EVALUATION OF BIOLOGICAL EFFECT OF PLATELET RICH FIBRIN ON THE POLIFIRATION AND OSTEOGENIC DIFFERENCIATION OF CULTURED HUMAN DENTAL PULP STEM CELL AN- IN VITRO STUDY

Dr. Akhilesh Tomar¹, Dr. Akanksha Bhatt², Dr. Vandana Pant³, Dr. A. B. Pant⁴

¹Assistant Professor, Department of Periodontics, Kantipur Dental College Teaching Hospital & Research Centre, Kathmandu, Nepal.
²Assistant Professor, Department of Conservative Dentistry & Endodontics, BBDCODS, BBD University, Lucknow, U.P, India.
³Professor, Department of Periodontics, BBDCODS, BBD University, Lucknow, U.P, India.
⁴Senior Scientist, Indian Institute of Toxicology and Research Center, Lucknow, U.P.

ABSTRACT
The height and density of alveolar bone is maintained by equilibrium, regulated by local and systemic influences between bone formation and resorption. Thus, during periodontal infection, the formation and resorption may lead to different defects on bony surface like, horizontal or vertical bone loss, osseous craters, ledges, exostoses etc. These defects need to be corrected or recontoured, which can be achieved by reconstructive bone surgeries using various graft materials or other procedures. Materials to be grafted can be obtained as autografts, allografts xenografts & alloplast. Graft materials should have the properties of biological acceptability, predictability, clinical feasibility, minimal operative hazards, minimal post-operative sequelae and patient acceptance. Most of the bone grafts which are available till date has the limitation in either their their osteoconductive property or the predictability of clinical outcome.

KEYWORDS: Platelet Rich Fibrin, Osteogen, Dental Pulp, Stem Cell, Osteoblasts.
INTRODUCTION

Blood supply provides the necessary cells, growth factors, and inhibitors to initiate the osteogenic biomineralization cascade.\(^4\) Injury to blood vessels during oral surgical procedures causes blood extravasation, subsequent platelet aggregation, and fibrin clot formation. Fibrin also provides a matrix for the migration of fibroblasts and endothelial cells that are involved in angiogenesis and responsible for remodeling of new tissue. Platelet activation in response to tissue damage and vascular exposure results in the formation of a platelet plug and blood clot as well as the secretion of biologically active proteins.\(^5\) Platelet alpha (\(\alpha\)) granules form an intracellular storage pool of growth factors (GF) including platelet-derived growth factor (PDGF), transforming growth factor\(\beta\) (TGF-\(\beta\)), vascular endothelial growth factor (VEGF), and epidermal growth factor (EGF).\(^6\) Insulin-like growth factor-1 (IGF-1), which is present in plasma, can exert chemotactic effects towards human osteoblasts.\(^7\)

After platelet activation, \(\alpha\) granules transforms some of the secretory proteins to a bioactive state.\(^8,9\) These active proteins bind to transmembrane receptors of target cells to activate intracellular signalling proteins\(^10\) which results in the expression of a gene sequence that directs cellular proliferation, collagen synthesis, and osteoid production.\(^11\)

So, concentrates of Platelets, Platelet-rich fibrin (PRF) represents a new step in the platelet gel therapeutic concept with simplified processing as compared to Platelet Rich Plasma (PRP) where biochemical modification\(^12\) like anticoagulants or bovine thrombin is required.\(^13,14\) In France Choukroun et al in 2001\(^15\) developed the PRF production protocol to accumulate platelets and released cytokines in a fibrin clot. The fibrin matrix supporting them certainly constitutes the determining element responsible for the real therapeutic potential of PRF.\(^12,16\)

The presence of stem cells in periodontal ligament and dental pulp has been already reported.\(^17,18\) Which are the actual regenerative cell in case of injury, repair and regeneration.

Till date no study has been conducted to see the effect of PRF on human dental pulp stem cells (hDPSCs) for it’s osteogenic potential. So this study was conducted with the objectives of-
1) To investigate the effect of platelet rich fibrin (PRF) on growth and proliferation of cultured human dental pulp stem cells (hDPSCs).

