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Keywords

Dental-derived; Stem cells; Secretome; Growth factors; Hypoxia

Abstract

Dental-derived stem cells (DSC) are important cells in tissue regeneration following tissue destruction. One of the environmental conditions in the injured tissue is reduce in oxygen level (hypoxia) but the effect of hypoxia on the DSC is not fully elucidated.

Objectives: This study aims to evaluate the effect of hypoxia on growth factor production and expression of dental-derived stem cells.

Methods: Rat periodontal ligament stem cells (PDLSCs) and dental pulp stem cells (DPSCs) were cultured in serum-free media for two or three days. When the cells achieved 70% confluence, they were incubated under normoxia (21%) or hypoxia (2%) conditions, before the conditioned media (CM) that contained the cells' secretomes were collected and compared with bone marrow stem cells (BMSCs).ELISA kits were used to analyze VEGF, TGF-*β*1 and IGF-1 levels in the collected CM. The reverse transcriptase-polymerase chain reaction (RT-PCR) was then used to determine the gene expression of the growth factors.

Results: Hypoxia incubation increased growth factor secretion by the dental-derived stem cells, and these findings were also supported by the gene expression analysis of *VEGF* and *TGF-β1*. Interestingly, IGF-1 was only detected in PDLSC CM, and these data were supported by prominent *IGF-I* gene expression and an inverse relationship with *IGF-BP1* expression by PDLSC, compared with DPSCs and BMSCs. TGF-*β*1 secretion by BMSCs was not influenced by hypoxic incubation.

Conclusion: Hypoxic incubation of the dental-derived stem cells alters growth factor content in the secretomes, and IGF-1 was only detected in the PDLSC secretome

Introduction

Periodontal tissue regeneration is a complex process involving the periodontal ligaments (PDL) and other complex structures, such as alveolar bone and cementum, which are usually diminished during periodontitis. In recent years, more research has been moving towards stem cells application in periodontal regeneration [1].

Mesenchymal stem cells (MSCs) are undifferentiated multipotent cells that can differentiate into mesoderm cell lineages, including osteogenic, adipogenic and chondrogenic lines. MSCs are widely distributed throughout the body, such as in the bone marrow stem cells (BMSC) and dental-derived stem cells [2], which include dental pulp-derived stem cells (DPSCs) and periodontal ligament stem cells (PDLSCs) [3,4]. However, most clinical trials using MSCs in humans are in the early stages [5], and very few are related to dental regenerative therapy [6-9].

Currently, there are several limitations in the clinical application of MSCs, including the possibility of ectopic tissue formation [10], injected MSCs being short-lived or removed by the circulation and clearance by the liver and lung [10]. The secretome is defined as cell secretions containing multiple growth factors, cytokines, enzymes, exosomes, micro RNA and other soluble mediators [14]. Many researchers have studied the bioactive molecules in MSC secretomes [15-19]. For instance, secretomes of mesenchymal and dental stem cells have been tested for tissue regeneration *in vitro* and *in vivo*, such as nerve and bone regeneration [14,15,20,21].

Despite the numerous attempts to identify the MSC secretome components, the exact content and factors contributing to their differential effects, including different oxygen concentrations during tissue culture remain inconclusive. Interestingly, disease-relevant conditions such as hypoxia have been shown to induce stem cells to increase growth factor expressions like VEGF, TGF-*β*1 and IGF-1, which are essential for cell survival and tissue regeneration [18,22-24]. Consequently, it has been concluded that stressed cells produce more protective secretomes to create an improved environment for cell survival. Furthermore, cell metabolic products may decline under serum deprivation conditions, resulting in a less cytotoxic environment [16]. However, to date, dental-derived stem cells studies on hypoxic incubation for secretome production are still limited [24-27]. Thus, this study aimed to evaluate VEGF, TGF-*β*1 and IGF-1 levels released in conditioned media from PDLSCs and DPSCs secretome compared with the more widely studied BMSCs secretome, particularly in hypoxic conditions.

