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The effect of platelet-rich plasma on osteoblast and periodontal ligament cell migration, proliferation and differentiation

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Background and Objective: Platelet-rich plasma is used to deliver growth factors, in a safe and convenient manner, for enhancing bone and periodontal regeneration. However, conflicting reports regarding its effectiveness suggest that further study of the relevant cellular mechanisms is required. The aim of this study was to investigate the *in vitro* effect of platelet-rich plasma on osteoblasts and periodontal ligament cell function.

Material and Methods: Various concentrations of platelet-rich plasma (100, 50, 20 and 10%) and platelet-poor plasma, obtained from human donors, were applied to primary cultures of human osteoblasts and periodontal ligament cells. [^{3}H]-Thymidine incorporation, crystal violet staining and MTT assays were utilized to assess DNA synthesis and proliferation. Migration was determined by assessing the cell response to a concentration gradient, while differentiation was assessed using Alazarin Red staining.

Results: Platelet-rich plasma and platelet-poor plasma had stimulatory effects on the migration of both human osteoblasts and periodontal ligament cells. At 24 h, DNA synthesis was suppressed by the application of the various concentrations of platelet-rich plasma, but over a 5-d period, a beneficial effect on proliferation was observed, especially in response to 50% platelet-rich plasma. Platelet-poor plasma resulted in the greatest enhancement of cellular proliferation for both cell types. At a concentration of 50%, platelet-rich plasma and platelet-poor plasma facilitated differentiation of both cell types.

Conclusion: Platelet-rich plasma can exert a positive effect on osteoblast and periodontal ligament cell function, but this effect is concentration specific with maximal concentrations not necessarily resulting in optimal outcomes. Platelet-poor plasma also appears to have the ability to promote wound healing-associated cell function.

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During the early stages of wound healing, platelet-released growth factors, including platelet-derived growth factor, insulin-like growth factor-1 and transforming growth factor-β, initiate a cascade of cellular and molecular events that result in wound healing in a highly regulated and coordinated manner (1–3). The understanding that

platelet-derived growth factors play an important role in wound healing has led to the development of recombinant growth factors aimed at influencing and enhancing repair and regeneration. The application of these growth factors to bone and periodontal regeneration has been investigated using *in vitro* and *in vivo* models with promising results obtained (4–9) that have provided the basis for subsequent human clinical studies (10,11).

In addition to the use of recombinant growth factors, concentrated formulations of platelets, known as platelet-rich plasma, have also been investigated as a potential source of autologous growth factors. Platelet-rich plasma is a volume of autologous plasma that has a platelet concentration approximately three to four times higher than baseline levels (12). Hence, platelet-rich plasma can be considered to contain higher ratios of all the platelet-derived growth factors and plasma components of an individual patient, including mediators of cell function during wound healing, such as platelet derived growth factor, transforming growth factor-β, insulin-like growth factor-1, epidermal growth factor, transforming growth factor-α, vascular endothelial growth factor, fibrin, fibronectin and vitronectin (13,14).

Whitman *et al*. first described the use of platelet-rich plasma in the dental setting and showed that platelet-rich plasma application to the underlying tissues allowed more predictable flap adaptation and hemostasis and ensured a more definitive seal than primary closure alone (15). Subsequently, there has been considerable interest in examining this method of promoting wound healing and regeneration. In particular, it has been proposed that platelet-rich plasma may be utilized alone or in conjunction with various graft materials to deliver growth factors to the wound site, especially in order to enhance bone and/or periodontal regeneration.

Many investigations have been conducted on the clinical effect of platelet-rich plasma on bone regeneration [for review see (3)] and periodontal regeneration (16–18), in addition to several *in vitro* studies (19–26) aimed at establishing the biological rationale for this treatment. However, there is a lack of consensus regarding the effectiveness of platelet-rich plasma, with early promising clinical reports (16,27–29) being tempered by subsequent negative findings (30–32). Hence, the aim of this study was to investigate the *in vitro* effect of platelet-rich plasma on the cells that are critical for periodontal and bone regeneration, namely periodontal ligament cells and osteoblasts, in terms of the key cellular functions associated with wound healing and regeneration, namely migration, proliferation and differentiation.

