Original Article

Human Dental Pulp Stem Cells: The Culture Optimization for Increased Growth

Baghaban Eslaminejad MR,[1] Nazarian H,[1] Shariati M,[2] Vahabi S,[2]

[1]Department of Stem Cell and Developmental Biology, Royan Institute for Stem Cell Biology and Technology, ACECR, Tehran, Iran
[2]Department of Periodontics, Dental Scool of Shahid Beheshti University, Tehran, Iran

Corresponding author: Mohamadreza Baghaban Eslaminejad,PhD
Department of Stem Cell and Developmental Biology
Royan Institute
Tehran, Iran
P.O. Box: 19395-4644
Tel No: +98(21)23562000
09012-5479957
Fax: +98(21)22310406
E-mail: eslami@royaninstitute.org
bagesla@yahoo.com

Abstract

Introduction: Dental pulp-derived mesenchymal stem cells (MSCs) have emerged as a promising tool for use in regenerative medicine. The in vitro growth kinetic and culture requirement of the cells derived from human dental pulp, which is the subject of this present study, is poorly described.
Materials and Methods: Stem cells were derived from human third molar and then characterized. The in vitro growth kinetics of the cells was examined by colonogenic assay and a determination of the population doubling number (PDN). Finally, the culture conditions were optimized for pulp stem cell maximum proliferation.
Results: Propagated dental pulp cells tended to differentiate into odontoblast, osteoblast, adipose and cartilage cells. Typically surface antigens were expressed as mesenchymal stem cells. The cells tended to be very proliferative with a PDN value of about 11. The colonogenic efficiency was about 60% and an average colony size was about 10.75±1.58 mm^2. The best culture condition for enhanced proliferation was achieved when the cells were seeded at 100 cells/cm^2 in a the presence of 20% FBS in a medium ($P<0.05$).
Conclusion: Taken together, the optimal culture conditions for human dental pulp-derived MSCs were determined. This information is helpful with respect to cell in vitro propagation which is greatly needed prior to their transplantation.

Keywords: Dental Pulp, Human Third Molar, Mesenchymal Stem Cells, Odontoblast, Proliferation, Skeletal Cell.

Received: 19, Aug., 2009
Accepted: 26, Sep., 2009

Introduction

Mesenchymal stem cells are defined as multipotent cells having the potential to be differentiated into bone, cartilage and adipose cell lineages and having the capacity to self renew their population over a relatively extended time. These cells hold great promise as cellular candidates for use in regenerative medicine.(1) Indeed, several successful applications of these cells in the treatment of various tissue defects have so far been reported.(2-7) MSCs were first isolated from bone marrow tissue. The limitation associated with bone marrow MSCs, including the painful harvest of marrow, the limited number of these cells and the aging of these cells, has led to a search for MSCs-like cells in other tissues.(8-10) Multiple tissues, such as adipose tissue, cord blood, amniotic fluid, peripheral blood, bone, cartilage, and muscle tissues have then been found to contain MSC- like population.(11-17)

Dental pulp tissue is among those sources that have been reported to contain MSC-like population referred to as dental pulp stem cells (DPSCs). These cells were first isolated by Grontos, et al, who had collected the pulp tissue from human third molar, digested them with collagease I and seeded the pulp

single cells onto plastic culture plates. The authors described the cells as colonogenic cells capable of producing sporadic, but densely calcified nodules in vitro and dentin-like structure lined with odotoblast-like cells in vivo.(18) According to their observations, the cells failed to differentiate into adipose cells. Subsequent investigations have demonstrated the cells, intensive capacity of differentiation into neuron-like cells, adipocytic, osteocytic and chondrocytic cells, as well.(19-21)

The importance of dental pulp-derived stem cells, in particular, is regarded to be their potential ability in regenerating dentin and dental pulp. Pulp tissue regeneration is indeed a long quest. It has been shown that producing a blood clot in the canal space of mature teeth can encourage the growth of connective tissue into the canal space. But such therapy has not been effective since the growing tissue is not pulp tissue. With the emergence of stem cells from pulp tissue, attempts have been made to repair the tissue using stem cells and tissue-engineering sciences.(22-27)

The use of the cells in any regenerative purpose requires copious amounts of cells which must be prepared prior to their transplantation alone or combined with a scaffold. Considering the small quantity of pulp tissue and hence its low stem cell contents, the cell in vitro expansion will be an inevitable step in any attempts trying to prepare the cells for use in both tissue engineering and tissue regeneration. To our knowledge the growth kinetics and culture requirements of dental pulp derived stem cells for accelerated propagation have not yet been reported. The present study is designed to address this issue. The objectives of this study were to isolate and characterize stem cells from human third molar, to investigate their growth kinetics and to optimize the culture conditions for their increased proliferation. Studies like this will help to promote the use of dental pulp derived stem cells for any experimental, preclinical and even clinical settings.

