ISSN NO. 2320-5407

Journal homepage:http://www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH

RESEARCH ARTICLE

Comparing adult human periodontal ligament and dental pulp derived MSCs: colony forming unit fibroblast assay and flow cytometry

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Manuscript Info *Abstract*

Manuscript History:

Received: 14 November 2014
Final Accepted: 25 December 2014
Published Online: January 2015

Key words:
Dental pulp, periodontal ligament, CFU-F Assays, flow cytometry .

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The current study was performed on adult human impacted third molars where aim of the study was to compare mesenchymal stem cells (MSCs) from cultures of periodontal ligament (PDL) and human dental pulp. The clonogenic potential of the isolated cells in both groups; group I (PDL) and group II (pulp) was assessed via Colony forming unit-fibroblast (CFU-F) Assay. The percentage of cells in both groups positive to cell surface markers CD146 and CD 34 were analyzed and sorted by Flow cytometry.

The morphologic characteristics of the MSCs were similar for PDL derived and dental pulp derived. Generally, the MSCs isolated from both tissues showed fibroblastic morphology. However, the growth rate of the MSCs was slightly different where dental pulp derived cells showed a slightly increased rate of proliferation versus PDL derived ones. CFU-F Assays of both tissues showed slight increase in the colony counts for dental pulp derived MSCs when comparing the output in PDL derived MSCs. However, this difference was proven statistically insignificant using student (t) test. Flow cytometry revealed that cells in both groups were negative for hematopoietic markers CD (34) and were positive for the CD (146).

Based on their CFU-F assay and flow cytometry results both tissues didn't show significant statistical difference.

INTRODUCTION

The first successful isolation of fibroblast-like colonies from bone marrow, i.e., MSCs, was described about 4 decades ago by Friedenstein et al. [1970]. Mesenchymal stem cells (MSCs) are defined as self-renewable, multipotent progenitor cells with the capacity to differentiate into several distinct mesenchymal lineages [Caplan et al., 1991].

During tooth formation, interactions between epithelial and dental papilla cells promote tooth morphogenesis by stimulating a subpopulation of mesenchymal cells to differentiate into odontoblasts, which in turn form primary dentin. These odontoblasts are thought to arise from the proliferation and differentiation of a precursor population, residing somewhere within the pulp tissue [Ruch, 1998].

Although it is evident that stem cells or progenitor cells are involved in dental development, the identification of dental stem cells was easier said than done. Opposing to rodents, who show a constant regeneration of their incisors, human teeth do not form new enamel after tooth eruption. Irma Thesleff's group identified these dental ectodermal stem cells in tissue explants of adult mouse incisors for the first time [Harada et al., 1999].

Studies have revealed the presence of adult stem cells in tissues of dental origin as well. Namely, primary cell cultures containing progenitor cells originating from both adult and deciduous dental pulp as well as periodontal ligament were described [Seo et al., 2004].

Progenitors and transit amplifying cells have a limited lifespan and therefore can only reconstitute a tissue for a short period of time when transplanted. In contrast, stem cells are self renewing and thus can generate any tissue for a lifetime. This is a key property for a successful therapy. The capacity to expand stem cells in culture is an indispensable step for regenerative medicine, and a considerable effort has been made to evaluate the consequences of the cultivation on stem cell behavior [Bluteau et al., 2008].

To utilize this potential, it is necessary to gain further insight into the characteristics of postnatal stem cells of dental origin and examine their full developmental potential first in vitro then in vivo [Kadar et al.,2009].

In a trial to model the epithelial-mesenchymal interactions which take place during tooth germ differentiation and to focus on the importance of DPSCs at this level, Kadar and co-workers [Kerkis et al.,2006] used cultures of mesenchymal cells isolated from adult human dental pulp and undifferentiated epithelial cells from salivary origin, and it has been shown that coculturing of DPSCs and epithelial cells resulted in dramatic changes of cell shape and migrating activity of DPSCs, while no obvious morphological change can be observed in the epithelial cells.

Another study has provided evidence that DPSCs and their osteoblast differentiated cells can be safely recovered after long-term cryopreservation [Papacciu et al., 2006].

It could be stated that within the periodontal ligament of both healthy and diseased teeth, cells have been identified consistent with their identification as putative stem cells [Grimm et al., 2008; Grimm et al., 2007]. The presence of an inflammatory reaction associated with periodontitis may enhance the number of these cells, and their role in periodontal tissue regeneration might be of great importance as a step on the road of dental tissue regeneration [Dannan et al., 2009].

All these features and abilities make these cells attractive for therapeutic 3-dimensional tissue reconstruction, with the potential of tailoring storage and recovery to the needs of the patient.

Hence, it is important to identify the properties of pulp and periodontal ligament derived stem cells in culture in order to gain a more profound insight of utilizing these properties in clinical and ex vivo trials in regenerative dentistry. Consequently the aim of this study was to identify and compare the properties of multipotent adult stem cells in the in-vitro cultures of human dental pulp and periodontal ligament which was partially fulfilled requesting further investigations.

