Non-Thermal Bio-Compatible Plasma Induces Osteogenic Differentiation of Human Mesenchymal Stem/Stromal Cells With ROS-Induced Activation of MAPK

YING LI¹,², (Member, IEEE), YA JIE LIU¹, SHU BIN WANG³, EUN HA CHOI², AND IHN HAN²

¹Department of Radiation Oncology, Peking University Shenzhen Hospital, Shenzhen Peking University–Hong Kong University of Science and Technology Medical Center, Shenzhen 518036, China
²Department of Plasma Bio-display, Plasma Bioscience Research Center, Applied Plasma Medicine Center, Kwangwoon University, Seoul 01897, South Korea
³Department of Oncology, Peking University Shenzhen Hospital, Shenzhen 518036, China

Corresponding author: Ihn Han (hanihn@kw.ac.kr)

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ABSTRACT The application of non-thermal bio-compatible plasma (NBP) for stem cell differentiation is promising in tissue engineering. However, the differentiation efficiency of NBP treatment on various types of human tissue-derived stem cells and the underlying mechanisms is yet understood. This study is the first time to investigate the role of NBP in inducing differentiation and its potential molecular mechanism by using human bone marrow-derived stem cells (hBMSCs) and human periodontal ligament-derived stem cells (hPDLSCs). Our results showed that NBP promote osteogenic differentiation of hPDLSCs more effectively than hBMSCs under the same treatment condition, indicating a tissue-dependent manner of NBP interacts with stem cells. Furthermore, an increase of intracellular reactive oxygen species (ROS) production of hPDLSCs and antioxidant enzymes activation was observed after NBP treatment. Particularly, mitogen-activated protein kinases (MAPKs) level was also increased and in consistent with ROS level increase. Taken together, this study revealed that with NBP induction, hPDLSCs is a more suitable stem cell source than hBMSCs for bone regeneration and tissue engineering, and ROS-induced activation of MAPKs are possibly involved in the osteogenic differentiation process.

INDEX TERMS Non-thermal bio-compatible plasma, plasma applications, reactive species, stem cell, tissue engineering, osteogenic differentiation.

I. INTRODUCTION

During recent years, non-thermal atmospheric pressure bio-compatible plasma (NBP) has been studied in the medical field for sterilization, anti-cancer, promoting wound healing etc. [1]–[3]. More recent, NBP also emerged as a powerful tool for stimulating progenitor cells to differentiate into osteoblasts, chondrocytes, myocytes, and neurocytes [4]–[8].

NBP generated reactive oxygen species (ROS) and reactive nitrogen species (RNS) are considered to play the main role during NBP interacting with stem cells. Stem cells reside in the specialized niches with low ROS level, while physiological levels of ROS are required to maintain genomic stability [9] and serving as second messengers in stem cell differentiation [10]. The exogenous ROS from NBP generation is one of the possible methods to stimulate the cellular signal pathways in stem cells. However, whether ROS would serve as signaling messengers or could cause oxidative
damage to the cells depends on the delicate equilibrium between ROS production, and their scavenging. The intracellular detoxification proteins are critical system that detoxify xenobiotics by scavenging the excess ROS to maintain redox homeostasis [11], [12].

Mechanically, mitogen-activated protein kinases (MAPKs) are well known for controlling a wide range of cellular process for proliferation, survival, apoptosis and differentiation, especially under oxidative stress, the ROS-responsive MAPK family, consisting of p38, c-Jun N-terminal kinase (JNK), and extracellular signal-regulated protein kinase (ERK) subfamily factors, were activated after NBP treatment [13]–[15].

Adult mesenchymal stem cells (MSCs) as a main cell source for bone tissue engineering, due to their characteristic of self-renewal and multipotency [16], [17], its high efficient differentiation is demanded. MSCs could be easily isolated from a variety of tissue in the human body, such as umbilical cord, bone marrow, adipose tissue, and dental tissues, among others [18]–[20]. The characteristic of human bone marrow-derived mesenchymal stem cells (hBMSCs) has been mostly used for researches on multiple differentiation potential [21]–[24]. However, considering the shortcomings of obtaining hBMSCs of invasive and pain from bone marrow aspiration and a low number of harvested cells, some researches attempt to harvest alternate MSCs from dental-derived tissues, such as periodontal ligament, dental pulp, dental follicles, gingiva and apical papilla [25]–[29]. Between these dental-derived stem cells, human periodontal ligament-derived stem cells (hPDLSCs), which reside in the perivascular space of the periodontium, possess characteristics of MSCs and could be a promising tool for periodontal bone and tissue regeneration [30], [31]. The multipotent differentiation capacity of hPDLSCs was well studied for periodontal tissue regeneration through transplanting hPDLSCs for replacing dead osteoblasts, fibroblasts and cementoblasts, promoting angiogenesis and modulation of the alveolar bone-PDL-cementum structure remodeling [32]–[34]. hPDLSCs-based therapeutics may be a step towards predictable periodontal regeneration and may have alternative potential applications in bone regeneration and maintenance [35].