Material and methods

Cell culture

During surgical extraction of third molars, teeth and bone chips were collected in explant media comprising Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 units/mL of penicillin, 100 μg/mL of streptomycin, 2.5 mg/mL of fungizone and nonessential amino acids. The study was approved by the human ethics committee of the University of Queensland and informed consent was obtained prior to the collection of the samples.

Periodontal ligament cells and osteoblasts were obtained as previously described (33,34). Briefly, the bone chips and periodontal ligament fragments obtained from the middle third of the root surface were minced into smaller tissue portions, transferred to 25-cm^2 tissue culture flasks (Corning Inc., Corning, NY, USA) and incubated in explant media at 37°C and a humidified atmosphere containing 5% CO_2. One week following establishment of the explants, the explant medium was changed to standard medium comprising Dulbecco's modified Eagle's medium, 10% fetal calf serum, 50 units/mL of penicillin, 50 μg/mL of streptomycin and nonessential amino acids.

Following cell growth from the explants, the cells were detached from the plate using 0.2% trypsin and 0.02% EDTA (Sigma Chemical Co., St Louis, MO, USA), and subsequently propagated by passaging in a 1:3 split until sufficient numbers were obtained to carry out the required experiments. Cells from passages three to five were used in this study.

Each experiment described in this study was carried out using three individual primary osteoblast and periodontal ligament cell lines from different donors. Each combination of cell line/platelet-rich plasma preparation was repeated in triplicate.

Platelet-rich plasma preparation

Platelet-rich plasma was prepared from blood drawn from healthy patients and applied to the cells within 30 min. Each experiment utilized platelet-rich plasma from three donors (two allogenic and one autogenous). Whole venous blood was collected in lithium heparin-coated collection tubes (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and initially centrifuged at 350 ***g*** for 10 min to separate the red blood cell portion from the platelet-rich and platelet-poor plasma. The upper layer of the red blood cell portion was included as the platelets containing the largest amount of growth factors, and hence having the greatest potential biological activity, are larger and mix with the upper 1 mm of the red blood cells. The inclusion of this small red blood cell layer imparted a red tinge to the platelet-rich plasma (28,35). The platelet-rich plasma and platelet-poor plasma portions were then extracted and centrifuged again at 1000 ***g*** for 10 min to separate the platelet-rich plasma from the platelet-poor plasma.

Dilutions of the platelet-rich plasma and platelet-poor plasma for the various experiments were obtained by mixing with standard serum-free medium (Dulbecco's modified Eagle's medium, nonessential amino acids and penicillin/streptomycin). Both autogenous and allogenic platelet-rich plasma/platelet-poor plasma preparations were used, with no differences on cell functions being observed (data not shown), and hence the results were combined.

Migration

Using Transwell Permeable Supports (Corning Inc.) (26,36) of 6.5 mm diameter and 8 μm pore size, migration from one side of a membrane to the other was examined after 6 h in response to five treatments: 100% platelet-rich plasma; 50% platelet-rich plasma; 10% platelet-rich plasma; 50% platelet-poor plasma; and serum-free medium. Three-hundred microlitres of treatment medium was placed in each well of a 24-well plate and allowed to incubate for 30 min. Care was taken to ensure that the treatment medium was settled at the bottom of the well, and then serum-free medium was gently applied on top until the layer just contacted the underside of the Transwell Permeable supports. Cells were seeded at a concentration of 2×10^3 cells/μL onto the outer surface of the Transwell Permeable supports and were incubated for 6 h.

At the end of the incubation period, the Transwell Permeable supports were removed and washed with phosphate-buffered saline solution, and the outer surface was carefully wiped dry with a flattened cotton bud and a microbrush to remove nonmigrated cells. The Transwell Permeable supports were then placed in a fresh 24-well plate containing 300 μL of crystal violet, incubated for 15 min and then removed, washed by flooding with tap water until free dye was no longer visible and allowed to air dry. This stain was then solubilized and extracted with 33% glacial acetic acid and the absorbance was read in a spectrophotometer at 570 nm. The absorbance reading is directly proportional to the number of cells that migrated from the outer to the inner surface of the Transwell Permeable supports, in response to the various treatments placed in the 24-well plates.

