Platelet-rich plasma influence on human osteoblasts growth

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Abstract
Objective: The influence of progressively high concentrations of platelet-rich plasma (PRP) on human osteoblast hFOB1.19 proliferation was evaluated.

Material and methods: The PRP was obtained from a human source. Two experiments were conducted. In the first one, PRP was diluted to 50%, 25%, 12.5% and 6.125% (v/v) with culture medium (Modified Eagle’s Medium (MEM) : Ham’s F12 Medium (HAM-F12%) and 1% antibiotics–antimicotic) supplemented with 10% of fetal bovine serum (FBS). In the second experiment, all conditions were identical except for the absence of FBS in the culture medium.

Results: The results of the osteoblast proliferation test were higher when stimulated by the 50% PRP dilution, with or without FBS. A further study is suggested to determine if concentrations above 50% could cause higher rates of osteoblast proliferation. In this study, the results were not statistically different (P<0.05) with 12.5% and 6.125% PRP dilutions. Additionally, it was shown that FBS is not necessary for PRP-mediated induction of osteoblast proliferation.

Conclusion: This study concluded that PRP promotes osteoblast proliferation and suggested its clinical application to bone graft procedures in implant dentistry.

Scientific studies have established platelet-rich plasma (PRP) as a therapeutic strategy in orthopedics [Ganio et al. 1993], in implant dentistry [Anitua 1999; Kassolis et al. 2000; Gruber et al. 2002], and as an aid for bone graft compaction as well as for hemostasis. PRP is a non-toxic, autogenous material that does not cause immune reactions when applied to the original donor. PRP possesses the advantage of being immediately available preoperatively, and consequently histocompatible, and being incapable of transmitting infectious diseases [Whitman et al. 1997]. PRP also contains high amounts of mitogenic polypeptide proteins, platelet-derived growth factors (PDGFs), β-transforming growth factors (TGF-β’s) and I-insulin-like growth factors, which appear to accelerate the osteogenesis in oral and maxillofacial surgery, as well as platelets, which are a source of growth factors that induce bone, epithelial and connective tissue repair. It has been successfully used since the 1970s. Matras (1970) used plasma to form fibrin ‘glue’ preparations obtained from blood centrifugation for skin graft procedures in mice. Studies showed the beneficial effects of PRP to repair cutaneous ulcers [Knighton et al. 1990; Lynch 1991]. PRP promotes acceleration of surgical wound repair by means of growth factors present in the platelets, which are the universal initiators of the healing process [Ganio
accelerate mineralization in oral and maxillofacial surgeries, (Ganio et al. 1999). Its benefits were also confirmed by Tayapongsak et al. 1993; Anitua et al. 2004; Schlegel et al. 2004; Wiltfang et al. 2004; Roldan et al. 2003). Modified Eagle's medium (MEM, Gibco, Invitrogen Corporation, Carlsbad, CA, USA) and Ham’s F12 medium (HAM-F12) [Cultilab, Campinas, SP, Brazil] (1 : 1) supplemented with 10% of fetal bovine serum (FBS, Gibco BRL, Campinas, Brazil), and 1% of antibiotics-antimycotic (penicillin G sodium – 100 U/ml, streptomycin – 100 μg/ml and amphotericin B – 0.025 μg/ml; Gibco BRL). The cells were cultured in a humidified incubator with 5% CO2 at 37°C. When cells reached confluency they were subcultivated using 0.1% of trypsin (Sigma, St. Louis, MO, USA; 5 g/ml) and 1% of calcium- and magnesium-free Dulbecco’s phosphate-buffer saline solution (Gibco, Invitrogen Corporation).

Platelet collection
Plasma was obtained from the venous blood of a healthy male volunteer. Blood was drawn into a 5 ml Vacutainer® (BD, Curitiba, Paraná, Brazil) containing 500 μl of the anticoagulant sodium citrate. PRP was obtained following the protocol developed by Macedo et al. (2003). Briefly, blood was centrifuged twice at 120 × g in an ALC centrifuge (Centrifuge 4206 ALC, ACE Surgical Supply Company Inc., Brockton, MA, USA) for 10 min at 20°C to remove red blood cells [first time] to obtain the PRP [second time]. Three hours later, the PRP was diluted in the cell culture medium for proliferative assessment of the osteoblasts. A previous study revealed that the activity of growth factors present in PRP decreased 4 h after blood was drawn (Ledent et al. 1995). Consequently, the PRP used here was obtained approximately 3 h after blood collection. This procedure was repeated three times, in a weekly interval, to obtain mean values. Therefore, the blood was drawn three times from the same male volunteer.

