Available online at http://www.journalcra.com

International Journal of Current Research
Vol. 8, Issue, 03, pp.28920-28927, March, 2016

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

ISSN: 0975-833X

RESEARCH ARTICLE

ASSESSMENT OF IDEAL CONCENTRATIONS OF PLATELET RICH PLASMA IN REGENERATION OF HUMAN PERIODONTAL LIGAMENT CELLS

[1]Dr. Santosh Kumar Verma, [2]Dr. Madhu Ranjan, *[,3]Dr. Barun Dev, [4]Dr. Abhik Sen, [5]Dr. Sankalp Verma and [6]Dr. Marilia Marceliano-Alves

[1]Department of Periodontics, Hazaribag College of Dental Sciences and Hospital, Demotand, Hazaribag, Jharkhand, India
[2]Reader, Department of Prosthodontics, Hazaribag College of Dental Sciences and Hospital, Demotand, Hazaribag, Jharkhand, India
[3]Senior Lecturer, Department of Orthodontics, Hazaribag College of Dental Sciences and Hospital, Demotand, Hazaribag, Jharkhand, India
[4]Assistant Professor, Department of Orthodontics, MGM Medical College, Kishanganj
[5]Department of Oral Medicine and Radiology, Bhabha College of Dental Sciences, Bhopal, MP, India
[6]Professor of Specialization Course in Endodontics at Brazilian Dental Association - Niterói - RJ; Professor of specialization course in Endodontics at Santos Dumont Air Force\Dental Clinic - Rio de Janeiro, Brazil

ARTICLE INFO

Article History:
Received 10th December, 2015
Received in revised form
26th January, 2016
Accepted 14th February, 2016
Published online 31st March, 2016

Key words:

Platelet rich plasma,
Periodontium,
Periodontal ligament cells.

ABSTRACT

Background: Platelet-rich plasma (PRP) has been used in periodontal therapy for past two decades. However, results obtained are conflicting. Moreover, the optimum concentrations of PRP to promote wound healing and regeneration still remains uncertain.
Objectives: The purpose of this study was to evaluate the biological effects of platelet-rich plasma (PRP) on human periodontal ligament cells (hPDLCs) in vitro. This study was an attempt to find ideal concentration for clinical uses.
Materials and method: Primary cultures of hPDLCs were obtained from healthy premolars. PRP was isolated by two-step centrifugation. Platelet gel releasate (PGR) was prepared by activating PRP with thrombin and CaCl2. The functional parameters of hPDLc (proliferation, alkaline phosphatase) were analyzed in vitro for 14 days in the presence of different concentrations (100%, 75%, 50%, and 25%) of platelet gel releasate (PGR). Statistical analysis was performed using one-way ANOVA.
Results: There was time-dependent increase in cell count in all groups till 14th days. The number of cell was maximum in 100% PGR on 1st (n=15$\pm$4.3) and 3rd (n=17$\pm$6.1) day. 25% PGR showed maximum proliferation on 7th and 14th day. Spectrophotometric ALPase activity assay showed time dependent increase in ALPase activity till 7th day and decrease afterwards. 25%PGR showed maximum ALPase activity on 7th day.
Conclusions: PGR is an effective stimulator of hPDLc. Higher concentrations (100% & 75%) of PGR results in excessive proliferation of cells in the initial stage but lower concentration (25%) showed controlled and gradual proliferation. Moreover, lower concentration of PGR caused differentiation (ALPase activity) of cells in the late stage. Thus, it seemed that PRP with 25% concentration was an ideal.

Citation: Dr. Santosh Kumar Verma, Dr. Madhu Ranjan, Dr. Barun Dev, Dr. Abhik Sen, Dr. Sankalp Verma and Dr. Marilia Marceliano-Alves, 2016. "Assessment of ideal concentrations of platelet rich plasma in regeneration of human periodontal ligament cells", *International Journal of Current Research,* 8, (03), 28920-28927.

INTRODUCTION

Periodontal regeneration requires restitution of the periodontal attachment apparatus i.e., new bone formation, new cementum deposition upon the denuded root surface and insertion of functionally oriented new collagen fibers of the periodontal ligament. Currently, osseous grafting and guided tissue regeneration (GTR) are the two most commonly employed techniques. (Bowers *et al*., 1989; Nyman *et al*., 1982) Other regenerative therapies like root conditioning, specific substances such as growth/differentiation factors or enamel matrix derivatives (Emdogain) have also provided a significant improvement in clinical parameters of treated defects. However, only limited histological evidence of true regeneration has been demonstrated with the majority of these therapies. The results of periodontal regeneration are still not entirely predictable. So, this study is an attempt to find cells and factors considered important for promoting periodontal regeneration. The periodontium has the capacity to regenerate a functional new attachment through the coordinated activity of

