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Platelet-Rich Plasma: Quantification of Growth Factor Levels and the Effect on Growth and Differentiation of Rat Bone Marrow Cells

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ABSTRACT

Platelet-rich plasma (PRP) is a new application of tissue engineering and a developing area for clinicians and researchers. It is a storage vehicle of growth factors (GFs) such as platelet-derived growth factor (PDGF)-AA, -BB, -AB; transforming growth factor (TGF)-β1 and -2; platelet-derived epidermal growth factor (PDEGF); platelet-derived angiogenesis factor (PDAF); insulin growth factor-1 (IGF-1); and platelet factor-4 (PF-4), which are known to influence bone regeneration. However, animal and clinical studies reveal different results with the use of PRP and its effect on bone healing. This could be due to the differences between species, that is, differences between species in GF concentrations or variation in presence of GFs between the various PRPs. In this study, rat bone marrow cells were cultured in PRP-coated wells or in uncoated wells for 16 days in osteogenic medium, and analyzed on cell growth (DNA content) and cell differentiation (alkaline phosphatase [ALP] activity, calcium content, scanning electron microscopy, and QPCR). The concentrations of TGF-β1, PDGF-AA, PDGF-AB, and PDGF-BB in rat, goat, and human PRP were subsequently determined. The results showed that PRP stimulated initial cell growth and had no effect on ALP activity. The calcium measurements showed a significant increase in calcium at days 8, 12, and 16. The real-time PCR results showed that PRP upregulated osteocalcin at day 1 and collagen type I at day 8. Overall, the immunoassays revealed that human PRP contained higher concentrations of growth factors per platelet compared to rat and goat PRP. Goat PRP showed higher concentrations of growth factors per platelet as compared to rat PRP except for PDGF-BB, which had a higher concentration in rat PRP. TGF-β1 was the most abundant growth factor in all 3 PRPs. On the basis of our results, we conclude that platelet-rich plasma contains osteo-inductive growth factors, which are probably species related. However, we cannot generalize the results because of large intraspecies variations. Further, we conclude that rat PRP gel stimulates initial growth and differentiation of rat bone marrow cells *in vitro*.

INTRODUCTION

Autologous bone or bone derivatives and substitutes for jaw and periodontal reconstruction have significant limitations in terms of availability, morbidity, efficacy, immunologic reaction, and disease transmission. Therefore, tissue engineering is an alternative approach, which includes, among others, the use of a synthetic material for the delivery of osteogenic (osteoinductive) morphogens.

Platelet-rich plasma (PRP) as a storage vehicle of growth factors (GFs) is a new application of tissue engineering and a developing area for clinicians and researchers. The GFs, such as platelet-derived growth factor (PDGF [AA, BB, AB]), transforming growth factor-β1 and -β2, platelet-derived epidermal

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growth factor (PDEGF), platelet-derived angiogenesis factor (PDAF), insulin growth factor-1 (IGF-1), and platelet factor-4 (PF-4) are known to influence bone regeneration. In addition, some authors have reported the presence of basic fibroblast growth factor (bFGF), epithelial cell growth factor (ECGF), interleukin-1 (cytokine), and osteonectin (major protein in mineralized bone) in the α-granules of the platelets.[1–4] All of this implies that platelet concentrates might influence bone formation through a variety of pathways.

The effects of PRP on osteoblast lineage have been confirmed by a few *in vitro* studies. Weibrich *et al.*[5] observed mitogenic effects of PRP on osteoblast-like cells. Annunziata *et al.*[6] and Lucarelli *et al.*[7] showed an increase in growth and differentiation of PRP-treated periodontal ligament cells and stromal stem cells as indicated by alkaline phosphatase (ALP) activity and collagen type I gene expression. Additional studies revealed that PRP stimulated the mitogenic response of bone cells derived from human trabecular[8] and rat bone marrow.[9] Yet, it is still unknown whether the stimulatory effects of PRP is related to the GFs present or to other microparticles from the cytoplasm or cell membrane that are released from activated platelets.[8]

The action mechanism of GFs is very complex, because each GF may have a different effect on the signal transduction of bone matrix mineralization. GFs may also interact with each other and consequently may form a cascade of different signal proteins with multiple pathways, which will ultimately lead to activation of gene expression followed by protein production. This specific characteristic of PRP, having multiple GFs, acting on multiple pathways, distinguishes PRP from single recombinant GFs that focus on a single signal transduction pathway.[10,11]

However, the number of *in vitro* studies is very limited and additional questions needs to be answered. For example, animal and clinical studies reveal different results with the use of PRP and its effect on bone healing. Some researchers reported an additional effect on bone regeneration by using PRP in combination with bone grafts;[12,13] others revealed no differences.[14,15] This could be due to the differences between species, that is, differences in GF concentrations between species or variation in the presence of GFs between the various PRPs.