Materials & Methods

Cell Cultures

MSCs were isolated from six-week-old Wistar-Han rats (Pharmaceutical Sciences Animal House, Aston University, Birmingham, UK) with an average weight of 120g. BMSCs were isolated from rat femurs, PDLSCs from the PDL tissue surrounding the roots of molar teeth and DPSCs were obtained from pulp tissue of incisor teeth, as previously described by [28,29]. The isolated cell populations were initially cultured at 37°C with 5% carbon

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dioxide (CO_2)(RS Biotech) in alpha minimum essential medium (α-MEM) (Biosera, UK) containing 10% Fetal Bovine Serum (FBS) (Gibco) and1% penicillin/streptomycin/amphotericin (100 units/mL penicillin with 100 μg/mL streptomycin and 2.5 μg/mL amphotericin) (Sigma-Aldrich, UK). The multi-potentiality of the cultures, including osteogenic and adipogenic differentiation, were verified based on previous studies and were validated in the laboratories [29].

Polymerase Chain Reaction (PCR) Analysis

Stem cell markers expression were analysed using semi-quantitative PCR [30]. RNA was isolated using the RNeasy kit (Qiagen, UK), and cDNA synthesis was generated using the TetroTM cDNA Synthesis Kit (Bioline,UK). The primers used for gene expression analysis are listed in Table 1. The specific gene band intensity was normalised to the GAPDH band intensity, and comparisons were made between the three cell types.

MSC growth in Hypoxia

The MSCs from passages three to five were seeded into 35mm dishes with a cell density of 2.5 x 104 cells/ml to evaluate the cell numbers and growth. The cells were either cultured in "normal"21% oxygen incubation or were incubated under hypoxia conditions (2% oxygen). The culture media in each well was refreshed every three days. In addition, the cells were cultured in serum-free media.

Viable cell counts were performed to monitor cell growth. The cell suspension was mixed with Trypan blue cell stain in a micro centrifuge tube and incubated for 10 min at room temperature to allow dye uptake by the cells. The viable and non-viable cells were counted manually under a microscope (Zeiss, Germany). Five counts per sample were recorded, and an average value was calculated. The cell count was repeated every three days until day 12. Experiments were performed in quadruplicate.

CM Collection

The BMSCs, PDLSCs and DPSCS of passage three to five were cultured with 10% FBS culture media until 70-80% confluent. Cultures were washed three times with 3ml PBS before 15 ml serum-free media were added into each flask. The cells were incubated in either a standard incubator with 21% oxygen or in a hypoxic incubator (Galaxy 48R, New Brunswick), in which the oxygen concentration was set to 2%. CM was collected on the second and third days of culture, filtered with a 0.2μm membrane filter (Sigma-Aldrich) and stored at -20^{0}C until ELISA analysis was performed. The experiment was conducted in triplicates.

Growth Factor Analysis

The VEGF, TGF-B1 and IGF-1 levels in each CM were determined using commercially available rat ELISA kits (R&D Systems) according to the manufacturer's guidelines.

Statistical analysis

The data obtained in this study were analysed using SPSS Version 22 for Windows. Independent sample t-test was used for experiments involving two groups, while One-way ANOVA for analysis of more than two groups, along with Bonferroni test as a post-hoc analysis. The findings were statistically significant at $p < 0.05$.

Results

The BMSCs, PDLSCs and DPSCs used in this study demonstrated typical MSC characteristics such as multi lineage differentiation and stem cell-related markers expression, including CD105, CD29, CD44 and CD90 as shown in the qRT-PCR analysis [30]. Furthermore, c-myc was expressed, which is considered a stem cell-related gene for cellular metabolism and proliferation. The expression of CD105, CD90 and CD29 in PDLSCs and DPSCs were significantly lower than BMSCs, however CD 29, CD44 and c-myc expression appeared similar across all MSCs (Figure 1).

Table 1: Primer sequence and conditions used for qRT-PCR analysis.

