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Isolation and Characterisation of a Dental Pulp-Derived Human Mesenchymal Stem Cell Line – CKC-Endeavour-2 and its Products Under Xeno- and Serum-Free Conditions

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Abstract

Background: The aim of this study was two folds first to isolate, culture and characterize dental pulp stem cells (DPSCs) derived from dental pulp tissue from human teeth under xeno-free and serum-free conditions using an explant method that is in compliance with good manufacturing practice guidelines (GMP). The derived cell line was used to obtain conditioned media and exosomes.

Methods: Molar teeth from healthy volunteers were collected with informed consent between the age of 18-25 years old, from the participating dentists and transferred to the lab in 10 ml of sterile phosphate buffer solution (PBS) within 30-45 min. Dental pulp tissue was retrieved from each tooth sample using a tweezer and the spoon excavator, washed in PBS and sliced into three to four pieces by using a disposable scalpel. The dental pulp explant tissue was transferred into the fresh 6 well plate containing complete MSC Expansion Media and cultured using an isolator (Biospherix).

Results: We have produced the proprietary cell line, CKC Endeavour-2 from dental pulp tissues that is fully characterised for surface markers, karyotype, and differentiation potential towards ectodermal, mesodermal, and endodermal pathways. In addition, we have characterised both the CM and exosomes for various grown factors and cytokines (total 105) by protein array. CKC Endeavour-2 CM significantly ($p < 05$) enhanced the wound closer in an *in vitro* scratch assay. Both the CKC Endeavour-2 and exosomes are being used for cosmeceutical and therapeutic developments.

Conclusions: The newly derived DPSC line, CKC Endeavour 2 is stable after repetitive propagation for 6 passages, is fully characterised based on International Society of Cell Therapy (ISCT) Guidelines. Both the cell line and the derived conditioned media thereof are xeno-free and serum-free used for therapeutic developments.

Keywords: Dental pulp stem cells (DPSCs); Fetal bovine serum (FBS); good manufacturing practice (GMP); conditioned media (CM)

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Introduction

Mesenchymal stem cells can be isolated from various tissues such as bone marrow derived, adipose tissue, umbilical cord derived tissue and dental pulp from teeth. These multipotent stem cells that can differentiate into various cell types such as fat, bone, cartilage and neural cells [1]. Dental pulp is a promising source of mesenchymal stem cells with the potential for cell mediated therapies and regenerative medicine. Dental pulp is particularly interesting in regenerative medicine because of the accessibility and differentiation potential of the tissue. Dental pulp has an early developmental origin with multi-lineage differentiation potential due to its development during childhood and adolescence. Dental pulp stem cells (DPSCs) were first isolated and shown to have multilineage differentiation potential [2]. The type of tooth to be used in isolation has been intensely investigated. Impacted third molars were used in the original studies but exfoliated deciduous ('baby') teeth could be an excellent source of cells for banking [3].

A remarkable amount of research and resources have been expended towards optimizing the protocols, freezing media composition, cooling devices and storage containers, as well as developing good manufacturing practices [4]. In order to ensure that dental pulp tissue retain their viability for culture and propagation, standardized protocols are devised. Cells selection by sorting methods has been proposed to enrich the cultured cell population in stem/progenitor cells [5]. Additionally, research has been done to retain their therapeutic characteristics following cryopreservation and that they are safe for clinical use [6]. In addition, cell isolation by explant culture appears to be easier, faster, safer, less expensive and more in line with GMP guidelines to obtain the clinical grade of DPSCs [7].

Exosomes are small extracellular vesicles and are involved in cell to cell communication. Exosomes derived from conditioned media (CM) are nano-sized and have been found to have significant role in cutaneous wound healing and to rejuvenate skin [8]. Stem cell therapy is a safe, more feasible and effective source for repairing the damaged tissue. Much of the functional improvement can be introduced by cell free conditioned media derived from MSCs or exosomes which contains essential growth factors and cytokines for skin regeneration and repair.

Therefore, present investigations were undertaken with the primary aim to develop a mesenchymal stem cell line derived from human dental pulp tissue (CKC endeavour-2) under xeno- and serum-free environments and that is fully characterised. These cells were then used to generate CM and exosomes containing 105 growth factors and cytokines for further therapeutic studies.

Material and Methods

DPSCs isolation and characterisation

Molar teeth from healthy volunteers were collected with informed consent between the age of 18-25 years old, from the participating dentists and transferred to the lab in 10 ml of sterile phosphate buffer solution (PBS). Each tooth was transferred into a petri dish. Dental pulp tissue was retrieved from each tooth sample using a tweezer and the spoon excavator. The excavated dental pulp tissue was transferred into the fresh 6 well plate. The dental pulp tissue was washed with PBS. The complete culture media was prepared following the manufacturer's protocol consisting of MSC Expansion Media Kit XF, human (500 mL bottle) (Miltenyi Biotec Australia Pty Ltd) plus MSC Expansion Media XF with added supplement (StemMACS), 1% Antibiotic/Antimycotic and 10% FBS. Dental pulp tissue was sliced into three to four pieces by using a disposable scalpel. The dental pulp tissue was plated on a 6 well plate. Initially, the tissue was cultured in complete culture media containing 10% FBS and after p-1 cell culture media was replaced to serum free and xeno-free media. The cell growth from dental pulp tissue explant can be seen within 3-5 days of culture. The cells were primarily cultured under an isolator (Biospherix) following the Good Lab Practice (GLP) compliance. The derived cell line was repetitively propagated for 6 passages, characterised based on International Society of Cell Therapy (ISCT) Guidelines and cryopreserved.