Proliferation

Proliferation of the periodontal ligament cells and osteoblasts was assessed using three methods, which were based on different outcome measures. The treatments utilized were 0% fetal calf serum (negative control), 10% fetal calf serum (positive control), 50% platelet-rich plasma, 20% platelet-rich plasma, 10% platelet-rich plasma and 50% platelet-poor plasma. All treatments were diluted with serum-free medium. A concentration of 100% platelet-rich plasma was not utilized as it resulted in total loss of cell viability (data not shown).

[^{3}H]-Thymidine incorporation (DNA synthesis) assay — The [^{3}H]-thymidine incorporation assay is based on the incorporation of radiolabelled [^{3}H]-thymidine into the replicating DNA strands during mitosis and therefore measures DNA synthesis. As previously described (37), the cells were seeded at a concentration of 2×10^4 cells per well in 24-well (Nunclon, Denmark) plates in standard cell culture medium. These cells were allowed to attach overnight. Then, the cells were incubated for 48 h in medium containing 0% fetal calf serum in order to synchronize the cells in the G0 phase of the cell cycle. At the end of this incubation period, the treatment medium was added and the cells were incubated for a further 24 h. For the last 4 h of this incubation period, the cells were pulse-labelled with 10 μCi/well of [^{3}H]-thymidine. Cells were then lysed with 1.5% sodium dodecyl sulfate for 15 min, after which they were combined with scintillation fluid and the radioactivity measured using a liquid scintillation counter (Beckman Instruments, Fullerton, CA, USA).

Crystal violet (colorimetric) assay — The crystal violet colorimetric assay directly measures cell numbers that are present. As previously described (37), cells were seeded into 96-well plates at a concentration of 2×10^3 cells per well and allowed to attach overnight in standard medium. This medium was then removed and the treatment media were added followed by incubation for 5 d. At the end of this incubation the media were removed, the wells were washed with phosphate-buffered saline and the cells stained with crystal violet for 30 min. Following the removal of excess stain, solubilization of the bound crystal violet was carried out with 33% glacial acetic acid solution and the absorbance was measured at 570 nm using a spectrophotometer. The relative spectrophotometry readings are directly proportional to cell numbers.

MTT proliferation (cell viability) assay — The MTT™ (Roche Diagnostics GmbH, Mannheim, Germany) proliferation assay was also used to assess the proliferative potential of platelet-rich plasma (26). This is a colorimetric assay where the amount of colour produced is directly proportional to the number of viable cells. Cells were seeded into 96-well plates at a concentration of 2×10^3 cells per well and allowed to attach overnight in standard medium containing 10% fetal calf serum. Treatment medium was added and the cells were incubated for a further 5 d. The MTT labelling reagent was then added to each well and the cells were incubated for a further 4 h, after which they were washed with phosphate-buffered saline and solubilization solution added. The plates were then incubated overnight and subsequently read at 570 nm using a spectrophotometer.

Differentiation

Differentiation of the periodontal ligament cells and osteoblasts was assessed by quantification of Alazarin red staining (38). Cells were plated in six-well plates at a concentration of 1×10^5 cells per well and incubated with Dulbecco's modified Eagle's medium containing 10% fetal calf serum (standard medium) and allowed to attach overnight. Subsequently, treatment media of 50% platelet-rich plasma, 20% platelet-rich plasma, 50% platelet-poor plasma, mineralization medium (consisting of standard medium with the addition of 50 μg/mL of ascorbic acid, 10 mM glycerophosphate and 10^{-8} M dexamethasone), standard medium and medium containing 0% fetal calf serum were added and the cells incubated for 5 d. At the end of this incubation period, the media were removed and the cells were washed and fixed with 95% ethanol for 15 min at 4°C. The cells were then stained with 2% Alizarin Red S (pH 4.1–4.3) for 15 min. Calcium forms an alizarin red

S–calcium complex in a chelation process, and red staining is evident in the well. This stain was then solubilized with 300 μL of 33% glacial acetic acid solution and the absorbance was measured at 415 nm using a spectrophotometer.

Statistical analysis

For the purposes of the statistical analysis, all repeated experiments were treated as individual events. One-way analysis of variance was used to assess whether there was a statistically significant effect of the different treatments on cell function. In order to identify statistically significant differences between the various treatments, post-hoc analysis was carried out using SPSS software (SPSS Inc., Chicago, IL, USA) and the Bonferroni correction for multiple comparisons. Statistical differences between groups were accepted for *p*-values lower than 0.05.