Corresponding author: Dr. Sankalp Verma,
Department of oral Medicine and Radiology, Bhabha College of dental sciences, Bhopal, MP, India

cells from the gingiva, alveolar bone, and periodontal ligament. (Melcher, 1972; Isaka *et al*., 2001) Periodontal ligament cells (PDLC) can synthesize the constituents of the periodontal ligament as well as differentiate into cementum and bone-forming cells. (Isaka *et al*., 2001; Lekic *et al*., 2001; Murakami *et al*., 2003) The expression of osteoblast-like properties by PDL cells, such as the production of alkaline phosphatase, has been widely described and suggested to be of pivotal importance for the regeneration of periodontal tissues. (Isaka *et al*., 2001; Murakami *et al*., 2003; Pitaru *et al*., 1994) Applying the principles of tissue engineering, periodontal regenerative capacity of growth factors alone or in combination with suitable allograft/alloplast as scaffold might be assessed by using human periodontal ligament cells (hPDLc). Consequently, over the past two decades, the potential use of growth factors in bone and periodontal regeneration has been investigated and viability has been demonstrated. (Cochran and Wozney, 2000) One way of delivering concentrated amounts of growth factors to the wound site is via the use of platelet-rich plasma (PRP). (Oates *et al*., 1993) PRP is a concentration of platelets in a small volume of plasma. In addition to haemostasis and inflammation, platelets are involved in wound healing and repair of growth factor (PDGF), transforming growth factor (TGF), insulin-like growth factor (IGF), epidermal growth factor (EGF) and many more. Concentrated platelet products can be applied as a solution containing the released growth factors (Platelet Gel Releasate, PGR). (Borzini and Mazzucco, 2005) Enriched platelet preparations have been claimed to promote rapid bone healing and regeneration when added either to injury sites in isolation, or when combined with autologous bone and bone substitute materials. (Oates *et al*., 1993) By concentrating platelets and delivering them locally, PRP provides a higher level of growth factors that stimulate the healing process in the periodontium in a more physiological way. (Anitua, 1999; Kim *et al*., 2002)

Studies investigating the effect of PRP on cell function *in vitro* have utilized a wide variety of cell types and have obtained conflicting results. Initially, it was reported that PRP has a positive effect on bone regeneration in mandibular defect reconstruction. (Marx *et al*., 1998) Kanno *et al*. demonstrated a favorable effect of PRP on human osteoblast-like cells. They also found that PRP acts as an activator in wound healing as well as enhances bone regeneration. (Kanno *et al*., 2005) Soffer *et al*. summarized that short-term human platelet Iysate exposure (up to 24 h) promotes the proliferative and chemotactic bone cell functions while long-term human platelet lysate exposure results in a decrease of both alkaline phosphatase activity and mineral formation. (Soffer *et al*., 2004) Recently, Choi *et al*. in 2005 showed that the viability and proliferation of alveolar bone cells were suppressed by high (30-100%) PRP concentrations, but were stimulated by low PRP concentrations (1–5%). (Choi *et al*., 2005) Till now limited information is available concerning the interaction of platelet concentrates with PDLc, and its role in periodontal regeneration. In particular, it is still uncertain which concentrations of PRP are optimal in promoting wound healing and regeneration. It is assumed that PRP would act in a similar manner to individual growth factors and that preparations containing maximal concentration of growth factors are ideal. However, there is little evidence to support this assumption, which may be flawed on the basis that PRP is a combination of different growth factors, each of which exerts a unique influence on the complex cascade of events that occurs during wound healing and tissue regeneration. With this aim, currently study was planned.

MATERIALS AND METHODS

Platelet rich plasma (PRP) used in this study was prepared and delivered from the Blood Bank, Government Medical College and Hospital, Trivandrum. The study setting was at Rajiv Gandhi Center for Biotechnology Jagathy, Trivandrum. The extracted tooth for this study was collected from the department of Oral Surgery, Dental College, Trivandrum. It was an *in vitro* study of 6 months duration. The study consisted of 200 wells in 12-well culture plates of human periodontal ligament cells (hPDLc). Culture wells were divided into 5 groups and each group had 10 culture wells. Each culture wells was sacrificed for experiment. Readings were taken on 1st, 3rd 7th and 14th days after adding platelet gel releasate (PGR) medium.

The study comprises of five groups with four different concentration of Platelet Gel Releasate (PGR) viz :Group I:100 % PGR; Group II: 75 %PGR; Group III:50 % PGR; Group IV:25 % PGR; Group V:control (without PGR). Inclusion criteria included patients aged 13-20 years. Teeth with no caries and periodontal problems, extracted for orthodontic correction from patients without any systemic diseases were selected. Inclusion criteria for Platelet Rich Plasma was healthy donors without any history of transmissible diseases or systemic diseases An informed consent was taken from the patient who donated their extracted tooth for the study. This study has been approved by Institutional Ethical Committee. Blood was collected from a healthy volunteer after doing proper screening tests for mandatory transmissible diseases (Malaria, HIV, Hepatitis B & C and VDRL-Syphilis). Donor should not have any history of medical disorders and should not be taking certain medications. Whole blood (approximately 350 ml) was collected into the first bag of a triple bag system (HL Haemopack Kerala, India). The bags should be sterile, pyrogen free and non toxic. The blood was drawn by venipuncture in the antecubital fossa. The whole blood was mixed gently and periodically with 49 ml of anticoagulant CPD (citrate-phosphate-dextrose solution) on a hemomixer to prevent clotting of blood. Platelet concentrate (PC) was obtained by means of a gradient density separator (Cryofuge 6000i, Heraus Instruments). Before centrifuging, the ABO group and D antigen was determined using Anti-A, Anti- B and Anti-D reagent. The packs were first centrifuged in a light spin for 10 minutes at 1600 × g. This resulted into fractionization of the whole blood into two basic components: Red blood cells (RBC) at the bottom and Platelet rich plasma (PRP) on the top. Then, the supernatant platelet-rich plasma (PRP) was transferred to one of the integral transfer packs and the RBCs were mixed with 100 ml additive solution (SAGM – Saline Adenine Glucose Mannitol solution) from another transfer bag. The bag containing the PRP was then centrifuged under heavy spin for 7 minutes at 4000×*g* to obtain supernatant platelet-