Material and Methods

The current study was performed by using human impacted third molars (n=7) collected from adults (18-26 years of age) at the Department of Oral Surgery, Ain Shams University. Patients were informed about the nature of the study and they were asked to sign an informed consent. The proposal of the study was reviewed by the faculty Research Ethics Committee.

Both periodontal tissues and pulp tissues were extracted from each molar and groups were divided as following:

Group I (periodontal tissue): Tooth surfaces were cleaned and the periodontal tissue was removed with a sterile scalpel and collected [El Fattah MA et al., 2010].

Group II (pulp tissue): The tooth cut around the cemento-enamel junction by sterile dental fissure burs to expose the pulp chamber, and the pulp tissue was removed from the crown and root [Gronthos S et al.,2000].

Culturing of pulp derived MSCs was done according to Gronthos et al., (2000), while Culturing of periodontal derived MSC was done according to El Fattah et al. (2010) with some modifications:

1. The fetal calf serum was used in 15%.
2. Replacement of the culture media was done in the first time on day 7 then every 3 days.

Flow cytometry:

The specific surface antigens of cells in both groups were characterized by flow cytometry analysis. The cells in culture were trypsinzed and stained with Fluorescein isothiocyanate (FITC) – or phycoerythrin (PE)-conjugated antibodies against CD 146 and CD 34.

Colony forming assay:

The number of cells in a sample (1ml) of the cell suspension was counted using a hemocytometer. Using a sterile pipette different densities of cultured cells were obtained by adding 0.25, 0.5 and 1 ml of cell suspension solution of known density to individual wells of the culturing plate. For each group every density was cultured in 3 wells to obtain a mean value. Culture wells were incubated for 10–14 days at 37°C in a humidified 5% CO2 incubator. MSCs were fixed by using freshly prepared precooled (-20℃) mixture of acetone/methanol (A: M at 1:1 V/V) for 10 minutes at room temperature [Ahmed, 2000]. The fixed adherent MSCs were stained by Giemsa stain. Three replicate plates were used for the experiments to obtain a mean value. The cells were washed twice with distilled water, and all visible colonies ≥ 2 mm in diameter per dish were counted. Colonies ≤ 2 mm in diameter and faintly stained colonies were not included in the analysis [Sung et al., 2008].

Result and Discussion

Examination of the initial cultures of both groups with inverted microscope in the current study revealed that the initial cultures contained crowded cell population with a majority of small spherical cells. However, the number of round-shaped cells showed a gradual apparent decrease and the fibroblast-like cells showed a gradual apparent increase over time generally. The MSCs isolated from both tissues showed fibroblastic morphology .In cultures of both groups, in the present study, various populations were detected among the cultured cells. Populations of small round dividing cells were observed at the start of culturing period and faded by the first week. Colonies also contained both spindle-shaped cells and large flat cells possessing a star or triangular shape (Figs 1&2).

Flow cytometry:

MSCs from both groups were characterized by Flow cytometry with two markers after culture in media. PDL derived-MSCs were negative for hematopoietic markers CD34 1.35 % and were positive for the CD146 16%. Furthermore, all pulp derived-MSCs showed close results also, negative for hematopoietic markers CD34 1.5% and were positive for the CD 146 19.5 % (Table 1).

Colony forming unit fibroblast assay:

According to the observations in the current study we assume that cultures of both groups went through the same morphological stages. However, the growth rate of the MSCs was slightly different where Pulp derived cells showed a slightly increased rate of proliferation versus PDL derived ones. This observation has been confirmed by assessing the proliferative capacity of both groups using Colony Formed Unit assay-fibroblast.

In the present research, cultured cells from both groups were cultured at different densities in individual wells of the culturing plate. After 14 days of incubation cultures were fixed and stained with Giemsa stain. Cells were washed twice with distilled water, and all visible colonies more than 2 mm in diameter per dish were counted. Colonies less than 2 mm in diameter and faintly stained colonies were not included in the analysis. This protocol was conducted by [Sung et al., 2008] also.

The proliferative capacity of pulp derived MSCs in the present study was estimated by mean of 1.6 colonies per 103 cells plated when cultured at 3000 cells /cm2, 2.6 colonies/ 103 cells plated when cultured at 15000 cells/cm2 and 3.3 colonies/103 cells plated when cultured at 750 cells/cm2. Furthermore , the proliferative capacity of PDL derived MSCs was estimated by mean of 1.16 colonies per 103 cells plated when cultured at 3000 cells /cm2, 1.6 colonies/ 103 cells plated when cultured at 15000 cells/cm2 and 2.6 colonies /103 cells plated when cultured at 750 cells/cm2(tables 2 & 3) and (Figs. 3&4).

Upon investigation of the cultures of the current studies stained with Giemsa stain the cultures showed the formation of organized colonies of fibroblast like cells exhibiting plump cell bodies with granular cytoplasm and thin cytoplasmic processes. Their nuclei were purple, vesicular and some were binucleated (Fig. 5).