Results

Migration

The results of the migration assay indicated that platelet-rich plasma had a stimulatory effect on the cellular migration of periodontal ligament cells (Fig. 1). Concentrations of 100% ($p < 0.01$), 50% ($p < 0.001$) and 10% platelet-rich plasma ($p < 0.001$) significantly enhanced migration compared with the negative control of 0% fetal calf serum. A 50% concentration of platelet-poor plasma also significantly enhanced migration of the periodontal ligament cells ($p < 0.02$), but no difference was found compared with the various platelet-rich plasma concentrations. There were no significant differences between the various concentrations of platelet-rich plasma.

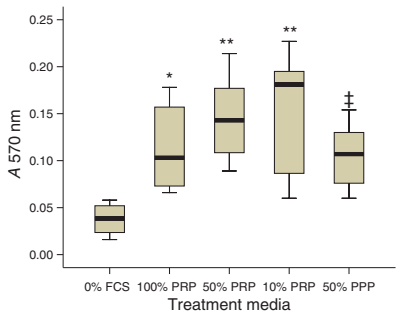

Fig. 1. Periodontal ligament cell migration (*A*, absorbance). FCS, fetal calf serum; PPP, platelet-poor plasma; PRP, platelet-rich plasma. ‡$p < 0.02$, *$p < 0.01$, **$p < 0.001$.

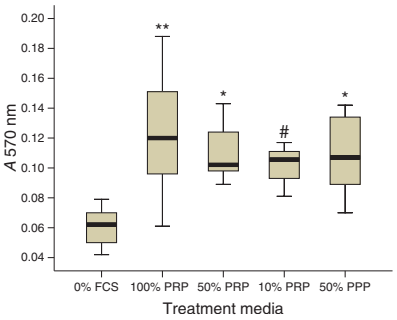

Fig. 2. Osteoblast migration (*A*, absorbance). $p < 0.05$, *$p < 0.01$, **$p < 0.001$. FCS, fetal calf serum; PPP, platelet-poor plasma; PRP, platelet-rich plasma.

The migration of osteoblasts was also significantly enhanced by the various concentrations of platelet-rich plasma, with 100% platelet-rich plasma ($p < 0.001$), 50% platelet-rich plasma ($p < 0.01$) and 10% platelet-rich plasma ($p < 0.05$) all showing significant stimulation compared with 0% fetal calf serum (Fig. 2). No statistical differences were seen between the various concentrations of platelet-rich plasma. A 50% concentration of platelet-poor plasma also had a significant migratory stimulus compared with 0% fetal calf serum ($p < 0.01$), but was not significantly different from the various platelet-rich plasma concentrations.

Proliferation

[^{3}H]-thymidine incorporation (DNA synthesis) assay — The [^{3}H]-thymidine proliferation assays showed similar effects of platelet-rich plasma on the DNA synthesis of both periodontal ligament cells and osteoblasts (Figs 3 and 4 respectively). Platelet-rich plasma exerted a statistically significant decrease in cellular DNA synthesis of periodontal ligament cells at all concentrations ($p < 0.01$) compared with the positive control. Furthermore, 50% platelet-poor plasma significantly increased DNA synthesis compared with all platelet-rich plasma concentrations ($p < 0.01$ for all concentrations).

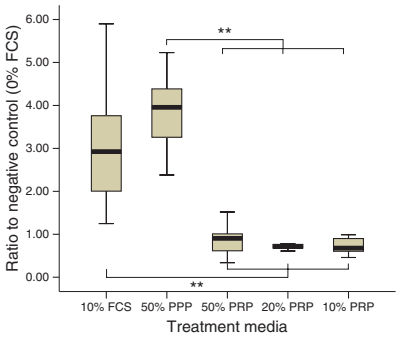

Fig. 3. Periodontal ligament cell DNA synthesis ([^{3}H]-thymidine incorporation assay). **$p < 0.01$. FCS, fetal calf serum; PPP, platelet-poor plasma; PRP, platelet-rich plasma.

