culture compared to that in the untreated control (0 sec) (Fig. 1b). These results suggest that APPJ that is irradiated for 120 sec can be considered as the optimized condition that affects osteoblasts.
Figure 3. Effects of APPJ on gene and protein expressions of ALP, COX-2, and iNOS. MC3T3-E1 cells were treated with or without APPJ (120 sec) for up to 14 days and mRNA expression of ALP (a), COX-2 (b), and iNOS (c). Data are expressed as the mean ± SD of three independent experiments performed in triplicate; *\(p<0.05\), ***\(p<0.001\), APPJ vs. untreated control. MC3T3-E1 cells were treated with or without APPJ (120 sec) for 7 or 14 days. Protein expression was assessed by Western blotting (upper images) and bar graph were performed to determine the protein band intensity of ALP (d), COX-2 (e), and iNOS (f) on 7 or 14 days of culture. The band intensity was measured five times; **\(p<0.01\), ***\(p<0.001\), APPJ vs. untreated control.
Effects of APPJ on the mRNA and protein expressions of Runx2 and osterix

We determined the effects of APPJ on the mRNA and protein expression of transcription factors, Runx2, and osterix on osteoblast differentiation. APPJ increased both the mRNA and protein expressions of osterix on day 7 of culture, compared to in the untreated control (Fig. 2b, c). In contrast, APPJ did not affect the mRNA expression of Runx2 (Fig. 2a).

Effects of APPJ on the mRNA and protein expressions of ECMPs

mRNA and protein expressions of ECMPs (Col I and OCN) were assessed to investigate the effects of APPJ on the bone formation ability in osteoblasts. APPJ increased the mRNA and protein expressions of OCN on day 14 of culture, compared to in the untreated control (Fig. 2d, f). In contrast, APPJ did not affect the mRNA expression of Col I (Fig. 2c).

Effects of APPJ on the mRNA and protein expressions of ALP, iNOS, and COX-2

To validate the effects of the APPJ-derived reactive species (ROS/RNS) on osteoblast differentiation, we assessed the mRNA and protein levels of ALP, iNOS, and COX-2. APPJ increased the mRNA and protein expressions of ALP on day 7 of culture, compared to in the untreated control (Fig. 3a, d). On the contrary, APPJ decreased the mRNA and protein expressions of iNOS and COX-2 on 14 days of culture compared to in the control (Fig. 3b, c, e, f).

Discussion

APPJ finds its application in various departments of the industry. Particularly, argon-APPJ in coagulation devices are routinely used to remove polyps and to stop bleeding during an open surgery in medical treatment. Currently, various APPJ sources are being clinically tested. In this study, indirect APPJ treatment was used because only the long-lived species could reach the target surface. A previous study on indirect APPJ treatment reported that at the target surface, most metastable and ions recombined to form neutral species. However, previous studies have investigated the cell viability or cell toxicity in vitro and described the biological effects of APPJ on osteoblasts. Canal et al. showed that APPJ treated medium causes apoptosis of bone cancer cells (Saos-2), but not the bone cells. Other in vitro studies have shown that treated APPJ with drug or peptide affects cells on the substrate such as poly (L-lactic acid) and hydroxyapatite enhanced osteoblasts cell adhesion. Thus, we investigated the effects of nitrogen-APPJ on the osteoblast differentiation in osteoblast cell line, MC3T3-E1.

Osterix is a major transcription factor containing three C2H2-type zinc-fingers, which act downstream of Runx2. However, in this present study, APPJ did not affect the mRNA expression of the gene encoding Runx2 (Fig. 2a). Several transcription factors that are involved in the osterix-mediated regulation of osteoblast differentiation include calcium-sensitive transcription factor, nuclear factor of activated T cells, cytoplasmic 1, and tumor suppressor p53. These results suggest that osterix plays an important role in osteoblast differentiation. We observed increased mRNA and protein expressions of osterix in response to nitrogen-APPJ (Fig. 2b, c). These results indicated that APPJ affects osteoblast differentiation. Hence, we next determined the effect of APPJ on the mRNA and protein expressions of ECMPs.

OCN is the most abundant non-collagenous protein (about 15%) of low molecular weight, found in bone. It comprises three to four residues of gamma-(gamma)-carboxyglutamic acid. Osteoblasts secrete OCN in the late differentiation stages. In this study, APPJ increased the mRNA and protein expressions of OCN (Fig. 2d, f). In osteoblasts, mineralized nodule formation involves two phases. The first phase of mineralization is the formation of the hydroxyapatite crystals within the matrix vesicles by the surface membrane of osteoblasts. In the second phase, hydroxyapatite accumulates in the extracellular matrix and deposits between the collagen fibrils. Although APPJ did not enhance the mRNA expression of the gene encoding Col I compared to that in the untreated control, it did detect the biological baseline expression level (Fig. 2c).

The ratio of inorganic phosphate (Pi) to inorganic pyrophosphate (PPi), which inhibits hydroxyapatite, holds significant importance in the second step of mineralization. Nucleotide pyrophosphatase/phosphodiesterase 1 (NPP1) produces Pi from nucleotide triphosphates. PPi remains localized within the membranes of the osteoblasts. Gerstenfeld et al. reported that high ALP activity remains associated with extracellular matrix formation in osteoblasts before the initiation of mineralization. APPJ not only increased the ALP mRNA but also enhanced the ALP mRNA and protein expressions (Fig. 1b, 3a, d). Indeed, these results indicated that APPJ enhances osteoblast differentiation that is associated with bone formation in osteoblasts in vitro.

