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Stem Cells from Human Extracted Deciduous Teeth Expanded in Foetal Bovine and Human Sera Express Different Paracrine Factors after Exposure to Freshly Prepared Human Serum

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Running Title: Expression of Paracrine Factors in Freshly Prepared Human Serum

Abbreviations: CPD, cumulative population doubling time; FBS, foetal bovine serum; FGF-2, fibroblast growth factor 2; G-CSF, granulocyte colony stimulating factor; HGF, hepatocyte growth factor; IGF-1, insulin like growth factor 1; iHS, individual human sera; IL, interleukin; ISCT, International Society for Cellular Therapy; LIF, leukaemia inhibitory factor; MSCs, mesenchymal stem cells; PDGF-BB, platelet derived growth factor BB; pHS, pooled human serum; SCF, stem cell factor; SDF-1A, stromal cell-derived factor-1A; SHED, stem cells from human extracted deciduous teeth; VEGF, vascular endothelial growth factor.

Abstract

Background: The response of stem cells to paracrine factors within the host's body plays an important role in the regeneration process after transplantation. The aim of this study was to determine the viability and paracrine factor profile of stem cells from human extracted deciduous teeth (SHED) pre-cultivated in media supplemented with either foetal bovine serum (FBS) or pooled human serum (pHS) in the presence of individual human sera (iHS).

Methods: SHED (n=3) from passage 4 were expanded in FBS (FBS-SHED) or pHS (pHS-SHED) supplemented media until passage 7. During expansion, the proliferation of SHED was determined. Cells at passage 7 were further expanded in human serum from four individual donors (iHS) for 120 hours followed by assessment of cell viability and profiling of the secreted paracrine factors.

Results: Proliferation of SHED was significantly higher ($p<0.05$) in pHS supplemented media compared to FBS supplemented media. pHS-SHED also maintained their higher proliferation rate compared to FBS-SHED in the presence of iHS. In iHS supplemented media, FBS-SHED expressed significantly higher levels of SDF-1A ($p<0.05$) after 24 hours compared to pHS-SHED. Similar results were found for HGF ($p<0.01$), LIF ($p<0.05$), PDGF-BB ($p<0.05$), SDF-1A ($p<0.01$), and IL-10 ($p<0.05$) when cell culture supernatants from FBS-SHED was profiled 120 hours post-incubation.

Conclusion: SHED expanded in pHS instead of FBS have higher proliferative capacity and show an altered secretion profile. Further studies are needed to determine whether these differences could result in better engraftment and regeneration following transplantation.

Keywords: mesenchymal stem cells; paracrine factors; proliferation; regenerative medicine; SDF-1.

1 Introduction

In the last few decades, cell based regenerative therapies have received considerable attention in the field. Multipotent mesenchymal stem cells (MSCs) are considered one of the best sources of stem cells for regenerative therapy. Several *in vitro* and *in vivo* studies have shown promising regenerative outcomes after transplantation of MSCs in different pathological scenarios (Gnanasegaran et al. 2017; Williams et al. 2013; Miyahara et al. 2006). However, most of the clinical trials using MSCs reported only short-term regenerative benefits that were linked to their low retention following transplantation (Haque et al. 2015; Trounson and McDonald 2015).

MSCs reside in the perivascular region of almost all tissues and organs of the human body (Kalinina et al. 2011; da Silva Meirelles et al. 2006). The number of MSCs within the tissues is relatively low; hence *in vitro* expansion prior to transplantation is needed to acquire therapeutically relevant cell numbers (Haque and Abu Kasim 2017). MSCs are often expanded in media supplemented with foetal bovine serum (FBS) that contains xenoantigens and could potentially impair the regenerative potential of MSCs (Haque et al. 2015). Recently, human platelet lysates, human cord blood serum, and allogenic pooled human serum (pHS) have been used as media supplements for MSCs expansion. Moreover, it has been suggested that these supplements are suitable for *in vitro* expansion of MSCs (Haque and Abu Kasim 2017; Bieback et al. 2012; Blazquez-Prunera et al. 2017b). Several recent studies suggested that the immunomodulatory properties of MSCs are dependent on the type of supplements such as FBS, pHS and commercially produced cell culture supplement that used for their expansion (Blazquez-Prunera et al. 2017a; Komoda et al. 2010). However, no major complications have been reported in clinical trials using MSCs expanded with different supplements (Yubo et al. 2017; Yim et al. 2016). Thus, we hypothesized that xeno-contamination might not be the only factor affecting engraftment and regenerative outcomes after MSC transplantation. Notably, regeneration represents a highly complex process and involves a large number of factors including paracrine signalling molecules, extracellular vesicles, and the extracellular matrix (Vunjak-Novakovic and Scadden 2011; Wagers 2012; Bassat et al. 2017). Moreover, endogenous cytokine gradient have been reported to play a vital role in the directional migration and engraftment of transplanted MSCs (Youn et al. 2011; Park et al. 2017). Hence,

following transplantation, appropriate response of the transplanted cells to the host`s microenvironment is important for successful engraftment of the cells and the subsequent regeneration of the targeted organ or tissue. Furthermore, secretion of paracrine factors by the graft cells could negatively impact the host's cytokine homeostasis.