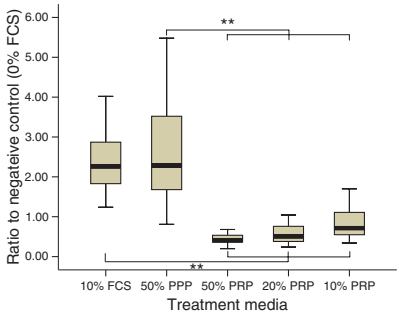

Fig. 4. Osteoblast DNA synthesis ([^{3}H]-thymidine incorporation assay). **$p < 0.01$. FCS, fetal calf serum; PPP, platelet-poor plasma; PRP, platelet-rich plasma.

Similar results were obtained using osteoblasts, whereby all platelet-rich plasma concentrations significantly inhibited DNA synthesis ($p < 0.01$) compared with the positive control (10% fetal calf serum). Platelet-poor plasma had a significant enhancing effect when compared with all concentrations of platelet-rich plasma ($p < 0.01$), but showed no difference compared with the positive control of Dulbecco's modified Eagle's medium containing 10% fetal calf serum.

Crystal violet (colorimetric) assay — Continuing the trend from the DNA synthesis assay, in the 5-d colorimetric proliferation assay, the periodontal

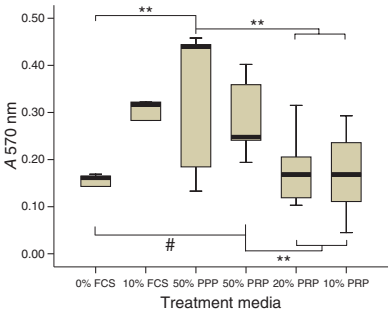

Fig. 5. Periodontal ligament cell proliferation (crystal violet/colorimetric) assay. *A*, absorbance. $p < 0.05$, $^{**}p < 0.01$. FCS, fetal calf serum; PPP, platelet-poor plasma; PRP, platelet-rich plasma.

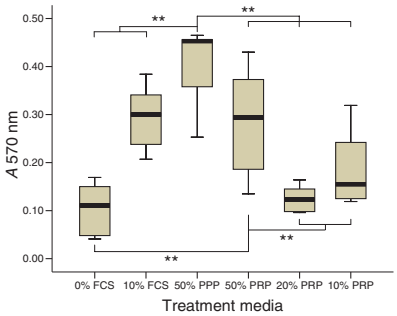

Fig. 6. Osteoblast proliferation (crystal violet/colorimetric) assay. *A*, absorbance. $^{**}p < 0.01$. FCS, fetal calf serum; PPP, platelet-poor plasma; PRP, platelet-rich plasma.

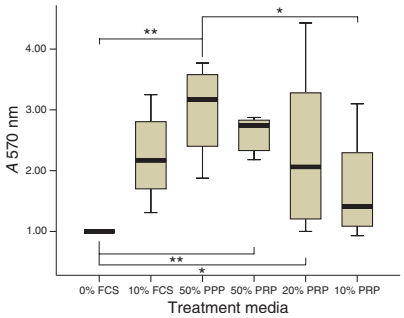

Fig. 8. Osteoblast proliferation (MTT/cell vitality) assay. *A*, absorbance. $^{*}p < 0.05$, $^{**}p < 0.01$. FCS, fetal calf serum; PPP, platelet-poor plasma; PRP, platelet-rich plasma.

ligament cells (Fig. 5) showed the greatest proliferative activity in the presence of 50% platelet-poor plasma, which was significantly higher than 20% platelet-rich plasma ($p < 0.01$), 10% platelet-rich plasma ($p < 0.01$) and the negative control of 0% fetal calf serum ($p < 0.01$). However, in this assay, 50% platelet-rich plasma induced a significant increase in cell numbers compared with 0% fetal calf serum ($p < 0.05$) and the lower concentrations of platelet-rich plasma, namely 20 and 10% ($p < 0.01$ for both). There was no significant statistical difference between 50% platelet-rich plasma, 50% platelet-poor plasma and 10% fetal calf serum. In addition, neither 20% nor 10% platelet-rich plasma showed a significant difference when compared with the negative control of 0% fetal calf serum.

Similarly, with regard to osteoblasts (Fig. 6), 50% platelet-poor plasma was the most beneficial in terms of proliferation compared with all platelet-rich plasma concentrations, as well as media containing 10 or 0% fetal calf serum ($p < 0.01$). A 50% concentration of platelet-rich plasma was significantly more mitogenic than 0% fetal calf serum ($p < 0.01$), 20% platelet-rich plasma ($p < 0.01$) and 10% platelet-rich plasma ($p < 0.01$). There were no statistical differences in proliferation between 10% platelet-rich plasma, 20%platelet-rich plasma and 0% fetal calf serum, and 50% platelet-rich plasma and 10% fetal calf serum.

