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Non-Thermal Atmospheric Pressure Plasma-Conditioned Root Dentin Promotes Attraction and Attachment of Primary Human Dental Pulp Stem Cells in Real-Time Ex Vivo

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Abstract: This study investigated if non-thermal atmospheric pressure plasma (NTAPP) treatment of root dentin surfaces promotes human dental pulp stem cell (hDPSCs) adhesion. Freshly extracted human single-rooted teeth ($n = 36$) were decoronated and cut (first vertically, then horizontally) into root dentin slices (3 mm thick). Primary hDPSCs cultures were seeded onto slices randomly assigned to pretreatment groups ($n = 9$/group): NaOCl (1.5%), EDTA (17%) then NTAPP (Group I); NaOCl then NTAPP (Group II); NaOCl then EDTA (Group III); and NaOCl alone (Group IV). Cell viability and proliferation were measured using MTT assay with log-linear statistical analysis. Cell attachment and spreading morphologies on dentin slices ($n = 3$/group) were examined through scanning electron microscopy. Early cell adhesion events and subcellular activities were observed in real time by live-cell imaging through holotomographic microscopy. Cell viability and proliferation were significantly higher on NTAPP-treated dentin ($p < 0.05$), without interactions with EDTA ($p > 0.05$). The attachment, spreading, extensions and multiple layers of hDPSCs were heightened on NTAPP-treated dentin. Cell adhesion, spreading, and dentinal tubule penetration were hastened on NTAPP-treated dentin surfaces in real-time, with elevated subcellular activities and intracellular lipid droplet formation. NTAPP-treated root dentin surfaces support enhanced cellular responses, potentially promoting pulp-dentin regeneration.

Keywords: dentin; human dental pulp stem cells; live-cell imaging; MTT assay; non-thermal atmospheric pressure plasma; scanning electron microscopy

1. Introduction

Non-thermal atmospheric pressure plasma (NTAPP) involves partially ionized gas containing electrically charged particles at atmospheric pressure. These include reactive oxygen and nitrogen species that induce various physiological effects, with promising clinical and preclinical applications in plasma medicine. Recent developments include direct and indirect applications of NTAPP for microbial biofilm eradication, tissue disinfection, wound healing, tissue rejuvenation, and surface modification of biological scaffolds. In dentistry, NTAPP has been studied for periodontal pocket decontamination, adhesion, caries treatment, root canal disinfection, implant surface treatment, and tooth bleaching [1,2].

In regenerative medicine, NTAPP may allow a versatile range of applications involving the manipulation of various cell types and processes, including stem cell attachment, proliferation, differentiation, and even apoptosis [3]. Tan et al. reported evidence of NTAPP improving stem cell attachment, proliferation, and differentiation [3]. It activated proliferation of various mesoderm-derived human stem cells [4], and enhanced proliferation of adipose tissue-derived stem cells without affecting their stem cell properties [5]. Yet, direct and indirect effects of NTAPP applications on human dental pulp stem cells (hDPSCs) have not been elucidated. These well characterized cell population with mesenchymal marker expression and potent multilineage differentiation are essential for tooth pulp-dentin regeneration in dentistry [6–9].

Pulp-dentin regeneration is both conceptually and clinically based on a cell homing approach to recruit endogenous stem cells into root canal system. It begins with cell recruitment, attachment, proliferation, and differentiation on root dentin surfaces, for which dentin conditioning is important. Conditioning with ethylenediaminetetraacetic acid (EDTA) alters chemical composition, topography, and wettability of root dentin [10,11], and thereby affects stem cell adhesion and proliferation on dentin surfaces [12–14]. Similarly, NTAPP was found to enhance dentin wettability and surface energy, significantly improving protein adsorption and cellular attachment [15]. However, the effects of NTAPP treatment on root dentin as cellular niche in regenerative endodontics are not fully understood.

The purpose of this study was to investigate the effects of NTAPP treatment of human root dentin surfaces in the recruitment, attachment and growth of hDPSCs. Cellular attachment and growth of primary hDPSCs on NTAPP-treated root dentin surface was quantitatively and qualitatively investigated, and initial recruitment and attachment events were monitored in real-time to find out subcellular changes.