Primer	Sequences (5' to 3')	Annealing T (°C)	Number of cycles	Accession no.	Product size
	Housekeeping Gene				
GAPDH	F-CCCATCACCATCTTCCAGGAGC R-CCAGTGAGCTTCCCGTTCAGC	60.5	27	NM_017008	1306bp
	Stem Cell Markers				
CD105	F-TTCAGCTTTCTCCTCCGTGT R-TGTGGTTGGTACTGCTGCTC	60.5	39	AY562420	2230bp
CD90	F-AGCTCTTTGATCTGCCGTGT R-CTGCAGGCAATCCAATTTTT	60.5	27	NM_012673	1328bp
CD29	F-AATGGAGTGAATGGGACAGG R-TCTGTGAAGCCCAGAGGTTT	60.5	27	NM_017022.2	3683bp
c-myc	F-CTTACTGAGGAAACGGCGAG R-GCCCTATGTACACCGGAAGA	60.5	36	AY294970	564bp
CD44	F-TGGGTTTACCCAGCTGAATC R-CTTGCGAAAGCATCAACAAA	60.5	36	M61875.1	2747bp
	Growth Factor Genes				
VEGF	F- TTCGTCCAACTTCTGGGCTC R- GCAGCCTGGGACCACTTG	60.5	39	NM_001287107.1	3546bp
TGF-β1	F- CGCCTTAGCGCCCACTGCTCCTGT R-GGGGCGGGACCTCAGCTGCAC	60.5	33	NM_021578	1482bp
IGF-1	F- GACCCGGGACGTACCAAAAT R-GTACTTCCTTTCCTTCTCCTTTGC	60.5	36	X06043.1	521bp
IGF-BP1	F- ACCTCAAGAAATGGAAGGAGCC R- ACACAGACCTGTGGGATTCG	60.5	42	J04486.1	1482bp

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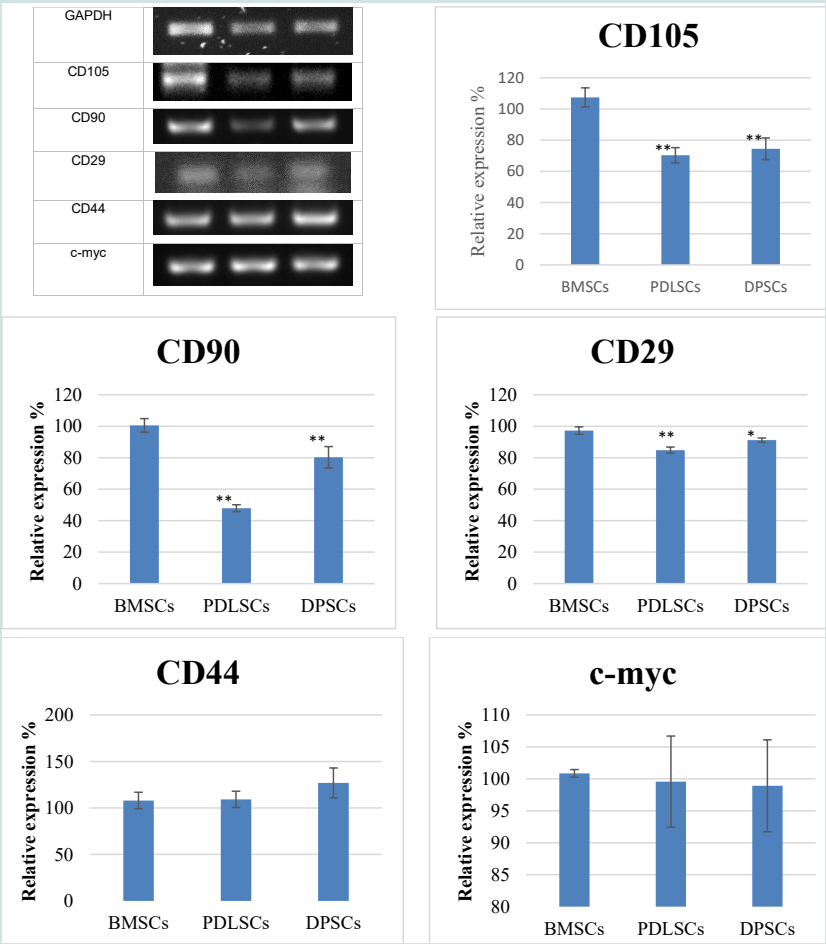

Figure 1: Stem Cell Markers. Stem Cell Marker Analysis. Semi-quantitative PCR analysis of gene expression of CD105, CD90, CD29, CD44 and c-mycin BMSCs, PDLSCs and DPSCs. Y-axis is the relative expression to GAPDH. Statistical comparison was performed using BMSCs as control with *p-value<0.05 and **p-value<0.001, n=3.

MSC growth

Hypoxic conditions

The DPSC cultures exhibited a significant increase in cell numbers under hypoxic conditions. On the other hand, the PDLSC growth rate increased significantly in the hypoxic environment until day 6 but decreased at day 9 (Figure 2). In contrast, the BMSC cultures had significantly increased cell numbers under normoxic compared to hypoxic conditions on day 12 (p-value<0.001).