Therefore, the aim of this study was to examine the presence and the concentrations of GFs in rat, goat, and human PRP. We examined the effect of rat PRP gel on rat bone marrow cells.

MATERIALS AND METHODS

PRP preparation

Rat PRP was obtained by drawing the whole blood of 4 male Wistar rats via cardiac puncture into tubes containing 3.8% sodium citrate. PRP was obtained from this anticoagulated blood after centrifugation at 800 rpm for 15 min at 25°C. Three milliliters of blood resulted in 150 μL of PRP. The platelets present in the whole blood and in PRP were counted automatically using a hematology analyzer. The concentration of platelets obtained in the PRP was 3 times higher than the baseline. This PRP was used to obtain a PRP gel. The platelet concentrate was activated with a 10% calcium chloride solution and 300 IU of bovine thrombin. One hundred and fifty microliters of PRP was mixed with 25 μL of thrombin (300 IU) (Fibriquik™ Thrombin, Bio-Merieux, Durham, NC) together with 25 μL of 10% $CaCl_2$. Goat PRP and human PRP (1 patient) were obtained from the Sanquin Bloodbank, Nijmegen, Regio Zuid-oost. The PRP fraction was obtained from venous blood of 1 goat. For this reason, 250 cm^3 of blood was drawn and centrifuged at a transfusion laboratory (Sanquin, Nijmegen, Netherlands) in various cycles (Clemmons *et al.,* 1983). Finally, 10–15 mL of PRP suspension was obtained. The platelet concentration was also counted (platelet count exceeding 1200×10^6/mL).

Quantitative sandwich enzyme immunoassay

A quantitative sandwich enzyme immunoassay (Quantikine, R&D Systems Europe Ltd., Abingdon, UK) was used for examining the amount of TGF-β1, PDGF-AA, PDGF-AB, and PDGF-BB in rat, goat, and human PRP. The samples were activated by adding 0.1 mL of 2.5 N acetic acid/10 M urea to 0.1 mL of plasma. After 10 min of incubation at room temperature, the samples were neutralized by adding 0.1 mL 2.7 N NaOH/1 M *N*,2-hydroxyethylpiperazine-*N′*-2-ethanesulfonic acid (HEPES). The immunoassays were performed following the manufacturer's instructions.

Cell isolation

Rat bone marrow (RBM) cells were isolated and cultured using the method described by Maniatopoulos *et al.*[16] Femora of male Wistar rats were washed in culture medium α-minimum essential medium (α-MEM, Gibco BRL, Life Technologies B.V. Breda, The Netherlands) with 0.5 mg/mL of gentamicin and 3 μg/mL of Fungizone (Sigma-Aldrich, St. Louis, MO). Epiphyses were cut off and diaphyses flushed out with 15 mL of osteogenic culture medium α-MEM, supplemented with 10% fetal calf serum (FCS, Gibco BRL, Life Technologies B.V. Breda, The Netherlands), 50 μg/mL of ascorbic acid, 50 μg/mL of gentamicin, 10 mM sodium β-glycerophosphate, and 10^{-8} M dexamethasone (Sigma-Aldrich). The cells were incubated in a humidified atmosphere of 95% air, 5% CO_2 at 37°C for 7 days. The medium was changed every 2 or 3 days.

Cell seeding

After 7 days of primary culture in osteogenic medium, the pre-osteoblast-like cells were harvested and seeded in 24-well plates at a concentration of 10,000 cells/well. The tissue culture polystyrene (TCPS) wells were first precoated with an PRP gel (150 μL of PRP mixed with 25 μL of thrombin (300 IU) and mixed with 25 μL of 10% $CaCl_2$ per well) or left uncoated. Cells were cultured for 16 days in the presence of osteogenic medium and incubated in a humidified atmosphere of 95% air, 5% CO_2 at 37°C. The medium was changed every 2 or 3 days.