The potential utility of NBP in bone regeneration has become attractive, however, most studies were limited to the behavior of committed progenitor cell lines. For application of NBP in tissue engineering, the effects of NBP in human tissue-derived stem cells has to be explored. Moreover, the underlying mechanism of interaction between NBP and osteogenic differentiation has not been well understood yet. In order to establish the concept of NBP application in stem cell-based tissue regeneration and find the more suitable stem cell sources for future clinical use, we applied NBP to two kinds of human tissue-derived MSCs, as hBMSCs and hPDLSCs. The effects of NBP on hBMSCs and hPDLSCs was determined and analyzed, more importantly, cell responses and involved molecular pathways during the NBP-induced stem cell osteogenic differentiation process was investigated in this study.

II. MATERIALS AND METHODS

A. PLASMA DEVICE SETUP

The plasma device we used in this study is a typical microdielectric barrier discharge (μ-DBD) type. The device consists of electrodes with a width of 200 micrometer (μm), a thickness of 5 μm and electrode gap distance of 200μm, and aluminum oxide (Al2O3) layer of 1 μm in thickness to prevent electrode hydration during discharge. Electric power for NBP generation was provided by alternating current (AC) power supply (15.4 kHz) using high voltage AC-AC inverter (PNP-1000, Daewkng Electric Company, Seoul, Korea). Nitrogen (N2) and air gas with 1.5 liter per minute (lpm) of flow rate was used as working gases, with 55 milliseconds on-time discharging (duty ratio is around 26.8%). The magnitudes of current and voltage were 13 milliamper (mA) and 500 voltage (V) (root mean square values), respectively. The plasma discharge area was about 35 millimeter (mm) in diameter. The treatment distance is about 2~3 mm from the discharge surface to the media surface (Fig. 1a).

The OES of micro-DBD discharging with N2 and air gas were measured by the spectrometer (HR4000, Ocean Optics, Dunedin, FL), respectively. The intensity of optical emission signals was recorded in terms of wavelength (range from 240 to 400 nm). The surface temperature of micro-DBD is measured using a thermal imager (Fluke, USA) and the concentration of O3 and nitrogen oxides (including nitric oxide (NO) and nitric dioxide (NO2)) during various NBP discharging time measured using a Gastec AP-20 gas sampling pump (Gastec Corp., Kitagawa, Japan) and Gastec detector tubes (No. 18M and 18L for O3, No. 11L for NO and NO2).

B. CELL CULTURE

According to our previous study method [36], human bone marrow tissue and human periodontal ligament tissues were isolated from jaw bone and periodontal ligament, respectively. The tissue was cut into 1~2 mm3 pieces, washed with DPBS, and enzymatically digested for 3~4 hours at 37 °C with 3 mg/mL collagenase type I (Thermo fisher scientific, USA). Samples were filtered using a 100 μm cell strainer, centrifuged at 2,500 rpm for 5 minutes, and the pellets were collected and cultured in vitro.

Cells were maintained in Minimum Essential Medium Eagle alpha-modified media (α-MEM, HyClone Laboratories, Logan, UT, USA), supplemented with 15% fetal bovine serum (FBS, Gibco, NY, USA), 1% antibiotics (penicillin/streptomycin, Welgene, Korea), basic fibroblast growth factor (bFGF, Corning, USA), insulin (Sigma, USA), GlutaMAX-1(100X, Gibco, Japan), MEM non-essential amino acids (100X, Gibco, USA) and ascorbic acid (Sigma, Japan). This media was used as basic growth media. Cells were kept in a humidified atmosphere of 5% CO2 at 37°C. The logarithmic phase the cells were detached by trypsin (ScienCell research laboratories, CA, USA) and seeded in 35 mm2 dishes with the density of 5 × 104 cells in each dish.

Y. Li et al.: NBP Induces Osteogenic Differentiation of Human Mesenchymal Stem/Stromal Cells
To induce osteogenic differentiation, 25 mg/ml ascorbic acid-2-phosphate, 5 mM β-glycerophosphate was supplemented to basic α-MEM growth media, and the media was designated as osteogenic induction (OI) medium.