poor plasma and the pelleted platelets (Platelet Concentrate, PC). The platelet poor plasma was transferred to the third integral bag leaving platelet concentrate, (PC) with approximately 60 ml of plasma at the bottom. All centrifugation and storage steps were carried out at 22°C to preserve the membrane integrity of platelets. Platelet counts were done on whole blood and platelet concentrate using a fully automated analyzer (LabLife DS Supreme) in the ACR laboratory. The Platelet Concentrate thus obtained is a straw colored liquid. This Platelet Concentrate liquid was converted into a gel form by the addition of 10% calcium chloride mixed with bovine thrombin (Sigma Aldrich, St Louis, MO, USA). Platelet gel was produced by mixing 10 ml platelet-rich plasma with 10U thrombin in 0.66 ml 10% Calcium Chloride in two 50 ml tubes and left for 5-10 min to clot. When the solution became gel, it was incubated for 3 hours at room temperature in a rotating shaker. This resulted into degradation of platelets and release of various growth factors. After degradation, platelets clumped together leaving a straw color liquid, known as Platelet Gel Releasate (PGR). PGR was collected into a separate container and this solution was defined as "100% PGR." Experiments were performed with 100% PGR, 75% PGR, 50% PGR and 25% PGR. Each dilution was obtained by supplementing PGR with Dulbecco's modified Eagle's medium (DMEM). Another control was taken which contained DMEM (Gibco New York USA) without PGR.

The extracted teeth were placed immediately in a tube containing Hanks' Balanced Salt Solutions (HBSS, Gibco New York USA) with composition potassium chloride, monobasic potassium phosphate, sodium chloride, dibasic sodium phosphate and D-glucose. The solution was supplemented with antibiotics: Penicillin G potassium (200 µ/l), streptomycin sulfate (200 µg/ml) and Amphotericin B (20 µg /ml) and adjusted to pH 7.4. After extraction, contact of teeth with hand or any surface was avoided to prevent its contamination. The teeth samples were immediately transported to Rajiv Gandhi Center for Biotechnology, Trivandrum. Under sterile conditions, the teeth were washed two times with Phosphate-buffered saline (150 mMNaCI, 20 mM sodium phosphate pH 7.2). The PDL tissue was mechanically removed by scraping the middle third of the root surface with a sharp blade no. 11 following proper guidelines. (Oates *et al*., 1993) The tissue samples were minced into small pieces using a sterile scissors or surgical blade and washed again with serum-free DMEM. The tissue was then digested in serum-free medium containing 2 mg/ml of collagenase at 37°C in a humidified atmosphere of 95% air and 5% CO_2 for 180 minutes. The supernatant was removed and the remaining tissue was placed in six-well culture plate, allowing cells to multiply from the explants at 37°C in a humidified atmosphere of 95% air and 5% CO_2. The dislodged cells in the supernatant were further centrifuged and re-suspended in fresh complete medium and seeded in 6-well plate. The cell cultures were observed periodically under microscope for visible growth and found to be mycoplasma free. Once the growth was achieved, the cells were trypsinized and transferred into 25 cm^2 tissue culture flask containing DMEM supplemented with 10% heat inactivated fetal bovine serum and 1% antibiotic antimycotic solution with penicillin G potassium (100µl), streptomycin sulfate (100 µg/ml) and amphotericin B (10µg/ml) at pH 7.4. Culture flasks were maintained in an incubator with the same culture medium at 37°C in a humidified atmosphere of 95% air and 5% CO_2 for 2-7 days. After incubation, the medium was replaced every alternate day until cell outgrowth created a subsequent monolayer. The cells were detached by trypsin / EDTA treatment and transferred to 25 cm^2 tissue culture flask for continued growth until the required number of cells was reached. Cells between the third and fifth passage were used in the experiments.

After the confluence was reached, cells were passaged using trypsin- EDTA (0.2%) treatment. Cells were collected and seeded in 12 -well plates at a density of 10 $x10^4$ cells/cm^2 in DMEM supplemented with 10% fetal bovine serum. Medium containing Platelet Gel Releasate (PGR) was added 24-48 hours after seeding. The day on which PGR medium and control medium was added, was considered as day 0 or baseline. On the day 0, the cells were divided into five different groups for adding mediums as follows:

I. 100% PGR group, in which the cells were cultured with the 1 ml of Platelet Gel Releasate (PGR)
II. 75% PGR group, in which the cells were cultured with 750 µl of Platelet Gel Releasate (PGR) plus 250 µl of serum-free DMEM
III. 50% PGR group, in which the cells were cultured with 500 µl of Platelet Gel Releasate (PGR) plus 500 µl of serum-free DMEM
IV. 25% PGR group, in which the cells were cultured with 250 µl of Platelet Gel Releasate (PGR) plus 750 µl of serum-free DMEM
V. Control group, in which the cells were cultured with 1 ml of serum-free DMEM

Cell proliferation was measured by direct cell count method wherein the number of viable cells present in each plate was determined in a Neubauers Hemocytometer by counting the number of cells present in the central grid area. Alkaline phosphatase activity was determined by measuring the release of p-nitrophenol from disodium p-nitrophenyl phosphate. ALP activity was assayed by incubating the cell lysates with a solution of p-nitrophenyl phosphate 16 mM, pH 10.2 (ALP reagent) (1:1 ratio). The reaction was carried out at 37°C for 15 minutes, and the production of p-nitrophenol was measured at 405 nm spectrophotometrically (Bio-Rad). The specific activity of alkaline phosphatase enzyme is expressed as Units/mg of protein. One unit is defined as the amount of enzyme that hydrolyses 1 nmol of p-nitrophenyl phosphate/min under the specified conditions.