Characterisation

Cell Surface Markers with Flow Cytometry Analysis:

The expression of cell surface markers both for positive (CD 44, 73, 90, 105) and negative (CD 34, 45) for MSCs was assessed at passage 4 by flow cytometry as per manufacturer's instruction (Attune NxT Flow Cytometer, Thermo Fisher Scientific US). Data were expressed both as mean percentage of positive cells and as median fluorescence intensity (MFI) ratio determined using the median fluorescence value of the specific marker analysed, divided by the median fluorescence value of the isotype control.

Multilineage differentiation (qualitative and quantitative)

Early passages of the DPSCs between P-1 and P-4 were cryopreserved and stored as the primary mother stock. DPSCs at

passage 4 were used to test the multilineage differentiation potential before cryopreserving. Cells were seeded at a density of 22,500 cells per well on a 6-well plate. After cell culture was about 70% confluent in about 2-4 days, the cells were subjected to differentiation media and cultured for 7- 21 days. These days were selected based on our preliminary experiments on differentiation for detecting early onset of differntation (data not shown):

Adipogenic differentiation using Oil Red staining

Briefly, aspirated the culture medium from each well. Gently washed the wells with 1 ml distilled water. Fixed the cells in 4% paraformaldehyde for 30 minutes at room temperature. Removed fixative and washed the wells with distilled water. Removed water completely and added 60% isopropanol for 5 mins. Discarded the isopropanol. Added 2 ml of 0.3% Oil O red solution and incubated at room temperature for 30 mins. Removed the dye and washed the cells with distilled water. Left the last wash in the wells. Inspected the cells using a phase microscope and taken images. After taking the images, followed the steps for quantitative analysis. Discarded the PBS wash from the wells. Added 2 ml of 100% Isopropanol solution to each well. Incubated at room temperature for 1 hours. After 1 hours, collected the Isopropanol solution from each well into Eppendorf tubes. Measured the absorbance using the spectrophotometer at 510 nm.

Chondrogenic differentiation using Alizarin staining

Aspirated culture medium from each well and gently washed cells 3 times with 1xPBS. Fixed the cells in 4% formaldehyde for 15 minutes at room temperature. Removed fixative and washed the cells 3 times with H_2O. Removed H_2O completely and added 1 mL of 40 mM ARS per well. Incubated at room temperature for 30 mins. Removed the dye and washed the cells 5 times with H_2O. If required, inspect the cells using a phase microscope and take images. Stored plates at -20°C prior to dye extraction.

For quantification, samples were treated according to the manufacturer protocol. Briefly added 800 µL of 10% acetic acid to each well of a 6-well plate and incubated at room temperature for 30 minutes with shaking (200 µL per well for a 24-well plate). Collected the cells using a cell scraper and transferred the cells in 10% acetic acid to a 1.5-mL microcentrifuge tube. Vortex for 30 seconds. Heated the samples at 85°C for 10 minutes. To avoid evaporation, the tubes were sealed with parafilm or added 500 µL of mineral oil to the top of the slurry. Incubated tubes on ice for 5 minutes. Centrifuged the slurry at 20,000g for 15 minutes. After centrifugation, transferred 500 µL (or 200 µL) of the supernatant to a new tube. Added 200 µL (or 75 µL) of 10% ammonium hydroxide to neutralize the acid. Read the absorbance at 405 nm with a plate reader.

Osteogenic differentiation using Alcian Blue staining

Aspirated the culture medium from each well. Gently washed the wells 3X with 1 ml PBS. Fixed the cells in 4% formaldehyde for 20 minutes at room temperature. Removed fixative and washed the cells 3X with 1 ml PBS. Removed PBS completely and added 1 ml of Alcian blue solution per well. Incubated at room temperature overnight. Removed the dye and washed the cells with 1 ml of PBS. Left the last wash in the wells. Inspected the cells using a phase microscope and taken images. After taking the images, followed the steps for quantitative analysis. Discarded the PBS wash from the wells. Added 1ml of 6M guanidine HCL solution to each well. Incubated at room temperature for 2 hours. After 2 hours, collected the guanidine HCL solution from each well into Eppendorf tubes. Measured the absorbance using the spectrophotometer at 650 nm.

RT-PCR

RNeasy Plus Mini Kit (Qiagen) was used to extract RNA from the DPSCs following the manufacturers protocol and the extracted RNA was stored at -80 degree before measuring the amount of RNA using Nanodrop. Following which cDNA kit (script™ cDNA Synthesis Kit, Bio-rad) was used according to the manufacturers protocol, after performing the cDNA synthesis, the resulting cDNA tubes are stored at -40ºC for performing PCR in the future. PCR mix (Thermo Fisher scientific, US) was prepared used the protocol provided by the manufacturer. The temperature was selected according to the primer sequences from literature used for the PCR reaction (Table 1). The PCR mix was run on a 2% agarose gel on a horizontal gel electrophoresis system. The results were recorded using the gel doc.

Table 1: Primers for RT PCR expression

Primers	Temperature	Sequence
CD44-Forward	60°C	CCTGGCAGCCCCGATTATTT
CD44-Reverse	60°C	AAGGACACACCCAAGCAAGG
PPAR gamma-Forward	50°C	CAGTGTGAATTACAGCAAACC
PPAR gamma-Reverse	50°C	ACAGTGTATCAGTGAAGGAAT
Osteocalcin-Forward	60°C	AGCAAAGGTGCAGCCTTTGT
Osteocalcin-Reverse	60°C	GCGCCTGGGTCTCTTCACT
Collagen 10A1-Forward	60°C	CACGCAGAATCCATCTGAGAATAT
Collagen 10A1-Reverse	60°C	GTTCAGCGTAAAACACTCCATGAA
RPLPO-Forward	60°C	AATCTCCAGGGGCACCATT
RPLPO-Reverse	60°C	CGTTGGCTCCCACTTTGT