C. CHARACTERIZATION OF CELLS
To determine the surface epitope profile of the isolated cells for MSCs surface markers, flow cytometric analysis was performed. hBMSCs and hPDLSCs from passage 2 were provided with 5 μl of fluorescein isothiocyanate (FITC)-conjugated antibody and 5 μl of blocking buffer and incubated at 4°C for 20~25 minutes in a dark place. The cells were added with 1 ml washing buffer consisting of PBS supplemented with 1% FBS and centrifuged at 1200 rpm. The pellet was suspended in 300~500 μl washing buffer and analyzed by flow cytometry (BioRad, USA). In this study, IgG was used as isotype control. The following antibodies were used to stain the cells: CD44, CD73, CD105, and the negative cocktail of CD45/CD34/CD11b/CD19/HLA-DR (Becton, Dickinson and Company, USA).

D. CELL VIABILITY DETECTION
[3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] (MTS) was used as a reagent to detect cell metabolic activity (viability). hPDLSC and hBMSC cells were seeded into 35 mm² culture dishes at a concentration of 5×10⁴ cells/dish in 2 ml of α-MEM basic media and incubated for 24 h prior to each experiment. Then the cells were treated by NBP for 1, 3, 5, 10, 20 min, respectively. N₂ and air gas was used as working gases. 24 h after treatment, MTS solution was added to each of the plates and incubated for 3 hours. The absorbance of the optical density (OD) was detected at 490 nm using a microplate reader (Biotek, VT, USA). All assays involved at least three independent sets of tests. The results are exposed as viability percentages to control (%).

E. COLONY FORMING UNIT-FIBROBLAS (CFU-F) ASSAY
CFU-F assay is used to assess the isolated hMSCs as the ability of a single cell to form a colony. hBMSCs and hPDLSCs were plated in a 6-well plate as the density of 1000 cells in
each well, after 24 hours incubation the cells were treated with NBP for 3 and 5 min, respectively. Then the cells were kept in the incubator to grow for 12 days until the colonies (defined as a group of cells more than 50) were formed. The cultures were terminated and stained with 2% (v/v) crystal violet (Sigma-aldrich, USA) for colony visualization. Then the colonies figures were captured by a scanner (Epson, Japan).

F. ALIZARIN RED S (ARS) STAINING
ARS staining is used to verify the state of osteogenic differentiation in terms of extracellular matrix mineralization. ARS dye can bind with calcium ions deposited in extracellular matrix to form an ARS-calcium complex as red nodules. hBMSCs and hPDLSCs were seeded in a 6-well culture plate at a cell density of $5 \times 10^4$ cells/well in basic $\alpha$-MEM media, 24 h after seeding the cells were treated by NBP 3 and 5 min, respectively. 24 h after NBP treatment, the basic media was changed to OI media. And then cultured for 5, 10 and 15 days, respectively, and followed by ARS staining at each time point. The cells were fixed with 4% paraformaldehyde (Biosesang, Korea) for 1 h at room temperature. After being washed with distilled (DI) water, they were stained with 60 mM ARS solution at pH 4.2 for 30 min at room temperature and followed by washing process with DI water to remove the unbound dye. The images of ARS staining were recorded using a scanner (Epson, Japan). For a quantitative calcium deposition, the stained cells were dried in the air and eluted with 5% acetic acid at room temperature for 20 minutes. The solution in each well was transferred into a 96-well plate and the absorbance was measured at 405 nm using a microplate, and data from 3 independent experiments are presented as the means ± SD.

G. QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION (qRT-PCR)
The osteogenic related gene expression levels were estimated by qRT-PCR. hBMSCs and hPDLSCs were seeded at a density of $5 \times 10^4$ cells/well in basic $\alpha$-MEM media. 24 h after seeding, the cells were treated by NBP 5 min. 24 h after NBP treatment, the basic media was changed to OI media, then cultured for 7 days after treatment and followed by the qRT-PCR process same as mentioned above. The osteogenic related gene expression, we prepared samples with NBP treatment of 3, 5 and 10 min, respectively, non-treated as a control group. The cells were incubated for 7 days at treatment and then followed by the qRT-PCR process same as mentioned above.

| Molecule                        | Sequences                  |
|--------------------------------|----------------------------|
| Alkaline phosphatase (ALP)     | F5′-ATGGGAATGGGTTGCACCACA-3′ |
| Runt-related transcription factor 2 (Runx2) | R5′-CCACGAAAGGGAACACTTGCT-3′ |
| Osteocalcin (OCN)             | F5′-GGGACCAAAAGGAAAGACATC-3′  |
| Collagen 1 (COL 1)            | R5′-GAGACCTCTCTGCTGCAACAAG-3′  |
| Osterix                        | F5′-ATGGGGAGGTTGAAGGTGCG-3′   |
| GAPDH                          | R5′-GGTGTTTGGATGCGAACAATA-3′  |

TABLE 1. The primer sequences for quantitative real-time polymerase chain reaction (qRT-PCR).