The morphologic characteristics of the MSCs were similar in PDL derived and dental pulp derived, whilest both groups morphology resembled the bone marrow mesenchymal stem cells (BMSCs) described in literature. This coincided with literature as [Gronths et al., 2000] described pulp derived MSCs were characterized by a typical fibroblast-like morphology analogous to the progeny of human bone marrow. Also, [Kadar et al., 2009 and El Fattah et al., 2011] stated that PDL derived MSCs possessed same morphologic and clonogenic properties as BMSCs.

The presence of various populations was studied by [Colter et al., 2001] and he concluded that cultures contained very small round cells that were primarily detected as they became highly reflective during replication.

Presence of spindle shaped and flat cells was also explained by [Lee et al., 2004 and Mok et al., 2003] who noticed heterogeneous populations of four distinct cell types which were spindle shaped , star shaped , petal shaped and flattened cells with granular cytoplasm.

Phenotypic analysis using flow cytometry was used as a rapid identification of a cell population Surface Ag expression. To assure that populations of MSC are not confounded by other cells, we examined the lack of expression of one of the hematopoietic Antigens (CD34) , as an additional criterion for MSC as they are not known to express these Antigens. CD34 was used as it is documented to mark primitive hematopoietic progenitors and endothelial cells [Dominici et al., 2006].

Furthurmore, presence of MSCs was confirmed in both tissues by the expression of the cluster of differentiation 146 (CD 146). CD 146 was documented as a marker of MSCs by a group of researchers as Ramalho-Santos et al.,2002; Alsalameh et al., 2004; Dominici et al., 2006 and Bongoso et al., 2010.

These findings coincided with literature as [Gronths et al.,2000] demonstrated that The frequency of colony-forming cells derived from dental pulp tissue (22–70 colonies/104 cells plated) was significantly higher in comparison to the incidence of bone marrow CFU-F (2.4–3.1 colonies /104 cells plated) over similar plating densities (0.1– 2.5 x 104 cells plated). Whilst, [Xu et al., 2004] concluded that BMSCs showed significantly higher colony forming efficiency than PDL MSCs cells. Where he determined the colony forming efficiency of BMSCs as (16.7%) which is significantly greater colony-forming efficiency than the PDL derived MSCs cells (6%). Also, El Fattah et al., 2010, stated that the formation of about 180 colonies, generated from 100,000 single cells when cultured at low density (105 cells per T 25 flask).

In accordance to this observation Gronths et al., 2000 described the cells within each colony pulp derived MSCs by being characterized with a typical fibroblast-like morphology. El Fattah et al., 2010, stated that the cells within each colony of PDL derived MSCs were characterized by a typical fibroblast-like morphology.

Moreover these findings coincide with the observations of Mok et al., 2003; Xu et al., 2004 and Urban et al., 2008, who noticed that the cultured MSCs stained with Giemsa stain showed bluish granular cytoplasm with vacuoles and showed signs of active division.

We recommend further studies to be carried out identifying unique markers for both PDL derived MSCs and pulp derived MSCs, in addition to Further development of carriers with appropriate shape and composition to be used in conjunction with ex vivo expanded DPSCs or PDLSCs makes the fabrication of a viable dental implant a real possibility in perhaps the not too distant future.