Conditioned media

DPSCs from p-3 to p-5 were cultured in T75 tissue culture flasks in xeno-free/serum-free (XF/SF) media (Miltenyi Biotec Bergisch Gladbach, Germany) at 37°C and 5% CO2 for harvesting conditioned media (CM). The confluency of the cultures at 75%-80% (3-5 days) was used as a criterion for starting the conditioning process. The supplement-containing media (Miltenyi Biotec, Bergisch Gladbach, Germany) was aspirated and the T75 flasks were rinsed twice with 5 mL prewarmed Phosphate Buffered saline (PBS) (Miltenyi Biotec, Bergisch Gladbach, Germany) to remove residual traces of supplement. Thereafter, 15 ml of fresh GMP grade xeno-free, serum-free, phenol red free and supplement-free media (Miltenyi Biotec, Bergisch Gladbach, Germany) was added to the T75 flask and incubated for 24 hrs for conditioning at 37°C at 5% CO2. The DP-CM was passed through a 0.45μm filter and stored in 15 mL falcon tubes at -80°C. Thereafter, CM were lyophilized and stored at +4°C.

Exosomes

Conditioned media were used to extract exosomes using exosome extraction kit (iZON, Germany) and following the manufacturer's protocol. The columns were washed with PBS and then 500 ul of CM was added to the top of the column, the first 3 mL of the elute was discarded. Following this 1.5 mL of elute was collected in an Eppendorf which contains the exosomes. The collected exosomes were stored at -80°. The extracted exosomes were measured using Nano sight NS300.

Cytokine array

The lyophilised powder of DPSCs-CM was weighed and reconstituted in 1mL of RO water. The protein concentration was measured using UV-based spectrophotometry. Manufacturer's instructions were followed to perform the cytokine array. Briefly, the array membranes were blocked with array buffer 6 at RT for 1 hour on a shaker. After blocking, 1.5mL samples containing 775uL of DPSCs-CM was mixed with 725uL array buffer 6 and added to the membranes for an overnight incubation at 4°C on a shaker. On the next day, the membranes were washed thrice with 1X wash buffer for 10 minutes on a shaker followed by the addition of the 1.5mL Detection antibody cocktail on the membranes. An incubation of 1hr at room temperature (RT) on the shaker was performed followed by 3 washes with 1X wash buffer for 10 minutes each. Following the washes, 2mL of streptavidin (1:2000) was added to each membrane for 30 minutes incubation at RT on a shaker. After streptavidin incubation the last 3 washes of 10 minutes each in 1X wash buffer was performed on a shaker followed by detection. Equal volumes of detection reagent 1 & 2 were mixed just before adding onto the membrane and detected by chemiluminescence using the FUJI LAS3000 Gel Documentation System and LAS3000 software. Analysis of the membranes were performed using the Array analyser in the Image J software. The reference spots were considered as positive controls. The background intensity of the negative control spots was subtracted from the sample values and graphs were plotted using Microsoft Excel.

Scratch Assay

The scratch assay was carried out as per protocol [16]. Briefly, the experiment was set up for CKC Endeavour 2 derived CM collected at passage 3-5 and lyophilised at varying concentration such as 20 mgs, 10 mgs and 6.6 mgs after reconstitution in phenol red free media (PRF media). Human skin fibroblast, HDF01 cell line were used for the assay, 100,000 cells were plated per well, after 72 hours of seeding the cells, a manual scratch was created using a tip of the pipette and specific areas were marked for observation post treatment, pictures were taken. Following which the wells were washed twice with PBS before adding the

treatment. PRF media was used a control, CM prepared in phenol red free media was used added to the wells, exosomes were diluted at 1:10 in PRF media. Lastly, 20 mgs, 10 mgs and 6.6 mgs of lyophilized CM from CKC-Endeavour-2 were reconstituted in 2 mL of PRF media and added to the HDF01 cells for 24 hours. After 24 hrs, scratch was observed, and pictures were taken at the same marked spot. The gap closure was analysed using Image J software.

Statistical Analyses

The statistical analyses of the data were performed with Graph Pad Prism 8. The data are presented as mean ±standard deviation (SD) and experiments were carried out in triplicates. All statistical analyses were performed at the significance level $p < 0.05$ using three-way analysis of variance (ANOVA) for multiple comparisons.

Results

Proliferation of DPSCs

DPSCs isolated by explant method started proliferation after 5 days and started extruding cells in culture dish (Figure 1A). The success of the explant method was depended on initial samples (early age samples were more amenable) and not all samples yielded cell lines and hence for initial isolation of cells from those samples, the addition of serum to the media was required. The serum was subsequently replaced with xeno-free, serum-free supplement containing media once the cells reached confluence and for the following passages.

DPSCs formed a monolayer after initial plating in tissue culture flasks. A consistent isolation procedure with routine yield of 2 to 4 $X10^6$ cells per 100mL of tissue processed was achieved. Under microscopic examination, the adherent cells displayed a fibroblast-like spindle-shaped morphology that was consistent with previous descriptions of DPSCs (Figure 1B). To confirm the purity of DPSCs, cells between passages 4 to 6 were examined by flow cytometry for expression of CD markers and their *in vitro* ability to differentiate into adipogenic, osteogenic, and chondrogenic lineages.

Cell Surface Markers

We have analysed the expression pattern of positive (CD44, CD90, CD105, and CD73) and negative (CD45, and CD34) markers of MSCs in our DPSCs derived from dental tissue. Cells (87.90 – 99.80 %) showed positive expression (Figure 2) thereby fulfilling the minimum criteria proposed by ISCT.