2) To investigate the PRF induced alterations in the expression of senescence and apoptosis markers.

3) To investigate the influence of platelet rich fibrin (PRF) on osteogenic differentiation potential of human dental pulp stem cells (hDPSCs).

**MATERIALS AND METHOD**

**Reagents and consumables**

All the chemicals, reagents, and kits used in this study were purchased from Stem Cell Technologies, Vancouver, BC, Canada, and Sigma, St Louis, MO, or unless otherwise stated. All the antibodies were procured from Chemicon International, CA, and Abcam, CA. Culture wares and plastic wares were procured from Nunc, Denmark, and Corning Inc., NY. Autoclaved endotoxin free Milli-Q water was used in all the experiments.

**Ethical statement for collection and transportation of human tissue**

Human dental pulp was extirpated from the healthy teeth of systemically healthy subjects undergoing a tooth extraction for impacted tooth or orthodontic procedures. Written informed consent was obtained from all the volunteers according to the Helsinki Declaration 1975, as revised in 2008. Approval by the Institutional Human Ethics Committee of BBD College of Dental Sciences, BBD University, Lucknow, India, was taken for all the procedures. Extracted pulp tissues were immediately placed in Minimum Essential Medium (MEM) with low glucose (Gibco BRL) + penicillin 10,000 I.U./100 ml, streptomycin 10,000 I.U./100 ml and fungizone 250 µg/100 ml medium (PSF) and transported to the Indian Institute of Toxicology Research (IITR), Lucknow, India, for further processing.

**Preparation of Platelet Rich Fibrin (PRF)**

The whole blood was drawn from the antecubital vein of 10 healthy male volunteers with no history of aspirin intake or other medications that might interfere with coagulation over the previous 2 weeks between age groups 18yrs to 35yrs. The centrifugation process was carried out as per Dohan et al method with certain modifications. In brief, PRF was extensively washed by perfusing it with 10 ml Tris-buffered saline (50 mM Tris-HCl, 100 mM NaCl, pH 7.4) containing aprotinin (100 KIU/ml) at 4°C. The fibrins were compacted by centrifugation at 1000xg for 10 minutes. The cream colored Platelet rich zone was separated from red blood cells rich zone using scissors (Fig 1). The PRF was washed with deionized water and fibrin-
bound proteins were extracted with 2000 µl rehydration buffer (7 M urea, 2 M thiourea, 4\% (w/v) CHAPS, 0.5\% (v/v) IPG 3–10 buffer) for 1 hour at room temperature and mixed with culture medium at a ratio of 1:10.

**Characterization of dental pulp stem cells**
The expression of the CD34, c-Kit and STRO-1 antigens were analyzed by indirect staining using mouse anti-CD34 IgG, rabbit anti-c-Kit IgG and mouse anti-STRO-1 IgM, followed by sheep anti-mouse-FITC, goat anti-rabbit-FITC and goat anti-mouse IgM-FITC. Non-specific fluorescence was assessed by using normal mouse IgG or IgM followed by the secondary antibody as detailed above. Analyses was done by Flowcytometer (BD FACSCanto™ II) immunocytochemical analysis and western blotting.

**Experiments with PRF**
**Cell Counting**
The proliferation rate was analyzed on the same hDPSCs population, seeded in 60 mm Petri dishes at the density of 4x10\(^3\) cells/cm\(^2\) and cultured for 1 week until reaching the confluence. Cell counting was performed in two culture conditions; one culture without PRF and another culture with PRF. The mean of cell number was calculated on three experimental samples for each condition and cell density was expressed as mean of cells/cm\(^2\) ± standard error (SE). The population doubling time (PDT) was calculated in the phase of exponential growth using following formula:

\[
PDT = \frac{\log_{10}(2) \times \Delta T}{\log_{10}(N_{7d}) - \log_{10}(N_{1d})}
\]