Methods and Materials

Pulp tissue and cell culture: Human third molars were collected from the patient at dental clinic of Shahid Beheshti University of Medical Sciences, under the guidelines approved by the Ethic Committee of Royan Institute. The patients from whom the teeth were obtained were young adults aged 20-25 years. Stem cells from the pulp tissue were isolated according to previously-published methods, with some modification.(18) At the Royan Institue cell culture lab, specimens were cut from around the root-enamel boundary using dental fissure burs. Pulp tissue was then gently removed from the chambers and was digested using an enzyme solution consisting of 3 mg/ml collagenase type I and 4 mg/ml dipase (both from Sigma, Germany) for 30 minutes at 37 °C. The digest was added with 3 ml DMEM (Dulbecco's Modified Eagle Medium, Gibco, Germany) supplemented with 15% FBS (Fetal Bovine Serum, Gibco, Germany) and centrifuged at 1200 rpm for 5 minutes. The pellet was then suspended in fresh medium, plated on 6-well culture plate at 10^3cells/well and incubated in an atmosphere of 5% CO2 and at a temperature of 37 °C. After 3 days, the medium was removed. The cells were washed with PBS (phosphate buffer solution) and provided with a fresh medium. The culture medium was changed twice weekly until confluency was achieved. About 80-90% confluent cultures were trypsinized and subcultured at 1:3 ratios (passage 1). Two additional subcultures were provided adequate cells for further following experiments. In all subsequent experiments, passaged-3 cells with more than 95% viable cells confirmed by trypan blue staining were used.

Flow cytometric analysis of cell surface epitopes: To study the surface epitopes of the isolated cells, flowcytometric analysis was used. The cells were prepared as follows: about 10^6 cells of passaged-3 cultures were counted and placed in 5 ml tubes and added with 5 µl of either PE or FITC-conjugated mouse antihuman antibody and 5 µl of blocking buffer. The cells were incubated at 4 °C for 20-25 min at a dark place. The cells were then washed with 1 ml washing buffer (PBS supplemented with 1% FBS) and centrifuged at 1200 rpm. The cell pellet was then suspended in 300-500 µl washing buffer and analyzed by flow cytometry (FACScalibur cytometer equipped with 488 nm argon lasers). In this study IGG2 and IGG1 were used as isotope control. WinMDI software was used to analyze the flow cytometric results. The following antibodies were used to stain the cells: FITC (fluorescein isothiocyanate)-conjugated CD31, CD33, CD45, CD90, CD105 and PE (propidium iodide)-conjugated CD11b, CD34, CD44, CD56, CD146 and CD73 (all purchased from Becton Dickenson, USA).

Multilineage Differentiation: ***Osteoblast differentiation:*** About 2× 10^5 cells/ml from passaged-3 cultures were plated in a 25-cm^2 culture flask and allowed to become confluent. The

medium was then removed and an osteogenic medium consisting of DMEM medium supplemented with 50 mg/ml ascorbic 2-phosphate (Sigma, USA), 10 nM dexamethazone (Sigma, USA) and 10 mM β glycerol phosphate (Sigma, USA) was added.(28) The culture was maintained for 21 days with the medium changes of twice weekly. At the end of this period, the osteogenic differentiation was examined by alizarin red staining and RT-PCR analysis.

Odontoblast differentiation: To direct pulp-derived stem cells to differentiate along odontoblast lineages, a confluent passaged-3 cell culture was provided with DMEM supplemented with 0.5 µM vitamin D3 (Sigma, USA), 50 mg/ml ascorbic 2-phosphate (Sigma, USA), 10 nM dexamethazone (Sigma, USA) and 10 mM β glycerol phosphate (Sigma, USA).(29) After 21 days, alizarin red staining and RT-PCR analysis were employed to evaluate the differentiation.

Adipocyte differentiation*:* Likewise, confluent cultures of passaged-3 cells were used to promote adipose differentiation. The differentiation-inducing medium was DMEM supplemented with 50 µg/ml ascorbic acid 3-phosphate, 100 nM dexamethazone and 50 µg/ml indomethcine.(28) After having been in a differentiation-inducing condition for about 21 days the cells were stained with Oil red and were further analyzed by RT-PCR for adipose-specific gene expression.

Chondroblast differentiation*:* For chondrogenic differentiation, 2.5×10^5 passaged-3 dental pulp cells were pelleted under 300 g for 5 minutes. These cells were provided with DMEM supplemented with 10 ng/ml TGF- ß3 (transforming growth factor-ß3)(Sigma, Germany), 10 ng/ml BMP6 (bone morphogenetic protein-6)(Sigma, Germany), 50mg/ml insulin transferin selenium+ premix(Sigma, Germany), 1.25 mg bovine serum albumin(Sigma, Germany) and 1% fetal bovine serum.(28) Differentiation of the culture was allowed to be extended for 21 days. At the end of this period, the pellets were processed for histological observations. For this process, the pellets were fixed with 10% formalin, dehydrated in ascending concentrations of ethanol, cleared in xylene, embedded in paraffin, cut into 5-µm-thick sections and finally stained with toluidine blue. Some pellet was used to extract mRNA in order to further examine cartilage-specific gene expression in the cells.