MTT proliferation (cell viability) assay — The MTT proliferation box-plots for the periodontal ligament cells and osteoblasts are shown in Figs 7 and 8, respectively. Treatment with 50% platelet-rich plasma significantly stimulated cell proliferation of periodontal ligament cells compared with media containing 0% fetal calf serum ($p < 0.01$), 10% platelet-rich plasma and 20% platelet-rich plasma (all $p < 0.01$), but there was no statistical difference compared with 10% fetal calf serum and 50%platelet-poor plasma. Similarly, 50% platelet-poor plasma was shown to increase proliferation significantly compared with 0% fetal calf serum, 10% platelet-rich plasma and 20% platelet-rich plasma (all $p < 0.01$).

Regarding osteoblasts, 50% platelet-rich plasma ($p < 0.01$) and 20% platelet-rich plasma ($p < 0.05$) significantly increased proliferation compared with 0% fetal calf serum. Furthermore, 50% platelet-poor plasma significantly increased proliferation compared with 0% fetal calf serum ($p < 0.01$) and 10% platelet-rich plasma ($p < 0.05$). No significant statistical differences were seen between the various platelet-rich plasma concentrations and 10% fetal calf serum, although there appeared to be trend towards reduced proliferative activity with lower platelet-rich plasma concentrations.

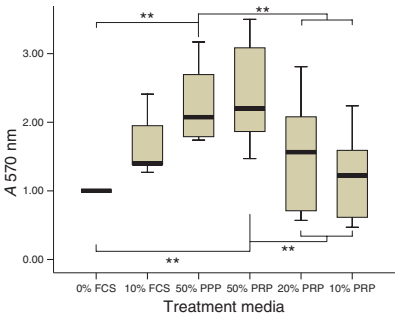

Fig. 7. Periodontal ligament cell proliferation (MTT/cell vitality) assay. *A*, absorbance. $^{**}p < 0.01$. FCS, fetal calf serum; PPP, platelet-poor plasma; PRP, platelet-rich plasma.

Differentiation

The results show that platelet-rich plasma and platelet-poor plasma are capable of inducing differentiation of the periodontal ligament cells and osteoblasts, as shown by the photographs of the wells with the Alizarin Red S staining of Ca^{2+} deposits (Fig. 9). This was reflected in the results obtained by solubilization of the stain and reading the absorbance thus obtained in the spectrophotometer at 415 nm (Figs 10 and 11).

In the periodontal cells (Fig. 10), 50% platelet-rich plasma ($p < 0.01$) and 50% platelet-poor plasma ($p < 0.05$) induced significant increases in differentiation compared with 0% fetal calf serum. In addition, 50% platelet-rich plasma significantly increased Ca^{2+} deposition compared with 10% fetal calf serum, mineralization medium ($p < 0.05$), 20% platelet-rich plasma ($p < 0.05$) and 10% fetal

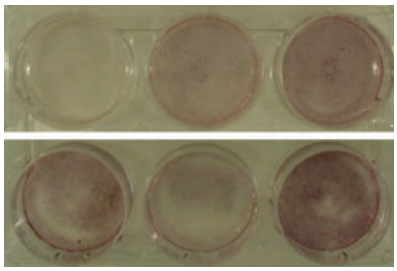

Fig. 9. Visual representation of osteoblast differentiation assessed by Alazarin Red staining. Top row, left to right: 0% fetal calf serum, 10% fetal calf serum, mineralization medium. Bottom row, left to right: 50% platelet-rich plasma, 20% platelet-rich plasma and 50% platelet-poor plasma.