SHED are neural crest-derived ectomesenchymal stem cells that, if cultivated in presence of FBS, exhibit all crucial properties of MSCs including the expression pattern, differentiation capacity, and immunotolerance after transplantation (Kaltschmidt et al. 2012; Sloan and Waddington 2009). In addition, it has been suggested that SHED are well tolerable and might have a proliferation and differentiation potential superior to MSCs from other sources (Wang et al. 2012; Nakamura et al. 2009).

As the paracrine factors play a vital role in the processes of proliferation, migration, and homing of transplanted stem cells including SHED, we to analyse the cell viability and expression of paracrine factors in SHED expanded in FBS and pHS supplemented media that have been exposed to freshly prepared human serum in an attempt to simulate the graft microenvironment. In this study we report that SHED expanded in the FBS have lower proliferative capacity and secrete higher level of paracrine factors in the presence of iHS compared to SHED expanded in pHS.

2 Materials and Methods

2.1 Ethics Approval

All the samples were obtained following an informed written consent. Sample collection procedures were approved by the Medical Ethics Committee, Faculty of Dentistry, University of Malaya (Reference #DF RD1301/0012[L] for blood collection; DFCD0907/0042[L] for teeth collection). An overview of the experimental strategy study is shown in the Figure 1.

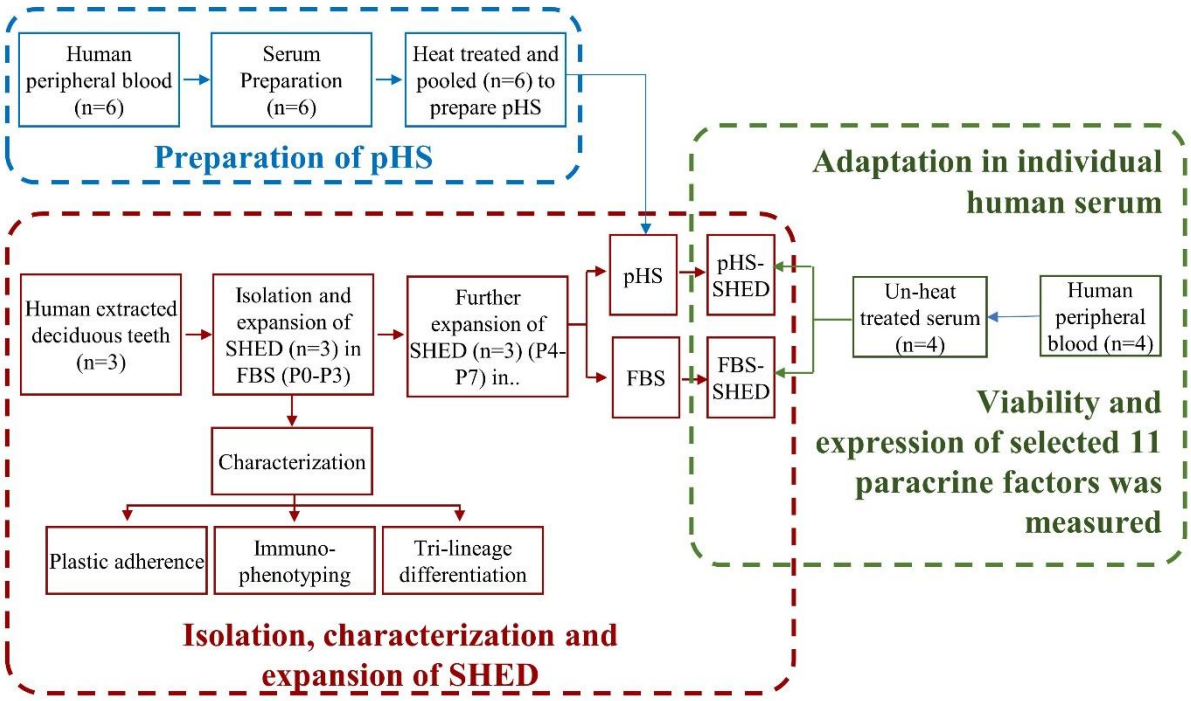

Figure 1 Schematic overview of the experimental strategy. (SHED, Stem cells from human extracted deciduous teeth; FBS, foetal bovine serum; pHS, pooled human serum; P, Passage; FBS-SHED, SHED expanded in FBS supplemented medium; pHS-SHED, SHED expanded in pHS supplemented medium).