RT-PCR analysis: Total RNA was isolated from the differentiated cells of osteogenic, odontoblastogenic, adipogenic and chondrogenic cultures using the RNX™ (-Plus) (RN7713C; CinnaGen Inc., Tehran, Iran). In order to eliminate residual DNA, the RNA sample was treated with 1U/ml of RNase-free DNaseI (EN0521; Fermentas,Opelstrasse 9,Germany) per 1 mg of RNA in the presence of 40 U/ml of ribonuclease inhibitor (E00311; Fermentasm, Germany) and 1×reaction buffer with MgCl2 for 30 min at 37°C. DNaseI was inactivated by adding 1ml of 25mM EDTA and incubated at 65°C for 10 min. Standard RT reactions were performed with 2 µg total RNA using oligo(dt) as a primer and a RevertAid™ First Strand cDNA Synthesis Kit (K1622; Fermentas, Germany) according to the manufacturer's instructions. For every reaction set, one RNA sample was prepared without RevertAid™M-MuLV Reverse Transcriptase (RT^- reaction) in order to provide a negative control of the subsequent PCR. To minimize variation in the RT reaction, all RNA samples from a single experimental setup were simultaneously reverse transcribed. Reaction mixtures for PCR included 2 ml cDNA, 1×PCR buffer(AMS™; CinnaGen Co., Tehran, Iran), 200 mM dNTPs, 0.5 mM of each antisense and sense primer (Table 1), as well as 1U Taq DNA polymerase.

Colonogenic assays: Colonogenic assay is being performed to estimate the growth potential of MSC-like populations in vitro. In the present study, this assay was employed to determine the proliferation capacity of the isolated stem cells from the pulp tissue. For this purpose, passaged-3 cells were counted using a hematocytometer, plated at 100 cells on a 10-cm Petri dish in DMEM supplemented with 15% FBS. These cells were allowed to grow for 10 days. At the end of this period, the cultures were observed with an inverted light microscope and the number of colonies were determined. Furthermore, the size of the colonies was also determined using a microscopic objective micrometer.

Calculation of population doubling number (PDN): PDN value is an important index indicating that how proliferative the given cells are in culture. To determine such an index for isolated pulp stem cells, passaged-3 cells were plated at 250 cells/well on 12-well culture plate in a DMEM, supplemented with 15% FBS, 100 IU/ml penicillin, and 100 µg/ml streptomycin and incubated for a period of 10 days, at the end of which, the cell were lifted and counted with hemocytometer. Using the equation $PDN= \log N/N0\times3.31$, PDN was determined.(30) In this

Table 1: Primers used in RT-PCR

Gene name	Direction	Sequence	Product size	A.T
DMP1	Forward	GCAGAGTGATGACCCAGAG	200	62
	Reverse	GCTCGCTTCTGTCATCTTCC		
DSPP	Forward	CCATTCCAGTTCCTCAAAGC	216	59
	Reverse	CTGCCCACTTAGAGCCATTC		
Aggrecan	Forward	TCAACAACAATGCCCAAGAC	193	58
	Reverse	AGCGACAAGAAGAGGACACC		
PPAR-Gamma	Forward	CTAAAGAGCCTGCGAAAG	202	62
	Reverse	TGTCTGTCTCCGTCTTCTTG		
PPAR- Alpha	Forward	TGCTATCATTTGCTGTGGAG	212	59
	Reverse	ACTCCGTCTTCTTGATGAT		
RunX2	Forward	CAAGTAGCAAGGTTCAACGA	165	58
	Reverse	CGGTCAGAGAACAAACTAGG		
Osteocalcin	Forward	GGCAGCGAGGTAGTGAAGAG	193	61
	Reverse	CAGCAGAGCGACACCCTAGAC		
GAPDH	Forward	CTCATTTCCTGGTATGACACC	245	60
	Reverse	CTTCCTCCTGTGCTCTTGCT		

equation, N_0 is the initiating cell number (in this study it was 250 cells), N the harvesting cell number (which was 512800 cells).

Optimizing the culture condition: In this study, initiating the cell seeding density and the FBS concentration of the culture medium was optimized in order to enhance extensive proliferation of the cells.
For this purpose, the isolated pulp stem cells (passaged-3) were plated on a 100-mm dish at varying densities of 10, 50, 100, 200 and 500 cell/cm^2 in DMEM, supplemented with 100 IU/ml penicillin, 100 mg/ml streptomycin and varying concentrations of fetal bovine serum including 5%, 10%, 15% and 20%, for a period of 10 days. At the end of this period, the cells were lifted and counted by a hemocytometer.
The data was used to calculate the fold increase in the cell number of different groups. One-way ANOVA was used to compare the results.