2. Materials and Methods

2.1. Primary Cell Cultures

hDPSCs were isolated from healthy human adult teeth. Following extraction, teeth were cleaned and their crowns separated with sterile water-cooled high-speed diamond burs. Pulp tissues were gently harvested by forceps and digested in 3 mg/mL collagenase type I (Sigma, St. Louis, MO, USA) at 37 °C for 60 min. Tissue digests were passed through a 70-μm cell strainer (Becton/Dickinson, Franklin Lakes, NJ, USA) to obtain single-cell suspensions that were seeded in 60- or 100-mm culture dishes with a control medium containing α-minimum essential medium (α-MEM; Life Technologies/GIBCO BRL, Gaithersburg, MD, USA) supplemented with 20% fetal bovine serum (FBS), 2 mM L-glutamine, 100 units/mL penicillin-G, 100 μg/mL streptomycin and 0.25 μg/mL fungizone (Gemini Bio-Products, Inc., Woodland, CA, USA), and maintained in 5% CO_2 at 37 °C. As colonies formed, cells were initially passed (1:3 ratio) at 80% confluence, and then continuously passed when confluent. These cells were passed, harvested, and stored in liquid nitrogen.

2.2. Root Dentin Slices

Human single-rooted freshly extracted teeth (*n* = 36) were cleaned by ultrasonic scaler (SH-2140, Saehansonic, Seoul, Korea) and stored in 0.5% sodium azide. Following decoronation, the roots were cut (first vertically then horizontally) and ground to achieve a smooth surface to make standardized root dentin discs (7 × 3 × 3 mm^3) slices of 3 mm thickness. The dentin slices were autoclaved at 121 °C for 15 min. Sterility was confirmed by anaerobic incubation at 37 °C overnight in BHI, trypticase soy, or MRS culture broth, followed by plating onto agar plates containing these respective culture media maintained anaerobically at 37 °C overnight. Dentin slices were randomly assigned (*n* = 9/group) for conditioning as follows: Group I, NaOCl (1.5%, 10 mL/5 min), then EDTA (17%, 10 mL/5 min), followed by NTAPP; Group II, NaOCl, then NTAPP; Group III, NaOCl, then EDTA; Group IV, NaOCl alone (control).

2.3. Non-Thermal Atmospheric Pressure Plasma (NTAPP) Treatment

Figure 1 shows schematic diagram of the experimental setup with air plasma jet system and a typical optical emission spectrum. The applied power for generating plasma was lowered to 5 W at 50 kHz. The amount of precursor monomers was set at 100 standard cubic centimeters, the flow rate of air as the working gas was set at 15 L/min and the treatment time was fixed at 1 min [16].
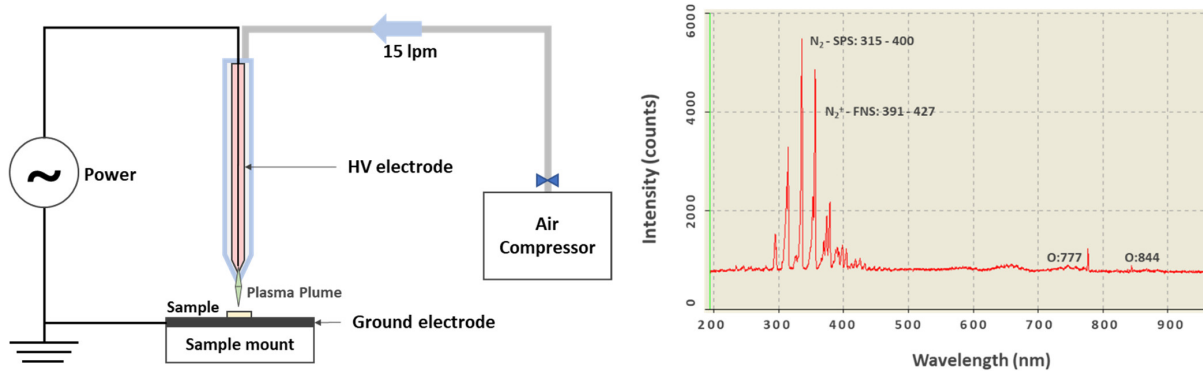

Figure 1. Schematic diagram of experimental setup with air plasma jet system (**left**) and the optical emission spectrum. (**right**). The spectrum is dominated by emissions from the second positive system (SPS) of molecular nitrogen (N_2 at 315–400 nm), the first negative system (FNS) of N_2^+ (391–427 nm), and atomic oxygen (777 and 844 nm).

