Comparison of the Direct and Indirect Non-Thermal Atmospheric Plasma Effect on Human Periodontal Ligament Mesenchymal Stem Cells

Abstract

This study determined the osteogenic potential of human periodontal ligament cells (hPDL-MSCs) when treated with nitrogen gas via a specially-designed plasma needle (non-porous type). A cell culture was prepared from extracted third molars, and the stem cell characteristics and phenotype were determined. The hPDL-MSCs were subjected to direct and indirect plasma treatment and were tested in terms of their viability, intracellular ROS activity, differentiation and genotyping using q-PCR. The results indicate that indirect treatment was of benefit to the viability and osteogenic differentiation of the cells while direct treatment was detrimental to periodontal cells due to its strong effect. This difference in the results of the direct and indirect plasma treatment may be used to develop future periodontal treatment.

Keywords: Human periodontal cells; Osteogenic differentiation; ROS activity; Stem cells; Plasma-jet

Research Article

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Abbreviations: hPDL-MSCs: Human Periodontal Ligament Mesenchymal Stem/Stromal Cells;

ROS: Reactive Oxygen Species; q-PCR: Quantitative Polymerase Chain Reaction; DMSCs: Dental Mesenchymal Stem Cells; RUNX2: Runt-related Transcription Factor 2; MTT: (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide)

Introduction

Periodontitis is an inflammatory condition that can result in chronic bone destruction and loss of teeth, and attending to such patients is a major cause of distress for dental practitioners. Periodontitis may be acute or chronic, with inflammation in the gums, detachment of apically migrated collagen fibres at the junctional epithelium, and bacterial presence within the alveolar bone [1]. Nevertheless, periodontal ligament can regenerate and form new attachment fibres between cementum and bone, which shows that progenitor cells, stem cells in periodontal ligament cell populations have a regenerative capacity. However, this regenerative property has not been determined for gingival connective tissue [2].

Stem-cell therapy is seen as a novel, promising option to regenerate periodontal tissue since such cells can be used to repair and/or regenerate defective tissues and organs, like bone [3,4]. The identification and characterization of dental mesenchymal stem cells (DMSCs) has been proven to be very beneficial to periodontal regeneration therapy, and at present, MSCs are defined as multipotential fibroblast-like cells that express CD73, CD105, and CD 90 and are negative for hematopoietic markers CD14, CD34, CD 38 and CD45 [2,5].

Recent studies has shown that plasma, the fourth state of matter, can effectively modify the surface of biomedical materials. Cold atmospheric-pressure plasma has been widely investigated for its potential medical and dental use, and non-thermal atmospheric pressure plasmas have been found to be very effective in killing bacteria. They are increasingly used for root canal sterilization, treating dental cavities and bleaching [6], sterilizing medical equipment, and treating wounds, all of which show a breadth of potential applications [7].

A plasma needle is a non-thermal atmospheric pressure plasma source that can be convenient particularly for biomedical applications. It is less aggressive and can be used effectively at room temperature [3]. The plasma needle [8] is small and precise in its operation and is also flexible. The small size of the plasma needle is very desirable in dental and medicinal practice, where high precision is necessary, and the bactericidal, non-destructive treatment of periodontal pockets appears to be one promising application. However, in addition to the fast and efficient bactericidal effect, plasma treatment can also affect other cells in the periodontal region [8]. Independent of our work, Miletic et al. [3] showed positive results of the direct effects of plasma on human periodontal cells.

This study identified the effect of indirect plasma treatment on dental stem cells and compared it to the effect of direct plasma treatment. We studied the direct and indirect interactions of the plasma needle with human periodontal ligament-derived mesenchymal stem cells (hPDL-MSCs) and checked the effects on the osteogenic differentiation of these cells.

Materials and Methods

Plasma source and Instrumentation

The source electrode parts used to generate the plasma jet consist of a hollow needle with a sharp tip and a cylinder type with a hole size between 1 and 2 mm. Also, a glass tube is installed between these electrodes to protect the electrical short-circuit. In particular, the discharge gas is allowed to flow through the inner hole of the hollow needle.