Results

Cell culture: The cells released after enzymatic digestion of the dental pulp established the primary culture in which several distinct colonies with fibroblastic morphology were observed.
A number of small, clear cells were also present on the fibroblastic cells (Figure- 1A). After 10 days, the primary culture reached confluency when all available surfaces of the culture vessel appeared to be covered by the fibroblastic cells (Figure- 1B). At the subcultures, it seemed that the cell proliferation became more rapid than that of the primary culture, so that the passaged cultures reached confluency in about 5-6 days. Fibroblastic morphology of the cells was maintained throughout the culture period.

Flow cytometry: More than 97% of the cells tended to express mesenchymal epitopes, including CD105, CD 90, CD 44, and CD 73. Endothelial-

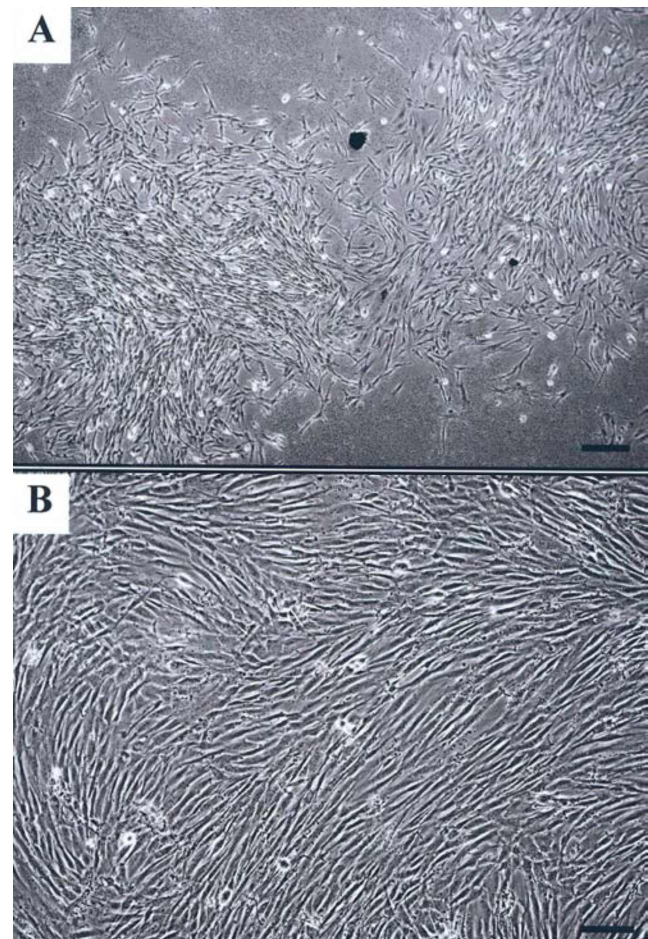

Figure- 1: The culture of pulp tissue cells. A) Primary culture: Two large colonies can be seen (bar=200µm). B) The same culture at confluency(bar=100µm).