2.4. Cell Viability and Proliferation

hDPSCs viability and proliferation on dentin were measured by MTT assay (reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to a purple formazan product). hDPSCs at passage 4 were seeded onto dentin slices from each group (5.0×10^2 cells/slice) and incubated at 37 °C in 5% CO_2 for 2 days. Absorbance was measured at 550 nm in a UV–vis spectrophotometer multiplate reader (VersaMax, Molecular Device, San Jose, CA, USA) with a reference wavelength of 690 nm.

2.5. Cellular Attachment and Spreading

hDPSCs attachment and spreading on dentin surfaces were examined by scanning electron microscopy (SEM). hDPSCs at passage 4 were seeded onto dentin slices (5.0×10^2 cells/slice) from each group (n = 3/group) and incubated at 37 °C in 5% CO_2 for 2 days. Dentin slices were washed thrice with PBS, fixed in 2% glutaraldehyde for 5 min. dehydrated in a gradient of ethanol and dried with hexamethyldisilane. Surfaces were gold coated and examined by SEM (Hitachi, Tokyo, Japan).

2.6. Cell Adhesion, Morphology and Intracellular Structures in Real-Time

hDPSCs adhesion, morphology and subcellular structure were observed in real-time through live-cell imaging with holotomographic microscopy (HTM) as previously reported [17]. For cellular morphology, hDPSCs at passage 4 were seeded onto NTAPP-treated dish (FluoroDish, World Precision Instrument, Sarasota, FL, USA) and incubated for 24 h. For cellular adhesion, hDPSCs at passage 4 were seeded (5.0×10^2 cells/slice) on dentin that had either been treated with NaOCl and EDTA alone (controls), or with NaOCl and EDTA followed by NTAPP (plasma-treated). HTM was performed with 3D Cell Explorer-fluo (Nanolive, Ecublens, Switzerland) using 60× air objective (NA = 0.8), wavelength (λ) of 520 nm (class 1 low power laser, sample exposure 0.2 mW/mm^2), and USB 3.0 CMOS Sony IMX174 sensor, with quantum efficiency (typical) 70% (at 545 nm), dark noise (typical) 6.6 e$^-$, dynamic range (typical) 73.7 dB, field of view 90 × 90 × 30 µm, axial resolution 400 nm, and maximum temporal resolution 0.5 3D RI volume per second. Physiological conditions for live cell imaging were attained with a top-stage incubator (Oko-

lab, Pozzuoli, Italy), and temperature (37 °C), humidity and CO_2 (5%) were maintained throughout image acquisitions.

An export was performed within the STEVE software, which controls the HTM microscope, to transform RI volumes into .tiff format. By doing so, RI volumes can be read by the FIJI program. The exported 3D .tiff files must be in float format to keep the explicit RI for each voxel value. The 3D RI volumes in .tiff format were then processed in batch within FIJI for performance purposes. Then 3D RI volumes were transformed into 2D RI maps using maximum intensity projections and were also saved as .tiff files. The resulting series of 2D frames could then be processed using CP3, which does not support full 3D data analysis yet. The CP3 pipeline was designed to load each 2D RI map, segment the contained objects using the primary objects detection module, and extract area, shape, and intensity features using the measurements modules. A critical point for proper object detection was to use a manual threshold value. The object size limits that we entered were designed to encompass the full spectrum of potential lipid droplet diameters from 1 to 5 pixels. The segmented objects were finally used to extract the area and the mean RI value of each lipid droplet in each frame of the time-lapse experiment. The data were exported as a .csv file, in which we used the extracted count, spherical volume, and surface area of lipid droplets [18].

Real-time changes of hDPSCs behaviors around NTAPP-treated dentin were observed. Dentin slice was trimmed to thickness of 0.05 mm for visualization under Nanolive. Pre-treated dentin slices with 1.5% NaOCl and 17% EDTA as aforementioned served as control. Cellular attraction and attachment images were acquired at 2 h interval until 8 h after thawing.