RESULTS

The study consisted of 200 samples which were randomly assigned to five groups (4 test groups and 1 control). After seeding and incubation of periodontal ligament cells, parameters were evaluated during the 1st, 3^{rd}, $7^{rh}$and 14^{th} day after seeding of cells. The two parameters, cell counting and alkaline phosphatase activity of periodontal ligament cells were evaluated during the 1^{st}, 3^{rd}, $7^{rh}$and 14^{th} day after adding their respective medium. The day on which the medium (Platelet Gel Releasate and Dulbecco's modified Eagle's medium) was added was considered as day 0 (zero) or

baseline. Results of this study were subjected for statistical analysis using one way ANOVA test.

Platelet rich plasma

In this study platelet counting were done on whole blood collected from the donors and the platelet concentrates. The values of platelet count on whole blood were 2,79,000 / µl and 3,24,000 /µl. The platelet counts in the platelet concentrates after centrifuge were 15,09,000 /µl and 13,95,000 respectively. So the percentage increase in platelet count over the normal venous blood count was 540% and 430% respectively.

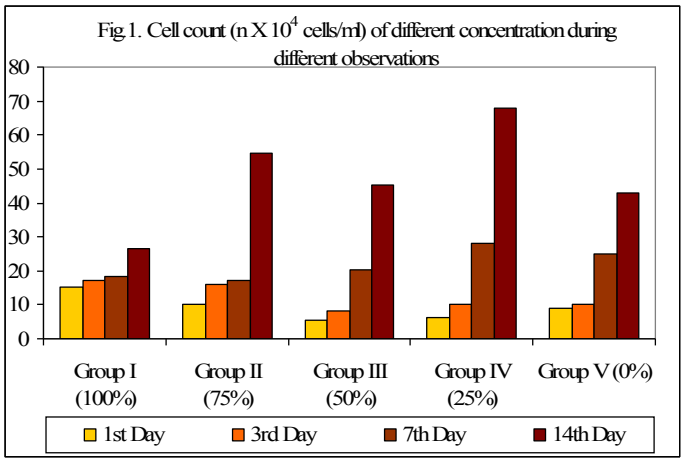
Cell counting

Cells in each culture well were trypsinized and collected. Cell proliferation was measured by direct cell count method using a NeubauersHemocytometer by counting the number of cells present in the central grid area **(Table 1)**

Table 1. Analysis of variance (One Way ANOVA) of cell count (n X 10^4 cells/ml) comparing different groups in different observations

Observation	Group	Mean	± SD	F value	P value
1st Day	Group I (100% PGR)	15.22^{c}	4.30	16.034	< 0.001
	Group II (75% PGR)	10.30^{b}	1.63		
	Group III (50% PGR)	5.58^{a}	2.42		
	Group IV (25% PGR)	6.15^{a}	1.51		
	Group V (0% PGR)	9.11^{ab}	1.56		
3rd Day	Group I (100% PGR)	17.20^{b}	6.10	10.556	< 0.001
	Group II (75% PGR)	16.05^{b}	1.88		
	Group III (50% PGR)	8.10^{a}	1.01		
	Group IV (25% PGR)	10.11^{a}	3.19		
	Group V (0% PGR)	9.97^{a}	1.84		
7th Day	Group I (100% PGR)	18.45^{a}	2.18	15.403	< 0.001
	Group II (75% PGR)	17.20^{a}	4.16		
	Group III (50% PGR)	20.28^{ab}	2.76		
	Group IV (25% PGR)	28.18^{c}	0.84		
	Group V (0% PGR)	24.78^{b}	6.74		
14th Day	Group I (100% PGR)	26.70^{a}	3.01	54.421	< 0.001
	Group II (75% PGR)	54.70^{c}	5.35		
	Group III (50% PGR)	45.15^{b}	3.82		
	Group IV (25% PGR)	67.95^{d}	5.98		
	Group V (0% PGR)	42.78^{b}	6.34		

a, b, c, d – Means with same super script do not differ each other (Duncan's Multiple Range Test)



The number of cells of the sample was derived as follows**:** C (in cells/mL) = n x 10^4 Comparing the cell count among test groups and control at different intervals (1st, 3rd, 7th and 14th day) demonstrate highly significant increase in number of cells in each concentrations (**Table 3).**The mean number of cells in group I (100%PGR) at the interval of 1st, 3rd, 7th and 14th day was 15.22 ±4.30, 17.20 ±6.10, 18.45 ±2.18 and 26.70 ±3.01 respectively. There was approximately two times increase in number of cells from 1st day to 14th day.

The mean number of cells in group II (75%) at the interval of 1st, 3rd, 7th and 14th days was 10.30 ±1.63, 16.05 ±1.88, 17.20±4.16, and 54.70 ±5.35 respectively. In group II (75% PGR) the increase in cell count was highly significant. Though the proliferation was faster on day 1 similar to group I but it became slower after 3rd day. In the final day (14th) the number of cells increased more than three times than that of 7th day.

GroupIII (50%)

The mean number of cells in group III (50%) at the interval of 1st, 3rd, 7th and 14th days were 5.58±2.42, 8.10±1.01, 20.28 ±2.76 and 45.15±3.82 respectively. There was highly significant increase in number of cells.

Group IV (25%)

The mean number of cells in group IV (25%) at the interval of 1st, 3rd, 7th and 14th days was 6.15±1.51, 10.11±3.19, 28.18±0.84 and 67.95±5.98 respectively.