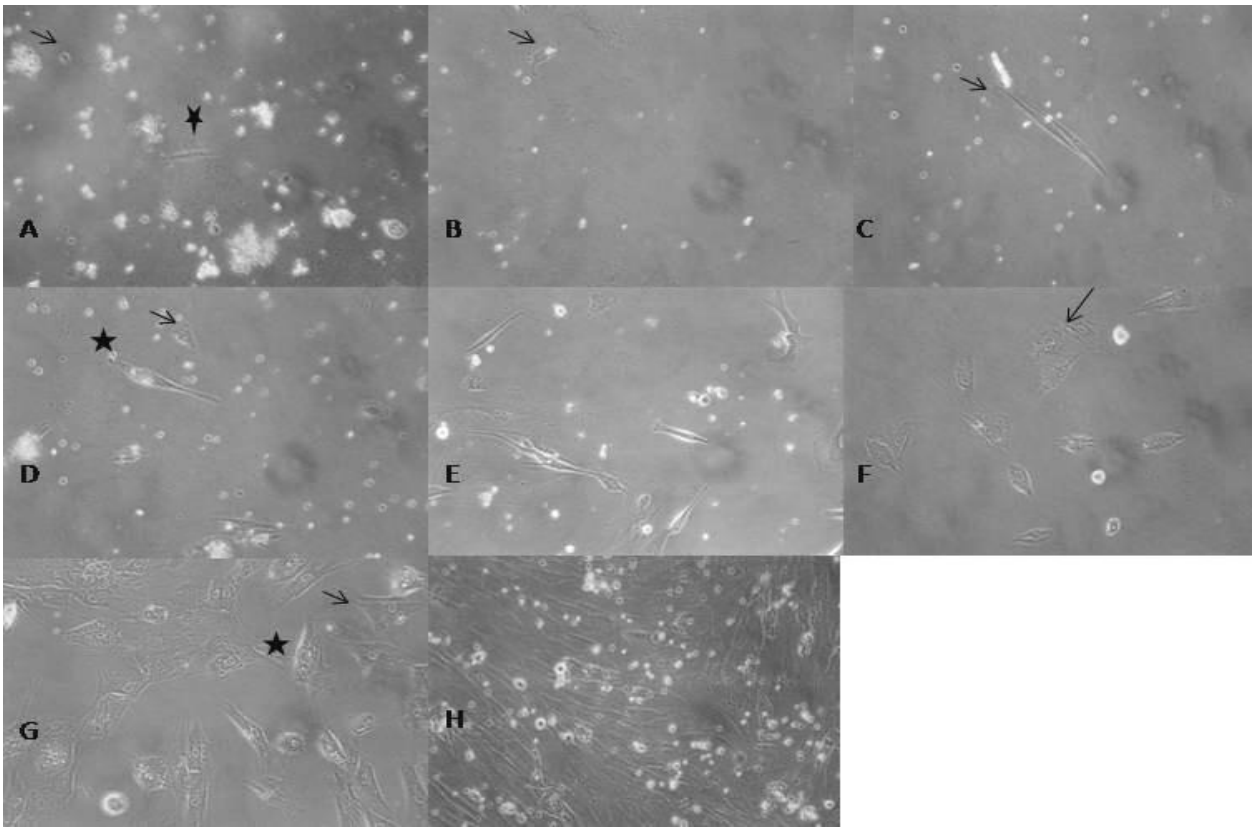

Figure 1: Photomicrographs of a primary culture of human PDL derived MSCs (**x200**), **A**: on day 1 of culture showing numerous small rounded cells. **B**: on day 3 of culture showing some cells started to acquire spindle shape (↓) while most cells retained their rounded morphology. **C**: on day 5 of culture showing clusters of small rounded cells along side with large fibroblast-like cells (↓) showing vesicular nucleus and granular cytoplasm. **D**: on day 7 of culture showing fibroblast-like cells exhibiting various morphologies, triangular shaped (↓) and spindle shaped (star) all retaining the vesicular nucleus and granular cytoplasm. Populations of small round dividing cells are still observed though less apparent. **E**: on day 9 of culture showing fibroblast-like cells retaining their characteristic plump cell body with granular cytoplasm, vesicular nucleus and thin cytoplasmic processes. **F**: on day 11 of culture showing an increase in the fibroblast-like cells is observed some of them are binucleated (↓). **G**: on day 1 3 of culture showing colonies of fibroblast-like cells with the typical morphology of mesenchymal stem cells with various cell shapes spindle, star (star) and triangular (↓). Populations of small rounded dividing cells are can still be observed in some sections. **H**: on day 20 of culture showing confluency.