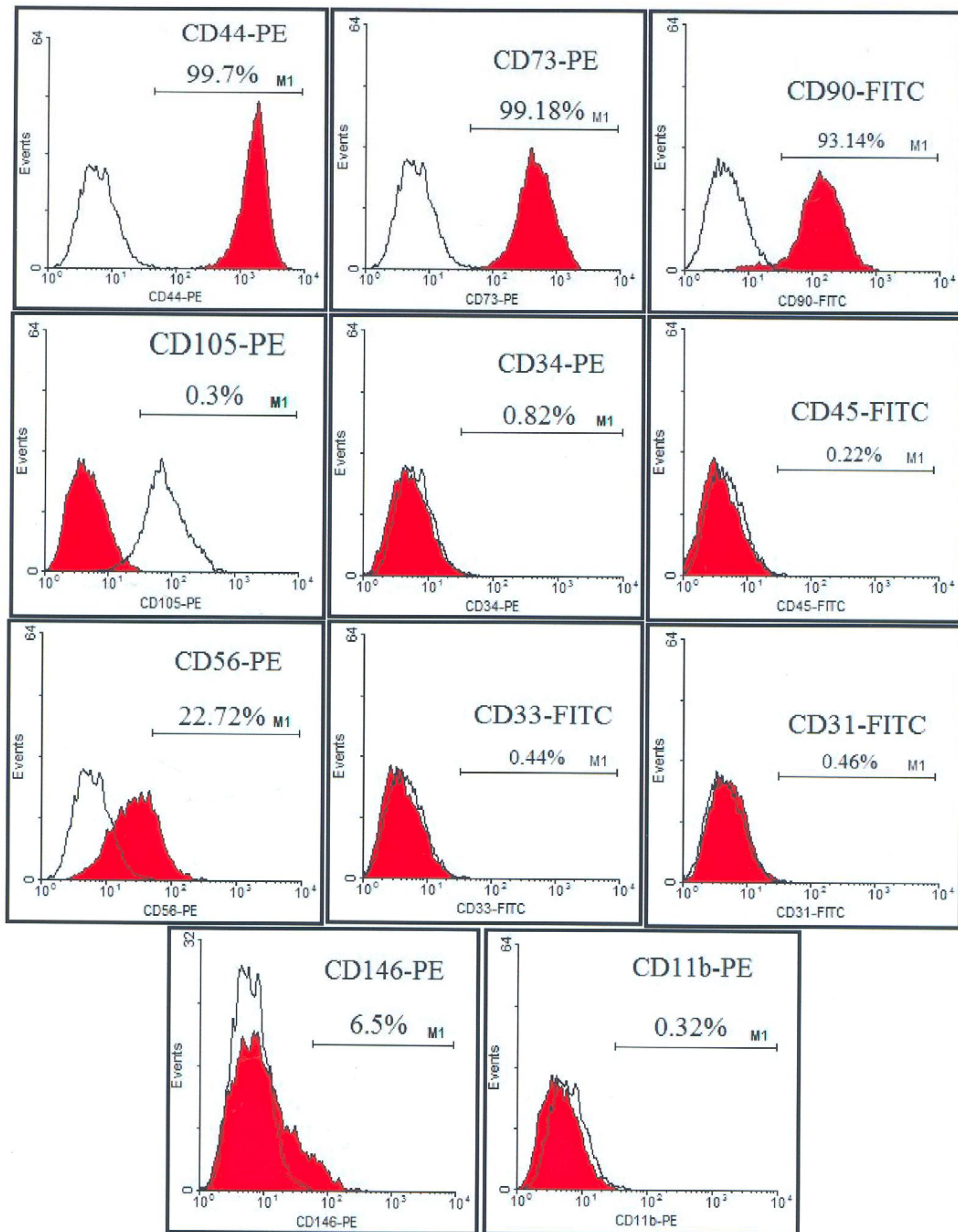

Figure- 2: Flow cytometry analysis of the dental pulp passaged-3 cells. The majority of the cells expressed mesenchymal antigens such as CD44, CD 73, CD90 and CD105 and not expressed endothelio-hematopoitic cell markers including CD34, CD45, CD56, CD33, CD31, CD146 and CD11b.

hematopoietic surface markers including CD 56, CD11b, CD 34, CD 31, CD 33, CD 45 and CD146 were expressed in very small percentages (less than 1-2%) of the dental pulp cells (Figure- 2).

Multilineage differentiation: The observations made of the osteogenic cultures revealed the formation of multiple nodule-like structures in this cultures. These nodules tended to be heavily stained with alizarin red which stains mineralized matrix which hade been deposited by the cells (Figure- 3A). RT-PCR analysis of bone related gene expression such as osteocalcin and Runx2 expression provided further evidences for successful bone differentiation of the studied cells (Figure- 3B).

Likewise, the dental pulp MSCs plated under odontoblastic conditions tended to form a number of cell aggregates on some area of the culture plate. Using alizarin red staining, those nodules high in mineralized matrix were stained intensively red (Figure- 3C). RT-PCR analysis demonstrated that the odontoblast-specific mRNA including DMP1 (Dentin matrix protein 1) and DSPP (dentin sialophosphoprotein) were largely produced in the differentiated cells (Figure- 3D).

The adipogenic differentiation potential of the studied cells appeared to be weak since only a few lipid droplet-containing cells were observed per microscopic field in the adipogenic cultures. These lipid droplets were stained red following the Oil red O staining (Figure- 3E). Furthermore, based on the RT-PCR results, adipose related-genes such as PPAR-alpha (Peroxisome proliferators activated-receptor-alpha) and PPAR-gamma (Peroxisome proliferators activated-receptor- gamma) were also expressed in the differentiated cells (Figure- 3F).

Toluidin blue staining of the sections prepared from chondrogenic pellets demonstrated that in the chondrogenic cultures, a metachromatic matrix was produced (Figure- 3G). Chondrogenesis by dental pulp cells was further analyzed and confirmed by RT-PCR method, which indicated the expression of cartilage-specific-genes, including aggrecan and collagen II, in the differentiated cells (Figure- 3H).

Colonogenic assay: According to the results, about 60% of the plated cells tended to form colony. The colonies displayed several to few hundred fibroblastic cells. The average size of the colonies, according to our measurement, was 10.75±1.58 mm^2.

Population doubling number: PDN calculation indicated that the dental pulp MSCs population doubled 10.96 times during the 10 day culture period.