Group V (Control)

The mean number of cells in Group V (Control)at the interval of 1st , 3rd , 7th and 14th days was 9.11 ± 1.56, 9.97 ± 1.84, 24.78 ± 6.74and 42.78 ± 6.34 respectively. There was highly significant increase in number of cells.

Regarding Group III (50% PGR) and group IV (25% PGR), a reverse trend was noticed compared to group I i.e., cell proliferation was slower in the initial stage (day 1 & 3) but became faster later (day 7 & 14). In these groups analysis of variance gave highly significant results. There were approximate two fold increase in the number of cells in Group III (50% PGR) on days 7 and 14th. In group IV (25% PGR) the increment in cell count was 2.8 times on 7th day and 2.4 times on 14th day compared to previous counting. Here the maximum count of cells was shown by group IV (25% PGR) compared to all other groups and in all days. In case of control it also showed a similar result as group III and IV i e., slower growth in the initial stage but faster proliferation in the late stage. **(Table 4)**

Alkaline phosphatase activity

Table 2 represent the alkaline phosphatase activity comparing different groups at different days of observations.

The results showed highly significant difference in alkaline phosphatase activity among different groups on days 1 and 14. At the same time it is significant on days 3 and 7.

Table 2. Analysis of variance (One Way ANOVA) of alkaline phosphatase comparing different groups in different observations

Observation	Group	Mean	± SD	F value	P value
1st Day	Group I (100% PGR)	5.012[b]	0.010	10.532	< 0.001
	Group II (75% PGR)	5.932[bc]	0.014		
	Group III (50% PGR)	3.826[a]	0.012		
	Group IV (25% PGR)	4.261[a]	0.006		
	Group V (0% PGR)	4.226[a]	0.027		
3rd Day	Group I (100% PGR)	6.901[bc]	0.074	2.036	< 0.05
	Group II (75% PGR)	7.921[c]	0.058		
	Group III (50% PGR)	5.508[b]	0.038		
	Group IV (25% PGR)	6.248[b]	0.059		
	Group V (0% PGR)	4.708[a]	0.035		
7th Day	Group I (100% PGR)	9.268[b]	0.057	2.191	< 0.05
	Group II (75% PGR)	8.348[a]	0.034		
	Group III (50% PGR)	9.568[b]	0.032		
	Group IV (25% PGR)	13.752[c]	0.277		
	Group V (0% PGR)	8.640[b]	0.074		
14th Day	Group I (100% PGR)	4.152[a]	0.002	11.806	< 0.001
	Group II (75% PGR)	5.948[ab]	0.028		
	Group III (50% PGR)	9.288[b]	0.037		
	Group IV (25% PGR)	12.488[c]	0.123		
	Group V (0% PGR)	7.101[b]	0.045		

a, b, c – Means with same super script do not differ each other (Duncan's Multiple Range Test)

Table 3. Analysis of variance (One Way ANOVA) of cell count (n X 10^4 cells/ml) comparing different observations in different groups

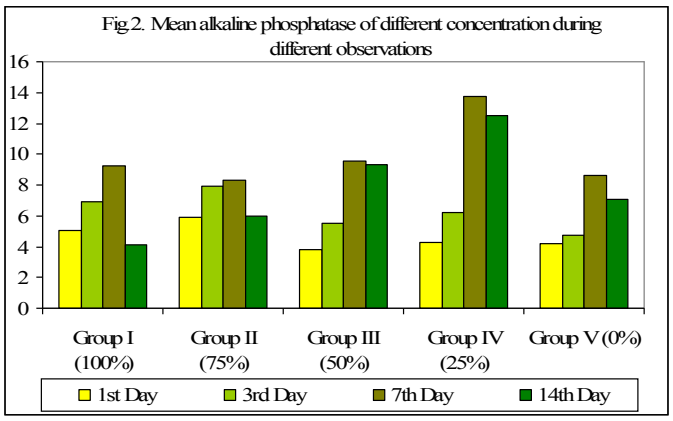
Group	Observation	Mean	± SD	F value	P value
Group I (100% PGR)	1st Day	15.22[a]	4.30	11.049	< 0.001
	3rd Day	17.20[a]	6.10		
	7th Day	18.45[ab]	2.18		
	14th Day	26.70[c]	3.01		
Group II (75% PGR)	1st Day	10.30[a]	1.63	189.965	< 0.001
	3rd Day	16.05[b]	1.88		
	7th Day	17.20[b]	4.16		
	14th Day	54.70[c]	5.35		
Group III (50% PGR)	1st Day	5.58[a]	2.42	277.306	< 0.001
	3rd Day	8.10[ab]	1.01		
	7th Day	20.28[c]	2.76		
	14th Day	45.15[d]	3.82		
Group IV (25% PGR)	1st Day	6.15[a]	1.51	448.903	< 0.001
	3rd Day	10.11[b]	3.19		
	7th Day	28.18[c]	0.84		
	14th Day	67.95[d]	5.98		
Group V (0% PGR)	1st Day	9.11[a]	1.56	65.708	< 0.001
	3rd Day	9.97[a]	1.84		
	7th Day	24.78[b]	6.74		
	14th Day	42.78[c]	6.34		