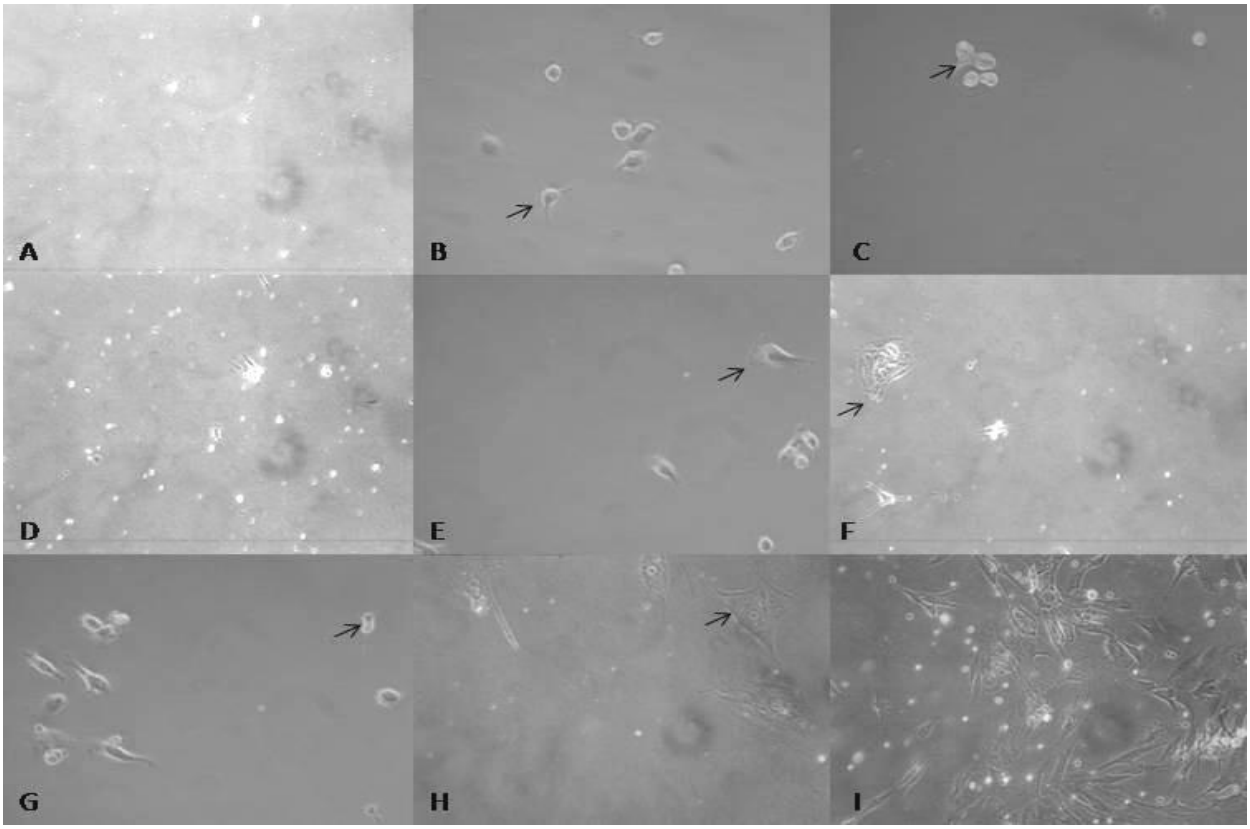

Figure 2: Photomicrographs of a primary culture of human pulp derived MSCs, **A**: on day 1 of culture showing disperse small rounded cells (x200). **B**: on day 3 of culture showing some cells started to form processes in order to adhere to the plastic flask (↓) (X 200). **C**: on day 3 of culture showing some of the small rounded cells undergo mitotic division demonstrating telophase of mitosis (X 400). **D**: on day 5 of culture showing clusters of small rounded cells shown along side with large fibroblast-like cells (x200). **E**: on day 7 of culture showing most rounded non adherent cells were removed leaving the adherent cells (↓) after changing the media (X200). **F**: on day 9 of culture showing fibroblast-like cells exhibiting various morphologies; some are star shaped others spindle shape all retaining the vesicular nucleus and granular cytoplasm. Populations of small round dividing cells are still observed though less apparent (↓) (X200). **G**: on day 11 of culture showing fibroblast-like cells can now be observed along side with small rounded dividing cells (↓) (X200). **H**: on day 13 of culture showing colonies of fibroblast-like cells demonstrating various cell shapes spindle, star (↓) and triangular all with granular cytoplasm, vesicular nucleus and thin cytoplasmic processes. Populations of small rounded dividing cells are can still be observed in some sections (x 200). **I**: on day 20 of culture showing confluency (x200).

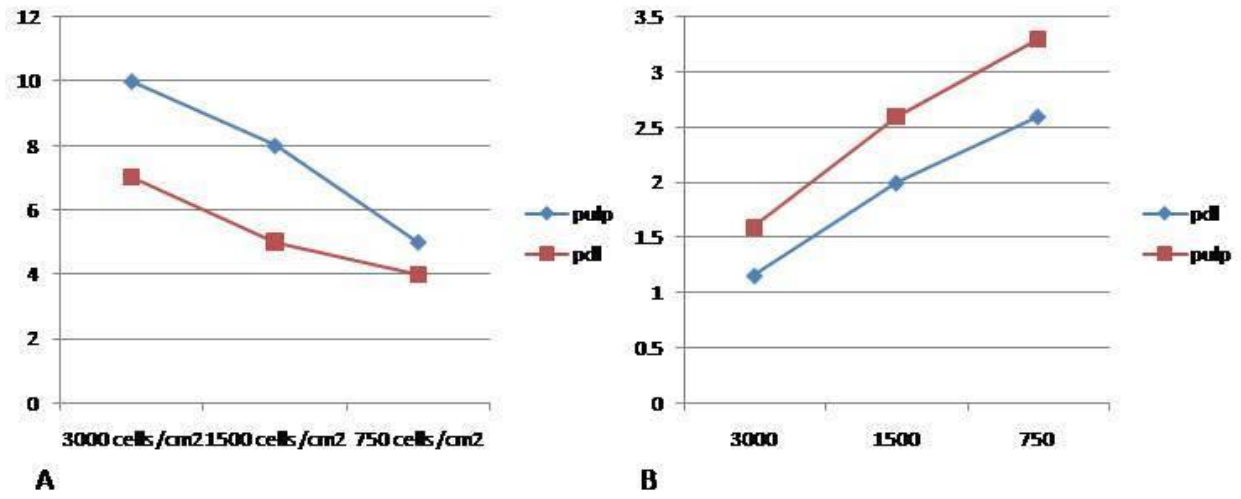

Figure 3: **A**: a graphic representation of the number of colonies created by each group in various cell concentrations, **B**: a graphic representation of the number of colonies created per 10^3 cells plated in each group with different culturing concentrations.