\(N_{7d}\) is the cell number at day 7 and \(N_{1d}\) is the cell number at day 1. To determine the population doubling (PD) rate, hDPSCs were initially seeded at the density of 4x10\(^3\) cells/Cm\(^2\) in culture medium supplemented with or without PRF. Cells were passaged and counted once they reached a sub-confluence of 80\%. At each passage cells were re-plated at the initial placement and culture were performed until passage 5. Three samples for each condition were used. The following formula was applied:

\[
P_D = \frac{\log_{10}(N) \times \log_{10}(N_{s})}{\log_{10}(2)}
\]
N is the harvested cell number and \( N_s \) is the initial plated cell number. Cumulative population doublings (CPD) index for each passage was obtained by adding the PD of each passage to the PD of the previous passages.

**Senescence and Cell Death**

To evaluate the presence of senescent cells in hDPSCs maintained in medium with PRF, cells at 5\(^{th}\) passage were seeded in 12-well plates and cultured until reaching the confluence. Samples were then processed by a senescence β-Galactosidase staining kit (Cell Signaling, USA), according to manufacturer’s instructions. Three samples for each culture condition were analyzed and percentage of senescent cells was calculated.

The presence of apoptotic cells in hDPSCs cultures was analyzed by detection of the cleaved form of poly (ADP-ribose) polymerase (PARP). Whole cell lysates of hDPSCs cultured in PRF containing medium and in regular medium at passages 1, 3 and 5 were processed for western blot analysis and PARP was detected by an anti-PARP specific Ab (Santa Cruz).

**Influence of PRF on osteogenic differentiation of hDPSCs**

hDPSCs were seeded at approximately 1x10^5 cells in 25 Cm\(^2\) culture flaks in the osteogenic differentiation medium for 36 days (α-MEM, supplemented with 10% FBS, 100 µM 2% ascorbic acid, 2 mM L-glutamine, rhTPO (25 ng/ml), rhSCF (25 ng/ml), rhFGF-basic (50 ng/ml), 100 U/mL penicillin and 100 µg/mL streptomycin, 100 nM dexamethasone, 10 mM β-glycerophosphate). hDPSCs were differentiated in two conditions; one in osteogenic differentiation medium without PRF and another in osteogenic differentiation medium with PRF.

**Real time PCR analysis**

Total RNA was extracted using TRIZol from osteogenic differentiating hDPSCs with and without the presence of PRF in the culture medium. RNA (1 µg) was reverse transcribed into cDNA by SuperScript III first-strand cDNA synthesis kit (Invitrogen Life Science, catalog no. 18080-051). Quantitative real-time PCR (qRT-PCR) for expression of marker genes of dental pulp stem cells and differentiating osteocytes were studied using SYBR Green dye (Applied Biosystems) using the gene specific primer sequences. The details of primer sequences, product size, annealing temperatures and gene bank accession numbers are given in table-1.
Western Blot
Whole cell lysates were obtained at different points of differentiation by using a hypotonic buffer (30 mM Tris-Cl, pH 7.8, containing 1% Nonidet P40, 1 mM EDTA, 1 mM EGTA, 1 mM Na$_3$VO$_4$, and freshly added Sigma-Aldrich Protease Inhibitor Cocktail). Lysates were cleared by centrifugation and the total lysate was immediately boiled in SDS sample buffer. The protocols of the Western blot were performed as described by (Kashyap et al, 2012). In brief, 60 µg of protein extract, quantified by a Bradford Protein Assay (Biorad), underwent SDS-polyacrylamide gel electrophoresis and were transferred to PVDF membranes. The following antibodies were used: rabbit anti-Runx2 diluted 1:500; anti-osterix (Osx) diluted 1:500, mouse anti-osteopontin (Osp) diluted 1:1000, mouse anti-osteocalcin and secreted osteocalcin (Ocn and sOcn) diluted 1:1000 (Millipore; MA, USA), rabbit anti-PARP (Santa Cruz) diluted 1:1000; peroxidase-labelled anti-rabbit and anti-mouse secondary antibodies diluted 1:3000 (Pierce Antibodies, Thermo Scientific; Rockford, IL, USA). Antibodies were diluted in TBS-T pH 7.6 containing 2% BSA and 3% free fatty milk. The membranes were visualized using ECL (enhanced chemiluminescence, Amersham, UK). Anti-actin antibody was used as control loading protein. Densitometry of cleaved PARP bands was performed by NIS software (Nikon). An equal area was selected inside each band and the mean of gray levels (in the scale of 0–256) was calculated. Data were then normalized to values of background and of control actin band.