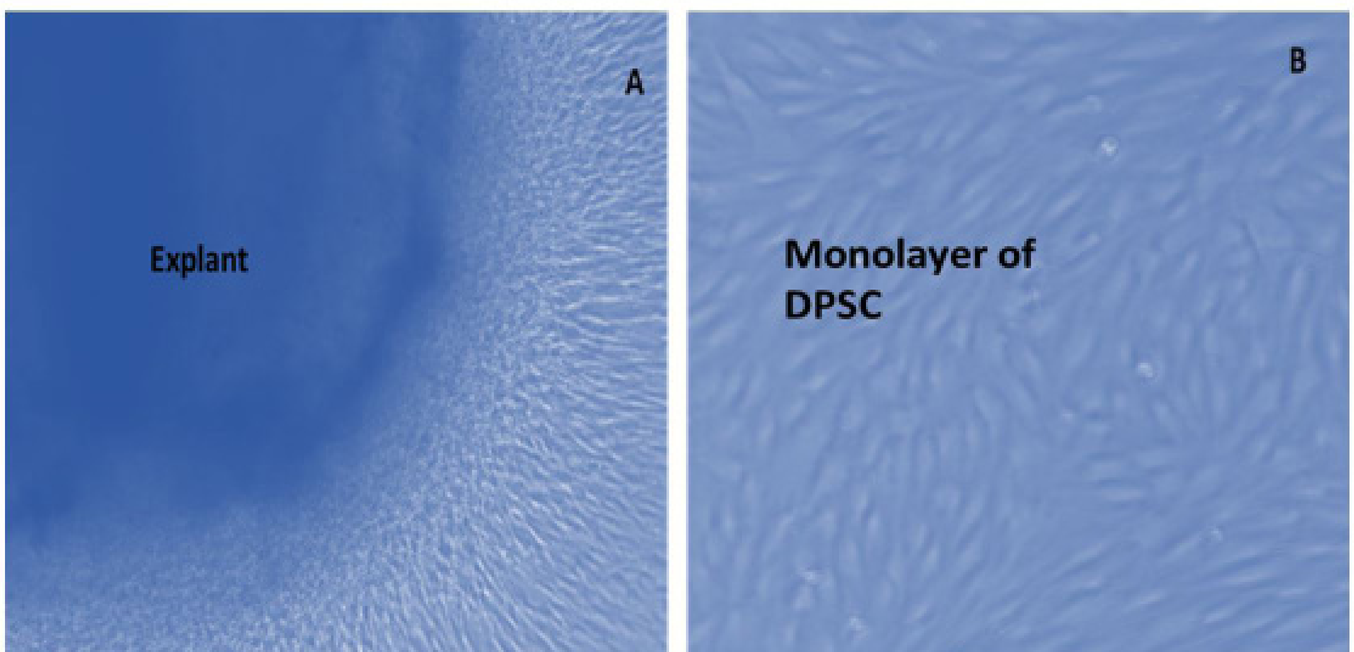

Figure 1: A: dental tissue explant extruding DPSC; **B:** a monolayer of spindle-shaped DPSC in tissue culture flask, day 4