Serum-free media culture conditions

MSCs cultured in serum-free media for three days showed no differences in cell viability and numbers under different oxygen incubations except for PDLSCs, which had significantly reduced cell numbers in hypoxia incubation. Additionally, no significant differences were recorded for viable cell numbers between BMSCs and DPSCs incubated in normoxia or hypoxia at three-time intervals, except the ones shown in Figures 3a and c. PDLSCs incubated in both normoxia and hypoxia incubations showed increased cell numbers at all time points, and the increases were statistically significant (Figure 3b). Generally, the result showed that cell viability was maintained in this study.

Levels of growth factors secreted in serum-free media cultures

Hypoxic-incubated BMSCs, PDLSCs and DPSCs secreted significantly greater VEGF than cells in normoxic conditions (Figure 4). Interestingly, IGF-1 was only detected in PDLSC cultures, where the hypoxic-incubated cells produced higher IGF-1 than the normoxic-incubated cells (Figure 4). Moreover, the TGF-β1 level was statistically higher in hypoxic PDLSCs and DPSCs.

These findings were subsequently corroborated by RT-PCR gene expression analysis. The differences in VEGF and TGF-β1 expression in all three cell types were not significant, although VEGF expression by BMSCs normoxia samples was higher than the hypoxia samples. BMSC and PDLSC hypoxic cultures expressed higher IGF-1, in contrast normoxic DPSCs expressed higher IGF-1(Figure 5).

All samples from the different cell types under different incubation conditions demonstrated higher VEGF, TGF-β1 and IGF-1 levels on day 3 compared today 2, except for TGF-β1 secreted

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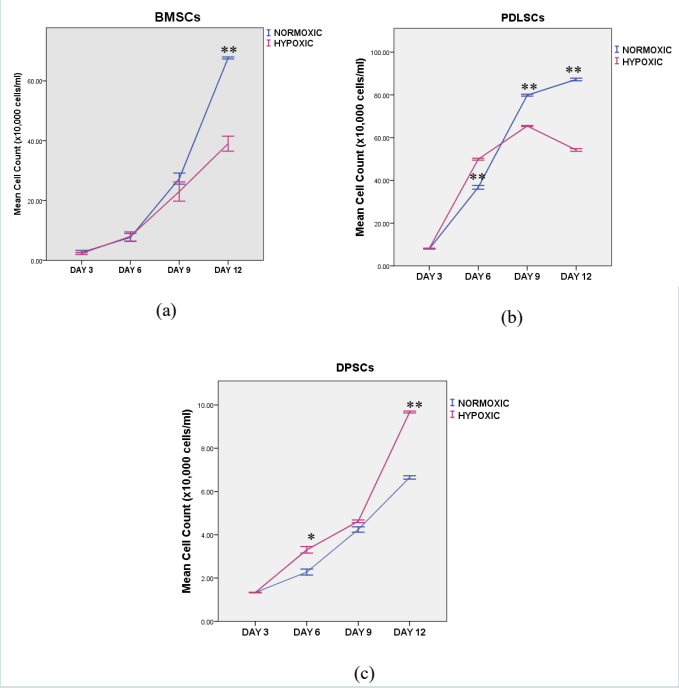

Figure 2: MSC growth under normoxic and hypoxic conditions. Viable cell count of the cells in each incubation environment was analysed and statistically compared using normoxic cultured cells as control with *p -value<0.05 and **p-value<0.001. Mean cell count +/- SD (n=4).