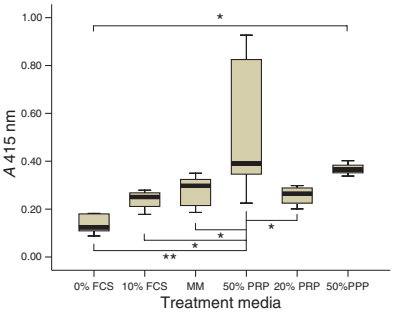

Fig. 10. Quantification of periodontal ligament cell differentiation (*A*, absorbance). $*p < 0.05$, $**p < 0.01$. FCS, fetal calf serum; MM, mineralization medium; PPP, platelet-poor plasma; PRP, platelet-rich plasma.

calf serum ($p < 0.05$). The 20% concentration of platelet-rich plasma showed no significant difference when compared with 50% platelet-poor plasma or mineralization medium.

The results obtained with the osteoblasts indicated that mineralization medium ($p < 0.05$), 50% platelet-rich plasma ($p < 0.05$) and 50% platelet-poor plasma ($p < 0.05$) induced a significant increase in differentiation when compared with 0% fetal calf serum (Fig. 11). No other significant differences were noted.

Discussion

Platelet-rich plasma is easily produced with minimal additional equipment using a two-spin technique to ensure complete separation of the blood fragments (12). In the present study,

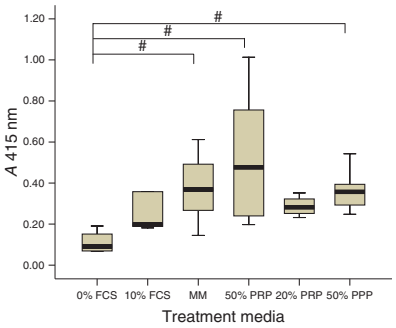

Fig. 11. Quantification of osteoblast differentiation (*A*, absorbance). $p < 0.05$. FCS, fetal calf serum; MM, mineralization medium; PPP, platelet-poor plasma; PRP, platelet-rich plasma.

the platelet-rich plasma was drawn from healthy volunteers immediately before application to the cell lines because platelets begin to release their growth factors immediately, with almost 70% of stored growth factors released within 10 min (12).

Several reports have previously assessed the effect of platelet-rich plasma on cell function *in vitro*. However, these studies utilized a wide range of methods for preparing the platelet-rich plasma formulation that was applied to the cells, and a wide variety of cell types was used. In this study, platelet-rich plasma was prepared and utilized in the same manner as for clinical use, with the various dilutions of platelet-rich plasma being directly applied to the cells. Furthermore, primary cell lines of periodontal ligament cells and alveolar bone-derived osteoblasts were used in order to replicate the clinical scenario. As individual growth factor concentrations in platelet-rich plasma preparations were not assessed in this study, and it is known that these can vary between individuals (14), it was important that the data were not biased by individual variation. Therefore, each experiment utilized platelet-rich plasma sources from three different donors, which was applied to three individual cell lines, thus minimizing the chance of generating biased data.

Both short-term (migration after 6 h, DNA synthesis after 24 h) and long-term (proliferation and differentiation after 5 d) effects on osteoblast and periodontal ligament cell lines were assessed, with similar results obtained for both cell types. However, different platelet-rich plasma concentrations had a significant effect on cell function.

In the short term, the various concentrations of platelet-rich plasma significantly enhanced migration for both osteoblasts and periodontal ligament cells. This finding was consistent with a previous report of platelet-rich plasma promoting migration of the SaOS osteoblastic cell line (26). The migration data showed that the various components of platelet-rich plasma possess biological activity, even at the lowest utilized concentration of 10%. Interestingly, platelet-poor plasma was also shown to possess biological activity.

The [^{3}H]-thymidine incorporation assay showed that platelet-rich plasma does not enhance DNA synthesis within 24 h. However, platelet-poor plasma significantly enhanced DNA synthesis in both cell types. This finding was in contrast to the findings of Celotti *et al*. (26), who showed that platelet-rich plasma increases DNA synthesis in osteblast-like cells. However, the platelet-rich plasma formulation used by Celotti *et al*. was the 'supernatant' derived from platelet-rich plasma, rather than the whole platelet-rich plasma preparation that is utilized clinically and was also used in the current study. Conversely, the 5-d proliferation assays (crystal violet and MTT), in keeping with other reports (19,21–23,26), showed that platelet-rich plasma enhanced cell proliferation, with a concentration of 50% being the most effective. As was the case with the DNA synthesis data, these assays showed that platelet-poor plasma significantly enhanced proliferation, with the formulation of 50% platelet-poor plasma having efficacy similar to that of the most effective platelet-rich plasma concentration of 50%.