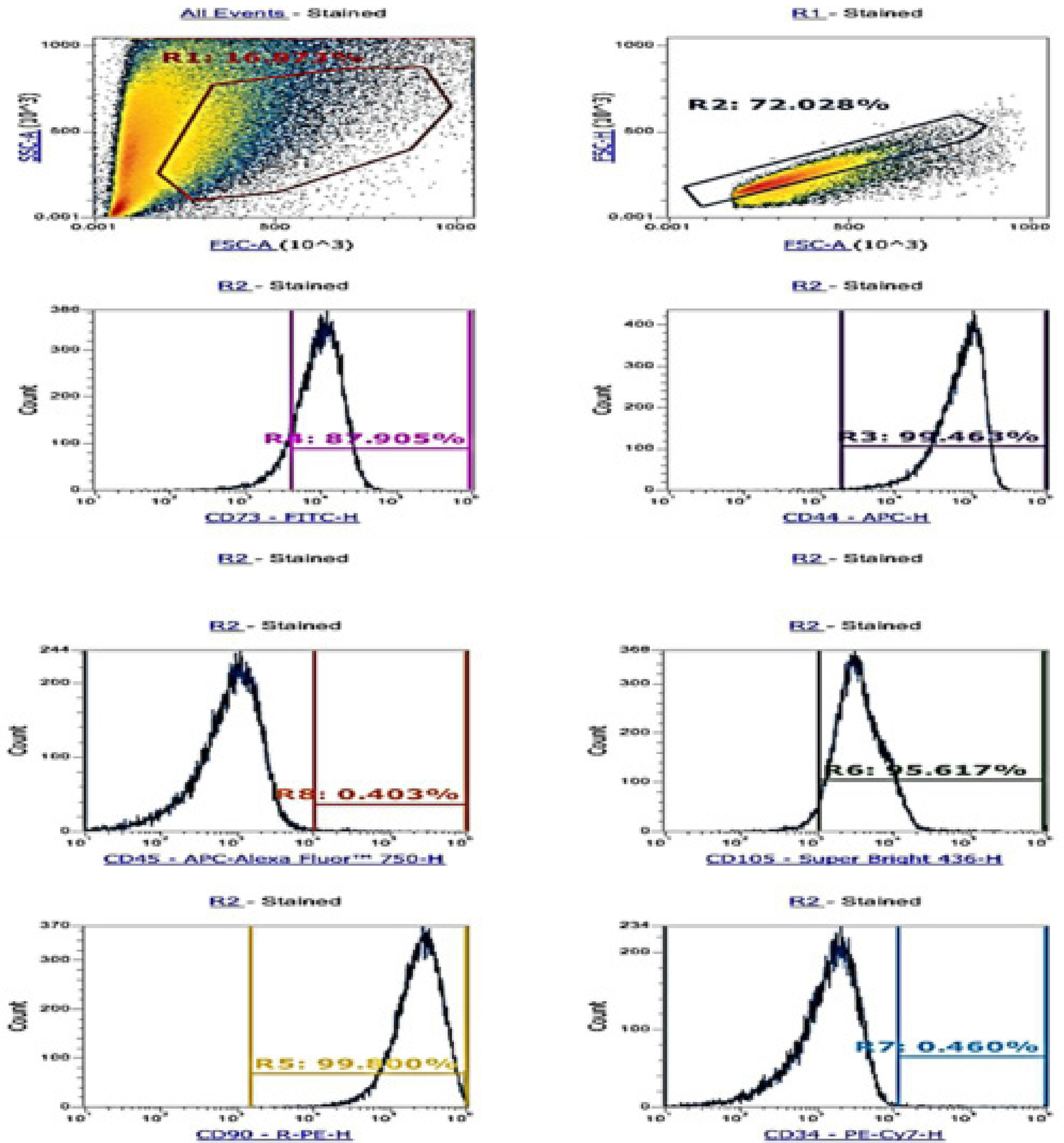

Figure 2: DPSCs expressed mesenchymal stem cell markers such CD44, CD73, CD 105 and CD 90. In addition, the cells did not express the haematopoietic markers such as CD34 and CD45

DPSCs multilineage differentiation

Adipogenic: At Day 9, the presence of lipid globules within the DPSCs was observed. Oil Red O stain was used to identify the neutral lipids (triglycerides) within the cells (Figure 3 top panel) followed by its quantification using isopropanol elution (Figure 3 bottom left panel).

Osteogenic: At Day 10, the presence of mineralised nodules was observed throughout the cell culture. Alizarin Red S stain was used to identify the calcium deposits in the mineralised matrix (Figure 3 top panel) followed by its quantification using acetic acid/ammonium hydroxide-based method (Figure 3 bottom middle panel).

Chondrogenic: At Day 21, the presence of proteoglycan-rich extracellular matrix in the cell micro masses was observed. Alcian blue stain was used to identify the carboxylated and sulphated proteoglycan-rich extracellular matrix (Figure 3 top panel C) followed by its quantification using guanidine hydrochloride-based method (Figure 3 bottom right panel).