Immunocytochemistry (Fluorescence Microscopy)
Fixed monolayer cells were permeabilized with 0.1% Triton X-100 in PBS for 5 minutes; samples were then blocked with 3% BSA in PBS for 30 minutes at room temperature and then incubated with the primary antibodies diluted 1:50 [rabbit anti-alkaline phosphatase (Alp), osteocalcin (Osc), osteopontin (Osp); mouse anti-collagen type-1 (Coll-1), osteonectin (Osn), Runx2] in PBS containing 3% BSA, for 1 hour at room temperature. After washing in PBS containing 3% BSA, the samples were incubated for 1 hour at room temperature with the secondary antibodies diluted 1:200 in PBS containing 3% BSA (goat anti-rabbit FITC, sheep anti-rabbit FITC, donkey anti-mouse Cy3). After washing in PBS samples were stained with 1 µg/ml DAPI in PBS for 1 minute, and then mounted with anti-fading medium (0.21 M DABCO and 90% glycerol in 0.02 M Tris, pH 8.0). The cells were visualized under an upright fluorescence microscope. For each marker, 20 randomly selected microscopic fields were captured and analyzed for fluorescence intensity with the help of Leica Qwin 500 Image Analysis Software (Leica, Germany).
RESULT

Purification and characterization of human dental pulp stem cells
The data of stem cell markers (c-Kit⁺, CD34⁺ and STRO-1⁺) of isolated and purified population of human dental pulp stem cells are summarized in Figure-2-5. The data of Flowcytometric analysis shows that majority of cells have expressed the stem cell markers viz., CD34 (66%), c-Kit (87%) and STRO-1 (99%) Figure3. Further purification was done using Robosep automated cell sorter for cells isolated from all the colonies derived from eight different clones. The analysis shows that the marker based selection further improve the purity of cell population significantly i.e., CD34⁺ (> 20%) and c-Kit⁺ (> 11%), while percentage of STRO-1⁺ cells remained almost similar as earlier Western blot analysis also confirms the purity of cells as evidenced by the expression of CD34 (115 kDa), c-Kit (145 kDa) and STRO-1 (60 kDa) proteins (Figure-4). These purified cells were allowed to proliferate further as adherent cells and maintained up to passage-5 in α-MEM culture medium supplemented with growth factors as described in Materials and Methods section. The makers of stem-ness were analysed at each passage. After the passaging, the rounded small cells (2-3 µm) increased in size and formed large colonies within 7 days time. These colonies were disaggregated by trypsinization and used in further passaging and experimentations (Figure-5 A & B).

PRF induced alterations in the cell counting and proliferation
The results of altered cell proliferation under the influence of PRF in culture medium are depicted in Figure-9 a-c. Time dependent (day 1, 4 and 7) gradual increase in the green fluorescence of 6-CFDA staining clearly indicates the differential population growth and proliferation in the cells. PRF exposure increases the proliferation rate in the cells exponentially till seven days of recording (Figure-9 a). The exponential increase in cell population reached to the level of significant difference at day 4 (p<0.01), when compared with cells grown in culture medium without PRF.