The supplied gas flows are controlled using a mass flow controller (Omega FMA 1000). The plasma jet can be generated using a high power supply connected a variable input voltage with the characteristics of an AC-AC inverter. This inverter can supply an output voltage of a maximum 15kV. The plasma needle was kept at a distance of 1.5 cm from the hPDLSC cells, which were treated at different time intervals (Figure 1).

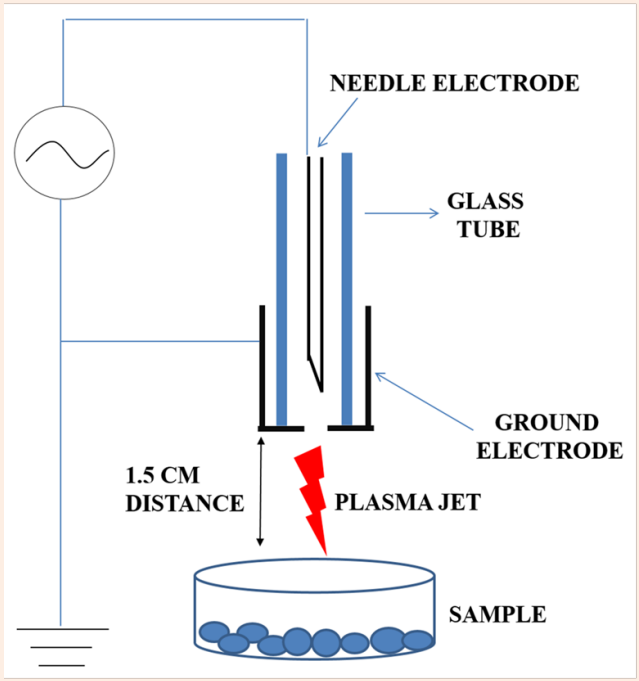

Figure 1: Schematic diagram of the plasma jet device. The plasma jet at a distance of 1.5 cm from the sample (hPDLSC cells). Nitrogen gas was allowed to flow through the inner hole of the hollow needle.

Plasma action mechanism

ROS/RNS (Reactive oxygen and nitrogen species) form in non-thermal plasma due to electron activity. The ROS is mainly comprised of free radicals like N_2, N_2^+, OH and O^-, and these reactive species can dissolve in liquid, but have a short life in a gaseous phase. Upon exposure to plasma, these free radicals engage in recombination, after which they are destroyed. They act on cell membranes by breaking cell adhesion molecules, and the membrane then acquire pores to penetrate reactive species into the cell that destroy mitochondria and nucleus, ultimately resulting in programmed (apoptosis) cell death [9].

Cell isolation and culture

Normal impacted third molars were extracted at the Department of Oral Surgery, Graduate School of Clinical Dentistry, Korea University, according to the approved ethical guidelines set by the Ethics Committee of the Faculty of Dental Medicine, Korea University (IRB AN16295-002). The periodontal ligament was gently separated from the teeth and was cultured in α minimal essential media (α-MEM, Hyclone; GE Healthcare, Utah) with 10% fetal bovine serum (FBS, Welgene, Deagu, South Korea) and 1% antibiotics. After a few days of culture, at 80% confluence, the cells were trypsinized and passaged regularly in growth medium (a-MEM with 10% FBS, 1% antibiotics).

Treatment of hPDL-MSCs with non-thermal atmospheric plasma

The cells were seeded in 24-well plates, 24 hrs before plasma treatment. After 24 hrs, the culture media was replaced with serum free media. For all experiments, the periodontal cells were treated with the plasma needle, directly and indirectly.

I. Directly- cells were treated with plasma without previous removal of serum free media (0.5 ml).

II. Indirectly- the serum free media (0.5 ml) was removed, treated with plasma, and then returned to the wells.

Identification and characterization of PDLSC

The characterization of hPDL-MSCs was performed using the BD Stem flow TM hMSC Analysis Kit. The Human MSCs were cultured (passage 2-3) and treated with accutase. Individual tubes containing $1x10^5$ cells were incubated with antibodies and were treated according to the manufacturer's recommendations. Following that, a Flow cytometry analysis was conducted.