2.7. Statistical Analysis

Differences in MTT assay results among the groups were examined using log-linear model analysis. Lipid droplet data were assessed for Gaussian distribution by the Shapiro-Wilk test, and paired t-test was performed. Statistical analysis was performed using statistical program SPSS 21.0 (SPSS, Chicago, IL, USA). Data are expressed as mean and standard deviations. Differences were considered to be statistically significant at $p < 0.05$.

3. Results

3.1. hDPSCs Attachment, Spread, and Proliferation

hDPSCs displayed fibroblast-like morphologies in 7-day cultures, and reached 95% confluence during first passage, showing epithelioid and polygonal shapes and forming colonies. Relative numbers of viable cells attached to dentin differed significantly between treatment groups ($p < 0.05$) (Figure 2). There was significantly more viability on NTAPP-treated dentin than controls (NaOCl), and EDTA pretreatment before NTAPP showed significantly more than other surfaces ($p < 0.05$). There was no interaction between NTAPP and EDTA treatments ($p > 0.05$).
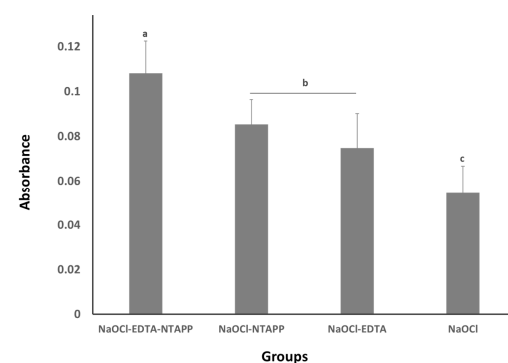

Figure 2. Human dental pulp cells viability and proliferation on dentin was measured by MTT assay. Different lowercase letters indicate statistically significant differences ($p < 0.05$).