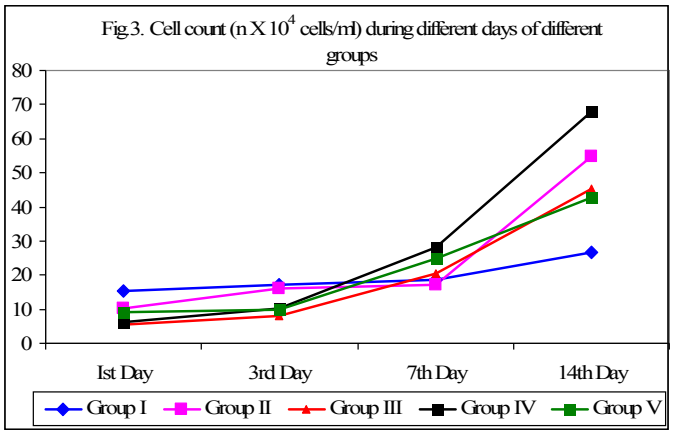
a, b, c, d – Means with same super script do not differ each other (Duncan's Multiple Range Test)

Table 4. Analysis of variance (One Way ANOVA) of alkaline phosphatase comparing different observations in different groups

Group	Observation	Mean	± SD	F value	P value
Group I (100% PGR)	1st Day	5.012[b]	0.010	8.771	< 0.001
	3rd Day	6.901[bc]	0.074		
	7th Day	9.268[c]	0.057		
	14th Day	4.152[a]	0.002		
Group II (75% PGR)	1st Day	5.932[a]	0.014	4.481	< 0.05
	3rd Day	7.920[b]	0.058		
	7th Day	8.348[b]	0.034		
	14th Day	5.948[a]	0.028		
Group III (50% PGR)	1st Day	3.827[a]	0.012	30.023	< 0.001
	3rd Day	5.508[ab]	0.038		
	7th Day	9.568[c]	0.032		
	14th Day	9.288[c]	0.037		
Group IV (25% PGR)	1st Day	4.260[a]	0.006	3.384	< 0.05
	3rd Day	6.248[b]	0.059		
	7th Day	13.752[c]	0.277		
	14th Day	12.488[c]	0.123		
Group V (0% PGR)	1st Day	4.220[a]	0.027	40.221	< 0.001
	3rd Day	4.708[ab]	0.035		
	7th Day	8.640[c]	0.074		
	14th Day	7.101[b]	0.045		

a, b, c – Means with same super script do not differ each other (Duncan's Multiple Range Test)





Day 1

The mean alkaline phosphatase activity in group I (100%), group II (75%), group III (50%) and group IV (25%) at day 1 was 5.012 ±0.010, 5.932 ±0.014, 3.826±0.012 and 4.261±0.006 respectively. The mean number of cells in group V (control) was 4.226± 0.027.

Day 3

The mean alkaline phosphatase activity in group I (100%), group II (75%), group III (50%) and group IV (25%) at day 3 was 6.901±0.074, 7.921 ± 0.058, 5.508± 0.038 and 6.248 ± 0.059 respectively. The mean number of cells in group V (control) was 4.708 ± 0.035.

Day 7

The mean alkaline phosphatase activity in group I (100%), group II (75%), group III (50%) and group IV (25%) at day 7 was 9.268 ±0.057, 8.348 ± 0.034, 9.568 ± 0.032 and 13.752 ± 0.277 respectively. The mean number of cells in group V (control) was 8.640 ± 0.074.

Day 14

The mean alkaline phosphatase activity in group I (100%), group II (75%), group III (50%) and group IV (25%) at day 14 was 4.152±0.002, 5.948 ±0.028, 9.288 ± 0.037 and 12.488 ± 0.123 respectively. The mean number of cells in group V (control) was 7.101 ± 0.045.

DISCUSSION

Although the biological rationale for the use of PRP is theoretically sound, the precise methodology that will result in the optimal benefit is yet to be elucidated. Thus, this study was an attempt to find out optimum concentration of PRP for periodontal regeneration. Platelet concentration and growth factor levels in PRP preparation are important aspects to be considered in evaluation of the validity of a method as variations may lead to inconsistent clinical results. In this study, platelet counting was done on whole blood collected. The percentage increase in platelet count over the normal venous blood count was 540% and 430% respectively. This is in agreement with studies done by Marx. (Marx, 2004) In this study, two parameters were observed i.e., cell count and alkaline phosphatase (ALPase) activity of cells. Cell count represents the proliferation of cells which is a critical event during early wound healing. Alkaline phosphatase activity is a representative marker of differentiation of cells. In both histochemical and biochemical studies, PDL has been shown to have intense ALP activity. (Somerman *et al*., 1988) In this study periodontal ligament cells were cultured and utilized for experiment because PDL contains cells which can form a new connective tissue attachmentand differentiate into osteoblasts and/or cementoblasts. (Nojima *et al*., 1990) The finding of this study indicated that platelet Gel Releasate (PGR) stimulates human periodontal ligament cells (hPDLc) proliferation, which is consistent with findings of previous studies. (Okuda *et al*., 2003; Graziani *et al*., 2006; Kawase *et al*., 2003; Han *et al*., 2007)

Results from the present study showed that the number of cells was maximum in group I (100% PGR) on 1st (n = 15 ±4.3) and 3rd day (n = 17± 6.1) compared to all other groups. On 7th and 14th day, the count of cells in group I (100% PGR) became less compared to the other groups (except 75% PGR on 7th day). This showed that the proliferation of cells was faster in group I in the initial stage (1st& 3rd day) but later (7th& 14th day) it became slower compared to other groups. This is because the high concentration of PGR had more effect on proliferation of cells in early stage as it contained maximum amount of growth factors but as the time passed growth factor concentration decreased. So the proliferation rate declined. The results showed that the proliferation was more with higher (100% and 75%) PGR concentrations in the initial stage (1st and 3rd day), but in the late stage (7th& 14th day) the higher (100% and 75%) PGR concentrations did not result in increasing proliferation compared to other groups. This is similar to the findings of other studies. (Soffer *et al*., 2004; Lucarelli *et al*., 2003; Gruber *et al*., 2002) They found a dose dependent effect of PRP on cell proliferation. The reason for decrease in proliferation in the late stage could be the over-confluence of cells due to excessive proliferation of cells under the higher concentration of growth factors in 100% and 75% PGR. Another reason may be the differentiation of cells into other types of cells. The differentiation of cells into other functional cells is at the cost of their ability to proliferate.