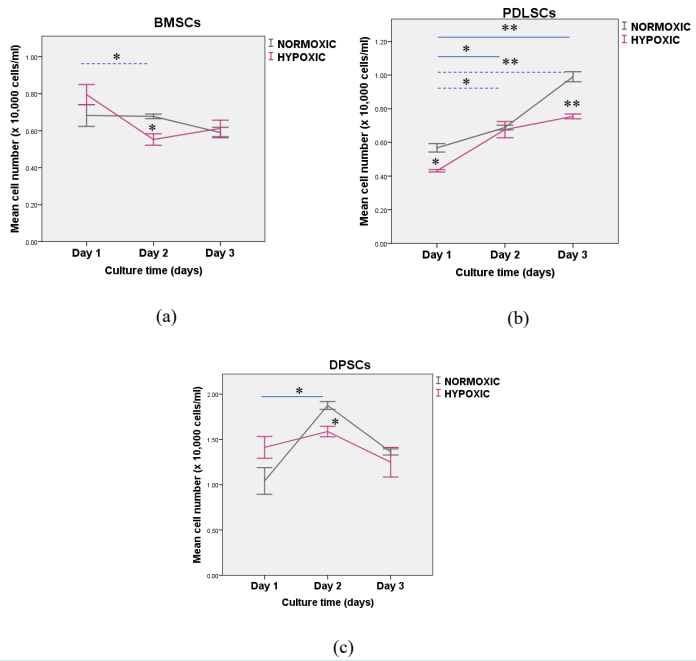

Figure 3: MSC number in serum-free media and different oxygen concentration. Data show mean cell numbers for the three cell types. The statistical analysis was performed between 1) between normoxic and hypoxic cells at all time points, and 2) between day 1 and day 2 or day 3. (n=5).
_____Comparison between normoxic cells
----------Comparison between hypoxic cells

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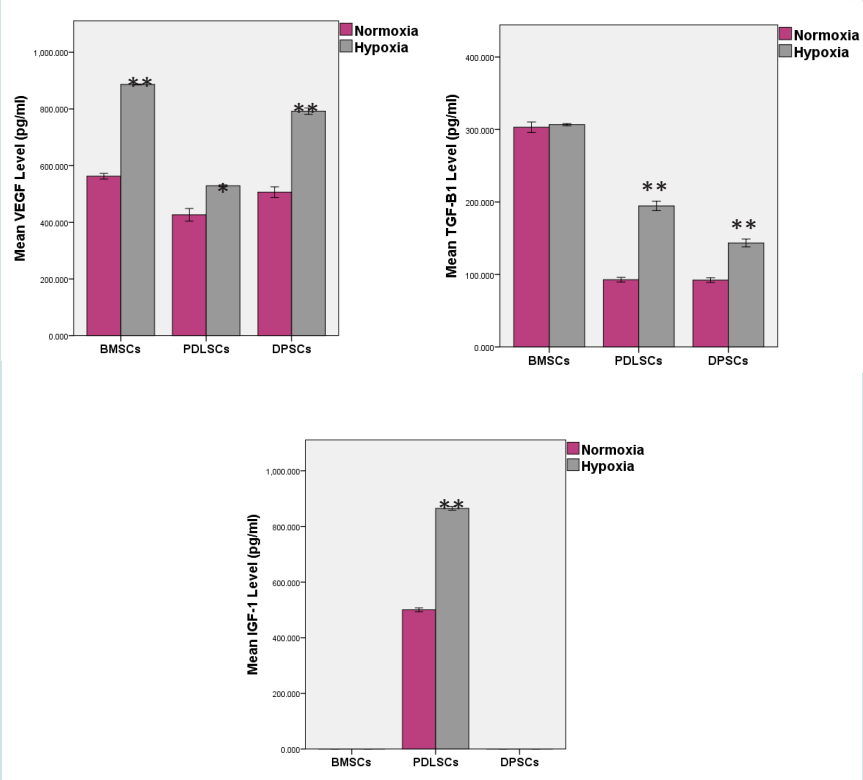

Figure 4: The level of growth factors released by the cell after 3 days in serum-free media and different oxygen incubations. Statistical comparison was performed using normoxic cultured cells as control with *p -value<0.05 and **p-value<0.001. n=3 (technical replicates).

by BMSCs (Figure 6). Apart from that, the filtrated CM contained significantly lower growth factors except for VEGF in BMSCs **under** hypoxic conditions (p-value =0.81, Figure 7) and TGF-β1 in DPSCs under hypoxic conditions(p-value = 0.215, Figure 7).

Discussion

The hypoxia-stimulated proliferation of MSCs has been reported in previous studies [31,32]. A low oxygen environment has been proposed to maintain their stemness by preserving the undifferentiated state of the cells. Conversely, increased oxygen concentration can promote MSC differentiation [31]. Similar findings were recorded for dental-derived stem cells in the current study. The PDLSC numbers increased in a hypoxic environment at the onset of the culture period; however, cell growth decreased by day 9. Progenitor cells residing in the periodontal ligament are speculated to have a reduced oxygen environment, as demonstrated by a previous *in vivo* study of tooth root development [33]. The increased DPSC numbers under hypoxia conditions are consistent with other studies that utilised animal and human samples [34,35].