I. ROS AND RNS DETECTION
Extracellular $H_2O_2$ and NO level were detected using the QuantiChrom Peroxide assay kit and Nitric oxide assay kit (BioAssay System, CA, USA), respectively. Serum-free $\alpha$-MEM media was treated with NBP for 1, 3, 5, 10, and 20 min, respectively, and non-treated group is as blank. Immediately after NBP treatment, the media was detected for $H_2O_2$ and NO level following each kit instructions.
cells were washed with phosphate buffered saline (PBS), intracellular ROS was determined by loading with 20 μM H$_2$DCFDA and incubated for 20 min in dark condition. The cells were then washed twice with PBS to remove the extra H$_2$DCFDA, and cells were collected for detecting on flow cytometry. To confirm the change with short incubation time, cells were treated with NBP for 5 min and incubated for 2, 4, 6, 8, 12, and 24 h, respectively, and the intracellular ROS was determined by H$_2$DCF DA using same procedure.

**J. WESTERN BLOT**

In order to extract protein, hBMSCs and hPDLSCs were treated with NBP for 5 min and incubated for 2, 4, 6, and 8 h, cells were then collected. For Western Blot analysis, the proteins were separated using 4-15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (BIO-RAD), and then was transferred to nitrocellulose membrane (Millipore, MA, USA). After blocking with 0.5% bovine serum albumin (BSA), the membrane was incubated with respective primary antibodies specific for glutathione S-transferase (GST), glutathione S-transferase alpha 1 (GST A1), superoxide dismutase (SOD) (all from Abcam, USA) and p38, phospho-p38 (p-p38), c-Jun N-terminal (JNK), p-JNK, extracellular signal-regulated kinases (ERK), p-ERK, GAPDH and β-actin (all from Cell Signaling, MA, USA). Subsequently, horseradish peroxidase (HRP)-conjugated secondary antibodies of goat anti-rabbit and goat anti-mouse (AbD Serotec, USA) were used to detect the corresponding primary antibody. Immunoblot was developed using enhanced chemiluminescence (Thermo Scientific, USA).

**K. STATISTICAL ANALYSIS**

Graphs are represented by the mean ± standard deviation of replicates. Student’s t-test was used for establishing significance between data points. The statistically significant differences were based on *p* < 0.05, **p** < 0.01, and ***p*** <0.001. Graphing, calculation and statistical analysis were performed using GraphPad Prism version 7.00 (GraphPad Software, USA). All assays involved at least three independent sets of tests for each group.

**III. RESULTS**

**A. PLASMA DEVICE PARAMETERS**

Fig. 1(a) showed the schematic diagram of μ-DBD device from the front view and the bottom view. NBP was generated with a breakdown voltage of 500 V and an electric current of 13 mA. The OES measurement revealed that discharging with N$_2$ gas achieving higher N$_2$ emission from second positive system (300-400 nm, with the dominant peak at 337.1 nm) and NO signals (240-280 nm) (Fig. 1b), as compared to air discharging OES (Fig. 1c). In Fig. 1(d), the surface temperature of micro DBD was below 30 degree Celsius even after 10 min continuous discharging. The discharging of micro DBD using air gas generated large amount of ozone while using N$_2$ gas produced low level ozone (Fig. 1e).

In addition, when discharging using N$_2$ gas, NO+NO$_2$ concentration increased as discharging time proceeding (Fig. 1f), which is consistent with the OES results of dominant N$_2$ exited peaks. It should be mentioned that the discharge chamber is not vacuum sealed. Thus due to the diffusion of the surrounding air, there is tracing amount of O$_2$ in the discharge region, which is the source of oxygen. The effects of N$_2$ and air gas on cell metabolic ability was further determined.

**B. CELL CHARACTERIZATION**

As shown in Fig. 2 (a), both isolated hBMSCs and hPDLSCs were positive for MSCs membrane surface markers of CD44, CD73 and CD105 (>80%) and lack of expression of CD34, CD45, CD11b, CD19, and HLA-DR surface molecules (<5%), which confirmed the isolated cells are mesenchymal stem cells. In Fig. 2(b), for hBMSCs both N$_2$ and air gas NBP treatment until 5 minutes the cell viability decreased less than 10%, but more than 10 minutes NBP treatment was toxic hBMSCs, especially after 20 minutes air gas NBP treatment, cell viability decreased around 50%. The viability of hPDLSCs was not affected by N$_2$ or air gas NBP until 20 minutes of treatment. Considering the 50% toxicity of air gas NBP to hBMSCs, we considered using N$_2$ as the working gas in the following experiments.