3.2. Treatments Altered Dentin Surface and hDPSCs Response

Dentin surfaces appeared different between treatment groups (Figure 3). Intertubular dentin appeared sleek and dentinal tubule orifices were intact on NTAPP-treated dentin (Group I and II). Smear layers were absent on EDTA-treated dentin (Groups I and III). Irregular scaly surfaces and eroded tubule orifices appeared on dentin treated with NaOCl alone (Group IV).
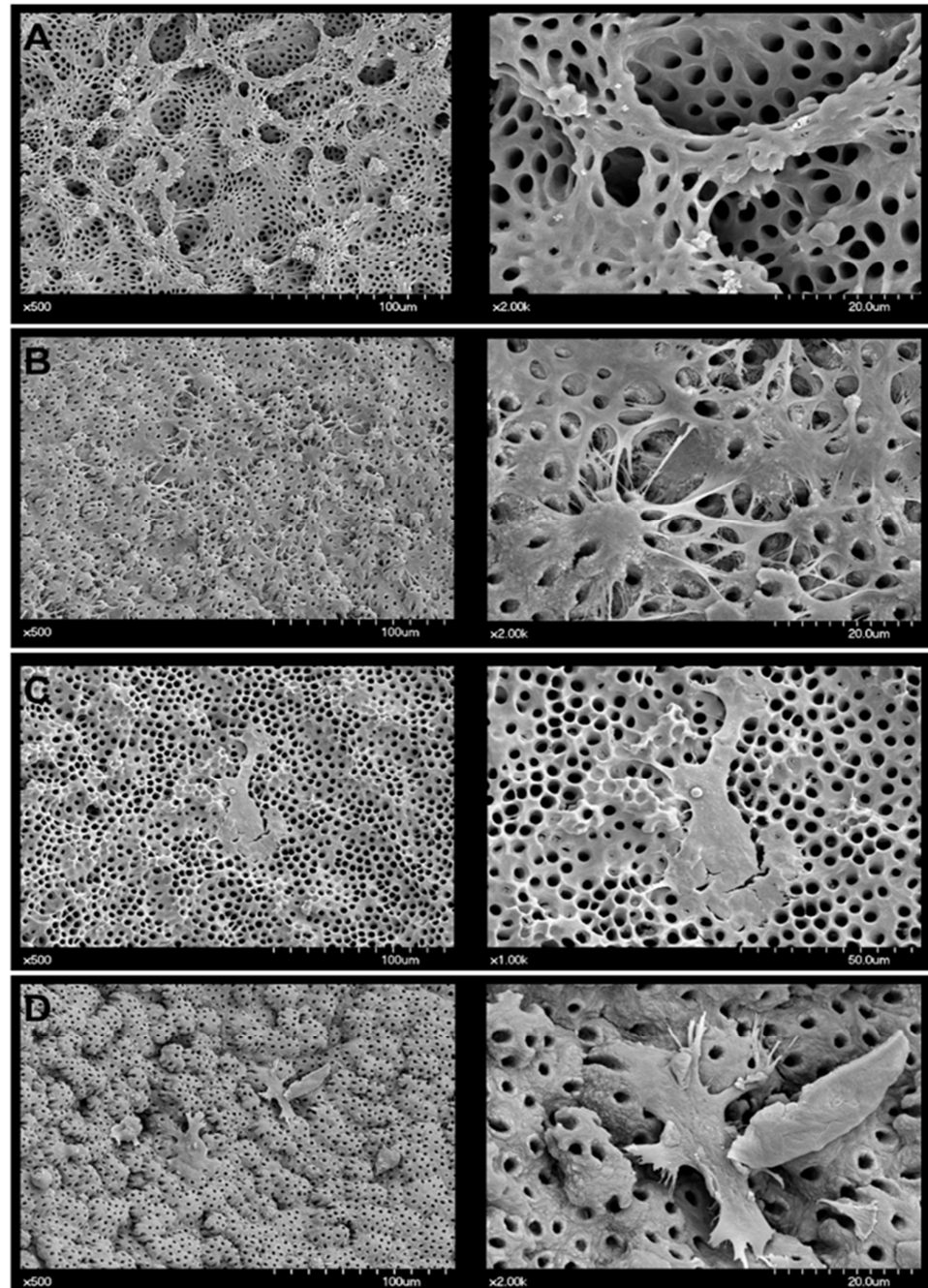

Figure 3. hDPSCs attachment and spreading on dentin slices observed through scanning electron microscopy (left, ×500; right, ×2000). (**A**) Group I (NaOCl + EDTA + NTAPP): Numerous hDPSCs had attached and spread out onto dentin surfaces in multiple layers, entangled by long cytoplasmic processes. (**B**) Group II (NaOCl + NTAPP): hDPSCs had formed multilayer structures on dentin. (**C**) Group III (NaOCl + EDTA): Fewer hDPSCs had spread out onto dentin without multilayers. (**D**) Group IV (control, NaOCl only): Few isolated hDPSCs had attached to dentin.

hDPSCs attached and spread out on all dentin surfaces (Figure 3), with differences in densities between treatments that reflected differences in viability (Figure 2). There was more attachment, spreading, longer cytoplasmic processes and higher densities from multiple layers that had merged to form clusters on NTAPP-treated dentin. Instead, markedly fewer isolated cells, much less spreading and shorter processes were seen on dentin controls (NaOCl-only).