2.2 Isolation and Expansion of SHED

SHED were isolated as described earlier (Govindasamy et al. 2010; Haque and Abu Kasim 2017).

2.3 Assessment of MSC Characteristics in SHED

According to the guidelines by the International Society for Cellular Therapy (ISCT) MSCs should fulfil the following criteria: they must adhere to plastic, express specific surface markers, and be capable of tri-lineage differentiation in vitro (Dominici et al. 2006).

Plastic adherence of SHED was confirmed by using an inverted microscope (Primo Vert, Carl Zeiss, Germany). Expression of specific surface antigens on SHED and tri-lineage differentiation potential were determined as described earlier (Haque and Abu Kasim 2017).

2.4 Preparation of Human Serum

Blood was collected from healthy male donors aged 21-35 years. Exclusion criteria were as follows: smoking, alcohol consumption, drug and/narcotics addiction, chronic diseases and diseases diagnosed within four weeks prior to the blood collection, major surgical treatment in the last 24 months, and immunotherapy. Blood was collected by trained health nurses at the Oro-Maxillofacial Surgical and Medical Sciences Department, Faculty of Dentistry, University of Malaya. Serum was prepared as described before (Haque and Abu Kasim 2017).

Briefly, 20 ml of blood was collected from each donor (n=6), transferred into a 50 ml sterile centrifuge tube (Falcon®, Corning, NY, USA) containing no anticoagulant and allowed to stand at room temperature for an hour to facilitate coagulation. The coagulated blood was centrifuged at 400×g for 15 minutes. After subsequent centrifugation at 1800×g for 15 minutes, the final serum supernatant was incubated at 57±2 °C for 30 minutes to obtain a complement inactivated human serum. Six heat inactivated sera (n=6) were combined to prepare the pHS.

iHS from four donors was prepared according to the procedure mentioned above with the minor modification. In particular, after the final round of centrifugation, the serum supernatant was left untreated to retain complement activity. pHS and non-inactivated iHS were sterilized by filtration through a 0.2 μm membrane filter (Nalgene™, Thermo Fisher Scientific, NY, USA).

2.5 Cultivation of SHED

SHED (n=3) were maintained in 10% FBS supplemented Knockout™-DMEM (Gibco®,Thermo Fisher Scientific) until passage 3. Subsequently (from passage 4-7), cultures were maintained in Knockout™-DMEM supplemented with either 10% FBS (Gibco®, Thermo Fisher Scientific, Lot No. 10270) or 10% pHS. Animal derived component free TrypLE™ express (Gibco®, Thermo Fisher Scientific) was used as cell dissociation reagent.

2.6 Effects of FBS and pHS on Proliferation of SHED

Cells from passage 5 to passage 7 were counted by using Trypan Blue (Gibco®, Thermo Fisher Scientific) dye exclusion method. Three technical replications for each biological samples (n=3) were

performed and the average number of cells were used to determine the population doubling time (PD) of SHED from each donor. Data were analysed and plotted using Microsoft Excel. PD at each passage was calculated by using the following equation:

$$X = \frac{\log_{10}(N_H) - \log_{10}(N_I)}{\log_{10}(2)}$$

where X = population doublings, N_I = inoculum number, and N_H = cell harvest number. To obtain the cumulative population doubling time (CPD), the PD increase at the given passage was added to the PD of previous passages (Cristofalo et al. 1998; Li et al. 2015).

2.7 Viability of FBS-SHED and pHS-SHED in Individual Human Serum

SHED maintained in FBS and pHS supplemented medium (FBS-SHED and pHS-SHED respectively) were seeded (passage 7) at a density of 100000, 50000, 25000, 12500 and 6255 cells/well in 96 well plates containing Knockout™-DMEM supplemented with 10% iHS (n=4). After 24 hours of incubation at 37 °C in 95% humidified air, and 5% CO_2, the viability of SHED was analysed using PrestoBlue™ Cell Viability Reagent (Invitrogen™, Thermo Fisher Scientific). In brief, all the media were discarded after 24 hours of incubation and the wells were washed twice with DPBS. Knockout™-DMEM with 10% PrestoBlue™ reagent (v/v) was added to each well and the plates were further incubated for 2 hours. Absorbance was measured at 570 nm with reference wavelength set to 600 nm using a microplate reader (Infinite 200 PRO, Tecan, Switzerland). The absorbance values were converted to the corrected absorbance of PrestoBlue™ reagent.