Viability assay

The viability of the hPDL-MSCs was measured using an MTT assay (Promega, Madison, WI, USA). The PDLSC cells were divided into control and treatment groups and were seeded at a concentration of $5x10^4$ cells/well (24 well plate), which were then treated (directly and indirectly) with plasma N_2 gas for 1, 3, 5 and 5x2 (cells were treated for 5min with a resting period of 2min and treatment again for 5min) minutes, respectively. The cell growth on polystyrene without any plasma treatment was examined as control. After plasma treatment, the medium was replaced with serum free media (0.5mg/ml) and an MTT substrate was added 24hrs after seeding. MTT was added (1:10) to each well, following incubation for 2h at 370˚C. The absorbance at 450 nm was determined using a microreader (BIOTEK, Synergy HT, Gangnam, South Korea).

Alkaline phosphatase activity

The effect on the hPDL-MSCs differentiation potential was analysed by measuring the activity of alkaline phosphatase (ALP) using the Alkaline Phosphatase Activity Colorimetric Assay kit (Bio vision). Cells were seeded at $1x10^5$ cells/well (24-well plate) and were treated directly and indirectly with plasma N_2 gas after 24 hrs for 3 minutes each, respectively. Once the cells were directly and indirectly exposed to plasma N_2 gas, they were divided in two groups. One group was cultured in basal media and the other group was cultured in osteogenic media for 7, 14 and 21 days each. The ALP activity was then measured by checking the absorbance at a wavelength of 405 nm using a spectrophotometer.

Intracellular ROS activity

The intra-cellular levels of ROS were measured using 5-(and-6)-chloromethyl-2′, 7′-dichlorodihydro-fluorescein diacetate (CM-H2DCF-DA; Molecular Probes, Eugene, OR, USA). The cells were seeded at $1x10^5$ cells/well (24 well plate) and were treated directly and indirectly with N_2 plasma gas after 24 hrs for 1, 2, 3 minutes, respectively. 21 hrs after treating with plasma, positive control was prepared by treating one group of cultured cells with H_2O_2. The cell growth on polystyrene without any plasma treatment was examined as normal control. Following this, incubation was conducted for 3hrs. Then 5μM CM-H_2DCF-DA was added. After 15 min, DCF fluorescence was determined using a flow cytometer.

q-PCR reaction

The cells were treated with plasma (direct and indirect) at a 3-minute time interval and were then cultured for 5 days. Following this, the total RNA was isolated from hPDL-MSCs using an RNA easy mini kit (Qiagen, Valencia, CA, USA). Two-microgram aliquots of RNAs were used to synthesize cDNAs using random hexamers and reverse transcriptase according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). The real-time polymerase chain reaction (PCR) was performed using a Real-time PCR Detection System (Bio-Rad, Hercules, CA, USA.

Statistical analysis

All data were expressed as mean + standard deviation (SD). Student's t-test was carried out for parametric homogenous data, and a Welch's t-test was used for non-homogenous data. Values of $p \leq 0.05$ were considered to be statistically significant, and each experiment was repeated at least three times. The data analysis was performed using Microsoft Excel and Sigma Plot.

Results and Discussion

Results

Identification and Characterization of PDLSC: The MSCs were identified using the BD Stemflow™ hMSC Analysis Kit. They were found to be positive for the mesenchymal markers CD44, CD90, CD73 and CD105 (Figure 2), and these results are similar to those reported in previous studies, thus establishing their credibility as periodontal ligament cells [2].