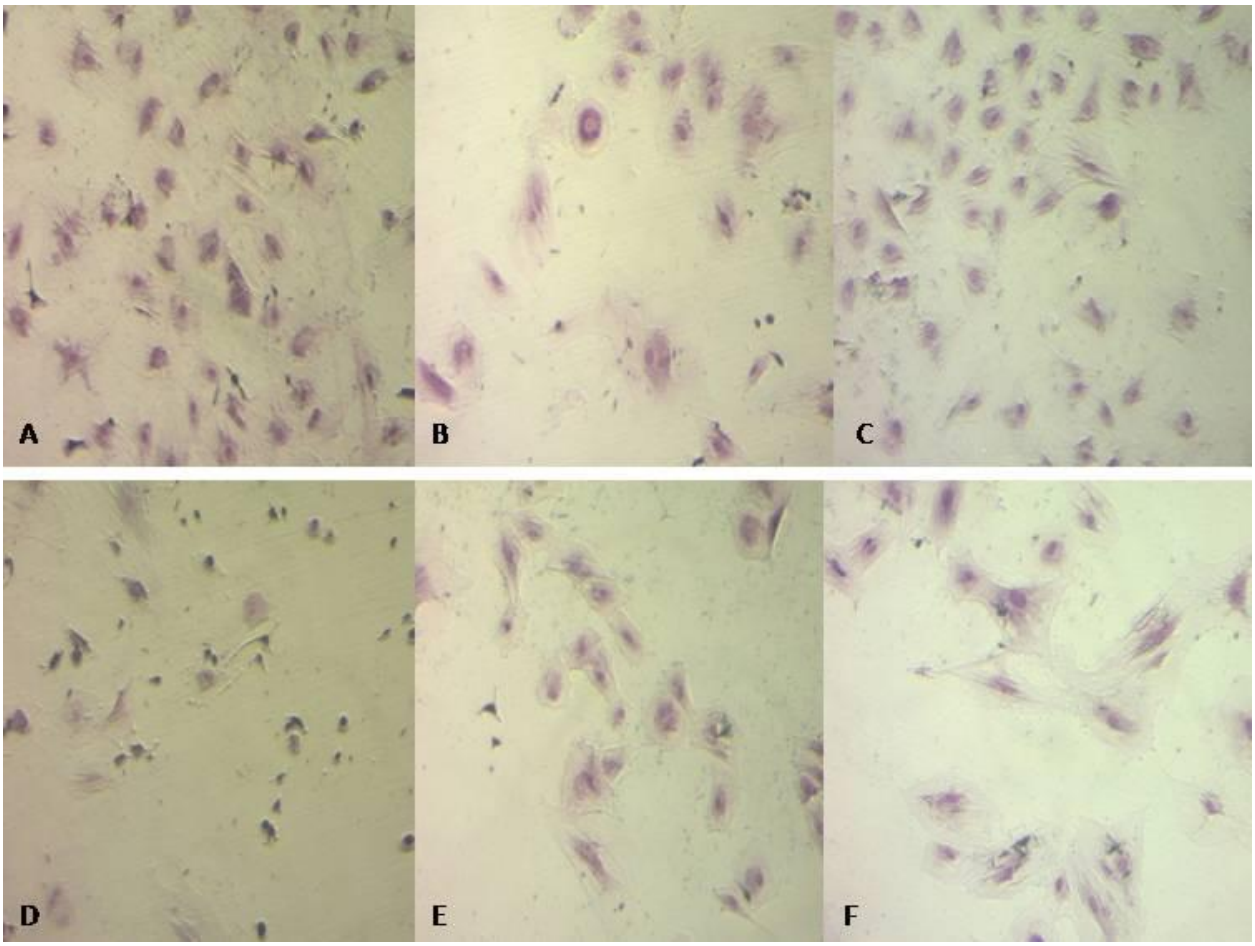

Figure 4: **Giemsa stain** (x100), **A**: Photomicrograph of colonies formed by human pulp derived MSCs of initial concentration 3000 cells /cm^2. **B**: Photomicrograph of colonies formed by human pulp derived MSCs of initial concentration 1500 cells/cm^2. **C**: Photomicrograph of colonies formed by human pulp derived MSCs of initial concentration 750 cells/cm^2. **D**: Photomicrograph of colonies formed by human PDL derived MSCs of initial

concentration 3000 cells /cm^2. **E**: Photomicrograph of colonies formed by human PDL derived MSCs of initial concentration 1500 cells/cm^2. **F**: Photomicrograph of colonies formed by human PDL derived MSCs of initial concentration 750 cells/cm^2.