2.8 Immunoassay

At passage 7, FBS-SHED and pHS-SHED were seeded in Knockout™-DMEM supplemented 10% iHS and supernatants were collected after 24 and 120 hours post-incubation. Supernatants were used to measure the amount of selected paracrine factors by using Luminex-based ProcartaPlex human cytokine/chemokine 11plex immunoassay kit (affymetrix, e-Bioscience, Vienna, Austria). The analysed paracrine factors were selected based on their involvement in cell survival and regulation of regeneration (Table 1)

Table 1: Selected paracrine factors analysed in the current research

Name of the paracrine factor	Function (References)
Fibroblast growth factor 2 (FGF-2)	Shows mitogenic effect (Salcedo et al. 1999; Werner and Grose 2003); enhances proliferation of human BM-MSCs by activation of JNK signalling (Ahn et al. 2009); slows down telomere shortening and the ageing process of MSCs (Yanada et al. 2006; Bianchi et al. 2003); provides cytoprotection (Werner and Grose 2003); induces CXCR4 expression on cells and helps angiogenesis (Salcedo et al. 1999)
Granulocyte colony stimulating factor (G-CSF)	Enhances cellular proliferation, migration, chemotactic properties (Murakami et al. 2013); prevents apoptosis (Murakami et al. 2013); induces HSCs and MSCs mobilization from bone marrow (Kawada et al. 2004)
Hepatocyte growth factor (HGF)	Induces proliferation, survival, migration and site-specific homing of various cell types including MSCs (Forte et al. 2006; Son et al. 2006).
Leukaemia inhibitory factor (LIF)	Supports self-renewal and maintains multi-differentiation potential of MSCs and other stem or progenitor cells (Metcalf 2003; Kolf et al. 2007).
Platelet derived growth factor BB (PDGF-BB)	Enhances expansion, migration and survival of MSCs (Fierro et al. 2007; Tamama et al. 2006; Krausgrill et al. 2009).
Stem cell factor (SCF), KIT ligand	Regulates proliferation, differentiation, migration and homing of several cell types including HSCs and MSCs (Lennartsson and Ronnstrand 2012; Pan et al. 2013).
Stromal cell-derived factor-1a (SDF-1A)	Enhances site specific migration and homing of MSCs by regulating SDF1/CXCR4 pathway (He et al. 2010; Yu et al. 2015).
Vascular endothelial growth factor A (VEGF-A)	Enhances proliferation and survival MSCs (Pons et al. 2008); promotes angiogenesis, anti-apoptotic and immunomodulatory properties (Sulpice et al. 2009; Wang et al. 2006)
Interleukin 4 (IL-4)	Induces activation of B cells; stimulates proliferation of T cells; regulates differentiation of T-lymphocytes to T helper cell 2 (T_{H2}) (Choi and Reiser 1998).
Interleukin 6 (IL-6)	Stimulates acute phase proteins production (Fattori et al. 1994); induces chronic inflammatory responses by stimulating T- and B- lymphocytes (Gabay 2006).
Interleukin 10 (IL-10)	Promotes immunosuppressive functions by inhibiting activities of T_{H1} cells, natural killer cells, and macrophages (Couper et al. 2008; Pierson and Liston 2010);.induces antibody production from activated B cells and amplifies humoral responses (Rousset et al. 1992).

2.9 Data Analysis

Data were analysed using independent sample t-test (SPSS version 22) and $p < 0.05$ was considered significant.

3 Results

3.1 SHED Exhibit MSC Characteristics

Morphological analysis using an inverted microscope revealed a homogenous monolayer soft plastic adherent cells, typical for MSCs (Figure 2 A, B). Using flow cytometry, we validated the expression of MSC positive markers (CD73, CD90, CD105) on the 95% SHED. Moreover, absence of CD14, CD20, CD34, and CD45 in majority of SHED was confirmed (Figure 2 C). The tri-lineage differentiation potential (adipogenic, chondrogenic and osteogenic) of SHED was confirmed following directed differentiation (Figure 2 D-F). Notably, osteogenic, adipogenic and chondrogenic cells were detected only in cells subjected to differentiation conditions while no differentiation was seen in control culture. These properties confirm that the isolated cells were SHED that have the MSCs like properties.