DNA analysis

After 2, 4, 8, and 12 days samples were used for DNA analysis. Medium was removed and cell layers were washed twice with phosphate-buffered saline (PBS). One milliliter of MilliQ was added to each sample. The samples were frozen and thawed repeatedly. DNA standard curve was made with λ DNA. One hundred microliters of sample or standard was added to 100 μL of Picogreen working solution (Molecular Probes, Leiden, Netherlands) and the samples were incubated for 10 min at room temperature in the dark. After incubation, DNA was measured using a fluorescence microplate reader (Bio-Tek instruments, Abcoude, Netherlands) with excitation filter 365 nm and emission filter 450 nm. The same samples were also used for the ALP activity assay.

ALP activity

ALP activity was measured at days 4, 8, and 12. For the assay (Sigma-Aldrich), 96-well plates were used. Eighty microliters of sample and 20 μL of buffer solution (5 mM $MgCl_2$, 0.5 M 2-amino-2-methyl-1-propanol) were added to the wells. Subsequently, 100 μL of substrate solution (5 mM *p*-nitrophenylphosphate) was added to the wells and the plate was incubated for 1 h at 37°C. The reaction was stopped by adding 100 μL of stop solution (0.3 M NaOH). For the standard curve, serial dilutions of 4-nitrophenol were added to final concentrations of 0–25 nM. The plate was read in an enzyme-linked immunosorbent assay (ELISA) reader (Bio-Tek instruments, Abcoude, Netherlands) at 405 nm.

Calcium content

Calcium content in the samples was measured by the ortho-cresolphthalein complexone (OCPC) method (Sigma-Aldrich). Calcium content was examined at 8, 12, and 16 days of incubation. Cell layers were washed twice in PBS. Five hundred microliters of 0.5 N acetic acid was added to the wells and the samples were incubated overnight. Samples were frozen at −20°C until use.

OCPC solution was prepared as follows: Eighty milligrams of OCPC was added to 75 mL of demineralized H_2O with 0.5 mL of 1 M KOH, 0.5 mL of 0.5 N acetic acid. To prepare sample solution, 5 mL of OCPC solution was added to 5 mL of 14.8 M ethanolamine/boric acid buffer (pH 11), 2 mL of 8-hydroxyquinoline (5 g in 100 mL of 95% ethanol), and 88 mL of demineralized water. Three hundred microliters of sample solution was added to 10 μL of sample. To generate a standard curve, serial dilutions of $CaCl_2$ were made (1–200 μg/mL). The plate was incubated at room temperature for 10 min and then read at 575 nm.

RNA isolation

RNA was isolated from the cells with the aid of a RNA isolation kit (RNeasy kit, QIAGEN, Venlo, Netherlands). The cells were then disrupted by adding 350 μL of RLT lysis buffer. The samples were stored at −70°C until use. The immunoassays were performed following the manufacturer's instructions.

The RNA obtained was quantified by adding 1 μL of sample to 49 μL of RNase-free water and the absorbance was measured in a spectrophotometer (Bio-Rad Laboratories, Veenendaal, Netherlands) at 260 nm.

Reverse transcriptase polymerase chain reaction (RT-PCR)

After isolation of the RNA, the RT reaction was performed. One microgram of total RNA, 1 μL of 100 ng of random primers, 1 μL of dNTP mix (10 mM each), and 10 μL of distilled water was added to a nuclease-free microcentrifuge tube. The mixture was heated to 65°C for 5 min and quickly chilled on ice. The contents of the tube were collected by brief centrifugation. Four microliters of 5×first-strand buffer (250 mM Tris-Cl, pH 8.3; 375 mM KCl; 15 mM $MgCl_2$) and 2 μL of 0.1M dithiothreitol (DDT) were added. This was incubated at 25°C for 10 min, and the contents of the tube were mixed gently and incubated at 42°C for 2 min. Finally, 1 μL (200 U) of Superscript II (Invitrogen, Breda, Netherlands) was added and the mixture incubated at 42°C for 50 min. The reaction was inactivated by heating the mixture at 70°C for 15 min. The obtained cDNA was used as a template in the PCR.