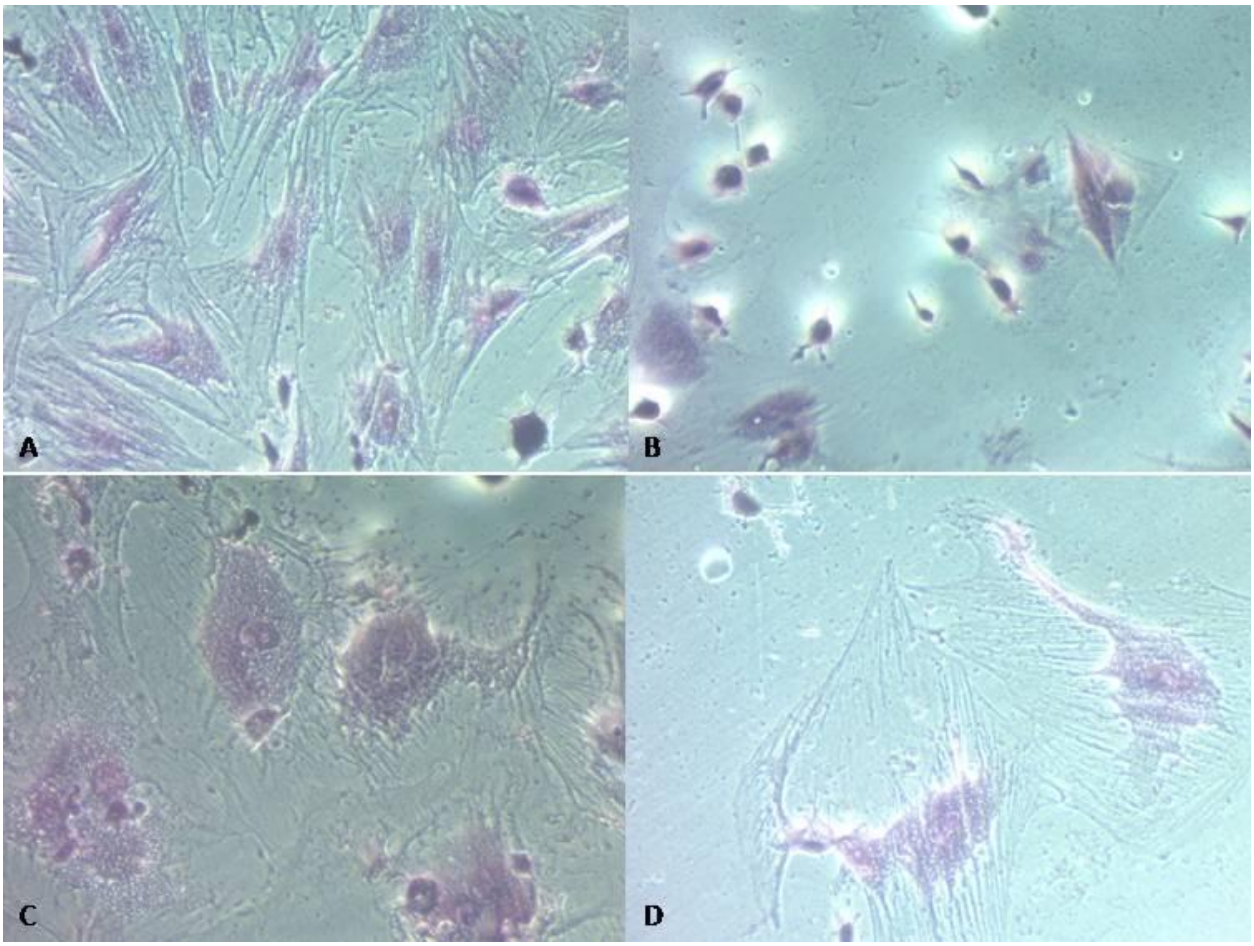

Figure 5: **Giemsa stain** (x200), **A&B**: Photomicrographs of colonies formed by human pulp derived MSCs showing cells with typical fibroblast-like appearance. **C & D:** Photomicrographs of colonies formed by human PDL derived MSCs showing cells with typical fibroblast-like appearance.

Table (1): Summary of Flow cytometric analysis results for both groups:

Marker	CD 34	CD 146
Group I	1.35% (negative)	16% (positive)
Group II	1.5% (negative)	19.5% (positive)

Table (2): Summary of the number of colonies created by each group in various cell concentrations:

Culturing density	3000 cells /cm^2	1500 cells/cm^2	750 cells/cm^2
Group I	7	4	4
	6	5	5
	9	6	3
Mean	7	5	4
S.D.	±1.5	±1	±1
Group II	9	9	7
	12	8	5
	10	7	4
Mean	10	8	5
S.D.	1.527525	1	1.527525

Table (3): Mean number of colonies formed per 10^3cells:

Culturing density	3000/cm	1500/cm	750/cm
Group I	1.16	1.3	2.6
	1	1.6	3.3
	1.5	6	2
Mean	1.6	2.6	3.3
SD	0.264575	0.351188	1.014889
Group II	1.5	3	4.6
	2	2.6	3.3
	1.6	2.3	2.6
Mean	1.16	2	2.6
SD	0.255343	2.631223	0.650641
t test	0.010958	0.466871	0.077634

References

Ahmed G, 2000: Studies on the proliferative potential in the culture of mouse embryo fibroblasts of different embryonic ages .Ph.D thesis in Histology university of Essex.

Alsalameh S, Amin R, Gemba T and Lotz M (2004) Identification of mesenchymal progenitor cells in normal and osteoarthritic human articular cartilage. Arthritis Rheum 50: 1522-1532.

Bluteau G, Luder H-U, De Bari C and Mitsiadis T.A. (2008): Stem cells and tooth engineering, European cells and Materials V o l. 16 (pages 1 - 9)

Bongso A and Lee E h (2010): Stem Cells: Their Definition, Classification and Sources , in STEM CELLS - From Bench to Bedside © World Scientific Publishing Co. Pte. Ltd.