3.3. NTAPP-Treatment Altered Cell Morphologies and Intracellular Structures

hDPSCs attached and spread out onto NTAPP-treated and untreated dishes (Figure 4A). They showed well spread fibroblast-like morphologies with intracellular organelles, including nucleus with nucleoli, lipid droplets, and vesicles. Adherent cell morphologies with newly formed lipid droplets adjacent to the endoplasmic reticulum were prominent in cells seeded on NTAPP-treated dish ($p < 0.05$) (Figure 4B).
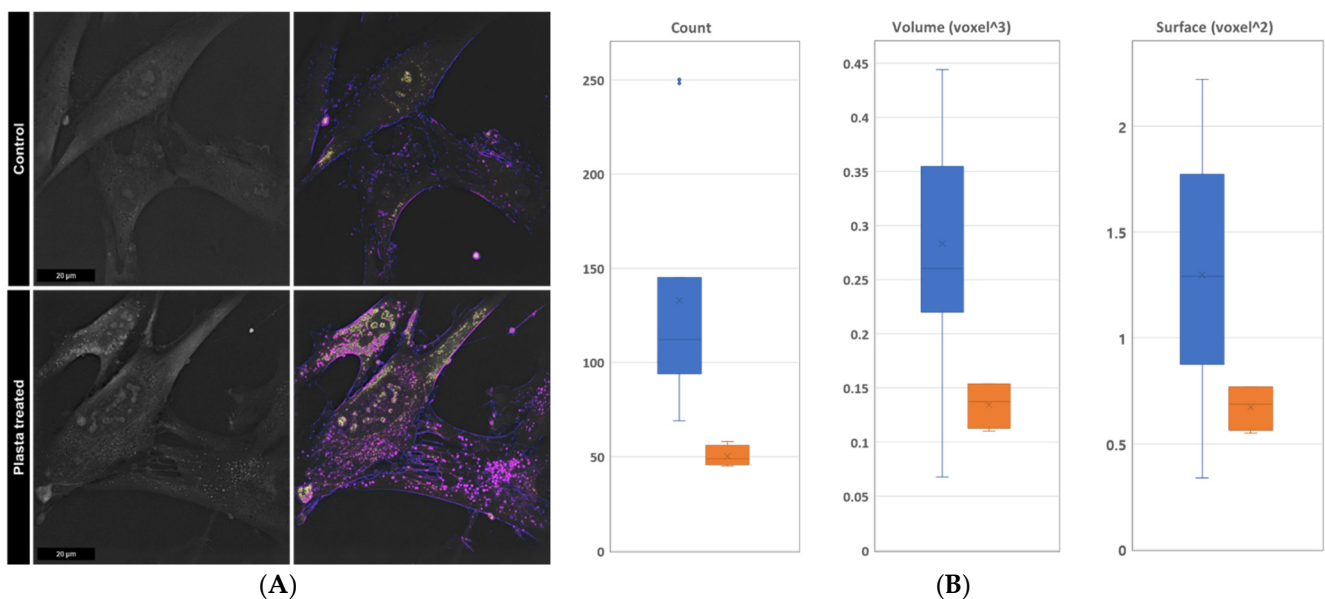

Figure 4. Morphology and intracellular structures observed by holotomographic microscopy. (**A**) Live-cell morphologies (left) and digitally stained subcellular structures (right, lipid droplets in pink) showed enhancement on NTAPP-treated surfaces (lower), compared to untreated controls (upper). (**B**) Lipid droplet count, volume, and surface area per cell were elevated on NTAPP-treated (blue) surfaces, compared to untreated controls (orange).