Quantitative PCR

The cDNA from the RT reaction was used as a template in PCR. To perform quantitative PCR, 4.5 μL of RNase-free water, 12.5 μL of SYBR® Green MasterMix (Eurogentec, Liege, Belgium), 5 μL of cDNA, 1.5 μL of reverse primer, and 1.5 μL of forward primer were added to a QRT-PCR 96-well plate. This plate was sealed, and the Q-PCR run was performed with the MyiQ Single-Color Real-Time Detection System for quantification with SYBR Green and melting curve analysis (Bio-Rad, Richmond, CA). DNA was PCR-amplified under the following conditions: 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C, with data collection in the last 30 s. The reference household gene used to normalize the amount of mRNA in the cultures was GAPDH ($\Delta C_T = C_{T,\text{gene of interest}} - C_{T,\text{GAPDH}}$ [1]). Gene expression was measured relative to the gene expression of cultures on the relevant substrates (PRP gel-coated or uncoated TCPS) that had not received antibodies, after day 1 of culture ($\Delta\Delta C_{T} = \Delta C_{T,\text{sample}} - \Delta C_{T,\text{control}}$ [2]). The-fold change in gene expression relative to the control was calculated via $2^{-\Delta\Delta CT}$ (3).[17]

Statistical analysis

This study was performed using 2 separate runs of experiments. In each run, samples were present in quadruple. In the Results section, data of both runs are described but only the first run is presented in the figures. Statistical analysis was performed for each run by using an unpaired *t*-test (GraphPad Software, San Diego, CA).

RESULTS

Presence and quantification of GFs in rat, goat and human PRP

The concentrations of GFs—TGF-β1, PDGF-AA, PDGF-BB, and PDGF-AB—in rat, goat, and human PRP were examined. Rat PRP was prepared by 1 centrifugation step and resulted in a 3-fold increase of platelets above baseline (rat PRP = 3000×10^6/mL). Human and goat PRP were prepared in 2 centrifugation steps and resulted also in a 3-fold increase for goat and 6-fold increase for human in platelets above baseline (goat and human PRP = 1200×10^6/mL). As shown in Table 1 and Fig. 1, various GFs were tested and different concentrations of GFs were obtained in rat, goat, and human PRP. For TGF-β1, 7.8 ng/mL was detected in rat PRP, 53 ng/mL was detected in goat PRP, and 2.6 μg/mL was detected in human PRP. For PDGF-AA, 9.7 pg/mL was detected in rat PRP, 561 pg/mL was detected in goat PRP, and 7.9 ng/mL was detected in human PRP. For PDGF AB, 10 pg/mL was detected in rat PRP, 108 pg/mL was detected in goat PRP, and 76.4 ng/mL was detected in human PRP. For PDGF-BB, 590 pg/mL was detected in rat PRP, 39 pg/mL was detected in goat PRP, and 66 ng/mL was detected in human PRP.

TABLE 1. CONCENTRATIONS OF THE GFS TGF-β1, PDGF-AA, -AB, AND -BB IN RAT, GOAT, AND HUMAN PRP

Growth factor	*PRP*	*Concentration*	*Concentration (pg/mL)/* p = *1000 * 10*^{6}
TGF-β1	Rat	23.3 ng/mL	7767
	Goat	63.5 ng/mL	52,917
	Human	3.1 mg/mL	2,580,000
PDGF-AA	Rat	29.2 pg/mL	9.73
	Goat	673.5 pg/mL	561
	Human	9.5 ng/mL	7916
PDGF-AB	Rat	30.2 pg/mL	10
	Goat	129.2 pg/mL	108
	Human	91.7 ng/mL	76,417
PDGF-BB	Rat	1.77 ng/mL	590
	Goat	47 pg/mL	39
	Human	79.3 ng/mL	66,083

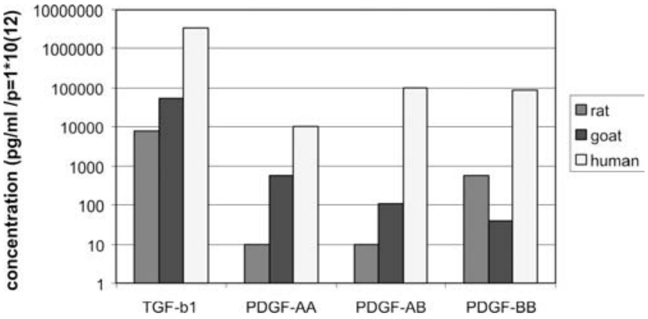

FIG. 1. Concentration of the GFs TGF-β1, PDGF-AA, -AB and -BB in rat, goat, and human PRP.