Senescence and Apoptosis
Cell senescence evaluated by detection of β-galactosidase activity in confluent culture of hDPSCs has shown no significant activity in both PRF exposed and non-exposed cells at 5-passage (fig. 9b-9c figure 10-11).
PRF induced alterations in the osteogenic differentiation

For osteogenic differentiation, hDPSCs were plated on a plastic surface at the density of $3 \times 10^3$ cells/cm$^2$ and then allowed to differentiate in the culture medium supplemented with growth factors required for osteogenic differentiation and with or without PRF. The cells were analyzed for a period up to 36 days.

Morphological and biochemical studies

Seeded cells got adherence and show sprouting even at 6 h of seeding the cells. Though, a slight, but difference in the cellular extensions was there in cells receiving PRF since 6 h of seeding, which became prominent in terms of number and morphological extension by day 6 and got sizable significant difference by day 12, cells receiving PRF exposure shows a significantly higher magnitude of mineral aggregation around the cells and more nodules were developed in comparison to the cells not receiving PRF exposure (Figure-1).

PRF induced alterations in the expression of osteogenic specific markers

PRF induced alterations at transcriptional level (Real Time-PCR)

With the progress of differentiation (day 6, 12 and 24), a gradual increase in the expression of mRNA of markers associated with osteogenic differentiation viz., alkaline phosphatase (Alp), collagen type I (Coll-1), osteocalcin (Osc), osteonectin (Osn), osteopontin (Osp) and RUNX2 was observed in both (PRF receiving and non-receiving) the cells. In cells growing without PRF exposure, the transcripts of Alp, Coll1, Osc, Osn, Osp and RUNX2 were detected after the onset of differentiation at day 6 i.e. 16.7±0.58, 22.5±1.15, 23.7±1.73, 25.5±1.15, 22.3±2.31 and 21.3±1.73 fold respectively in comparison to undifferentiated control. The expression of these markers viz., Alp, Coll1, Osc, Osn, Osp and RUNX2 were recorded highest at day 24 of differentiation i.e. 25.8±1.15, 28.4±1.73, 32.7±0.58, 32.5±1.16, 37.5±2.3, and 29.8±1.2 fold respectively in comparison to undifferentiated control. While, beyond day 24, there were no significant alteration in expression of these marker genes. An additive response of PRF was observed as it enhanced the fold expression of transcripts of osteogenic markers Alp, Coll1, Osc, Osn, Osp and RUNX2, when compared the profile that to non-PRF exposed cells at corresponding days of differentiation. By day 6 of differentiation in the cells receiving PRF, the levels of Alp, Coll1, Osc, Osn, Osp and RUNX2 were enhanced to 23.5±1.15, 31.5±0.59, 32.6±1.16, 33.3±1.2, 39.7±1.3, and 32.2±0.58 fold to their respective undifferentiated control cells. The fold induction was further increased by further differentiation on day 12 i.e., Alp (30.9±0.58), Coll1 (34.3±1.17), Osc (36.5±1.73), Osn
(37.2±2.3), Osp (42.6±1.7) and RUNX2 (37.8±1.6) fold of respective day controls. At late full maturity (day 24), the expression of Alp, Coll1, Osc, Osn, Osp and RUNX2 were increased to 33.5±1.7, 38.4±1.7, 39.4±2.31, 46.4±2.3 and 45.6±1.7 fold of controls. The expression induction difference was highly significant statistically (p<0.01) in PRF exposed cells at day 12 and 24 of differentiation, when the data were compared with the cells not receiving PRF (Figure-13).

**PRF induced alterations at translational level**

**Western blot analysis**

To confirm the differentiation of hDPSCs towards osteoblasts like cells, in both conditions, the presence of specific marker Runx2, osterix (Osx), Osp, osteocalcin and secreted osteocalcin (Ocn and sOcn) was analyzed by western blot in whole cell lysates of differentiating hDPSCs. Results show linearity in the expression pattern of markers studied at transcriptional levels all through the differentiation of cells. Data of western blot analysis are presented in figure-14 a & b. These markers were detected in both the differentiating conditions, and show an increase of expression during the differentiation.