3.4. NTAPP-Treated Dentin Enhanced Cellular Attachment in Real Time

hDPSCs attached and spread onto dentin surfaces progressively in real time over 8 h (Figure 5). Cells spread onto NTAPP-treated and untreated dentin slices with exposed tubular structures and complex surface topographies. They exhibited heightened mobility around NTAPP-treated dentin compared to naïve slices. hDPSCs around dentin slices occupied NTAPP-treated surfaces within 2 h, and naïve dentin over 4–6 h. Cells on NTAPP-treated dentin had stabilized and extended their cellular processes into dentinal tubules at 8 h, whereas cells on naïve dentin at 8 h appeared like cells on NTAPP-treated slices at 4–6 h.
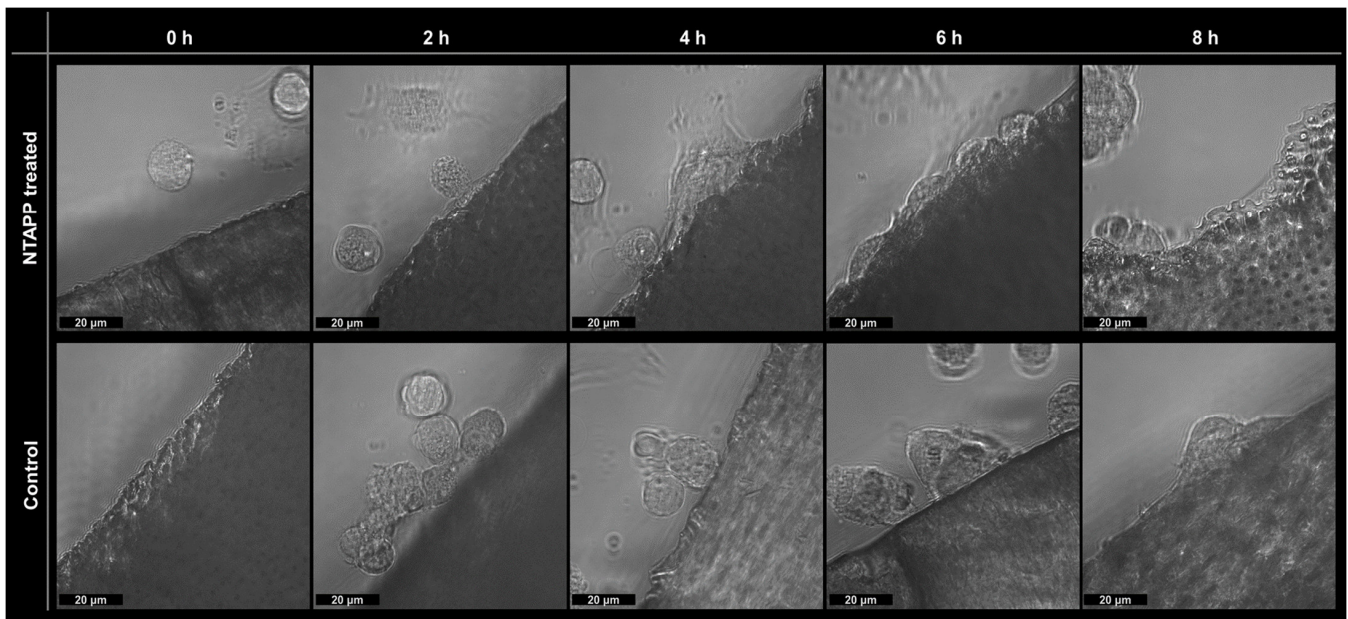
Figure 5. Live-cell imaging of hDPSCs attachment on root dentin. Holotomographic microscopy time series of NTAPP-treated (upper) and untreated controls (lower). Cells initiated attachment on NTAPP-treated dentin at 2 h, and on untreated controls at 4 to 6 h. hDPSCs on NTAPP-treated dentin had spread out over 4 to 6 h, and sprouted cellular processes into dentinal tubules at 8 h. Whereas, cells on untreated dentin were not spread until 6 to 8 h.

4. Discussion

Favorable effects of NTAPP on wound healing, immune responses, and stem cells involving increased expression of growth factors and cytokines have been reported in prior studies [19–21]. Similarly, this study demonstrated that NTAPP treatments of root dentin surfaces enhanced hDPSCs responses through cell adhesion, spreading and proliferation, which may benefit regeneration in endodontics. Pulp-dentin regeneration appears to be dependent on release of cell signaling molecules from reservoirs in root dentin [22,23]. Their stimulation of stem cell attachment and spreading on dentin surfaces constitutes the initial phase of cellular function [15]. These bioactive factors may be released by dissolution, as EDTA pretreatments reportedly enhanced pulp stem cell migration, whereas water had no effects, and NaOCl restrained migration [24]. Similarly, Pang et al. (8) showed increased cell density on EDTA-conditioned dentin than on untreated dentin. However, EDTA had no significant effect on hDPSCs counts in this study, and only NTAPP significantly increased cell viability. EDTA's effects may have been hidden by the impact of NTAPP treatment. Pre-rinsing dentin with NaOCl before EDTA was shown to reduce growth factor release, whereas chlorhexidine before EDTA increased their amounts [25].