Effect of rat PRP gel on rat bone marrow cells

The DNA results showed that PRP stimulated the initial cell growth significantly at days 2 and 4 ($p < 0.05$). A linear growth curve for uncoated control samples was observed. In contrast, the coated samples showed a linear growth from day 2 to 4, a steady state from day 4 to 8, and a decrease in DNA from day 8 to 12 (Fig. 2).

A negative effect of PRP on the ALP activity could be observed at days 4, 8, and 12. The ALP activity of the control samples showed an increase of activity from day 4 to 8 to 12 while the PRP-coated samples showed an ALP activity at a constant low level (Fig. 3).

A significant increase in calcium content was found at days 8, 12, and 16 for the samples coated with PRP compared to the uncoated samples ($p < 0.05$). The uncoated samples showed only a low amount of calcium at day 16 (Fig. 4).

Real-time PCR was performed to get some additional data about the gene expression of certain osteogenic markers. The results showed that PRP downregulated ALP and bone sialoprotein at days 1 and 8. It downregulated osteocalcin at day 8 and downregulated collagen type I at day 1. However, PRP upregulated osteocalcin at day 1 and upregulated collagen type I at day 8 (Fig. 5).

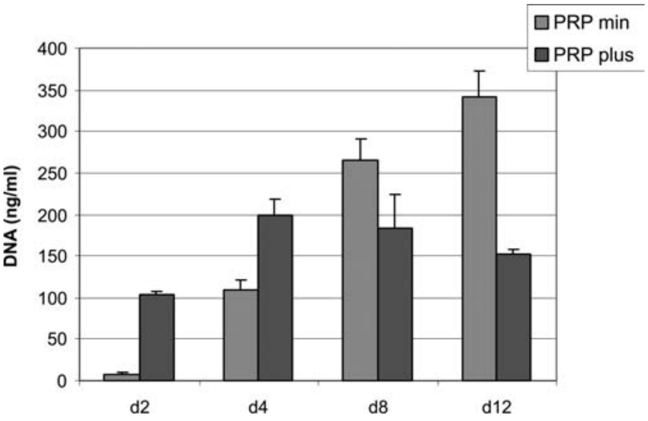

FIG. 2. Cell growth of rat bone marrow cells cultured on PRP-coated and noncoated wells (1st run).

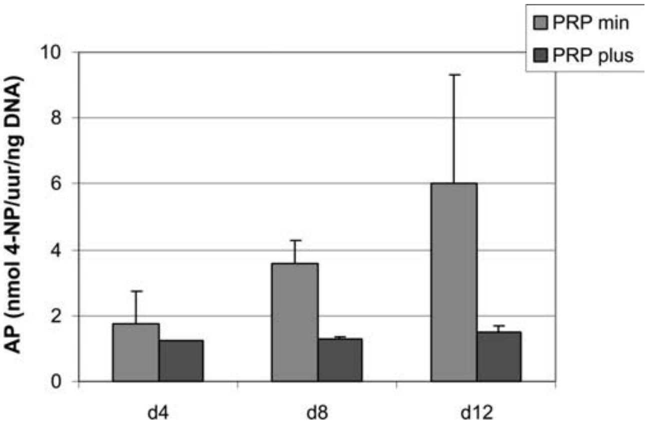

FIG. 3. ALP activity of rat bone marrow cells cultured on PRP-coated and noncoated wells (1st run).