Evaluation of human osteoblast proliferation by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay
hFOB1.19 cell cultures (3 × 10^4 cells/well) were prepared in 96-well tissue culture plates (Corning, NY, USA). After a 24 h period of incubation, the cell culture medium was replaced by one containing 50%, 25%, 12.5% and 6.125% of diluted PRP without or with 10% FBS.

Next, platelet gel was obtained upon addition of 5% calcium chloride to the medium. Four days after replacing original culture medium with another one containing PRP, the MTT method [Mossman 1983] was applied with modifications [Andrighetti-Fröhner et al. 2003]. After 4 days, at 37°C in humidified 5% CO2 atmosphere, 50 μl of MTT [Sigma, 1 mg/ml] solution prepared in MEM:HAM-F12 (1 : 1) cell medium was added to each well and the plates were incubated for 4 h at 37°C. Next, the medium was not removed with suction, and 100 μl of dimethyl sulfoxide (Merck Biosciences, Darmstadt, Germany) was added to each well to dissolve formazan crystals. After gently shaking the plates for 5 min, whereby crystals were completely dissolved, the absorbance was read on a multiwell spectrophotometer [Bio-Tek, Elx 800, Winoski, VT, USA] at 540 nm. The original MTT technique [Mossman 1983] was modified, the cell culture medium was not removed, for a gel clot was formed inside the wells, and, by means of light microscopy, the cells had migrated to the clot. Afterwards, the influence of the PRP was assessed by a scanning spectrophotometer [Ultraspex 3000-Pharmacia Bio-tek, Winooski, VT, USA] in the four concentrations used in this research. This evaluation showed no influence of the PRP in the cell culture medium when read at 540 nm. Therefore, PRP did not influence the results obtained from the spectrophotometer [Bio-Tek, Elx 800] at 540 nm. The percentage of cell growth was calculated as [(A – B)/ A × 100], where A and B are the absorbances of control and treated cells, respectively. In this calculation, B was subtracted from A and divided by the absorbance of the treated group multiplied by 100, resulting in the percentage of cell growth.
The percentages of cell growth by the PRP in relation to hFOB1.19 osteoblasts represent mean ± standard error of the mean values of three different experiments. Variance analysis and the Tukey test (P<0.05) were carried out as appropriate.

Results

Evaluation of human osteoblast proliferation by MTT assay

The results of the influence of different concentrations of PRP on hFOB1.19 osteoblast proliferation in MEM:HAM-F12 (1:1) without and with 10% FBS are expressed as percentages of cell growth that indicate mean values of three independent experiments. The results were evaluated statistically using analysis of variance and Tukey analysis (Fig. 1).

A comparison of the results of hFOB1.19 osteoblast proliferation by PRP with and without 10% FBS showed that cell growth increases as a function of the concentration of PRP. Cell growth is always higher in the groups receiving 10% FBS, showing the highest influence on cell growth between 675.7% and 824% with 695.7% as the median value for the groups receiving 50% PRP. Figure 1 shows the results of the variability considered in this research for the groups receiving 10% FBS and those not receiving FBS.

Discussion

Past investigations have established the influence of growth factors on cell proliferation [Gronthos & Simmons 1995; Kuznetsov et al. 1997; Lind 1998; Tian et al. 1999]. PRP, which contains many growth factors, has been shown to stimulate cell proliferation in vitro [Slater et al. 1995; Nakanishi et al. 1997; Weibrich et al. 2002; Kawase et al. 2003; Lucarelli et al. 2003; Okuda et al. 2003]. Whittman et al. (1997) described the preparation of platelet gel showing its advantages in bone graft procedures to accelerate the healing process. Literature on bone cell physiology shows many instances where PRP was in association with bone graft procedures. In these cases, PRP has demonstrated an acceleration of the formation of mature bone. In addition, a greater formation of cancellous bone was noted when compared with bone grafts that did not receive PRP when evaluated 4–6 months after the procedure [Marx & Garg 2003]. In vitro investigations have identified that the PDGF, a subcomponent of the PRP, has a significant effect on cell proliferation [Nakanishi et al. 1997; Weibrich et al. 2002; Kawase et al. 2003; Okuda et al. 2003].