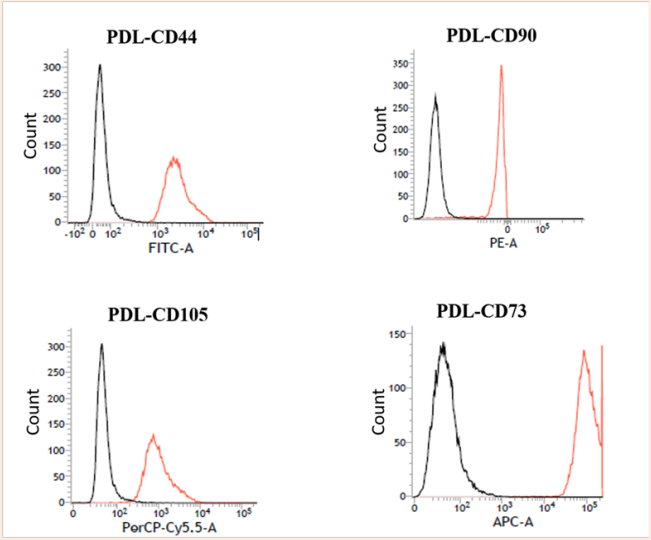

Figure 2: Flow cytometric analysis of MSC markers. PDLSCs were used passage 2, 3. PDLSCs were found to be positive for MSC markers CD 44, CD90, CD73 and CD105. MSCs identification was done using BD Stemflow™ hMSC Analysis Kit.

Effect of plasma on cell viability: The direct and indirect effects of the plasma were tested in terms of the viability of hPDL-MSCs. The primary cells isolated from periodontal ligament were cultured and treated (direct and indirect) with plasma N_2 gas for 1, 3, 5 and 5x2 minutes, respectively. Figure 3 depicts the microscopic analysis of the plasma treatment along with the graph. With direct treatment by plasma, effect was mostly on the central region of each treatment well and the cell survival rate decreased as the treatment time increased. A treatment time of 5x2 minutes was most detrimental to the cells. With indirect plasma treatment, the cells were uniformly affected throughout each well and the rate of cell death was less than that of the direct treatment. However, a high treatment time (5x2minutes) with the indirect method was also detrimental to the cell viability.

Alkaline phosphatase activity: To analyse the effect of the direct and indirect plasma treatment, we checked the supernatant of the cells after plasma treatment at 5, 7, 14 and 21 days. At, 5 days of treatment, significant changes were not seen in any of the treatment groups (as shown in Figure 4). After a treatment time of 10 days, the cells increased significantly in the basal media, but there was no such increase in the cells in the osteogenic media, indicating that basal media is beneficial for stem cell growth. The results also demonstrated that plasma treatment increased the osteogenic differentiation of hPDL-MSCs, and this effect was more significant with indirect treatment than with direct. In the osteogenic media group, there was a gradual change in both the direct and indirect group for osteogenic activity. Figure 5 shows the microscopic detection of mineralization (osteogenesis) by calcium deposits (not observed after 14 days of treatment). However, at 21 days, Alizarin Red Staining was detected in the osteogenic media for both direct and indirect treatment with more particle generation in the indirectly treated sample.

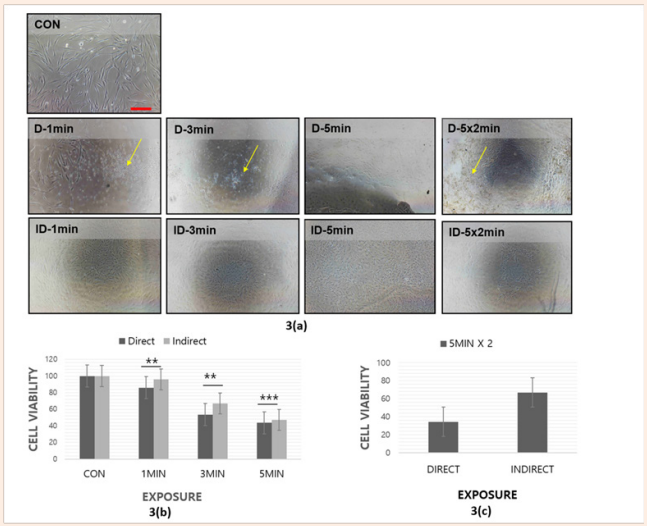

Figure 3: (a) Cell condition after treatment with plasma N_2 gas for 1, 3, 5 and 5x2 minutes respectively. The yellow arrow indicates the dead cells depending on the treatment time. Note that the number of dead cells is significant seen after direct plasma treatment.

(b) The graph indicates the decrease in cell viability with increase in treatment time.

(c) The treatment time of 5x2minutes was most detrimental for the cells after direct and indirect plasma treatment. The notation in the figure is: ∗p<0.05, ∗∗ p<0.01, ∗∗∗ p<0.001compared to the untreated control (Welch T-test).