When the cells were cultured in serum-free media for up to three days, there was a decrease in BMSC cell viability after two days and after one day culture for DPSCs, under normoxia and hypoxia conditions. In contrast, PDLSCs were constantly viable, which may be contributed by active IGF-1as a survival-promoting and anti-apoptotic factor in the culture. Previous studies have reported that the epigenetic programming of the IGF-1 gene in MSCs may occur when cultured in serum-deprived conditions, and IGF-1-depleted CM demonstrated higher cell apoptosis compared with cells cultured in non-depleted IGF-1 CM [36]. Furthermore, IGF-1 is one of the growth factors involved in cell metabolism and regulates oxidative stress resistance [37]. Thus, this finding may be related to the demand for PDLSCs as they exhibit the fastest turnover rate in the body [38]. Similarly, the present study showed an increase in IGF-1 level by PDLSCs in hypoxic incubation.

IGF-1was exclusively expressed by PDLSC, while IGF-BP1 was present at relatively low levels in PDLSCs compared with BMSCs and DPSCs. This finding may be related to PDLSCs relatively high turnover rate and the metabolically active state of PDL cells [38,39]. Consequently, the presence and role of IGF-I and its multiple binding proteins in different MSCs require further investigation. Notably, a recent microarray study demonstrated relatively high levels of IGF-BP-6, -2 and -4, but lower IGF-BP1 and -3 in the secretome of human PDLSCs [40]. In addition, a higher level of IGF-2 in PDLSC CM was reported compared to IGF-1 [40].

The relatively low IGF-1 gene expression in the DPSC samples aligned with previous reports. An increase in IGF-BP2 and IGF-BP3 gene expression was observed, although there was no mention of IGF-BP1 expression [41]. Furthermore, the low level of IGF-1 concomitant with the high level of IGF-BP1 in BMSC may be related to the role of BP in protecting IGF-1 degradation within the bone marrow niche [42]. Apart from IGF-BP1, other IGF-BPs were also

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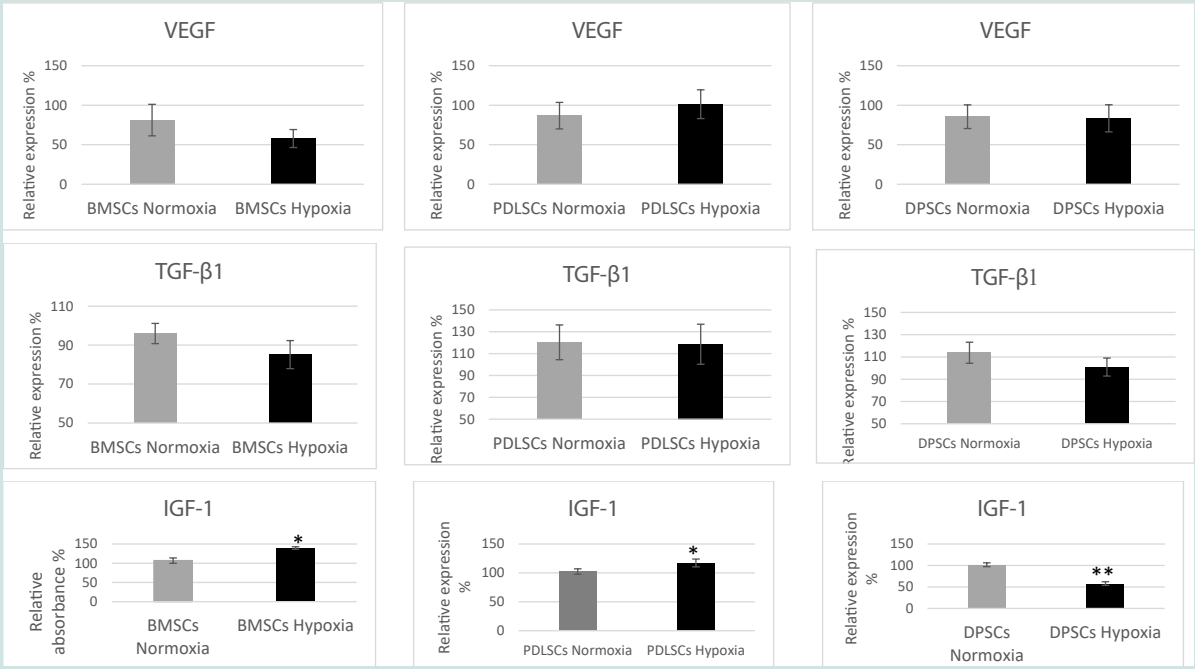