MSCs were originally referred to as fibroblastoid colony-forming-unit fibroblast cells because one of their characteristic features is adherence to tissue culture plastic and generation of colonies when plated at low densities [37]. The CFU-F assay showed that NBP treatment reduced the colony forming ability of hBMSCs and hPDLSCs (Fig. 2c). Especially for hBMSCs, the colony forming ability of NBP treatment of 3 and 5 min was significantly lower than that of the control group. It is worth noting that in the 5 min treatment group of hBMSCs, although there are only few number of colonies observed, the cells were still survive in the culture dish. 5 min NBP treatment only inhibited the colony forming ability of hBMSCs, but did not induce toxicity on cells, which was consistent with viability assay of 5 min treatment in Figure 2 (b). Whereas, for hPDLSCs, the number of colonies in the NBP 3 and 5 min treatment groups decreased slightly, indicating that NBP had little effect on the colony forming capacity of hPDLSCs. Taken together, Figure 2 revealed that both hBMSCs and hPDLSCs belong to MSCs, but hPDLSCs had stronger survival and colony forming ability than hBMSCs under NBP treatment.

**C. OSTEOGENESIS WITH NBP AND CONDUCTIVE MEDIA**

In order to detect the effect of NBP on the osteogenic differentiation of hBMSCs and hPDLSCs under osteogenic-inducing condition, calcium deposition during bone matrix mineralization was determined by ARS staining with red nodules. The results showed that NBP had different effects on osteogenic differentiation of hPDLSCs and hBMSCs. Facilitation of matrix mineralization by NBP was shown in a treatment time and incubation time dependent manner in hPDLSCs. Particularly, after 15 days culturing, the mineral calcium...
FIGURE 2. Characterization of isolated cells and NBP effects on cell ability. (a) Flow cytometry results of mesenchymal stem cells (MSCs) markers CD44, CD73, CD105 and CD34. The black lines represent isotype control IgG expression and red lines depict the marker expression. (b) MTT assay on hBMSCs and hPDLSCs with N2 and air gas discharging. Cell metabolic ability was detected at various treatment time of 1, 3, 5, 10 and 20 min, respectively. The relative viability of hBMSCs and hPDLSCs were decreased as a NBP treatment time-dependent manner, while hBMSCs were more vulnerable than hPDLSCs with long time treatment (10 and 20 min). (c) Colony forming unit-fibroblast (CFU-F) assay of hBMSCs and hPDLSCs with 3 and 5 min treatment using N2 gas. The effect of NBP treatment on colony forming ability of hBMSCs was greater than that of hPDLSCs. The results are representative of at least 3 independent experiments. Data results correspond to mean ± standard deviation. The results revealed that both hBMSCs and hPDLSCs were MSCs, but hPDLSCs had stronger survival and colony forming ability than hBMSCs under NBP treatment.

FIGURE 3. NBP co-treatment with osteogenic inductive medium promoted osteogenesis in hPDLSCs than in hBMSCs by facilitating extracellular mineralization (a) and (b), and up-regulation of osteogenic gene expression (c). (a) ARS staining for calcium deposition of hBMSCS and hPDLSCs treated with NBP for 3 and 5 min, respectively. ARS staining with red nodules indicated more calcium deposition and higher extracellular mineralization in cells. (b) Quantification of calcium deposition through the elution of ARS staining, hPDLSCs had more calcium deposition with co-treatment. (c) Real-time PCR analysis for osteogenic genes expression of ALP, OCN, Runx2, Osterix and COL 1 in hBMSCs and hPDLSCs. Cells were treated with NBP 5min and incubated for 1, 7 and 14 days, respectively. Data results correspond to mean ± standard deviation.

deposition in hPDLSCs increased 1.3-fold of 3 min and 1.5-fold of 5 min treatment with NBP, as compared to non-treated group at 15 days. However, in hBMSCs the mineralized calcium deposition was only shown increasing in control group according to incubation time, NBP treatment groups was not showing increasing pattern (Fig. 3a and 3b).
Y. Li et al.: NBP Induces Osteogenic Differentiation of Human Mesenchymal Stem/Stromal Cells

**Figure 4.** NBP promoted osteogenesis in hPDLSCs than in hBMSCs by up-regulation of osteogenic markers expression and cell morphology change. (a) Real-time PCR analysis of osteogenic gene (ALP, Runx2, Osterix and Osteocalcin) expression in hBMSCs and hPDLSCs. Cell morphology of (b) hBMSCs and (c) hPDLSCs were identified by staining of actin with Alexa Fluor 488-phalloidin (green) and nuclei are counterstained with Hoechst 33342 (blue) and merged together with two channels (merged). Scale bar = 50 μm. Cells were treated with NBP for 3, 5, and 10 min, respectively, and repeated treatment on day 0, 2, 4, and at day 5 cells were collected for real-time PCR and immunofluorescence staining analysis. NBP induced higher up-regulation of osteogenic maker and typical osteoblast morphology in hPDLSCs as compared to hBMSCs, revealing that NBP promoted osteogenic differentiation in hPDLSCs, rather than in hBMSCs.