The differentiation results indicated that both platelet-rich plasma and platelet-poor plasma are capable of inducing differentiation of periodontal ligament cells and osteoblasts, as shown by Alizarin Red S staining, which identifies calcium deposits. This finding was in agreement with reports that platelet-rich plasma enhanced the

expression of mineralized tissue-associated proteins by osteoblasts and periodontal ligament cells (39,40).

A major consideration in the interpretation of the results of this study, especially with regard to the difference in DNA synthesis and cell proliferation results, is that the platelet-rich plasma and platelet-poor plasma preparations were directly applied to the cells to mimic the clinical scenario. Indeed, following application of the 100% platelet-rich plasma gel-like preparation, the cells acquired an altered morphology, with some cells detaching, suggesting that cell viability was being compromised and necessitating the exclusion of this formulation (100% platelet-rich plasma) from the proliferation and differentiation assays. Although the other preparations of platelet-rich plasma (50, 20 and 10%), did not appear to affect cell vitality, they also did not demonstrate a mitogenic effect at 24 h. As platelet-poor plasma also formed a gel-like complex in the plates, yet demonstrated a markedly greater mitogenic effect compared with the various concentrations of platelet-rich plasma, it appears that the concentrated cocktail of active components present in platelet-rich plasma are inferior in terms of promoting wound healing-associated functions, especially in the short term and on the cells that come into direct contact with the formulation.

When platelets are activated, they release their growth factors almost immediately, with nearly 70% released in the first 10 min and almost 100% within the first hour (12). Platelets can then be expected to continue to synthesize additional growth factors for several days, after which they lose vitality (12). Therefore, it can be postulated that the highly concentrated cocktail of molecules present in the 100% preparation of platelet-rich plasma may be initially detrimental to the cells that it directly contacts, but at lower concentrations and over time, as its activity diminishes, it begins to promote cell function, as seen with the stimulation of cell proliferation by 50% platelet-rich plasma at 5 d. The suggestion that maximal (100%) concentrations of platelet-rich plasma may not be ideal and that better outcomes may be obtained by 'intermediate' concentrations of platelet-rich plasma, is supported by both *in vitro* (19,41) and *in vivo* (42) data. Indeed, in one of the few studies to investigate the clinical efficacy of different concentrations of platelet-rich plasma, Weibrich *et al.* (42) showed that intermediate concentrations were the most effective in terms of bone regeneration.

Platelet-poor plasma significantly enhanced wound healing-associated cell function, which is consistent with previous reports that have shown a clinical benefit of using platelet-poor plasma (15). Furthermore, it has been suggested that the clinical action of platelet-rich plasma itself is a result of the fibrin content (20), which is essentially platelet-poor plasma. It has also been demonstrated that platelet-poor plasma preparations contain significant amounts of certain growth factors, namely insulin-like growth factor-1 and bone morphogenetic protein-2, at levels comparable to those found in platelet-rich plasma (43).

The results from the current study show that, in the short term, platelet-rich plasma appears to downregulate DNA synthesis, with high concentrations adversely affecting cell viability in the immediate area of placement. This is accompanied by a strong migratory stimulus towards distant cells at all platelet-rich plasma concentrations (10–100%). Over the longer term, and at intermediate concentrations (50%), platelet-rich plasma appears to enhance the proliferation of periodontal ligament and osteoblast cells, and these cells are then induced to differentiate. The loss of cell viability at concentrations of 100% platelet-rich plasma may be a problem in periodontal regeneration or bone grafting where a limited number of progenitor cells are present in the ligament and graft particles, respectively. This may account for the results of several recently published reports of a lack of clinical benefit following the use of platelet-rich plasma (30–32). Furthermore, the possibility that high concentrations may adversely affect cell viability is important in the context of platelet-rich plasma being proposed as a delivery vehicle for stem and progenitor cells in tissue engineering-based clinical therapies.

Interestingly, platelet-poor plasma demonstrated a better overall effect on cell function *in vitro*. Platelet-poor plasma encouraged cellular proliferation in the short and long term, promoted cell migration and stimulated differentiation. Appropriately designed clinical studies comparing different concentrations of platelet-rich plasma, as well as platelet-poor plasma, are indicated to determine the effect of various platelet-rich plasma concentrations on clinical outcomes.

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