Figure 5: Growth factor gene expression in MSC cultured normoxia and hypoxia.
Semi-quantitative PCR analysis of gene expression profiles of BMSCs, PDLSCs and DPSCs showed the expression of VEGF, TGF-β1 and IGF-1 between normoxia and hypoxia cultures. Statistical comparison was performed using normoxic cultured cells as control with *p-value <0.05 and **p-value<0.001 n=3.

identified in CMs from BMSCs, including IGF-BP-2, -3, -4 and -6 in their microarray profiles [17,23]. However, the specific roles of this IGF-BPs within this context have not been fully understood.

Hypoxia incubation can alter VEGF secretion. In this study, VEGF production was higher in hypoxia conditions for all three cell types. Nonetheless, this outcome was contradictory at the transcriptome levels since there were no significant differences between normoxia and hypoxia conditions for all cell types. This observation may be the result of translational differences and cellular storage of VEGF [43].

VEGF was highly secreted by BMSCs inhypoxia after three days of culture, whereas PDLSC CM demonstrated the lowest VEGF levels, although hypoxia incubation increased its production. The effect of hypoxia incubation on VEGF production corroborated with other studies. For instance, VEGF production was higher by PDLSCs and DPSCs incubated in hypoxia conditions with 1-2% oxygen concentration [34]. The relatively high production of VEGF by BMSCs compared with DPSCs and PDLSCs could reflect the significance of cell origin from the bone marrow. This tissue is closely associated with the regulation of angiogenesis and provides a known source for endothelial progenitor cells, not only under normal physiology but in any pathological conditions in the body [44].

TGF-β1 is a critical growth factor in tissue repair and regulates cell differentiation [45]. In this study, TGF-β1 was highly expressed by BMSCs compared to PDLSCs and DPSCs, and hypoxic incubation induced both cell types to produce more TGF-β1. Nevertheless, there was no difference in TGF-β1 production by BMSC under different oxygen concentrations, indicating that oxygen is not essential for TGF-β1 production by BMSCs.

There are various methods available for CM collection to study the secretome from cultured cells. Most studies have opted for 48 h [14,17,19,20,24], while the others collected the CM on the third day or after. The CM was collected after two days to obtain richer growth factors or bioactive molecules, but the media could be a limiting factor because of the cells metabolic activity [16]. One study collected the CM of rat DPSCs, BMSCs and ADSCs after three days for mass spectrometry analysis and reported various proteomic profiles associated with MSC secretome and angiogenesis, cell migration and inflammatory response [46]. Another study reported the collection of CM from DPSC cultures on every 4th day up to 24 days culture. Notably, it was found that the cell viability was higher at shorter collection periods [16]. In the present study, CM was collected at less than four days to reduce the chances of contamination with by-products of the cell metabolic activity. The data indicated that a longer culture time allowed more growth factors to accumulate in the CM.

Another variable in the CM processing technique is filtration, as reported in the literature [10,16,20,46]. The current study recorded lower growth factor concentrations in media filtered through a 0.2μm membrane, commonly used for sterile filtration. This finding suggested that bioactive molecules such as VEGF, TGF-β1 and IGF-1 are entrapped and filtered out.

Important growth factors were detected in BMSCs CM, PDLSCs CM and DPSCs CM compared to serum-free media. However, the serum utilised during cell expansion in the laboratory is usually animal-based, thus, carrying the risk of cross-infection from animals to other species and eliciting adverse reactions [47]. Furthermore, clinical studies using stem cell secretomes are limited and only involve