Magnification 100X scaled to 100µm

CON: Control; D: Direct; ID: Indirect (plasma treatment)

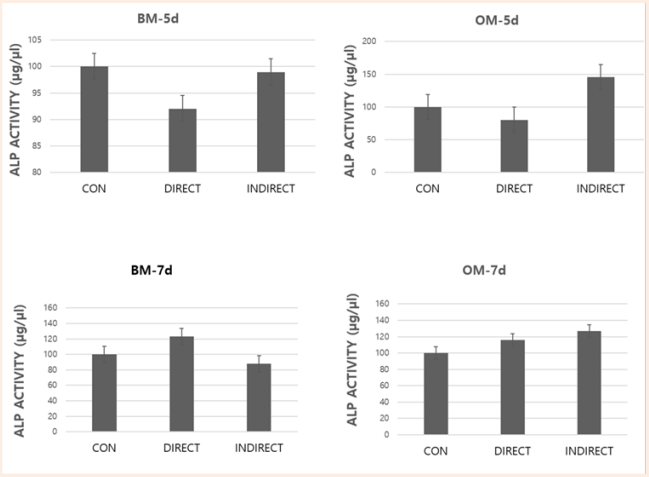

Figure 4: Alkaline phosphatase activity (ALP) for the hPDLSCs. After direct and indirect plasma treatment at 5, 7, 14 and 21 days the ALP activity was measured. At, 5 days of treatment, no significant change seen. With increase in time the osteogenic differentiation of hPDL-MSCs increased, the effect more significant with indirect treatment than with direct.

CON: Control; BM: Basal Media; OM: Osteogenic Induction Media

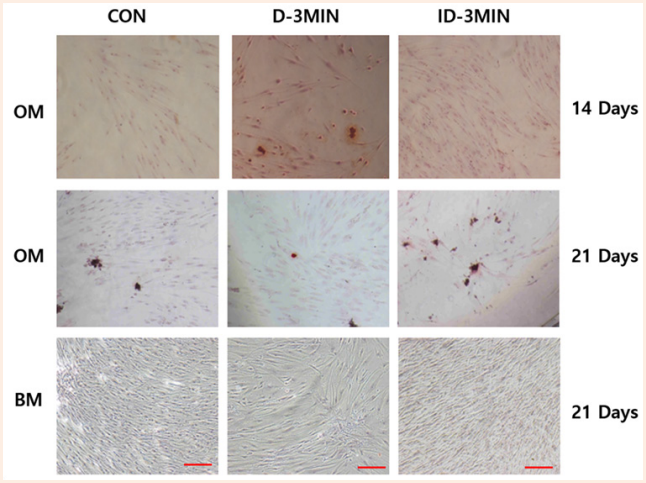

Figure 5: Alizarin Red S staining (ARS) for mineralization on PDLSCs by plasma treatment. Effect of direct and indirect plasma treatment on the hPDLSC compared at 14 and 21 days.

Magnification 100X scaled to 100µm

CON: Control; BM: Basal Media; OM: Osteogenic Induction Media

Intracellular ROS activity: The intracellular ROS levels were measured for different treatment times (both direct and indirect) at 1, 2 and 3 minute time intervals (Figure 6). The ROS levels in the control medium (without plasma treatment) were elevated but decreased when plasma treatment with N_2 gas was finished. When compared to highly elevated levels of H_2O_2, the ROS levels after direct and indirect plasma treatment were found to be quite low. However, indirect plasma treatment resulted in an increase in ROS generation when compared to direct treatment.