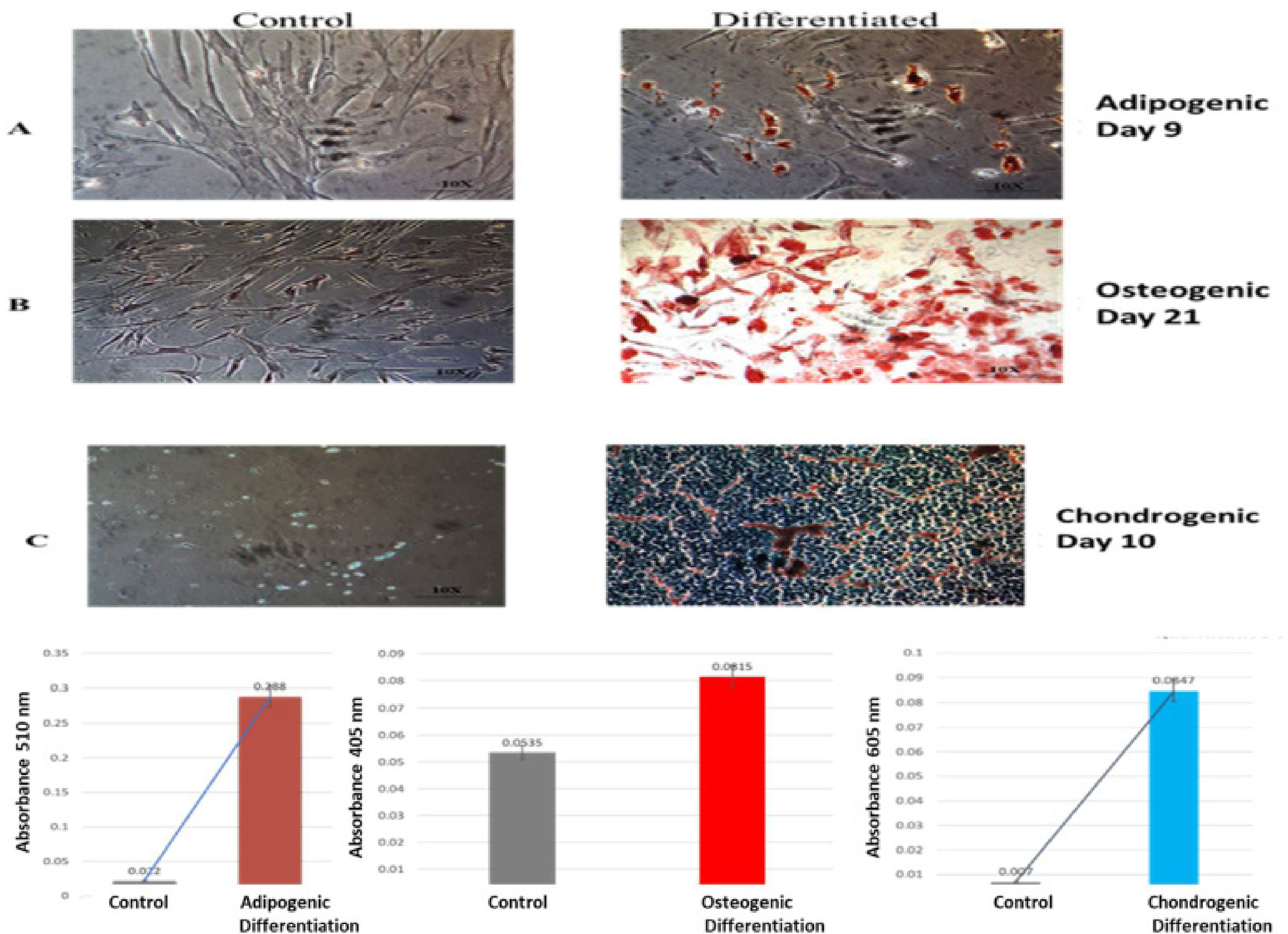

Figure 3: Top panel, tri-lineage differentiation potential of DPSCs. Presence of lipid globules stained with Oil Red O demonstrated in adipogenic-differentiated DPSCs (A). The presence of mineralised matrix in osteoblast differentiated DPSCs stained by Alizarin Red S stain (B). The presence of carboxylated and sulphated proteoglycans in chondrocyte differentiated DPSCs stained by Alcian blue (C). Bottom panel from left, quantification of adipogenic, chondrogenic and osteogenic differentiation

RT-PCR expression in DPSCs

DPSCs were analysed for the expression of mesenchymal marker CD44 and multi-differentiation markers. Undifferentiated DPSCs expressed CD 44 marker specific to mesenchymal cells (3D). Adipogenic marker PPARγ showed a very weak expression at day 7 (3A) in comparison to the staining results at day 9 (2A). On the other hand, osteogenic differentiation marker osteocalcin showed a stronger expression (3C). Likewise, the chondrogenic differentiation marker collagen 10A1 was expressed in chondrogenic differentiated DPSCs (3D) (Figure 4).

Exosomes concentration

Exosomes particles were measured using Nano sight, the concentration ranged between 1.07X10^{8} ± 2.11X10^{7} particles/ml (Figure 5). DPSCs- conditioned media exosomes displayed that the size of the exosomes varied, 70% range from 25 to135 nm, 20% range from 255 to 315 nm and 10% range from 588 to 765 nm.

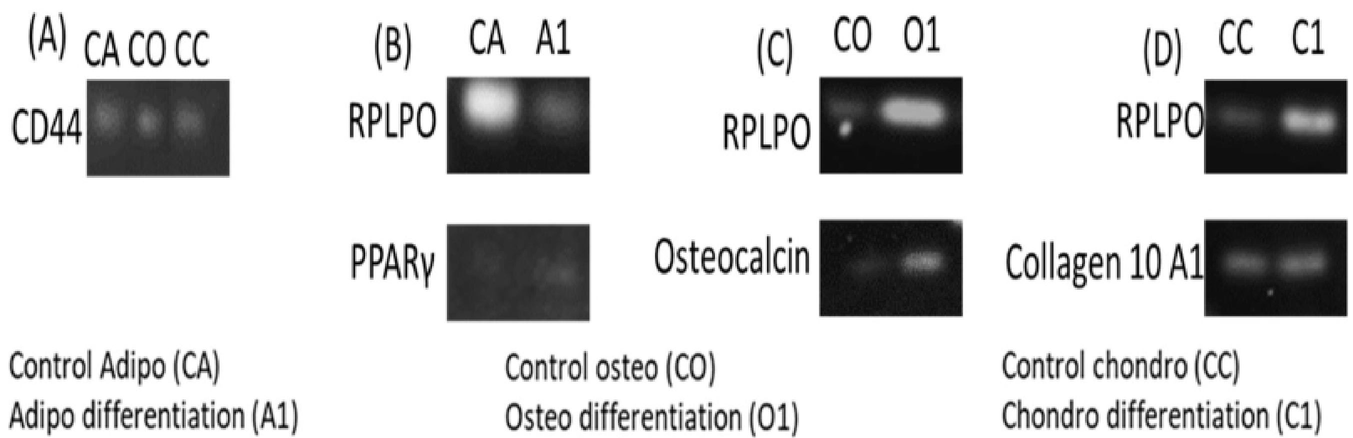

Figure 4: RNA expression of undifferentiated DPSCs (A) CD44, Adipogenic differentiated (B) PPARγ, Osteogenic differentiated (C) Osteocalcin (OC), and Chondrogenic differentiated (D) Collagen 10A1 at day 7