A gradual increase in the expression of osteogenic marker proteins was observed all through the differentiation and peaked by day 24. Data show the significant alterations in osteogenic markers viz., Runx2 (1.34, 2.9 and 2.2 fold of undifferentiated control), Osx (4.1, 5.2 and 6.6 fold of undifferentiated control), Osp (1.9, 2.5 and 3.0 fold of undifferentiated control), Ocn (1.3, 2.2 and 2.5 fold of undifferentiated control) and sOcn (4.8, 7.7 and 5.3 fold of undifferentiated control) at day 6, 12 and 24 of differentiation respectively. Addition of PRF in differentiation medium also enhanced the protein expression of these markers. PRF increased the expression of osteogenic markers viz., Runx2 (7.8, 9.7 and 12.2 fold of undifferentiated control), Osx (6.2, 7.7 and 8.1 fold of undifferentiated control), Osp (1.8, 3.7 and 10.2 fold of undifferentiated control), Ocn (1.5, 3.7 and 4.2 fold of undifferentiated control) and sOcn (6.5, 8.7 and 9.3 fold of undifferentiated control) at day 6, 12 and 24 of differentiation respectively.

**Immunocytochemical analysis**

The images of immunocytochemistry in figure-15-17 are representative of the marker proteins assessed to analyze the osteogenic differentiation. The immunocytochemical localization analysis at day 24 shows the expression of alkaline phosphatase (Alp) together with other specific markers of osteogenic commitment, such as Osc, Osp, Coll-1, Osn,
Runx2. Double immunofluorescence labelling was used to carry out the simultaneous analysis of two marker proteins in hDPSCs derived differentiating osteoblasts in differentiation medium with or without PRF supplementation. There was a linear correlation in the data of immunocytochemistry with western blot analysis and data of Real Time PCR for the expression of these osteogenic marker proteins. The immunocytochemical localization also confirms the significant functional expression of marker proteins of osteoblasts. A gradual increase in the expression of Alp and Coll1 markers was observed following the onset of differentiation at day 6 (18.7 and 22.3 % area of expression respectively) and peaked at day 24 (39.4 and 44.4% area of expression respectively), when compared with the data of undifferentiated control i.e. day 0 (14.2 and 16.5% area of expression respectively). The percent area of expression of Alp and Coll1 was found to be enhanced in PRF supplemented cells (25.5 and 31.2% area of expression respectively) at day 6 to 44.3 and 48.7 % area of expression respectively at day 24 as compared to control 19.2 and 26.5 % area of expression respectively at day zero (Figure-15).The expression induction difference was significantly (p<0.01) higher in PRF exposed cells at all the time points studied, when compared with the cells of respective day of differentiation without PRF (Figure-15).

Similar trends were also recorded for the expression of marker proteins viz., Osc, Osn, Osp and Runx2 (Figure-16-17).

Platelet rich fibrin with a portion of bottom layer

Platelet rich fibrin

Figure-1
Figure 2. Human dental pulp stem cells (hDPSCs) selection. Diagram representing the percentage of c-Kit, CD34 and STRO-1 positive cells in eight different dental pulp cell colonies derived from clonal density plated cells.

Figure 3. Human dental pulp stem cells (hDPSCs) selection Flowcytometric analysis of hDPSCs sorted by MACS. The percentage of positive cells is indicated.

Figure: 4. Human dental pulp stem cells (hDPSCs) selection Western blot analysis of whole cell lysates of sorted hDPSCs by anti c-Kit, anti-CD34 and anti-STRO-1 protein antibodies.
Figure 5. Microphotographs of cultured human dental pulp stem cells. Isolated and purified population of hDPSCs in culture at day zero in undifferentiated stage. A full grown colony of cells with loosely adhered cells in the periphery and dissociating cells. Images were captured at 200x magnification using Leica phase contrast microscope equipped with Leica IM50 software (Leica Microsystems, USA).