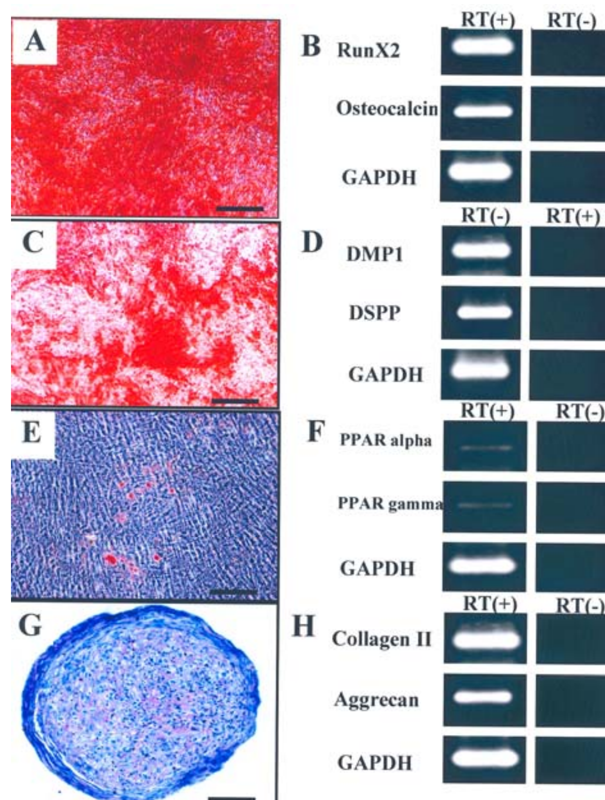

Figure- 3: Multilineage differentiation potential of the pulp derived stem cells. A) osteocytic differentiation and B) RT-PCR data. C) Odontoblastic differentiation and D) RT-PCR analysis. E) Adipose differentiation and F) RT-PCR results. G) Cartilage differentiation and H) RT-PCR data. The scale bar of all parts is 100µm.

Cell seeding density: The results of this part are indicated in Figure- 4. According to the data, significantly more fold increase occurred in the culture initiated at 100 cell/cm^2 ($P<0.05$).

FBS concentration: Based on the results indicated in Figure- 4, 20% FBS creates more fold increase than other FBS concentrations.

Discussion

In this study, attempts were primarily made to investigate the culture needs of mesenchymal stem cells taken from human third molar for their maximum proliferation in vitro. Studies like this, help render stem cell investigation applicable to regenerative medicine. To reconstruct the defects in tissue, cell therapy needs a copious number of regenerating cells to transplant into lesion site. Regenerative stem cells tend to occur in very few quantities in their tissue of origin. Therefore, there is a need to increase their numbers before transplantation as an inevitable step in any cell-based treatment of tissue defects. The present work had been conducted to determine the optimal culture condition for a better proliferation of stem cell from human dental pulp which was considered as promising candidate for both tooth regeneration

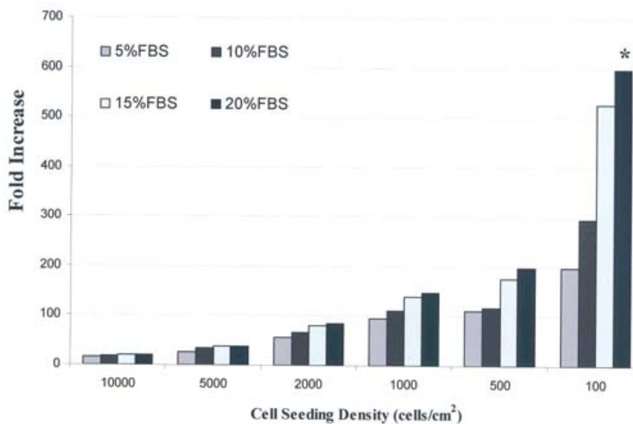

Figure- 4: Culture optimization for maximum proliferation. The human pulp-derived MSCs exhibited maximum fold increase when plated at 100cells/cm^2 in a medium supplemented with 20% fetal bovine serum. * indicates a P<0.05.

as well as bone tissue repair. To our knowledge, this has not been reported before.

Stem cells of dental pulp are of particular importance since pulp from third molar, from which stem cell can be derived, are discarded if not used. Dental pulp stem cells have the capacity to generate dentin-producing cells, termed odontoblast. Dentin lesions are a common problem associated with human teeth. Many attempts have so far been made to cure this problematic condition. Emerging stem cells from dental pulp are a promising tool to achieve this goal.(22-27) Furthermore, having the ability to be differentiated from other tissue including bone, cartilage and neural cells, pulp derived stem cells can be used to implement regeneration of skeletal and neural tissue, as well. These cells, with such a multipotential ability, which is comparable to stem cell from umbilical cord blood, can be used to establish a cell bank for their future application in tissue regeneration. One basic necessity in order to establish pulp stem cell banks is to propagate the cells in considerable quantities. In the present study, culture condition for the maximum proliferation of the cells was investigated.

One aspect relating to dental pulp stem cells which has been poorly addressed is their exact identity.(18-21) It is not clear whether the cells are of the mesenchymal stem cell population residing in pulp tissue or are from stem cells with a distinct identity other than the MSC population. To address this issue, stem cells isolated from dental pulp, were examined, if they had met the criteria describing mesenchymal stem cells isolated from elsewhere.(31) According to our results, stem cells isolated from human dental pulp succeeded in differentiating down to three typical skeletal cell lineages of bone, cartilage and adipose cells, these being one of the gold standard used to identify MSCs. Moreover, according to flow cytometric data, the cell expressed a number of mesencymal lineage surface epitopes and did not express endothelio-hematopoietic surface markers, a feature characteristic of MSCs. Considering these evidences, however, it can be concluded that they are of the MSCs population residing in pulp tissue.