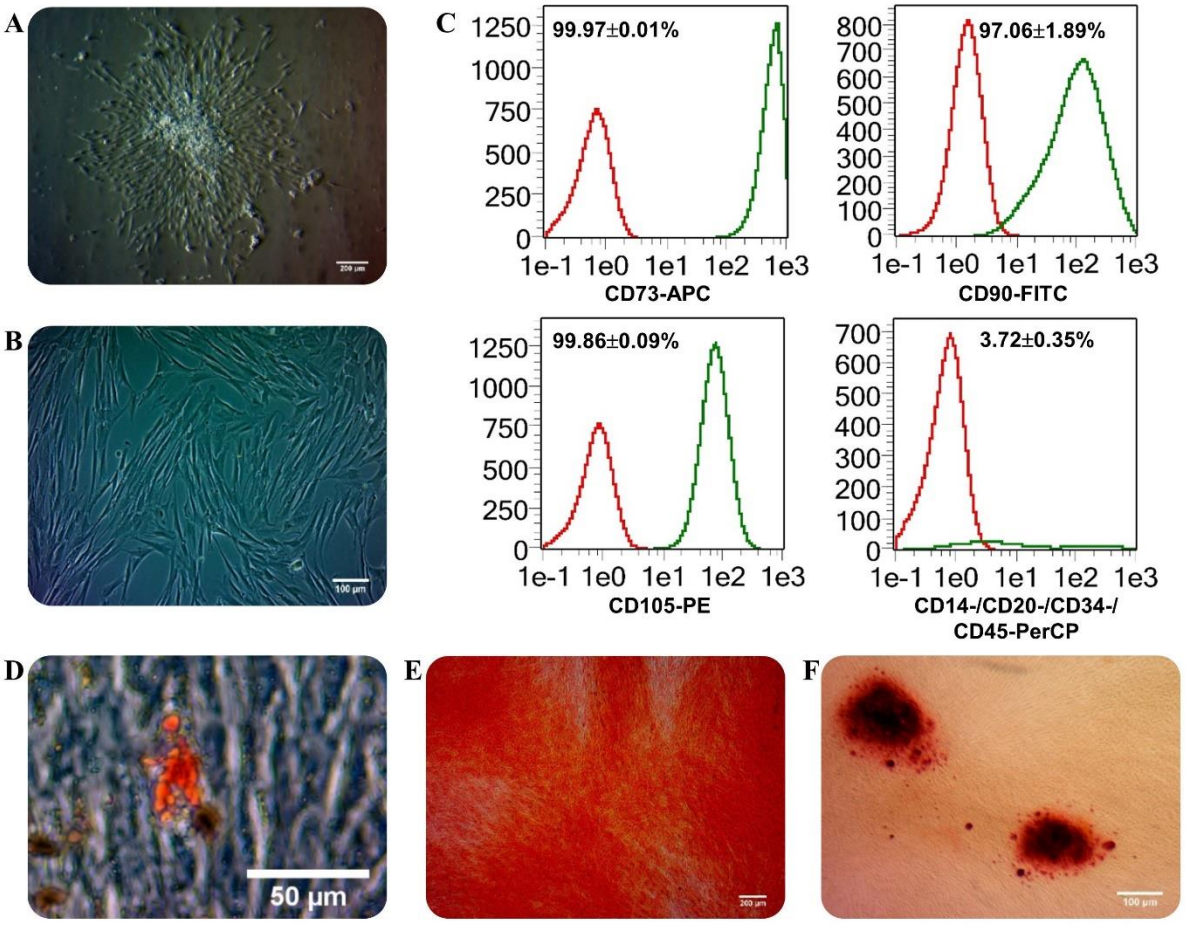

Figure 2 MSCs like properties of SHED. (A,B) SHED adhere to plastic surfaces. (C) Cells were immuno-labelled with a cocktail of fluorochrome-conjugated monoclonal antibodies: allophycocyanin (APC)-conjugated CD73, fluorescein isothiocyanate (FITC)-conjugated CD90, phycoerythrin (PE)-conjugated CD105, and peridinin-chlorophyll-protein complex (PerCP)-conjugated CD14, CD20,

CD34 and CD45. FACS analysis reveals that SHED are positive for CD90, CD105 and CD73 and do not express CD14, CD20, CD34 and CD45. (D) Oil Red O positive lipid droplets reveal adipogenic differentiation potential of SHED. (E) Safranin O positive staining confirms chondrogenic differentiation potential of SHED and (F) Alizarin red positive extracellular calcium deposition indicates osteogenic differentiation potential of SHED.