Caplan AI. (1991): Mesenchymal stem cells. J Orthop Res 9:641_50.

Colter DJ, Sekiya I and Prockop DJ (2001): Identification of a subpopulation of rapidly self-renewing and multipotential adult stem cells in colonies of human marrow stromal cells. Proc Natl Acad Sci USA 98: 7841-7845.

Dannan A. (2009): Dental-derived Stem Cells and whole Tooth Regeneration: an Overview ,Journal of clinical medicine research, Volume 1, Number 2, June 2009, Pages 63-71 Publish ahead of Print, March 31, 2009 doi:10.4021/jocmr2009.03.1230

Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, et al. (2006): Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 8:315-317.

El Fattah MA, Ding G, Wei F, Zhang C, Ezz EA and Wang S (2010): Identification and Cementoblastic/ Osteoblastic Differentiation of Postnatal Stem Cells from Human Periodontal Ligament. Maced J Med Sci. doi.10.3889/MJMS.1957-5773.2011.0147.

Friedenstein AJ, Chailakhjan RK and Lalykina KS (1970): The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells. Cell Tissue Kinet 3:393Ð403

Grimm W-D, Arnold WH, Becher S, Dannan A, Gassmann G, Kaltschmidt B, Levermann S, et al.(2008): Stammzellbasierte Therapieformen in der parodontalen Regeneration Zahnrztliche Mitteilungen 2008;98:40-48.

Grimm W-D, Dannan A, Gassmann G, Kaltschmidt B, Varga G, Widera D and Kaltschmidt C (2007): Periodontium derived stem cells as multipotential adult progenitor cell. J Dent Res 2007;86, Spec Iss A

Gronthos S, Mankani M, Brahim J, Robey PG and Shi S (2000): Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. Proc Natl Acad Sci USA 97:13625–13630.

Harada H, Kettunen P, Jung HS, Mustonenm T, Wang YA and Thesleff I (1999): Localization of putative stem cells in dental epithelium and their association with Notch and FGF signalling. J Cell Biol 147:105–120

Kadar K, Kiraly M, Porcsalmy B, Molnari M, Racz MG, Blazseki J, Kallo K, Szabo EL, Gera I, Gerber G and Varga G(2009): Differentiation potentialof stem cells from human dental origin-promise for tissue engineering :Journal of physiology and pharmacology : 60, Suppl 7, 167-175.

Kadar K, Kiraly M, Porcsalmy B, Molnari M, Racz MG, Blazseki J, Kallo K, Szabo EL, Gera I, Gerber G and Varga G(2009): Differentiation potentialof stem cells from human dental origin-promise for tissue engineering :Journal of physiology and pharmacology : 60, Suppl 7, 167-175.

Kerkis I, Kerkis A, DozortsevD, Stukart-ParsonsGC,MassironiSM, Pereira LV, Caplan AI and Cerruti HF (2006): Isolation and characterization of a population of immature dental pulp stem cells expressing OCT-4 and other embryonic stem cell markers. Cells Tissues Organs 184:105–116

Lee OK, Kuo TK and Chen WM (2004): Isolation of multipotent mesenchymal stem cells from umbilical cord blood. Blood, 103: 1669-1675.

Mok, leng CF and Cheng SK (2003): isolatin and identification of putative stem cells from bone marrow .Malysian journal of pathlgy .25:121-27.

Papaccio G, Graziano A, d'Aquino R, Graziano MF, Pirozzi G, Menditti D and De Rosa A. (2006): Long-term cryopreservation of dental pulp stem cells (SBP-DPSCs) and their differentiated osteoblasts: a cell source for tissue repair. J Cell Physiol 2006;208:319-325.

Ramalho-Santos M, Yoon S, Matsuzaki Y, Mulligan RC and Melton DA (2002): "Stemness": transcriptional profiling of embryonic and adult stem cells. Science 298: 597-600.

Ruch JV. (1998): Odontoblast commitment and differentiation. Biochem Cell Biol ;76:923-938.

Seo BM, Miura M, Gronthos S, Bartold PM, Batouli S, Brahim J, Young M, Robey PG, Wang CY and Shi S (2004): Investigation of multipotent postnatal stem cells from human periodontal ligament. Lancet 364:149–55.

Sung J H, Yang H M, Park J B, Choi G S, Joh J W, Kwon C H, Chun J M, Lee S K and Kim S J (2008): Isolation and characterization of mouse mesenchymal stem cells . Transplntation proceedings ,40, 2649-2654.

Urban V S, Kiss A J, Kovas A J, Goczae V, Monostori A and Uher D (2008): Mesenchymal stem cells cooperate with bone marrow cells in the therapy of Diabetes stem cells. 26(1):244-53.

Xu W, Zhang X, Qian H, Zhu W, Sun X, Hu J, Zhou H and Chen Y (2004): Mesenchymal stem cells from adult human bone marrow differentiate into a cardiomyocyte phenotype *in vitro*. Exp Biol Med (Maywood) 229: 623-631.