In a preliminary study, Gronthos, et al, had reported that the fibroblastic cells from human dental pulp were capable of differentiating into odontoblast, similar to cells in vivo and neural cells in vitro. According to their results, these cells have not displayed adipogic potential.(18) In contrast, other research work has reported the successful differentiation of dental pulp cells into adipose cells (21). In the present study, adipose differentiation of stem cells from dental pulp was examined. Our data indicated that such potential was far less extensive, so that a few lipid-containing cells were observed in each microscopic field of adipogenic cultures. This point, however, needs more investigation to elucidate an underlying cause of such a weak adipogenic potential displayed by dental pulp stem cells.

Since stem cells occur in only a small quantity in tissue, their in vitro expansion is an essential step prior to their application in any experimental, preclinical and clinical set-ups. According to the previous investigation, ex vivo expansion of stem cells is largely dependent on the presence of FBS in a culture medium. Stem cells propagated in culture containing FBS are immunogenic and may transfer bovine pathogens upon transplantation.(32-33) While the serum-free expansion of stem cells has been a subject of several investigations, the propagation of stem cells with FBS is still practiced in many laboratories. In this study, pulp stem cells were examined in terms of their FBS need for maximum proliferation. Our findings demonstrated that 20% FBS is an optimal concentrations. Also, 15% FBS still exhibited a reasonable proliferative effect on the cells. Therefore, it is wise to apply 15% concentrations rather than 20% concentration to help reduce the possible complications associated with serum.

In most cell therapy strategies, it is desierable to prepare the cellular materials in as short a period as possible. To accelerate cell proliferation in culture, there are two crucial parameters: FBS concentrations and an initial cell seeding density. In the current study, the best cell seeding density with

maximum cell yield was determined for the isolated cells. Based on our findings, a maximum increase in cell number was achieved when the cells were plated at a low density of 100 cells/ cm^2. In a study by Bartmann, et al, it has been reported that there is an inverse correlation between human MSC seeding density and their proliferation.(34) Our results are consistent with this data.

Taken together, we determined the optimal culture conditions for human dental pulp-derived MSCs. Under low-density condition of 100 cells/cm^2 and the presence of 20% FBS, the cells tended to proliferate rapidly ending with the formation of a monolayer covering all available surfaces of the culture vessels. A high proliferation rate, along with a multilineage differentiation potential, render the dental pulp stem cells a suitable substitute for marrow-derived MSCs; more importantly, this results in a promising tool for use in regenerative medicine of dental and skeletal tissue.

Acknowledgement

This work has been done in Royan Institute and by financial support provided by Royan Institute.