3.2 SHED Expanded in pHS Maintain a Highly Proliferative Phenotype in iHS

Significantly higher proliferation of SHED was observed in pHS compared to FBS supplemented media (Figure 3 A, B). SHED seeded at density of 25000cells/well or lower showed significantly higher viability in pHS supplemented medium after 24 hours of incubation (Figure 3 C). In the presence of iHS, significantly higher viability of pHS-SHED was also observed (Figure 3 D).

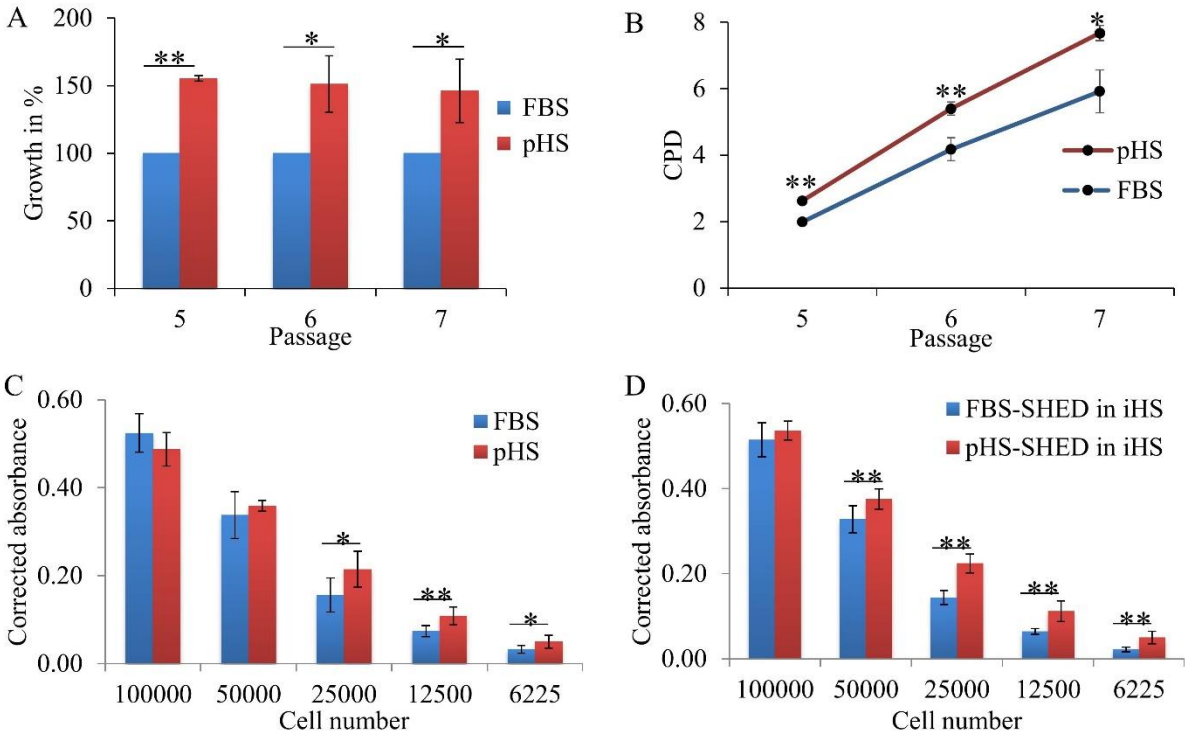

Figure 3 Proliferation of SHED. A) Comparative growth of SHED (n=3) in pooled human serum (pHS) and foetal bovine serum (FBS) supplemented media. B) Cumulative population doubling (CPD) of SHED in pHS and FBS supplemented media. C) Viability of SHED in the pHS and FBS supplemented media after 24 hours of incubation. D) Viability of SHED yielded from pHS and FBS supplemented media (pHS-SHED and FBS-SHED respectively) in the presence of iHS (n=4) after 24 hours of incubation. (* = $p<0.05$, ** = $p<0.01$)

3.3 SHED Expanded in FBS Express Higher Levels of Paracrine Factors in iHS

After 24 hours of incubation in the presence of iHS, significantly higher expression of SDF-1a was detected in FBS-SHED cell culture supernatants compared to that in pHS-SHED. The expression levels of FGF-2, HGF, LIF, PDGF-BB, VEGF, and IL-6 were higher in FBS-SHED supernatants. In contrast, marginally higher expression levels of SCF, G-CSF and IL-10 were observed in the cell culture supernatants of pHS-SHED (Figure 4).