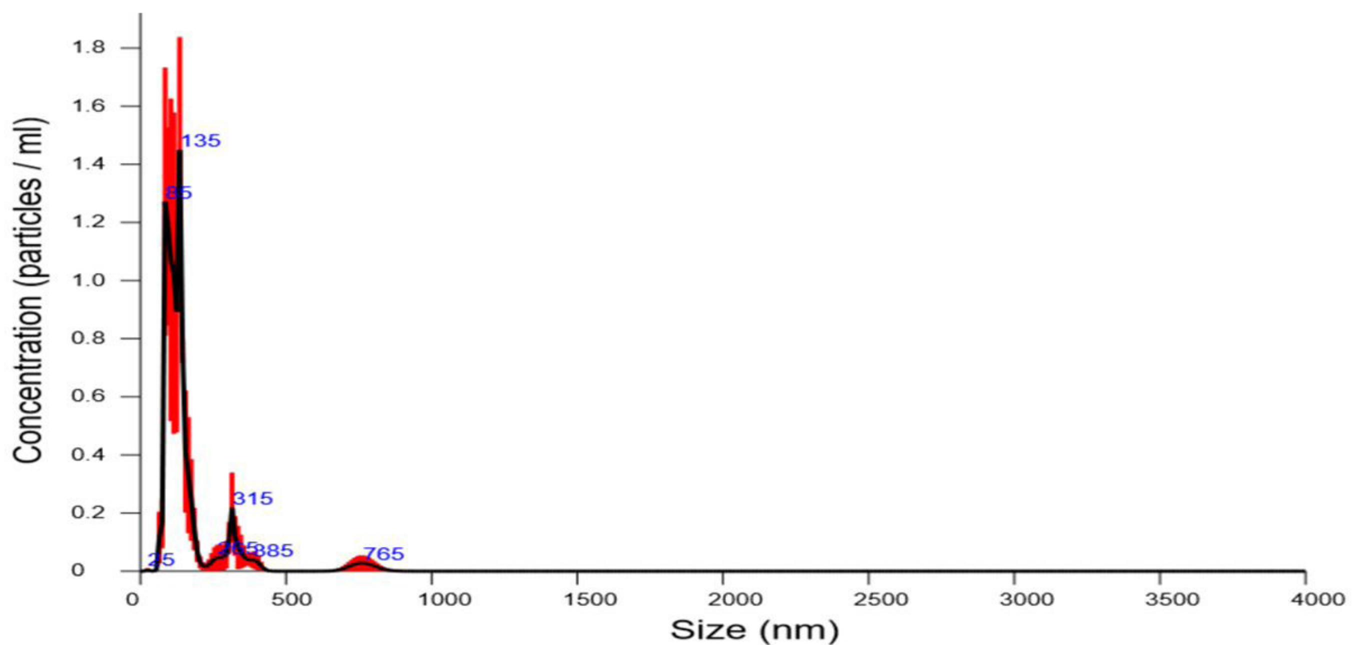

Figure 5: Exosome concentration and particle size distribution of DPSC exosomes displayed about 70% range from 25 to135 nm, 20% range from 255 to 315 nm and 10% range from 588 to 765 nm

Cytokine profile

Quantification of 105 human cytokines and growth factors by protein array showed that DPSCs CM harbour a variety of these factors relevant for various conditions particularly skin related Serpin E1 cytokine and other growth factors important for anti-inflammatory and immunomodulatory roles (Figure 6).

Wound healing- Scratch Assay

Our data show that the lyophilized DP-CM at 10 mgs and 20 mg concentration resulted in a significant (P< 0.05) closure of the gap at 24hour time point (Figure 7).

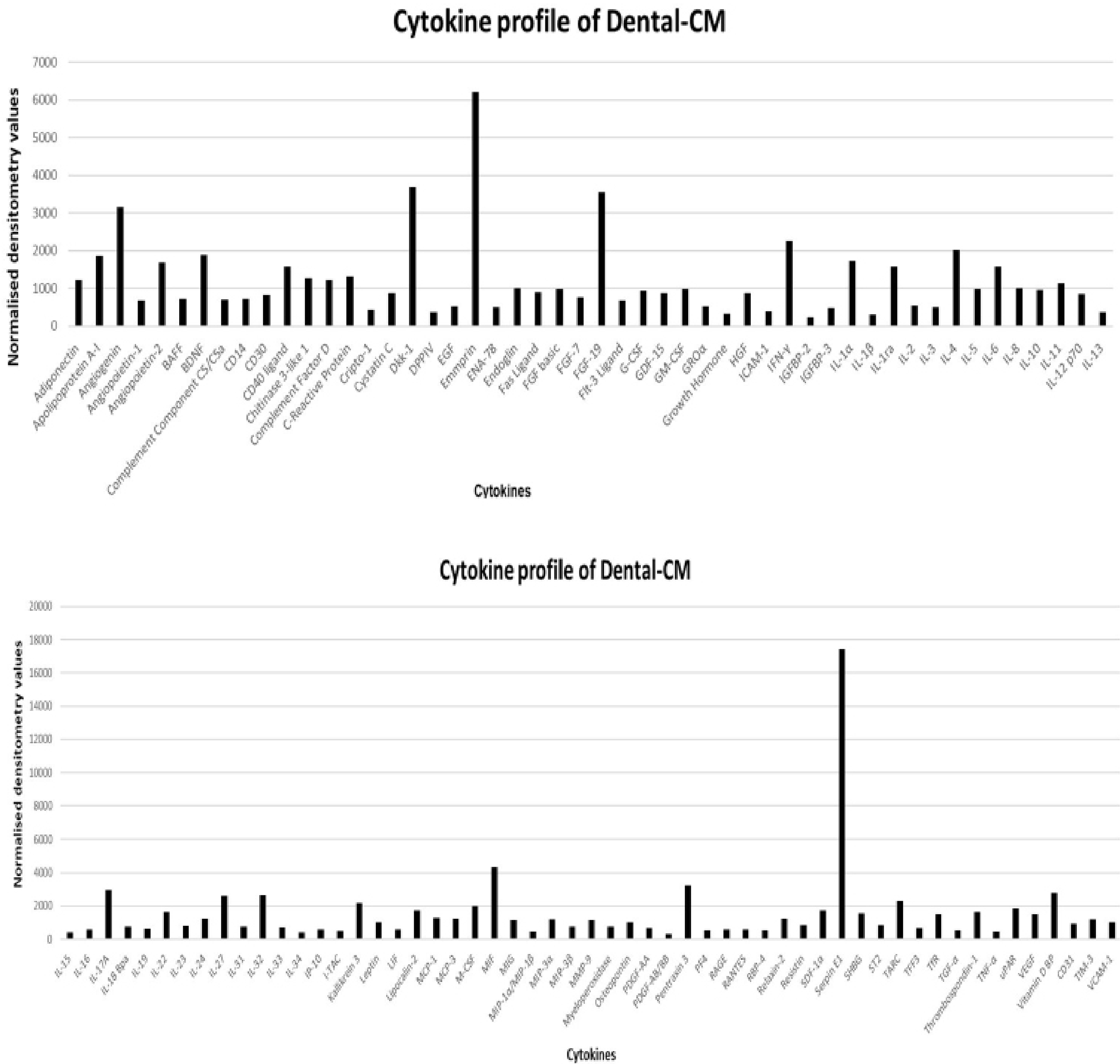

Figure 6: Human cytokine array analysis of DPSCs-CM. Densitometric analysis of proteins associated with regenerative potential