Increasing the platelet Gel Releasate (PGR) concentration did not result in increasing cell proliferation. Optimal results were obtained by final concentration of 25% PGR in the late phase

of healing. This is little different from the study by Graziani *et al* where the optimum result was obtained with 16.5% (but the study was conducted on oral gingival fibroblasts). (Graziani *et al*., 2006) This study has found the high level of cell proliferation was obtained by diluting the maximally concentrated PGR with serum free DMEM, giving a final concentration of 25% PGR. Proliferation stimulated by 25% PGR was at a slower rate than with 100% PGR on 1st and 3rd day but became faster on 7th day and 14th day. An excessive mitogenic effect could result in out of control cell proliferation and the loss of maturation features characteristic of the cell lineage. This correlates with the study of Liu *et al*. (2002). Aside from proliferation, this study also examined osteogenic differentiation of human periodontal ligament cells (hPDLc) following exposure to different PRP concentrations, by assessing alkaline phosphatase activity (ALPase). Interestingly, reports of the effect of PRP on hPDLc on ALP activity are contradictory, perhaps as a result of different methods or cell models used; Some studies indicating stimulation (Graziani *et al*., 2006; Kawase *et al*., 2003) and others showing inhibition (Gruber *et al*., 2002; Arpornmaeklong *et al*., 2004) This study found that PRP stimulate osteoblastic differentiation of human periodontal ligament cells. These findings correlate with the previousstudies. (Graziani *et al*., 2006; Weibrich *et al*., 2003) They found that the use of platelet concentrate had a positive effect on bone regeneration only within an 'intermediate' concentration range. Indeed, the use of highly concentrated platelet preparations (6–11 times the normal platelet count) appeared to have an inhibitory effect on healing. In Graziani'sstudy, PRP-max (33%) stimulated osteoclastogenesis and osteoblast differentiation. (Gruber *et al*., 2002) Results showed that in all groups, there is time dependent increase in the alkaline phosphatase activity on 3rd and 7th day as foundbut ALPase activity decrease on 14th day. Similar findings were noted in another study. (Kawase *et al*., 2003) The reason could be that till 7th day cells had differentiated enough to show higher ALP activity. After 7th day, aging of cells started due to over confluence of cells and differentiation of cells stopped. It is also possible that short-term PGR (up to 7 days) promoted the differentiation while long-term PGR exposure resulted in a decrease of differentiation so as alkaline phosphatase activity. This is in agreement with the study of Soffer *et al*. 2004. The images of the periodontal ligament cells taken under phase contrast microscope (Nikon) showed that the morphological characteristic of cells was similar to fibroblasts. In the initial stage images showed few fibroblasts like cells but later on the surface was completely filled with cells showing the proliferative potential of hPDLc. The microscopic images also suggest that healthy morphologic feature is more evident in the cells cultured with PGR than control.

Summary

The present study establishes the 25% PRP concentration as ideal that would be promote maximum proliferation and differentiation of periodontal ligament cells.

Conclusion

The following conclusions were drawn from the study:

- PGR is an effective stimulator of hPDLC proliferation as there was time dependent increase in number of cells.
- Different concentrations of PGR exerted different effects on human periodontal ligament cells.
- PGR stimulated proliferation of cells in a dose-dependent manner in the beginning stage and not in the late phase.
- PGR can increase alkaline phosphatase activity (ALP) as well as stimulate osteoblastic differentiation of hPDLC to a certain extent.
- ALPase activity was maximal in 100% PGR group in the beginning but 25% PGR group showed maximum ALPase activity in the late stage.
- The ideal concentration of PRP is that which provided the most optimal environment for periodontal regeneration through a balance between proliferation and differentiation. In periodontal healing proliferation of cells is required in the initial stage and later cells should differentiate in other functional cell types for complete regeneration of lost tissues. In this study the optimum concentration is 25% PGR as it provides adequate cell proliferation in the initial stage and valuable differentiation later.
- However, multicentre clinical studies and histological evidence on *in vivo* models are required to support this study.

REFERENCES

Anitua E. Plasma rich in growth factors: preliminary results of use in the preparation of future sites for implants. *International Journal of Oral & Maxillofacial Implants,* 1999;14: 529–535.

Arpornmaeklong P, Kochel M, Depprich R, Kubler NR, Wurzler KK. Influence of platelet-rich plasma (PRP) on osteogenic differentiation of rat bone marrow stromal cells. An in vitro study. *Int. J. Oral Maxillofac. Surg*., 2004;33: 60–70

Borzini and Mazzucco. Platelet gels and releasates *Current Opinion in Hematology*, 2005;12:473—479

Bowers GM, Chadroff B, Carnevale R *et al*. Histologic evaluation of new attachment apparatus formation in humans. Part I, II & III. *J Periodontol*., 1989; 60:664- 693.