After 120 hours of incubation, significantly higher expression levels of HGF, LIF, PDGF-BB, SDF-1 and IL-10 were detected in the FBS-SHED supernatants. Moreover, we detected higher expression of FGF-2 and IL-6. Higher expression of SCF, VEGF-A and IL-4 were detected in pHS-SHED supernatants (Figure 4).

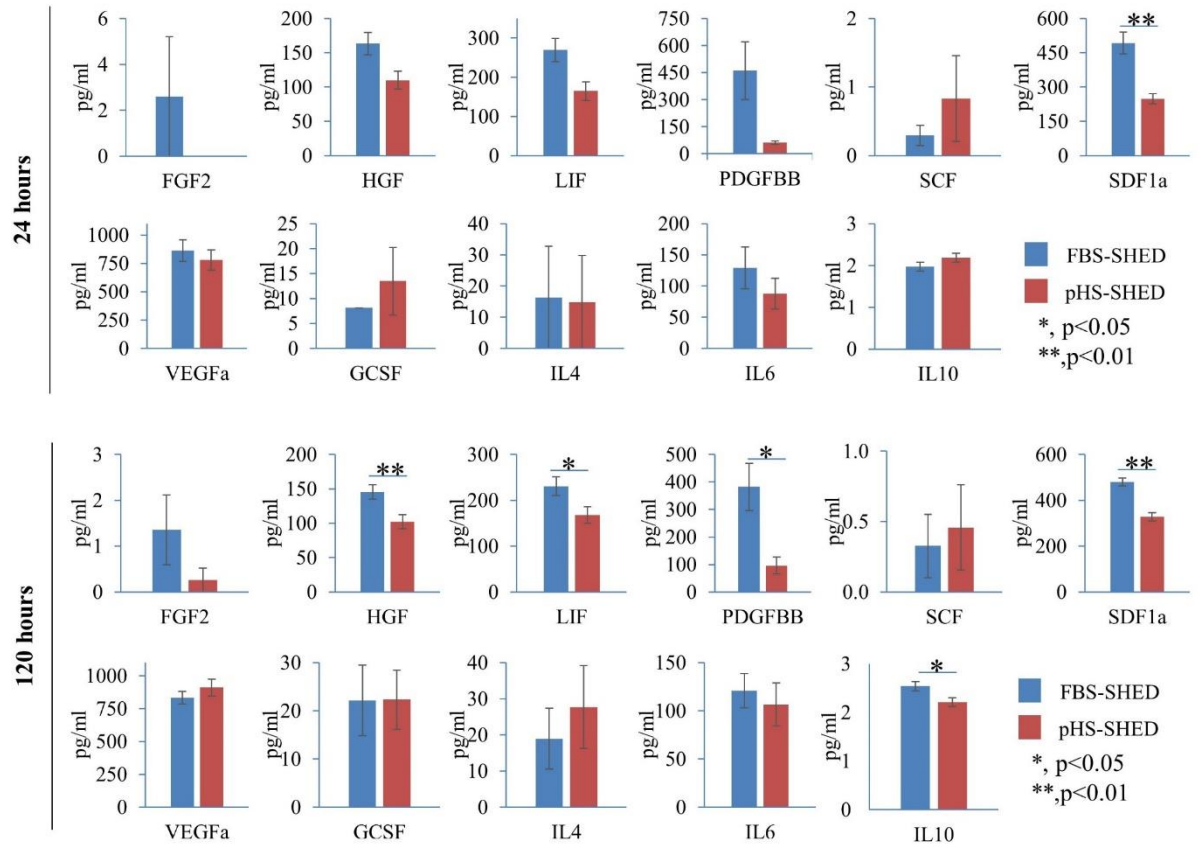

Figure 4 Comparative expression of paracrine factors in the cell culture supernatants collected from FBS-SHED and pHS-SHED in the presence of freshly prepared individual human serum (iHS) at 24 and 120 hours of incubation. (* = $p<0.05$, ** = $p<0.01$).

4 Discussion

Pulp tissue extracted from a deciduous teeth is very small in volume and not suitable to attempt to isolate and expand them in different media. Moreover, isolation of dental pulp derived stem cells (DPSC) was not successful at lower human serum concentration (20%<) (Khanna-Jain et al. 2012). The concern regarding xeno-contamination due to isolation and expansion of SHED in xenogeneic serum supplemented media can be minimized by culturing them in human serum supplemented media for two weeks only (Komoda et al. 2010). Hence, in this study SHED were isolated and expanded in FBS supplemented medium until passage 3 to get enough cells to use them for different experimental purposes.