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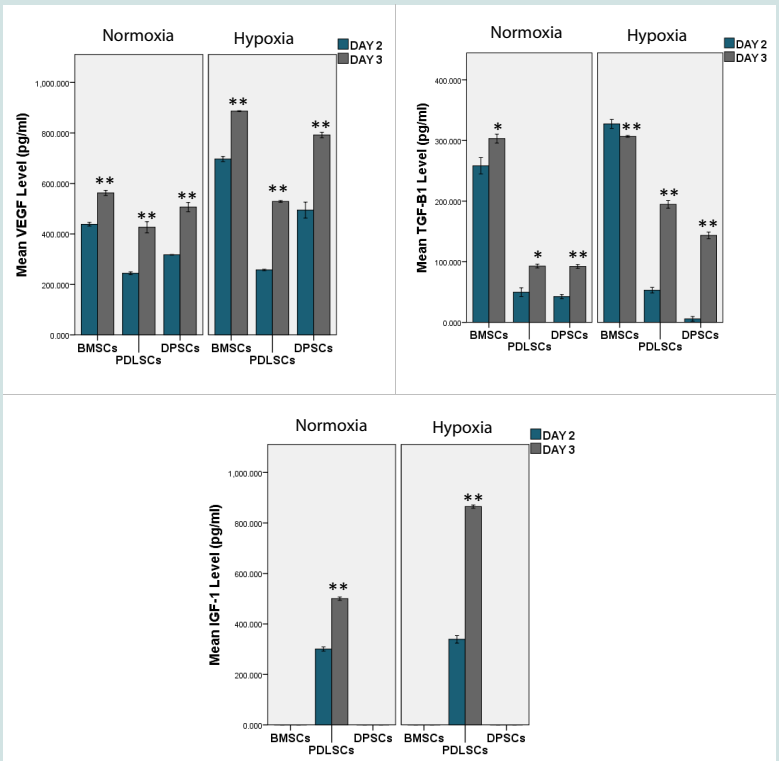

Figure 6: Growth factors levels in CM collected on different days.
The level of growth factors shown in the culture media were analysed in BMSCs, PDLSCs and DPSCs cultured for two- or three-days and. The statistical comparison was performed using Day 2 CM as control group with *p -value<0.05 and **p-value<0.001. n=3.

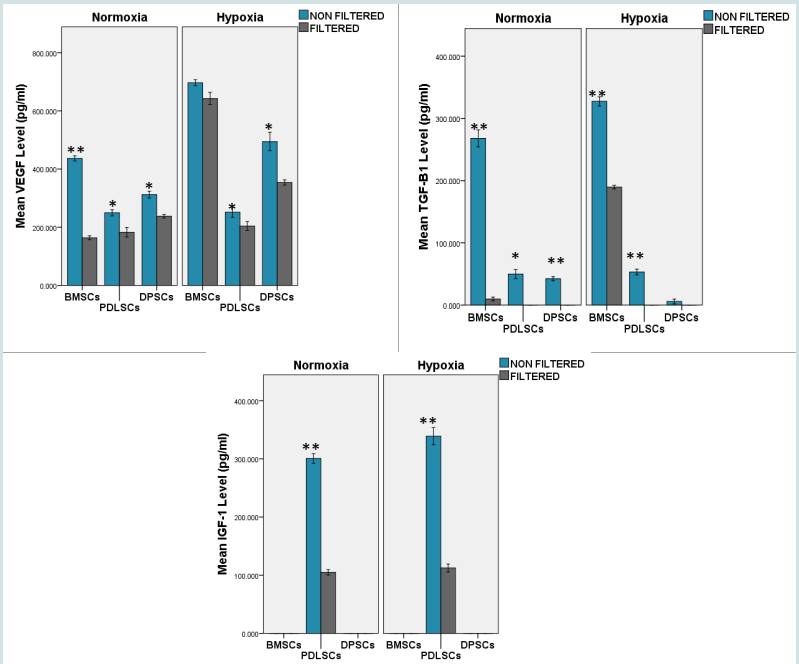

Figure 7: Effect of the filtration on the level of the growth factors in the collected CM.
Level of VEGF, TGF-β1 and IGF-1 secreted by the BMSCs, PDLSCs and DPSCs with and without 0.2μm filtration. Statistical comparison was performed using filtered samples as control with *p -value<0.05 and **p-value<0.001. n=3.

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the BMSC secretomes [48]. Therefore, since the cells secretome were retrieved in the form of CM and serum-free in this study, they are highly applicable for translational research and human clinical studies. Nevertheless, further investigations are crucial on dosages, storage, and long-term safety of the CM.

Conclusion

In summary, hypoxia incubation can potentially promote dental-derived stem cell cultures to generate optimum VEGF, TGF-β1 and IGF-1 within the secretome. In addition, it can be concluded that the secretome derived from PDLSCs was notably different compared to the other two cell types in IGF-1 production. Nevertheless, further study is required to determine the dental-derived stem cells secretomes mechanism of action to be translated into therapeutic application.

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