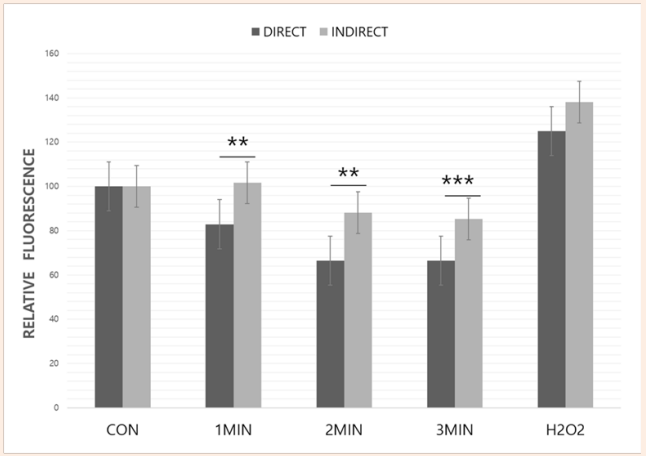

Figure 6: ROS levels in PDLSCs with direct and indirect plasma treatment. Intracellular ROS levels were measured at 1, 2 and 3 minute time interval. The notation in the figure is: ∗∗ p<0.01, ∗∗∗ p<0.001 compared to the untreated control (Welch T-test). Indirect plasma treatment resulted in increased ROS generation in comparison to the direct treatment.

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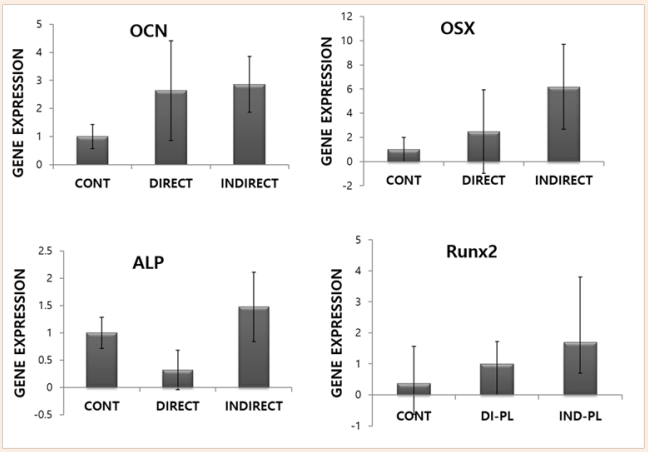
Gene expression analysis: The variation in gene expression was studied via real time q-PCR analysis after 5 days of cell culture following the direct and indirect plasma exposure to determine which genes played a significant role after each type of treatment. The presence of special markers, collagen, RUNX-2, osteocalcin, ALP and osteoblast specific transcription factor-osterix was determined. With N2 plasma treatment, osteocalcin, RUNX-2, and ALP upregulation occurred, but there was downregulation of the collagen marker. Gene expression increased more with indirect treatment than with direct treatment (Figure 7).



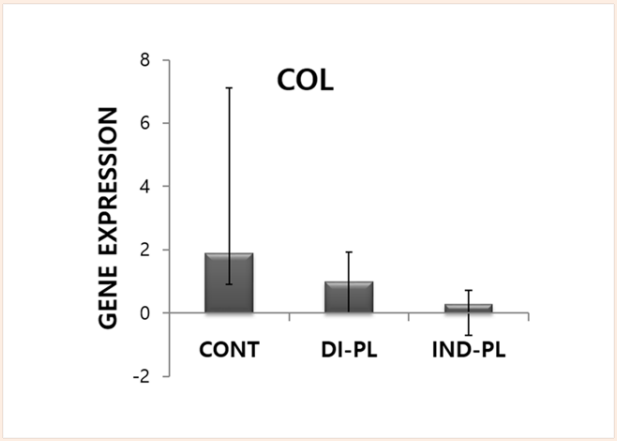


Figure 7: Analysis of osteogenic markers in PDLSCs using real-time q-PCR. Variation in gene expression studied using Real-time PCR Detection System after 5 days culture of cells following the direct and indirect plasma exposure to determine presence of gene markers.

OCN: Osteocalcin; OSX: Osteoblast Specific Osterix; ALP: Lkaline Phosphatase; COL: Collagen

Discussion

Mesenchymal stem cells are known for their ability to renew themselves and differentiate into multiple cell lineages. To find future uses for these cells in periodontal regeneration and to determine their therapeutic use with plasma treatment, hPDL-MSCs were treated with nitrogen plasma, and their behaviour was studied in cell culture conditions.