Enhanced hDPSCs attachment on NTAPP-treated root dentin may have been due to improved hydrophilicity. NTAPP reportedly improved dentin wettability [26], and 30s of NTAPP brush treatment made human dentin super hydrophilic [27]. Their XPS analyses showed the oxygen/carbon ratio increased dramatically after NTAPP brush, suggesting that new oxygen-containing polar moieties had formed [27]. Similarly, Koban et al. demonstrated improved human dentin wettability from NTAPP, which supported enhanced human osteoblast spreading [28]. Likely, NTAPP generates reactive spots or species through C-C and C-H bonds splitting from vigorous atom/ion bombardment of dentin surfaces, which increases their hydrophilicity. The resultant increased adsorption of cell adhesion proteins such as fibronectin enhances cellular attachment, spreading and proliferation. These indirect effects of NTAPP on the natural niche of stem cells could also be applied to synthetic biomaterials to promote cellular responses. For example, Yang et al. reported that NTAPP changed nanoscale topography and elasticity of polymeric substrates, which enhanced human mesenchymal stem cell adhesion and spreading [29].

Cell orientation, organization, morphology and function are strongly influenced by their underlying substrate [30], and the recruitment of odontoblast precursors to dentin surfaces, and their extension of cellular processes into dentinal tubules is particularly important in regenerative endodontics. In this study, hDPSCs seen through SEM had attached and spread out in the direction of dentinal tubules exposed by EDTA and NTAPP treatments. They sprouted microvilli on dentin, with cellular extensions and filopodia often penetrating NTAPP-treated dentinal tubules. Additionally, hDPSCs observed in real-time by HTM live-cell imaging were attracted and attached to NTAPP-treated dentin surfaces faster than controls. Cells with enhanced attachment to NTAPP-treated dentin may have preferential expression of focal adhesion kinase (FAK) and faster progression through the cell cycle [3].

Enhanced subcellular activities were clearly visible in hDPSCs attached to NTAPP-treated surfaces. This is in accordance with previous research showing that cold atmospheric plasma could support optimal pluripotent stem cell attachment by turning polystyrene cell culture dishes from hydrophobic to hydrophilic state [31]. It is also speculated that polystyrene surface had been changed in topography and elasticity at the nanoscale level, enhancing adhesion and spreading of cells through promoted focal adhesions [29]. In this study, image-based analysis of label-free live hDPSCs on NTAPP-treated dishes showed significantly higher lipid droplets than controls. It is worth emphasizing that this study demonstrated lipid droplets through live-cell imaging in real-time without labeling. Previously, intracellular lipid droplet formation was visualized by fluorescence microscopy using dyes, which are phototoxic, and require reduced acquisition length and frequency to limit perturbations. Lipid droplets play a key role in physiological processes involving gene expression and cellular signaling, which may influence metabolism and differentiation. Therefore, further lipidomic analyses of hDPSCs and related NTAPP applications are warranted [32].

There are limitations in this study. In addition to current findings focused on these initial hDPSCs behaviors, in depth investigations on cell cycle analysis and secretion profiles are necessary to investigate odontogenic, osteogenic, chondrogenic, neurogenic, or angiogenic differentiation of dentin-attached hDPSCs. Furthermore, elucidating the underlying mechanism of NTAPP-induced tissue-specific differentiation of stem cells including adipose-derived stem cells, bone marrow-derived mesenchymal stem cells, and hDPSCs will require reactive oxygen and nitrogen species analysis. For instance, NTAPP on human adipose tissue-derived stem cells generated nitric oxide (NO), which increased cytokine and growth factor expression, and downregulated intrinsic apoptotic pathways [5,19]. NO at a low concentration increased pluripotent genes (Oct4, Sox2, Nanog) expression, and at high concentrations promoted human embryonic stem cell differentiation independently of its second messenger role [33]. Exogenous NO promoted DPSC differentiation into odontoblasts and induced tertiary dentin formation in rats [34]. Therefore, NTAPP's effects on differentiation could be expanded to guide endocrine/paracrine conditioned cells toward various osteogenic/chondrogenic lineages [35,36]. Long term observation using HTM will be required to trace these subcellular changes during differentiation.

As these NTAPP-induced cell- and dentin- changes may concurrently direct array of lineages in pulp-dentin regenerations, cell- and dentin-specific applications for NTAPP will need to be thoroughly characterized and standardized, so that they can be customized for clinically applications in regenerative endodontics.

5. Conclusions

NTAPP-treated root dentin surfaces support enhanced hDPSCs attachment, spreading, subcellular activity and proliferation, which may promote pulp-dentin regeneration in clinical applications.

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