References

1. Baksh D, Song L, Tuan RS. Adult Mesenchymal Stem Cells: Characterization, Differentiation and Application in Cell Therapy. Mol Med, 2004; 8: 301-136.
2. Horwitz EM, Gordon PL, Koo WK, et al. Isolated Allogenic Bone Marrow- drived Mesenchymal Cells Engraft and Stimulate Growth in Children with Osteogenesis Imperfecta: Implications for Cell Therapy of Bone. Proc Natl Acad Sci, USA, 2002; 99: 8932-8937.
3. Koe ON, Gerson SL, Cooper BW, et al. Rapid Hematopoietic Recovery after Confusion of Autologous - Blood Stem Cells in Advanced Breast Cancer Patients Receiving High Dose Chemotherapy. J Clin Oncol, 2000; 18: 307-316.
4. Quarto R, Mastrogiacomo M, Cancedda R, et al. Repair of Large Bone Defect with the Use of Autogenic Bone Marrow Stromal Cell. N Engl J Med, 2001; 344: 385-386.
5. Barry FP. Mesenchymal Stem Cell Therapy in Joint Disease. Novartis found symp, 2003; 249: 86-96.
6. Sugaya K. Potential Use of Stem Cells in Neuro-Replacement Therapies for Neurodegenerative Diseases. Int Rev Cytol, 2003; 228: 1-30.
7. Chapel A, Bertho JM, Bensidhoum A, et al. Mesenchymal Stem Cells Home to Injured Tissues when co-Infused with Hematopoietics Cells to Treat a Radiation- Induced Multi-Organ Failure Syndrome. J Gene Med, 2003; 5: 1028-1038.
8. Pittenger MF, Mackay AM, Beck SC, et al. Multilineage Potential of Adult Human Mesenchymal Stem Cells. Science, 1999; 284: 143-147.
9. Baxter MA, Wynn RF, Jowitt SN, et al. Study of Telomere Length Reveals Rapid Aging of Human Marrow Stromal Cells Following in Vitro Expansion. Stem Cells, 2004; 22: 675-82.
10. Nishida S, Endo N, Yamagiwa H, et al. Number of Osteoprogenitor Cells in Human Bone Marrow Markedly Decreases after Skeletal Maturation. J Bone Miner Metab, 1999; 17: 171-177.
11. Noth U, Osyczka AM, Tuli R, et al. Multilineage Mesenchymal Differentiation of Human Trabecular Bone-Derived Cells. J Ortho Res, 2002; 20: 1060-1069.
12. Wickham MQ, Erickson GR, Gimble JM, et al. Multipotent Stromal Cells Derived from Infrapatellar Fat Pad of Knee. Clin Ortho, 2003; 196-212.
13. Jankowsk RJ, Deasy BM, Huard J. Muscle-Derived Stem Cells. Gene therapy, 2002; 9: 642-647.
14. Zvaifler NJ, Marinova-Mutafchieva L, Adams G, et al. Mesenchyamal Precursor Cells in the Blood of Normal Individuals. Artheritis Res, 2000; 2: 477-488.
15. You Q, Tong X, Guan Y, et al. B J Int Med Res, 2009; 37: 105-112.
16. Erices A, Conget P, Miguell JJ. Mesenchymal Progenitor Cells in Human Umbilical Cord Blood. Br J Haematol, 2000; 109: 235-242.
17. Eslaminejad MB, Taghiyar L. Mesenchymal Stem Cell Purification from the Articular Cartilage Cell Culture. Iran J Basic Med. Sci, 2008; 3: 146-153.
18. Gronthos S, Mankani M, Brahim J, et al. Postnatal Human Dental Pulp Stem Cells (DPSCs) in Vitro and in Vivo. Proc Natl Acad Sci, U S A, 2000; 97: 13625-13630.
19. Laino G, Carinci F, Graziano A, et al. In Vitro Bone Production Using Stem Cells Derived from Human Dental Pulp. J Craniofac Surg, 2006; 17: 511-515.
20. d'Aquino R, Graziano A, Sampaolesi M, et al. Human Postnatal Pulp Cells co-Differentiate into Osteoblast and Endotheliocytes: A Pivotal Synergy Leading to Adult Bone Tissue Formation. Cell Death Differ, 14: 1162-1171.
21. Zhang W, Walboomers XF, Shi S, et al. Multilineage Differentiation Potential of Stem Cells Derived from Human Dental Pulp after Cryopreservation. Tissue Eng, 2006; 12: 2813-2823.
22. Huang GT, Gronthos S, Shi S. Mesenchymal Stem Cells Derived from Dental Tissues vs those from other Sources: Their Biology and Role in Regenerative Medicine. J Dent Res, 2009; 88: 792-806.
23. Buurma B, Gu K, Rutherford RB. Transplantation of Human Pulpal and Gingival Fibroblasts Attached to Synthetic Scaffolds. Eur J Oral Sci, 1999; 107: 282-289.
24. Gu K, Smoke RH, Rutherford RB. Expression of Genes for Bone Morphogenetic Proteins and Receptors in Human Dental Pulp. Arch Oral Biol, 1996; 41: 919-923.
25. Myers WC, Fountain SB. Dental Pulp Regeneration Aided by Blood and Blood Substitutes after Experimentally Induced Periapical Infection. Oral Surg Oral Med Oral Pathol, 1974; 37: 441-450.
26. Ostby BN. The Role of the Blood Clot in Endodontic Therapy. An Experimental Histologic Study. Acta Odontol Scand, 1961; 19: 324-353.
27. Mooney DJ, Powell C, Piana J, Rutherford B. Engineering Dental Pulp-like Tissue in Vitro. Biotechnol Prog, 1996; 12: 865-868.
28. Eslaminejad MB, Nikmahzar A, Thagiyar L, et al. Murine Mesenchymal Stem Cells Isolated by Low Density Primary Culture System. Dev Growth Diff, 2006; 48: 361-370.
29. Tonomura A, Sumita Y, Ando Y, et al. Differential Inducibility of Human and Porcine Dental Pulp-derived Cells into Odontoblasts. Connect Tissue Res, 2007; 48: 229-238.
30. Piera-Velazquez S, Jimenez SA, Stokes D. Increased Life Span of Human Osteoarthritic Chondrocytes by Exogenous Expression of Telomerase. Arthritis Rheum, 2002; 46: 683-693.

31. Dominici M, Le Blanc K, Mueller I, et al. Minimal Criteria for Defining Multipotent Mesenchymal Stromal Cells. The International Societry for Cellular Therapy Position Statement. Cytotherapy, 2006; 4: 315-317.

32. Spees JL, Gregory CA, Singh H, et al. Internalized Antigens must be Removed to Prepare Hypoimmunogenic Mesenchymal Stem Cells for Cell and Gene Therapy. Mol Ther, 2004; 95: 747-756.

33. Pittenger MF, Mackay AM, Beck SC, et al. Multilineage Potential of Adult Human Mesenchymal Stem Cells. Science, 1999; 284: 143-147.

34. Bartmann C, Rohde E, Schallmoser K, et al. Relationship between Donor Age and the Replicative Lifespan of Human Cells in Culture: A Reevaluation. Proc Natl Acad Sci, USA, 1998; 95: 10614-9.