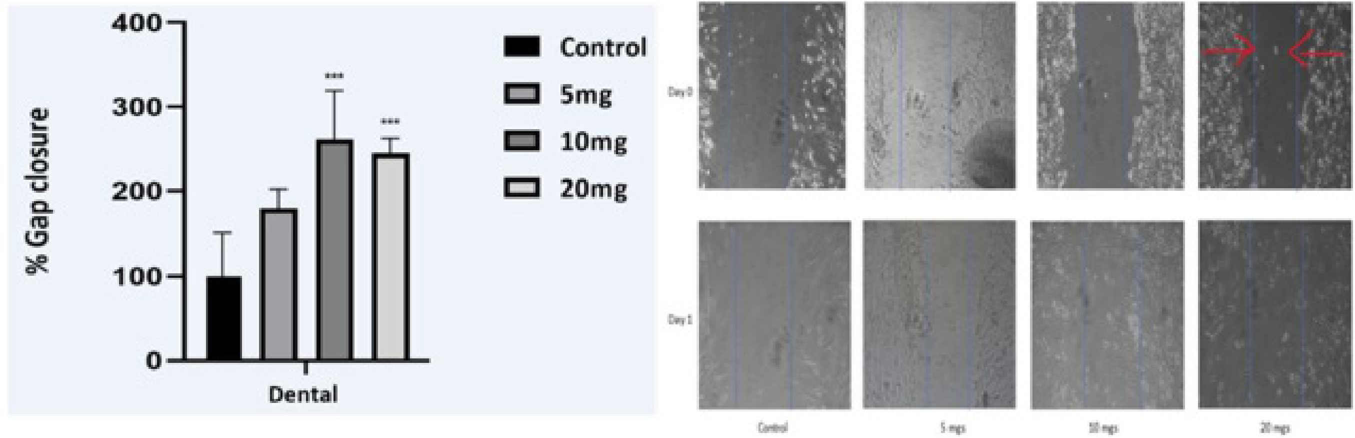

Figure 7: Left: The dose response of lyophilised DP-CM and AD-CM in scratch wound assay representing significant percentage gap closure that corresponds to the distance migrated by the human dermal fibroblast cells. **Right:** A representative scratch wound assay images, showing the migration (red arrows) of human dermal fibroblast cells (From Jain, *et al.* 2019) treated with 5mgs, 10 mgs and 20 mgs DPSCs-CM in comparison to the control at 0 hrs and 24 hrs

Discussion

Over the past years, research has been more focused on the need to develop safer cell culture protocols for easy translation to clinics. DPSCs have been reported to have stem cell-like properties [2]. They are proven to be a good and easily accessible source for cell therapy in regenerative medicine for dental pulp regeneration [9], bone [10], nerve and vascular disease [11]. DPSCs can be easily accessed and extracted from human third molars or deciduous teeth. However, there are limitations to their use in cell therapy products as serum is used for cell isolation and culture. Additionally, serum has the potential risk of viral contamination and lot to lot variability in serum vary depending on batch [12]. Therefore, for safer and stable cell therapy we have tried to establish a serum-free culture system for DPSCs culture and cryopreservation. In our study, isolated DPSCs (CKC endeavour-2) were well characterized and differentiated into three lineages such as adipogenic, osteogenic and chondrogenic. Moreover, human DPSCs were successfully cultured in a serum-free condition and we were able to develop serum-free and xeno-free conditioned media to make small molecules such as exosomes. In the current study, DPSCs after primary culture proliferated and differentiated at a similar rate in serum free, xeno-free media than in 10% FBS containing media. Similar results have been shown in other studies, the cells were expanded in serum-free medium, where the use of FBS for the isolation of a primary cell culture is still needed [13].

The conditioned medium can be harvested from various kinds of MSCs. Most studies show data with different sets of growth factors and other cytokines/factors. It has been well documented that MSCs release rich secretomes containing massive amounts of cytokines, chemokines, and growth factors, together with extracellular nanoparticles. The present studies identified 105 different growth factors and cytokines based on protein array. It has been widely reported that Serpin E1, HGF and bFGF plays an important role in re-epithelialization, granulation tissue formation and increases fibroblast proliferation. Similarly, the role of MSCs in angiogenesis is of great interest since inadequate vessel growth leads to wound healing disorders [8]. Collectively, these essential growth factors were sufficiently expressed in our study and suggests the potential applicability in wound healing. Further studies on animal models and the clinical trials are required to substantiate data. The most important nanoparticles in the CM secreted by MSCs are exosomes, which is researched extensively [14]. Exosomes are extracellular vesicles of 30–120 nm in diameter secreted by cells that act as messengers by communicating with other cells. After internalization into recipient cells, exosomes derived from these MSC considered to specifically differentiated cells [15]. These exosomes form the basis of developing future acellular therapy.

In conclusion, our study presents a novel application of the xeno-free, serum-free medium in DPSC culture, and shows that is practical approach to proliferate and differentiate cells to reduce the effects of the problems caused by FBS. Nevertheless, many challenges and obstacles still need to be resolved for their

ultimate use in the clinics, However, for expansion of cells and cryopreservation serum-free and xeno-free culture system could be used, which meets the DPSC bio-banking standards. DPSCs derived CM and extracellular vesicles are an attractive acellular tool for therapeutics in regenerative medicine. These finding may promote clinical applications of stem cells in acellular repair and regeneration therapies like wound healing [16].

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