Some previous studies have been reported that both direct and indirect APPJ cause cell death or apoptosis through ROS/RNS in various cells in vitro. Nitric oxide synthase (NOS) generates nitric oxide (NO), which is a short-lived free radical. Bacterial endotoxin or inflammatory cytokines including interleukin-1 (IL-1), tumor necrosis factor (TNF), and interferon-γ (IFN-γ) expressed by various mammalian cells induces iNOS. Thus, we examined the effects of ROS/RNS on APPJ stimulation in osteoblast differentiation. APPJ decreased the mRNA and protein expressions of iNOS (Fig. 3c, f). iNOS has also associated with COX-2 expression and prostaglandin E2 (PGE2) production in various cell types, including osteoblasts. PGE2 is a lipid mediator and belongs to the family of eicosanoids, and nearly all cells produce PGE2. In response to cell-specific trauma, stimuli, and signaling molecules, PGE2 is synthesized from arachidonic acid, via the actions of COX enzymes. In this study, APPJ decreased the mRNA and protein expressions of COX-2 (Fig. 3b, e). IL-1α is known to increase PGE2 production and inhibit mineralized nodule formation and ALP activity in the osteosarcoma cell line ROS 17/2.8. An anti-inflammatory peptide purified from seahorse inhibited effects of 12-O-tetradecanoyl-phorbol-13-acetate on the increased iNOS and COX-2 expression and induced human osteoblastic differentiation, and ALP activity. The current study results were consistent with those of the previous reports. Indirect APPJ with nitrogen may exhibit anti-inflammatory effects via the downregulation of the mRNA and protein expressions of iNOS and COX-2. The majority of the previous in vitro studies reported that direct or indirect APPJ induces apoptosis-mediated cell death. However, in such cases, the exposure time of APPJ has been longer compared to that in the present study. These observations suggested that the usage of APPJ to cells could be expanded depending upon the conditions.

In summary, indirect APPJ with nitrogen induced osteoblast differentiation-related transcription factors (Runx2 and osterix), OCN, ALP activity, and reduced the mRNA and protein expressions of enzymes iNOS and COX-2. Thus, it can be concluded that APPJ affects osteoblast differentiation. The study not only presented the potential of APPJ in the growth suppression of cancer cells or bacteria but also highlighted its applicability in the differentiation of normal cells, including osteoblasts in vitro.
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Conflict of Interest

The authors declare no conflict of interests with respect to the authorship and/or publication of this article.

References

1. Lackmann JW and Bandow JE. Inactivation of microbes and macromolecules by atmospheric-pressure APPI jets. Appl Microbiol Biotechnol 98: 6205-6213, 2014
2. Liebmann J, Scherer J, Bibinov N, Rajasekaran P, Kovacs R, Gesche R, Awakowicz P and Kolb-Bachofen V. Biological effects of nitric oxide generated by an atmospheric pressure gas-plasma on human skin cells. Nicotine Oxide 24: 8-16, 2011
3. Kaneko T, Sasaki S, Takashima K and Kanzaki M. Gas-liquid interfacial plasma producing reactive species for cell membrane permeabilization. J Clin Biochem Nutr 60: 3-11, 2017
4. Ahn HJ, Kim KII, Hoan NN, Kim CH, Moon E, Choi KS, Yang SS and Lee JS. Targeting cancer cells with reactive oxygen and nitrogen species generated by Atmospheric pressure air plasma. PLoS One 9: e86173, 2014
5. Long F. Building strong bones: molecular regulation of the osteoblastic and chondrocytic differentiation. Chem Biol Interact 184: 413-422, 2010
6. Epstein FH, Moncada S and Higgs A. The L-arginine-nitric oxide pathway. N Engl J Med 329: 2002-2012, 1993
7. Nathan C. Nitric oxide as a secretory product of mammalian cells. FASEB J 6: 3051-3064, 1992
8. Orimo H. The Mechanism of mineralization and the role of alkaline phosphatase in health and disease. J Nippon Med Sch 77: 4-12, 2010
9. Gerstenfeld LC, Chipman SD, Glowacki J and Lian JB. Expression of differentiated function by mineralizing cultures of chicken osteoblasts. Dev Biol 122: 49-60, 1987
10. Ryu B, Qian Z-J and Kim SK. Purification of a peptide from sea horse, that inhibits TPA-induced MMP, iNOS and COX-2 expression through MAPK and NF-κB activation, and induces human osteoblast-like cells by cell membrane permeabilization. J Cell Biochem Nutr 60: 3-11, 2017
11. Lee JH and Kim KN. Effects of a nonthermal atmospheric pressure plasma jet on human gingival fibroblasts for biomedical application. Biomed Res Int 2016: 2876916, 2016
12. Patelli A, Mussano F, Brun P, Genova T, Ambrosi, E Michieli N, Mattei G, Scopece P and Moroni L. Nanoroughness, surface chemistry, and drug delivery control by atmospheric plasma jet on implantable devices. ACS Appl Mater Interfaces 10: 39512-39523, 2018
13. Wolf G. Function of the bone protein osteocalcin: definitive evidence. Nutr Rev 54: 332-333, 2009
14. Gesche R, Awakowicz P and Kolb-Bachofen V. Biological effects of nitric oxide generated by an atmospheric pressure gas-plasma on human skin cells. Nicotine Oxide 24: 8-16, 2011
15. Epstein FH, Moncada S and Higgs A. The L-arginine-nitric oxide pathway. N Engl J Med 329: 2002-2012, 1993