We found that the cell viability of hPDL-MSCs was different with direct or indirect treatment. With direct plasma treatment, the physical force of the plasma jet was seen to affect the cells at the centre of each well within a confined area, resulting in direct harm to the cells with increasing time. The death of the cells in the central region may have affected the other cells as well, diminishing their survival potential. The cell number diminished with a treatment time of 5x2min, thus indicating that an increase in treatment time is detrimental to the stem cells. However, with indirect treatment, the effect of the plasma was uniform throughout each well and was less harmful than that of the direct treatment. Since central cells started to die after direct treatment for 3 minutes, but no such effect was seen at the same time interval after indirect treatment, we believe that indirect plasma treatment is a safer treatment mode. Therefore, at a 3 min interval, we decided to check the other parameters of our experiment.

Various studies, like that by Yonson et al. [10], have shown that plasma has no deleterious effect on cell viability. Miletic et al. [3] conducted a similar study using helium gas [3], and they indicated a strong antibacterial effect with no significant effects on the cell viability due to the plasma treatment. Another study reported negligible effects on cell viability on 3-D scaffolds after plasma jet treatment [11]. However, the treatment time conditions used in these studies were different than in ours.

Stem cells are a preferred group of cells for tissue engineering procedures due to their regenerative capacity [12]. The osteogenic potential of periodontal ligament stem cells has been studied before and has been shown to be useful for filling bony defects with successful results [13]. We examined the osteogenic effect of N_2 plasma on the stem cells. Alkaline phosphatase is an early marker for osteogenic differentiation, and in our experiment, indirect plasma treatment of hPDL-MSCs yielded better results than treatment with direct plasma, thereby indicating that (indirect) nitrogen plasma is safer for osteogenic differentiation of periodontal stem cells. This property may be used during bone regeneration/tissue engineering with these cells. The enhanced osteogenic differentiation of hPDL-MSCs after plasma treatment was studied before by Miletic et al [3]. Althaus et al. [14] also studied the osteogenic potential of cells on plasma-treated ketones, citing positive results. In this study, indirect treatment in osteogenic media showed greater differentiation than with direct treatment. In our study, we already mentioned that cell death was initiated at 3 minutes of direct treatment with plasma. Therefore, differentiation may be inferred to have been inhibited by direct treatment, but well-differentiated cells were seen with indirect treatment.

Many studies have shown that [15-17] the ROS generation within cancer cells increases upon plasma treatment, thereby causing apoptosis of cancer cells. The reported mechanism of cell death is the disruption of mitochondrial membrane potential [15]. In our study, the ROS levels within hPDL-MSCs decreased (compared to control) after treatment with plasma. The low ROS generation despite the plasma treatment may be due to the cells interacting with aqueous ROS and using up the ROS in the media, or it may be due to periodontal ligament cells showing a different response to the signalling molecules. The low generation of ROS may also be attributed to the high dose of plasma (N_2)

administered, as also reported by Arjunan et al. [18]. More studies are required to confirm this peculiar behaviour of hPDL-MSCs.

Real time-PCR uses one housekeeping gene for normalization, so the results can vary depending on what control has been applied, and this may result in unpredictable differences in their expression patterns for the same individual [5]. In our study, upregulation was observed for the osteogenic gene markers, osteocalcin, RUNX-2, and ALP, but there was downregulation of collagen. RUNX-2, considered as the main osteoblastic marker, is known to control the expression of collagen [19]. For the direct treatment, RUNX-2 and collagen had opposite expressions, but both increased for indirect treatment. Also, we already inferred that direct plasma treatment is an inhibitor for differentiation, which confirms the ALP downregulation seen in our experiment. Indirectly-treated cells expressed remarkable differentiation and hence may be more suitable for cell-based therapy.

Further studies may be required to conduct a molecular and immunochemical analysis of the osteogenic differentiation of plasma-treated periodontal mesenchymal cells. The results indicate that for both direct and indirect treatment with non-thermal plasma, there was decreased cell survival as time passed with a longer plasma treatment time, especially at 5x2min, which may be significantly detrimental to the cells.

Conclusion

We studied and analysed the characteristics, viability and differentiation status of hPDL-MSCs. Indirect, non-thermal plasma treatment was found to be safer than direct treatment (detrimental to the cells), and these findings may provide new insights to utilizing indirect plasma treatment methods for periodontal defect therapies, regeneration, and tissue engineering.

Acknowledgement

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