Furthermore, the gene expression of osteogenic markers, ALP, Runx2, Osterix and osteocalcin was significantly increased by NBP 5 min treatment after 14 days incubation, as for ALP increased 5.7 fold, Osterix 5.3 fold, OCN 4.6 fold, Runx2 2.2 fold (Fig. 3c), respectively. For hBMSCs, the expression was also increased at some level, but not as significant as in hPDLSCs, especially at 14 days culture. The similar pattern was not observed in COL 1 expression. These results indicated that co-treatment of NBP and osteogenic inducitive medium promoted osteogenic differentiation of hPDLSCs, rather than hBMSCs.

**D. OSTEOGENESIS WITH NBP**

Further to detect the NBP effect alone on osteogenesis of hMSCs, osteogenic markers were examined after 7 days incubation with NBP treatment of 3, 5, and 10 min, respectively. The results revealed the involved role of NBP during the osteogenic differentiation of hBMSCs and hPDLSCs. Compared with hBMSCs and hPDLSCs, as shown in Fig. 4(a), osteogenic specific marker of ALP, OCN, Runx2, Osterix increased depend on treatment time within 5 minutes treatment, especially 5 minutes treatment could induce highest gene expression. For 10 min NBP treatment group, gene expression decreased in hPDLSCs. Compared with hBMSCs, NBP treatment is more effective to promote osteogenic differentiation in hPDLSCs. Cell morphology was also observed during osteogenesis using confocal microscope. Undifferentiated hMSCs appeared fibroblast-like shape as shown in control group in both Fig. 4(b) and (c). Interestingly, after 3 and 5 min NBP treatment, the morphology of hPDLSCs changed from spindle to typical osteoblast polygonal shape, especially in 5 min treated group, marked with red arrows in Fig. 4(c). In addition, the differentiated hPDLSCs concomitantly presented a changed distribution of actin fibers compared to control group of hPDLSCs (Fig. 4c). However, this typical cell morphology change was not observed in hBMSCs as shown in Fig. 4(b). This finding revealed that NBP could induce the osteogenic differentiation of hPDLSCs rather than hBMSCs.
E. INTRACELLULAR ROS LEVEL

As shown in Fig. 5(a) and (b), extracellular H$_2$O$_2$ and NO level increased in a NBP treatment time-dependent manner, while intracellular total ROS level in hBMSCs was decrease and in hPDLSs was no significant change 24 h post treatment (Fig. 5c). Further to confirm the ROS level change within 24 h, we detected intracellular ROS level at 2, 4, 6, 8, 12, and 24 h post treatment, respectively. Interestingly we found that for hBMSCs, ROS level decreased after treatment until 24 h, however, for hPDLSs, ROS level increased to peak at 8 h post treatment, and then recover back to control level until 24h (Fig. 5d).

F. ACTIVATION OF MOLECULAR SIGNAL PATHWAY BY NBP

In Fig. 5 (e), NBP induced an increase of GST and GSTA1 enzymes after 4 h treatment, and keep increasing and significant higher than control until 12h, while a transient increase of SOD2 at 4h and then started to decrease. Meanwhile, mitogen activated protein kinases (MAPKs) family (p38, ERK and JNK) was also activated after 4 h treatment. Activated form of p38 and ERK increased significantly and reach to peak value at 8h, JNK only showed transient increase at 4h. Moreover, phosphorylated p38 sustained high expression until 12 h as compared with the control group. These results implied that a high p38 activity was associated with...
the signal transduction for hPDLSCs to differentiate into osteoblasts.

IV. DISCUSSION
Recent publications focused on the killing effect of NBP on microorganisms and cancer cells. Besides, the potential of NBP on facilitation wound healing, melanogenesis and osteogenic differentiation of stem and progenitor cells has gradually emerged. This study for the first time investigated NBP effects on osteogenic differentiation in two kinds of human tissue derived mesenchymal stem cells. The results revealed that NBP has the capacity to induce MSCs differentiate into osteoblast even without the presence of chemical inducting factors, and the induction effect showed a tissue-dependent manner, as hPDLSCs with more effective osteogenic differentiation than hBMSCs. Moreover, we found that the activation of antioxidant enzymes and MAPKs may also involve in NBP induced osteogenesis of hPDLSCs.