DISCUSSION

The results of our immunoassays revealed that human PRP contained higher concentrations of GFs per platelet as compared to rat and goat PRP. Goat PRP showed also higher concentrations of GFs per platelet compared to rat PRP except for PDGF-BB, which had a higher concentration in rat PRP. TGF-β1 was the most abundant GF in all 3 PRPs. As of yet no data are available about the differences among rat, goat, and human PRP. Apart from animal studies with successful outcomes for PRP, there are also contradictory results in which no benefit of PRP was found.[3,18] The present results can probably explain why it is currently very difficult to obtain a beneficial effect of PRP in experimental animal studies. The PRP applied was always prepared from blood of the experimental animals, while no data were available about the amount of platelets that need to be used in the studies. This could be species-related because of the differences in concentrations of the GFs in PRPs obtained from rat, goat, and human. Only a few studies have reported on the synergistic effects of multiple GFs in a defective site. There are still many questions to be answered before the exact mechanism of the action of PRP can be fully explained.

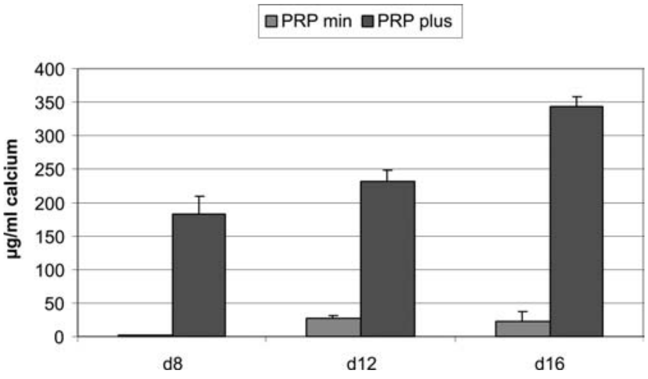

FIG. 4. Calcium content of rat bone marrow cells cultured on PRP-coated and noncoated wells (1st run).

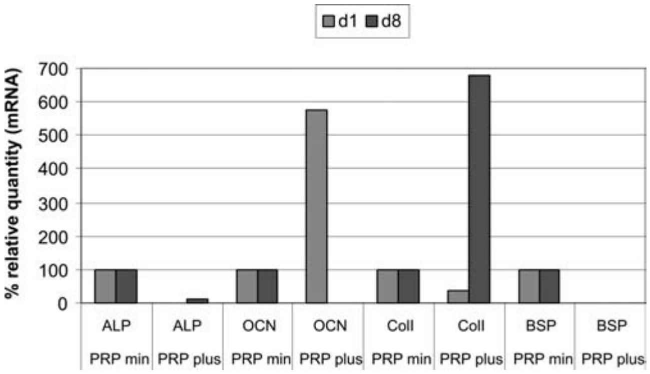

FIG. 5. Relative quantity of mRNA (%) of ALP (alkaline phosphatase), OCN (osteocalcin), ColI (collagen type I), and BSP (bone sialoprotein). The so-called $2^{-\Delta\Delta CT}$ method was used to evaluate the differences in gene expression between uncoated (PRP min) and PRP-coated samples (PRP plus). All Ct values were normalized to the C_T values of the housekeeping gene GAPDH. Second, the ΔC_T values of PRP were normalized to the ΔC_T values of the control group, the uncoated samples. Finally, the $2^{-\Delta\Delta CT}$ values of the control group were called 100% while the $2^{-\Delta\Delta CT}$ values of the PRP-coated groups were multiplied by 100%.

According to Marx *et al.*,[1] human PRP is an autologous concentration of human platelets above baseline in a small volume of plasma. Normal platelet counts in human blood range between 150×10^6/mL and 350×10^6/mL with an average of about 200×10^6/mL. A concentration of 1000×10^6/mL in a 5-mL volume of plasma could be the working definition of human PRP. Platelet concentrations of less than 1000×10^6/mL were not reliable to enhance wound healing, whereas higher concentrations did not show further enhancement of wound healing.[19] Weibrich *et al.*[20] examined the platelet concentration among different human donors and discovered that the platelet concentration of the human PRP, prepared in a blood bank, correlated with the platelet count in the donor whole blood. They found also a significant influence of gender on platelet concentration, but no influence of age. On the other hand, Paques *et al.*[21] demonstrated that the concentrations of the GFs present in human platelet lysates were not related to donor origin. Regarding the required platelet concentration for PRP efficiency, Haynesworth *et al.*[22] showed that the proliferation and differentiation of adult mesenchymal stem cells were directly related to the platelet concentration. They showed a dose–response curve indicating that a sufficient cellular response to platelet concentrations first began when a 4- to 5-fold increase over baseline platelet numbers was achieved. Platelet concentrations ranged from 800 to 1200×10^6 platelets/mL have been proposed to be effective by Weibrich *et al.* and Fennis *et al.*[23,24] As most individuals have a baseline blood platelet count of $200\pm75\times10^6$/mL, a platelet count of 1000×10^6/mL as measured in a volume of 5 mL plasma can be the "therapeutic dose" of PRP.[2,25] The PRP formulations that were used in our study had a 3-fold increase for rat and goat and a 6-fold increase for human PRP; that is, rat PRP had a concentration of 3000×10^6/mL (whole blood 1000×10^6/mL), goat PRP had a concentration of 1200×10^6/mL (whole blood 400×10^6/mL) and human PRP had a concentration of 1200×10^6/mL (whole blood 200×10^6/mL).