Figure: 6 (a) Human dental pulp stem cells (hDPSCs) growing in culture medium with or without Platelet Rich Fibrin (PRF). CDFA (6-Carboxyfluoresceine diacetate, Sigma Aldrich; St. Louis, MO, USA) vital staining dye was used to stain cultured viable hDPSCs at 1, 4 and 7 days. Green signal indicates viable cells.
Figure 6. (b) Human dental pulp stem cells (hDPSCs) growing in culture medium with or without Platelet Rich Fibrin (PRF). Values are mean in a Log Scale of three independent experiments (n = 3); Values are mean reported in a Log scale \(< 0.01^{**}, p < 0.001^{**}, p < 0.001^{***}\) X

Figure 7: Real Time PCR analysis for expression of osteogenic marker genes of human dental pulp stem cells derived differentiating osteogenic cells. Relative quantification of altered mRNA expression of osteogenic marker genes (Alp, Coll-1, Osc, Osn, Osp and Runx2) in differentiating osteogenic cells at day 6, 12 and 24 in differentiation medium.
supplemented with or without Platelet Rich Fibrin (PRF). β-actin was used as internal control to normalize the data and differentiation induced alterations in mRNA expression are expressed in relative quantity (mean±S.E) compared to the expression in the undifferentiated cells at day zero.

Figure 8: Western blot analysis of expression for osteogenic markers of human dental pulp stem cells derived differentiating osteogenic cells. Western blot and relative quantification of altered protein expression of osteogenic marker genes (Runx2 Osx, Opn, Ocn and sOcn) in differentiating osteogenic cells at day 6, 12 and 24 in differentiation medium supplemented with or without Platelet Rich Fibrin (PRF). The values obtained at day zero were considered as basal i.e., Relative quantification in the expression at day 6, 12 and 24 was done comparing the values at day zero of undifferentiated cells. β-actin was used as loading control to normalize the data.
Figure 9: Immunocytochemical localization for expression of osteogenic markers Alp and Coll-1 in differentiating osteogenic cells derived from hDPSCs. (a) Representative microphotographs of osteogenic marker Alp and Coll-1 proteins in hDPSCs derived osteogenic cells (without and with PRF) at various maturity i.e., day 0, 6, 12, 24 and 36. Images were captured at 400x magnification. (b) Quantification of altered expression of Alp and Coll-1 proteins in hDPSCs derived osteogenic cells at various maturity without PRF. (c) Quantification of altered expression of Alp and Coll-1 proteins in hDPSCs derived osteogenic cells at various maturity with PRF. Data are expressed in mean±S.E of percent area of expression in the snapped microscopic fields. A minimum of 20 microscopic fields were snapped for each group.

Figure 10: Immunocytochemical localization for expression of osteogenic markers Osc and Osn in differentiating osteogenic cells derived from hDPSCs (a) Representative microphotographs of immunocytochemical localization of osteogenic marker Osc and Osn proteins in hDPSCs derived osteogenic cells (without and with PRF) at various
maturity i.e., day 0, 6, 12, 24 and 36. Images were captured at 400x magnification. (b) Quantification of altered expression of Osc and Osn proteins in hDPSCs derived osteogenic cells at various maturity without PRF. (c) Quantification of altered expression of Osc and Osn proteins in hDPSCs derived osteogenic cells at various maturity with PRF. Data are expressed in mean±S.E of percent area of expression in the snapped microscopic fields. A minimum of 20 microscopic fields were snapped for each group.

Figure 11: Immunocytochemical localization for expression of osteogenic markers Osp and Runx2 in differentiating osteogenic cells derived from hDPSCs (a) Representative microphotographs of osteogenic marker Osp and Runx2 proteins in hDPSCs derived osteogenic cells (without and with PRF) at various maturity i.e., day 0, 6, 12, 24 and 36. Images were captured at 400x magnification. (b) Quantification of altered expression of Osp and Runx2 proteins in hDPSCs derived osteogenic cells at various maturity without PRF. (c) Quantification of altered expression of Osp and Runx2 proteins in hDPSCs derived osteogenic cells at various maturity with PRF. Data are expressed in mean±S.E of percent area of expression in the snapped microscopic fields. A minimum of 20 microscopic fields were snapped for each group.
Figure 12. Microphotograph of morphology of differentiating osteogenic cells at different stages of maturity derived from human dental pulp stem cells (hDPSCs) in differentiation medium supplemented with or without Platelet Rich Fibrin (PRF). Images were captured at 200x magnification using Leica phase contrast microscope equipped with Leica IM50 software (Leica Microsystems, USA). PLATE-8