First of all, we established the optical condition for NBP treatment on stem cell by cell viability tests. The results showed that NBP effect on cell viability as a dose-dependent manner and until 5 min treatment induced no cytoxicity to both hBMSCs and hPDLSCs, although it exhibited cancer cell killing effect in our previous study [38]. It has been known that plasma effects on cells depend on plasma doses and cell types, while intermediate doses could induce cell proliferation, growth factor release as well as apoptosis in cancer cells, and high doses causing normal cell death, this is consistent with other published literatures [39]–[41]. The colony forming ability is a special characteristic of stem cells and to exhibit the adaptability and independent survival ability of a single cell under culture condition [42]. In our study, the CFU-F assay showed a more obvious decrease of colony forming ability of hBMSCs than hPDLSCs with NBP 3 and 5 min treatment, indicating that the cellular function of hPDLSCs was more stable under NBP treatment.

The further osteogenic differentiation study showed a more suitable differentiation state in NBP-induced osteogenesis of hPDLSCs, which is characterized by high amount of calcium deposition and up-regulated osteogenic gene expression with a combination treatment of osteoinductive supplements and NBP. Besides, when hPDLSCs were treated with NBP treatment alone, the osteogenic marker genes expressed significant high than that in hBMSCs, and also a typical osteoblast morphology of polygonal change in hPDLSCs was observed.

Initially, NBP has been shown to have potential to induce osteogenic differentiation, even though NBP treatment is less effective than combined osteogenic differentiation inducers [4]. The findings that osteogenic differentiation in hPDLSCs with extracellular matrix maturation, osteogenic gene expression and polygonal morphology change suggests that NBP treatment is an effective method to induce osteogenic differentiation in hPDLSCs. Additionally, researches also showed that NBP can induce stem/progenitor cells to differentiate into neurons [43], myocytes [7] and osteoblasts [13], which are in consistent with our results to confirm the effect of NBP on stem cell differentiation.

It has been well known that the most dominant functional factors during interaction between NBP and stem/progenitor cells are the various RONS generated by NBP. For example, NO· radical acts as a neurotransmitter in nerve system [44], and also play an important role in neuronal differentiation regulation [45]. Similarly, NO· also significantly increased during osteogenic differentiation by the synthesis of endothelial NO· synthase (eNOS), indicating the regulation role of NO· in osteogenesis [46]. In our study, the extracellular detection showed an increase of NOx and H2O2 in the NBP treated media, which is in consistent with the result of Elsaadany et al. [5], with NBP treatment, ALP enzyme activity significantly increased. Moreover, Steinbeck et al. [4] found that the concentration of O2− and H2O2 in another osteogenic cell line (MLO-A5) increased immediately and 1 h after NBP treatment, and then returned to the initial level after 24 h. In our study, the similar pattern was also observed as intracellular ROS level in hPDLSCs reached to peak value at 8 h and then returned to the initial level after 24 h. It can be speculated that elevated level of intracellular ROS is associated with the increased number of exogenous ROS-stimulated mitochondria and more reactive species generation, while the decreasing of ROS level indicated the activation of antioxidant defense system to automatically regulate intracellular ROS levels and maintain homeostasis of intracellular environment.

Enzymatic and non-enzymatic antioxidants in intra- and extracellular space are the main mechanism to regulate the balance of intracellular redox homeostasis. Therefore, ROS production and antioxidants activity must be tightly controlled to maintain the homeostasis of intracellular redox status, and the proper amount of ROS can be a second messenger to mediate signal pathway without excessive ROS induced intracellular oxidative stress [47]. Glutathione (GSH) conjugation is the primary phase II reaction. The glutathione S-transferase (GST) and its subfamilies catalyze the conjugation of thiol group of GSH and are related with cancer susceptibility and patient survival [48], and are key enzymes that are responsible for the high-capacity metabolic inactivation of electrophilic compounds and toxic substrates, participating in the detoxification of xenobiotics and limiting oxidative damage [49]. Superoxide dismutase (SOD) mainly catalyze the conversion of O2− radicals to H2O2, and further decomposed into harmless H2O and O2 by hydrogen peroxide (catalase) or peroxidase.

The interaction between NBP and hPDLSCs started with generation of large amount of ROS, RNS and organic molecules taking place at the gas-liquid interface or in the bulk liquid [50], and finally existed in a liquid phase are mostly long-lived and stable species including H2O2, NO2 and NOx, etc. Many effects of NBP on biological cells and tissues are mediated by these RONS [51]. In our study, cells were kept in the medium and directly treated by NBP. In the medium, H2O2 and NOx level were significantly
elevated after NBP treatment, these species then induced a rising of intracellular ROS level of hPDLSCs at 8 h after treatment. However, 4 h after NBP treatment, the antioxidants GST, GST A1 and SOD were activated to scavenge excessive ROS, and the ROS level in hPDLSCs return to control level after 24 h. In addition, we hypothesized that a significant decrease of intracellular ROS in hPDLSCs at 4 h may associate with the transient increase of SOD. These results indicate that activation of the antioxidant enzymes regulates intracellular redox reactions and maintained the cell survive and cellular functions in hPDLSCs.