Choi BH, SJ Zhu, BY Kim, JY Huh, SH Lee, JH Jung. Effect of platelet rich plasma (PRP) concentration on the viability and proliferation of alveolar bone cells: an in vitro study. *Int. J. Oral Maxillofac. Surg*., 2005; 34: 420–424.

Cochran DL, Wozney JM. Biological mediators for periodontal regeneration. *Periodontol*., 2000;19:40–58.

Graziani F, Ivanovski S, Cei S, Ducci F, Tonetti M, Gabriele M . The in vitro effect of different PRP concentrations on osteoblasts and fibroblasts. *Clin. Oral Implants Res*., 2006;17, 212–219.

Gruber R, Varga F, Fischer MB, Watzek G. Platelets stimulate proliferation of bone cells: involvement of platelet derived growth factor, microparticles and membranes. *Clin. Oral Implants Res*., 2002;13: 529–535.

Han J, Meng HX, Tang JM, Li SL, Tang Y, Chen ZB. The effect of different platelet-rich plasma concentrations on proliferation and differentiation of human periodontal ligament cells in vitro. *CellProlif*,.2007; 40(2):241-52.

IsakaJ, Ohazama A, Kobayashi M *et al*. Participation of periodontal ligament cells with regeneration of alveolar bone. *J Periodontol*., 2001;72:314–323

Kanno T, Takahashi T, Tsujisawa T, Ariyoshi W, Nishihara T. Platelet-rich plasma enhances human osteoblastlike cell proliferation and differentiation. *Int. J. Oral Maxillofac. Surg*., 2005;63, 362–369.

Kawase T, Okuda K, Wolff LF, Yoshie H. Platelet-rich plasma-derived fibrin clot formation stimulates collagen synthesis in periodontal ligament and osteoblastic cells in vitro. *J Periodontol*., 2003; 74(6):858-64.

Kim SG, Chung CH, Kim YK, Park JC, Lim SC. Use of particulate dentin-plaster of Paris combination with/without platelet-rich plasma in the treatment of bone defects around implants. *Int. J. Oral Maxillofac. Imp*., 2002;17, 86–94.

Lekic PC, Rajshankar D, Chen H, Tenenbaum H, McCulloch CA . Transplantation of labelled periodontal ligament cells promotes regeneration of alveolar bone. *Anat. Rec*., 2001;262, 193–202.

Liu Y, Kalen A, Risto O, Wahlstrom O. Fibroblast proliferation due to exposure to a platelet concentrate in vitro is pH dependent. *Wound Repair Regen*., 2002;10:336–340.

Lucarelli E, Beccheroni A, Donati D, Sangiorgi L, Cenacchi A, Del Vento AM, Meotti C, Bertoja AZ, Giardino R, Fornasari PM, Mercuri M, Picci P. Platelet-derived growth factors enhance proliferation of human stromal stem cells. *Biomaterials*, 2003;24:3095–3100.

Marx R.E. Platelet-rich plasma: evidence to support its use. *International Journal of Oral and Maxillofacial Surgery*, 2004;62: 489–496.

Marx RE, Carlson ER, Eichstaedt RM, Schimmele SR, Strauss JE, Georgeff KR. Platelet-rich plasma: Growth factor enhancement for bone grafts. *Oral Surg Oral Med Oral Pathol Oral RadiolEndod*., 1998; 85(6):638-46

Melcher AH. On the repair potential of periodontal tissues. *J Periodontol*., 1972; 47:256-260.

Murakami Y, Kojima T, Nagasawa T, Kobayashi H, Ishikawa I. Novel isolation of alkaline phosphatase-positive subpopulation from periodontal ligament fibroblasts. *J Periodontol*., 2003;74:780–786.

Nojima N, Kobayashi M, Shionome M, Takahashi N, Suda T, Hasegawa K. Fibroblastic cells derived from bovine periodontal ligaments have the phenotypes of osteoblasts. *J Periodontal Res*.,1990; 25:179-185.

Nyman S, Lindhe J, Karring T, Rylander H. New attachment following surgical treatment of human periodontal disease. *J ClinPeriodontol*., 1982; 9:290-296.

Oates TW, Rouse CA, Cochran DL. Mitogenic effects of growth factors on human periodontal ligament cells in vitro. *J Periodontol*., 1993; 64: 142-148

Okuda K, Kawase T, Momose M, Murata M, Saito Y, Suzuki H, Wolff LF, Yoshie H. Platelet-rich plasma contains high levels of platelet-derived growth factor and transforming growth factor-beta and modulates the proliferation of periodontally related cells in vitro. *J Periodontol*., 2003; 74(6):849-57.

Pitaru S, Pritzki A, Bar-Kana I, Grosskopf A, Savion N, Narayanan AS. Bone morphogenetic protein 2 induces the expression of cementum attachment protein in human periodontal ligament clones. *J Periodontal Res*., 1994;29: 81–94.

Soffer E, Ouhayoun JP, Dosquet C, Meunier A, AnagnostouF . Effects of platelet lysates on select bone cell functions. *Clin. Oral Implants Res*., 2004;15, 581–588.

Somerman MJ, Archer SY, Imm GR, Foster RA. A comparative study of human periodontal ligament cells and gingival fibroblasts in vitro. *J Dent Res*., 1988;67:66-70.

Weibrich G, KleisWilfried KG, Buch R, Hitzler WE, Hafner G. The Harvest Smart PRePTM system versus the Friadent-Schütze platelet-rich plasma kit: Comparison of a semiautomatic method with a more complex method for the preparation of platelet concentrates. *Clin. Oral Implants Res*., 2003;14: 233–239.

****