Prior to conduct researches using MSCs from any sources, their minimum criteria set by the ISCT needed to be studied (Dominici et al. 2006). Usually, the differentiation potential and phenotypic markers expression on MSCs are not affected by the supplements (Blazquez-Prunera et al. 2017b; Oikonomopoulos et al. 2015). Hence, MSCs like properties of SHED was also studied using the cells expanded in FBS only. SHED with having fibroblast like morphology, plastic adhering capacity, tri-lineage differentiation potential, highly expressed (>95%) MSCs positive markers and negligibly expressed (4%<) MSCs negative markers further confirmed their MSCs like properties (Figure 2).

Properties of AB-blood group specific pHS as an alternative to FBS are frequently being studied in the field of stem cell research (Patrikoski et al. 2013; Aldahmash et al. 2011; Bieback et al. 2012; dos Santos et al. 2017). To widen the donor pool, previously we attempted to prepare pHS from blood irrespective of donors' blood group (Haque and Abu Kasim 2017). In that study, pHS was prepared by pooling serum from six donors to minimize donor to donor variation (Haque and Abu Kasim 2017). Another study also reported consistent results among different batches of pHS prepared by pooling sera from six donors (dos Santos et al. 2017). Therefore, in this study we used the pHS prepared by pooling sera from six donors irrespective to their blood groups to expand SHED prior analysing their proliferation and paracrine factor expression in the presence of iHS. However, to minimize batch to batch variations pooling of sera from a large number of donors could prove more useful (Diez et al. 2015).

Higher proliferation of MSCs in the media supplemented with pHS (prepared from AB blood group-typed donors) has been reported earlier (Turnovcova et al. 2009; Bieback et al. 2012). Recently, we have shown that pHS prepared with sera, regardless of the donors' blood group, maintain higher percentage of rapidly proliferating cells and significantly lower percentage of partially differentiated flat cells (Haque and Abu Kasim 2017). In the present study, pHS-SHED were found to maintain their proliferation potential in iHS supplemented media (Figure 3). Taken together, these results imply that pHS might represent a suitable supplement for the expansion of SHED prior to transplantation.

Paracrine factors play a vital role in the processes of regeneration by regulating the proliferation, migration, and homing of transplanted cells. In addition to the cell viability, expression of paracrine factors from FBS-SHED and pHS-SHED following exposure to iHS were also studied. By using the same iHS as supplement for both FBS-SHED and pHS-SHED we have tried to minimize the variations in the composition of paracrine factors in the media. Furthermore, the in vitro and in vivo half-life of paracrine factors are not more than couple of hours (Beutler et al. 1985; Peters et al. 1996). As the supernatants were collected at 24 and 120 hours post-incubation it is expected that the contribution of the paracrine factors in the supernatants by the sera would be negligible or not at all (Haque et al. 2017).

Induced chemotaxis of bone marrow derived MSCs towards IGF-1, PDGF-BB and SDF-1a indicates the importance of systemic and local inflammatory state on the migration and homing of cells to the site of injury (Ponte et al. 2007). The role of SDF-1a gradients on the directional migration of MSCs have also been reported earlier (Park et al. 2017). Significantly higher expression of SDF-1 in the supernatants of FBS-SHED collected at both 24 and 120 hours of incubation was observed. Significantly higher amount of HGF, LIF and PDGF-BB in the FBS-SHED supernatants at 120 hours of incubation was also measured (Figure 4). SDF-1, HGF and PDGF-BB are well-known chemoattractant and their role in the regenerative therapy has been acknowledged by several researchers (Li et al. 2017; van de Kamp et al. 2017; Ponte et al. 2007). Therefore, higher expression of paracrine factors from FBS-SHED could be a sign of disruption in the gradient of the paracrine factors following transplantation of MSCs expanded in FBS supplemented media that might lead to non-specific engraftment of the transplanted cells.

5 Conclusion

It has been reported that the main shortcoming of MSC-based therapy is low number of engrafted cells affecting the attainment of the long-term functional benefits of this therapy. Gradient of paracrine factors play a vital role in the tissue specific migration of transplanted cells. The expression of paracrine factors by FBS-SHED in iHS after 24 and 120 hours was higher compared to pHS-SHED. Higher expression of paracrine factors could lead to disruption of body's own paracrine factor gradient, non-specific engraftment of transplanted and circulatory cells, and eventually affect the process of regeneration. Our results indicate that SHED cultivated in human serum instead of FBS have higher proliferative capacity and show an altered secretion profile. Future studies will assess if these differences result in better engraftment and regeneration following transplantation.

Acknowledgment

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Conflicts of Interest

The authors declare no conflicts of interest.

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