However, questions are raised about the efficacy of PRP in dental applications. There are clinical studies where PRP was used for grafting the floor of the maxillary sinus in association with bone graft material for increasing alveolar bone height prior to the placement of endosseous dental implants in the posterior maxilla. To determine the efficacy of the sinus augmentation procedure compared with the results achieved with various surgical techniques, grafting materials and implants, a systematic review of clinical studies was conducted by Wallace & Froum (2003). In this investigation, the authors verified the effect on implant survival of maxillary sinus augmentation vs. implant placement in the non-grafted posterior maxilla. The authors concluded that there were insufficient data to recommend the use of PRP in sinus graft surgery, a very common procedure used in implant dentistry.

In another investigation, the effect of PRP on bone regeneration associated with autogenous bone graft was evaluated in animal models where bone defects received autogenous bone in association with PRP, and without PRP. Results of these studies showed that the addition of PRP does not appear to enhance new bone formation in autogenous bone grafts [Schlegel et al. 2003, 2004; Choi et al. 2004; Wiltfang et al. 2004].

Further, PRP was studied on bone augmentation procedures in vitro and was compared with bone morphogenetic protein when added to autologous bone grafts. The authors concluded that, by means of a histomorphometric study, PRP failed to enhance bone formation [Roldan et al. 2004].

Froum et al. (2002) studied the efficacy of PRP on bone growth and osseointegration in human maxillary sinus grafts when associated with grafts of anorganic bovine bone that contained minimal or no autogenous bone. The results of their studies showed that the effect of PRP did not show a significant difference either in vital bone production or in interfacial bone contact on the test implants.

The studies enumerated above suggest uncertainty in recommending the use of PRP in bone-regeneration procedures. However, the present investigation shows in vitro that PRP has a positive effect on osteoblast proliferation.

The results of this investigation are in accordance with the results obtained by Nakanishi et al. (1997) and Kawase et al. (2003) showing that PRP stimulates cell proliferation when added to culture medium containing osteoblasts. In this study, we observed a positive correlation between the increased concentration of PRP in culture medium and the rate of osteoblasts proliferation, consistent with the results published by Lucarelli et al. (2003), which
showed that 10% of PRP added to osteoblast culture medium was sufficient to induce evident cell proliferation. The present study showed that 50% PRP caused the best proliferative results in osteoblast culture. Further studies are needed to establish if progressively higher concentrations of PRP would have a corresponding influence on proliferation. In addition, our results showed that FBS is not necessary for PRP-mediated induction of hFOB1.19 osteoblast proliferation as shown by Lucarelli et al. (2003).

The content of PDGF and TGF-β present in platelet gel was studied during the preparation and storage of PRP. The content of PDGF and TGF-β decreases according to higher storage periods, showing less cell growth promotion 4 h to 3 days after blood is drawn. In this study, the experimental use of blood to supplement the cell growth media did not exceed 3 h after blood was drawn [Ledent et al. 1995].

Currently, cultivation of human osteoblasts in vitro is challenging [Lind 1998], and PRP has demonstrated a significant influence on osteoblast reproduction. The positive results of in vitro [Nakanishi et al. 1997; Weibrich et al. 2002; Lucarelli et al. 2003] studies encourage research into further refinement of methods for osteoblast cultivation. Methods conducive to high yields of osteoblast proliferation may contribute ultimately to the development of processes that promote growth or replacement of substitute bone material. They also could potentially have far-reaching benefits in orthopedics, maxillofacial surgery as well as implant dentistry by eliminating the need for surgically complex bone harvesting as well as reducing associated pain and costs. However, the effects of PRP associated with autogenous bone grafts in clinical situations require further investigation.

The purpose of the present study was to evaluate the influence of progressively higher concentrations of PRP on cell growth medium. The maximum amount of PRP added to the growth medium was 50%, showing the best proliferative results in human osteoblast culture. Therefore, further studies are suggested to answer if higher concentrations of PRP would alter the proliferative rate of human osteoblasts.

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