During the intracellular redox balance process, we also found that p38, JNK and ERK were elevated during incubation time, and especially p38 was significantly increased at 4 h until reach to peak value at 8 h and sustained until 12 h. Other studies have shown that NBP-induced elevation of intracellular ROS can activate MAPKs signaling, including apoptosis signaling pathways in a variety of cancer cells [52]–[54] and in osteogenic differentiation, indicating p38 is a ROS sensitive molecules, especially under NBP treatment.

In summary, our study is the first to investigate the effect of NBP on osteogenic differentiation of of hMSCs derived from two human tissues. By comparing the osteogenic differentiation, we found that the effect of NBP on osteogenic differentiation is tissue-dependent, and the stem cells derived from periodontal ligament have more pronounced osteogenic differentiation under the induction of NBP. Our findings suggested that NBP is a potential strategy to promote the osteogenic differentiation of stem cells, which lays a foundation for the application of NBP in bone regeneration and tissue engineering.

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**SHU BIN WANG** received the M.B.B.S. degree in clinical medicine from Dr. Norman Bethune Medical University, in 1990, the M.S. degree in oncology from Harbin Medical University, in 1997, and the M.D. and Ph.D. degrees in pharmacology from the Peking Union Medical College, in 2001.

He is routinely involved in clinical work and also research design. As a medical doctor, he had worked as an Oncologist with Heilongjiang Cancer Hospital (and Tumor Hospital of Harbin Medical University), from 1990 to 1998. From 2001 to 2013, he worked as a Deputy Chief Physician and the Deputy Director of the Oncology Department, Shenzhen People’s Hospital. He is currently the Director of the Oncology Department, Peking University Shenzhen Hospital. He has published more than 50 academic articles, mainly a series of research on the mechanism and clinical reversal of chemotherapy resistance of breast cancer, ranking the national leading level in the field of tumor resistance. He had in charge of several project works and is active to instruct students for research work. His research interest includes application of non-thermal atmospheric pressure plasma to reverse the treatment sensitivity of various chemo- and radiotherapy-resistant cancers.

**EUN HA CHOI** received the Ph.D. degree from the Korean Advanced Institute of Science and Technology, in 1987. He has participated to high power pulsed plasmas, high power microwave (HPM) generation, focused and gas filled ion sources, plasma display panels, and non-thermal atmospheric pressure plasma field with more than 500 SCI journal publications. During these research works, he developed many useful nonthermal atmospheric pressure plasma sources, which inevitably leads to new research area called plasma bioscience, medicine, and agricultures.

**IHN HAN** focused on researches on plasma biosciences and medicines on fundamental interactions of nonthermal bioplasma and biological cells/materials, such as stem cells, cancer cells, and biomaterials in tissue engineering. She is especially experienced on the research of mesenchymal stem cells, including isolation, characterization, and differentiation. She has published around 20 peer-reviewed articles in prestigious journals. She participated and co-directed organization of many times the international workshops and symposium for plasma biosciences and medicines. Her current research interests include cancer therapy, stem cell differentiation, regenerative engineering, and biomaterials in tissue engineering. She received the Young Research Award from Functional Materials Society, in 2018, in recognition of notable and outstanding performance in research development of functional materials.

His current research fields are plasma biosciences and medicines on fundamental interactions of nonthermal bioplasma and biological cells developing various plasma sources and producing about 430 SCI articles and 50 patents in the plasma bioscience area. His plasma bioscience works in last 8 years are bioplasma source development and analysis; micro-DBD, and plasma jets for plasma medicine and agriculture; bioplasma interactions with biological cells and cancer cells; OH measurement in bioplasma at atmospheric pressure; plasma density and temperature measurement in bioplasma at atmospheric pressure; OH generation mechanism in liquids induced by bioplasma at atmospheric pressure; and interactions between plasmas and biomaterials for dental applications. Organizing world-wide networking of scientists, he is actively working on plasma bioscience, medicine, and agricultures, in which he is a Chairman of the international symposium International Symposium of Plasma Bioscience (ISPB) and a Co-Chairman of AEPSE, ICMAP, and IFFM. In fact, as the Director of the PBRC and APMC, last 8 years, he organized about 22 times the international workshops and symposium for plasma biosciences and medicines. Also, he was working as a Chairperson of the Asian Joint Committee Member, Applied Plasma Science and Engineering, for plasma science networking and development from 2016 to 2017.