The present study revealed that PRP had a stimulating effect on the initial cell growth and matrix mineralization of rat bone marrow cells. This is in agreement with Lucarelli *et al.*[7] and Weibrich *et al.,*[5] who also observed increased cell growth and differentiation with osteoblast-like cells and stromal cells. Soffer *et al.*[26] observed in a study with fetal rat calvarial cells that short-term exposure (24 h) of PRP to the cells stimulated the growth and differentiation of these cells. However, a long-term exposure resulted in a decrease in both ALP activity and mineral formation. Choi *et al.*[27] and Arpornmaeklong *et al.*[28] showed in their study that the viability and proliferation of alveolar bone cells obtained from mongrel dogs and rat bone marrow cells were suppressed by high PRP concentrations, but were stimulated by low PRP concentrations. Arpornmaeklong *et al.*[28] observed also that the cell differentiation of rat bone marrow cells was suppressed with a high concentration of PRP. In the present study, the well plates were coated with a PRP gel. Therefore, the cells probably experienced a short-term exposure of GFs. As known from other studies the life span of a platelet in a wound and the period of direct influence of its GFs is less than 5 days in *in vivo* situations.[29] After the initial burst of GFs, the platelets synthesize and secrete additional GFs for the remaining days of their life span. However, it can be questioned whether this same GF release pattern will occur in an *in vitro* situation. Tsay *et al.*[30] showed in their *in vitro* study that PRP gels had a burst release of 80% of PDGF-AB and 82% of TGF-β1 in the medium after 24 h and a sustained release for another 14 days.

Additional studies revealed that PRP stimulated the mitogenic response of bone cells derived from human trabecular[8] and rat bone marrow.[9] Yet, it is still unknown whether the stimulatory effects of PRP is related to the GFs present or to other microparticles from the cytoplasm or cell membrane that are released from activated platelets.[8] However, our ELISA results and also Soffer *et al.*[26] showed that appropriate amounts of GFs can be detected. Previous *in vitro* studies with single GFs, such as TGF-β1, showed already that concentrations of 5–10 ng/mL in rat bone marrow cell cultures result in increased cell proliferation with no effect on cell differentiation.[31] Other authors reported that the single GFs PDGF-AA and BB enhanced cell proliferation of bone cells (10 ng/mL)[10,32,33] Antibodies against PDGF appeared to decrease the mitogenic activity of human bone cells with PRP.[8]

Real-time PCR results showed a downregulation for the ALP gene when PRP was used as the coating. This is in keeping with our ALP activity results, which showed a decrease in activity with PRP, while more calcium content was measured with PRP-coated samples. In accordance with the calcium results, the PCR results showed an upregulation for the genes osteocalcin (as a marker for the osteoblast phenotype) and collagen type I (as a marker for matrix mineralization).

The present study also contains some limitations. Rat PRP was obtained from 6 rats and was used for rat bone marrow cell culture and GF quantification, but the human and goat PRP samples for GF quantification were taken from 1 patient or 1 animal. Therefore, we cannot generalize the results obtained from GF quantification because of the large intraspecies variations.

CONCLUSION

On the basis of our results, we conclude that PRP contains osteo-inductive GFs, which are probably species related. However, we cannot generalize the results because of the large intraspecies variations. Further, we conclude that rat PRP gel stimulates the initial growth and differentiation of rat bone marrow cells *in vitro*. Evidently, PRP has the potential to be an osteoinductive factor for GF-based tissue engineering.

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