DISCUSSION

Recently, reports have begun to emerge demonstrating that populations of adult stem cells reside in the periodontal ligament and dental pulp of humans (K. Nagatomo et al.[20] 2006, Gronthos S et al.[19] 2002). Stem cell biology, an emerging field of research, provides promising methods in vitro as well as in vivo in animal models which make speculation about a future application in human dentistry reasonable.

Keeping the background knowledge, we are showing the biological effect of platelet rich fibrin (PRF) to increase the cell proliferation, effect on apoptosis and senescence, and enhance osteogenic differentiation of hDPSCs in the present study that may allow PRF therapy to be used in clinical application with minimal safety concerns. The PRF does not
dissolve quickly, instead the fibrin matrix is slowly remodelled the way natural blood clot resorb. (Dohan et al\cite{21} 2009, Dohan et al\cite{22} 2009).

Platelets are known to release a variety of growth factors on activation (Agis H.\cite{23} 2010). These factors have a positive effect on tissue repair. Success of second generation PRF for various defects may be due to its mechanical properties, its localized high platelet concentration and its elevated cytocompatibility. Few data indicate that PRF is more than 600 times stiffer than the stiffest fibrin clot obtained at ambient pressure and described in the literature to date (Zhang et al.\cite{24} 2002, Zhang et al.\cite{25} 2007).

Our results show that growth factors released from PRF can support hDPSCs proliferation and differentiation. This is consistent with published results that show that growth factors released from platelets have the potential to stimulate MSC proliferation (Doucet et al.\cite{26} 2005; Lucarelli et al.\cite{27} 2003). Their study also shows the same effect of growth factors on MSC. This finding is relevant in that it supports a therapeutic role of the PRF alone if implanted in a site in which local MSC could be recruited to heal the damaged tissue.

Our data demonstrate that PRF is an appropriate supplement for the in vitro expansion of hDPSCs, as its addition to culture medium promotes a good proliferation rate. Furthermore, PRF increases the hDPSCs doubling during culture indicating this as the optimal condition to obtain sufficient cell number soon and consequently to reduce the waiting for a therapeutical treatment.

We observed that PRF group shows significant higher activity of these osteogenic markers like Alp, Osn, Osp, Osc and Runx2. As David M. Dohan Ehrenfest in\cite{28} 2009 and Huang F.M. et al\cite{29} in 2010 found the similar result in their respective studies. There was a linear correlation in the data of immunocytochemistry with western blot analysis and data of Real Time PCR for the expression of these osteogenic marker proteins. The immunocytochemical localization also confirms the significant functional expression of marker proteins of osteoblasts In 2012 Clipet F et al\cite{30} found the similar result with PRF whereas Eun-Ju Kang\cite{31} in 2009 with Fibrin glue. After day 24, the number of hDPSC shows a slightly reduction probably due to increase of cell death that normally occur during late cell differentiation.
It can be conclude that PRF is efficacious material for cell proliferation and differentiation with minimal threat of disease transfer. PRF is an autologous preparation and found to be clinically effective and economical biomaterial than any other available regenerative materials including PRP. We can use PRF either independently or with other bone grafting materials to perform regenerative procedures attractive as it is found to be an excellent or strong agent for cell differentiation and proliferation.

Future studies are required to elucidate the precise mechanism of action of PRF for dental pulp stem cells regeneration both in-vitro and in-vivo. In-vitro experiment are helpful to essay the biological effect of PRF, but they might be limited in their ability to simulate the clinical condition.

REFERENCES


