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The *in vitro* effect of different PRP concentrations on osteoblasts and fibroblasts

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Abstract

Objectives: The aim of this study was to assess the biological rationale for the use of platelet-rich plasma (PRP) by evaluating the effect of different concentrations of PRP on osteoblasts (OB) and fibroblasts (FB) function *in vitro*.

Material and methods: PRP was obtained from volunteer donors using standard protocols. Primary human cultures of oral FBs and OBs were exposed to both activated and non-activated plasma as well as various concentrations of PRP (2.5 ×, 3.5 × and max (4.2–5.5 ×)). Cell proliferation was evaluated after 24 and 72 h using an MTT proliferation assay. Production of osteocalcin (OCN), osteoprotegerin (OPG) and transforming growth factor β1 (TGF-β1) was evaluated in OB after 24 and 72 h. Statistical analysis was performed using one-way ANOVA.

Results: PRP-stimulated cell proliferation in both OBs and FBs. The effect of different PRP concentrations on cell proliferation was most notable at 72 h. The maximum effect was achieved with a concentration of 2.5 ×, with higher concentrations resulting in a reduction of cell proliferation. Upregulation of OCN levels and downregulation of OPG levels were noted with increasing PRP concentrations at both 24 and 72 h. TGF-β1 levels were stimulated by increasing concentrations of PRP, with the increased levels being maintained at 72 h.

Conclusions: PRP preparations exert a dose-specific effect on oral FBs and OBs. Optimal results were observed at a platelet concentration of 2.5 ×, which was approximately half of the maximal concentrate that could be obtained. Increased concentrations resulted in a reduction in proliferation and a suboptimal effect on OB function. Hence, different PRP concentrations may have an impact on the results that can be obtained *in vivo*.

At a cellular level, wound healing and regeneration involves a complex cascade of events, including cell proliferation and differentiation. These processes are known to be modulated by growth factors. Consequently, over the past two decades, the potential use of growth factors in bone and periodontal regeneration has been investigated and in principle viability has been demonstrated (Cochran & Wozney 1999).

One way of delivering concentrated amounts of growth factors to the wound site is via the use of platelet-rich plasma (PRP). PRP is a concentration of human platelets in a small volume of plasma. It contains concentrated amounts of the seven growth factors shown to be actively

secreted by platelets – the three isomers of platelet-derived growth factor (PDGF-AA, PDGF-BB and PDGF-AB), two forms of transforming growth factor β (TGF-β1 and TGF-β2), vascular endothelial growth factor and epithelial growth factor. Initially, it was reported that PRP has a positive effect on bone regeneration in mandibular defect reconstruction (Marx et al. 1998). Subsequently, the use of PRP has been investigated in several studies, with some reporting success in promoting regeneration (Kassolis et al. 2000; Mazor et al. 2004), while others found no additional benefit (Aghaloo et al. 2002; Froum et al. 2002). Therefore, the clinical usefulness of PRP remains controversial (Sanchez et al. 2003; Tozum & Demiralp 2003; Marx 2004). In particular, the methods of preparation of PRP and consequently, the concentration of growth factors that are achieved are variables that may explain the different clinical results that are obtained.

Although individual, purified growth factors have been extensively studied *in vitro* (for a review, see Cochran & Wozney 1999) prior to use *in vivo*, PRP has only recently started to be assessed in a similar manner. However, studies investigating the effect of PRP on cell function *in vitro* have utilized a wide variety of cell types and have obtained conflicting results (Liu et al. 2002; Lucarelli et al. 2003; Gruber et al. 2004; Kilian et al. 2004; Soffer et al. 2004; Kanno et al. 2005). In particular, it is still uncertain which concentrations of PRP are optimal in promoting enhanced 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 occur during wound healing and regeneration. The aim of this study was to assess the effect of different concentrations of PRP on the proliferation and differentiation of human oral fibroblasts (FB) and osteoblasts (OB) *in vitro*. A controlled *in vitro* system was used in order to provide data that will assist in the future design of clinical studies utilizing PRP.

Material and methods

PRP preparation

PRP was prepared from venous blood obtained from three volunteer donors (age range 24–29 years) at the Department of Hematology, University of Pisa by a standard aphaeresis procedure using an MCS$^+$ (Haemonetics, Briantree, MA, USA) device and acid citric dextrose (ACD-A; 22 g/l sodium citrate, 24.5 g/l glucose monohydrate and 8 g/l citric acid monohydrate) at a ratio of 1 : 10. The donors' platelet counts in venous blood were 185,000–268,000 platelets/μl. The platelets were automatically leucodepleted by negative-charged pall filter.

Different platelet concentrations were obtained relative to the concentrated platelet count (range 800,000–1,375,000 platelets/μl, representing an increase of 420–550% over the baseline venous count) by diluting with Dulbecco's modification of Eagle's medium (DMEM) (Gibco Inc., Rockville, MD, USA). Platelet concentrations of 250% (PRP-2.5 ×), 350% (PRP-3.5 ×) and 420–550% (PRP-max) of the normal venous blood counts were achieved.

Cell culture

Human OB and gingival FB were obtained from a 57-years-old woman undergoing molar tooth extraction.

OBs cells were obtained according to the technique described by Mailhot & Borke (1998). Briefly, bone chips obtained during the extraction were collected in serum-free DMEM supplemented with penicillin/streptomycin (100 U/ml) (Sigma, St Louis, MO, USA) and adjusted to pH 7.4. After 30 min of digestion using 1 mg/ml of collagenase (Boehringer Mannheim, Indianapolis, IN, USA) at 37°C, the supernatant was extracted and transferred to another centrifuge tube. This treatment was repeated six times, with the first two fractions being discarded. The subsequent fractions of released cells, together with the remaining bone tissue, were pooled, centrifuged and plated onto six-well culture dishes.

Human gingival FBs were obtained according to the technique described by Specchia et al. (2001). Briefly, gingival tissue samples were minced into small pieces using microdissection scissors and washed with serum-free DMEM. The gingival tissue was then digested in serum-free medium containing 1 mg/ml of collagenase at 37°C in 5% CO_2 for 80 min. The supernatant was removed and the remaining gingival tissue was placed in six-well culture dishes, allowing cells to migrate from the explants.

Following explantation, both OB and FB cultures were maintained in DMEM supplemented with 10% feotal bovine serum and 100 U/ml of penicillin—streptomycin, and incubated in a humidified atmosphere at 37°C with 5% CO_2 until they formed monolayers. Cells were passaged when confluence was reached and utilized following the third passage.

Cell proliferation

Human OBs and FBs (1000 cells/well) were seeded subconfluently in 96-well plates in 100 μl of serum-free DMEM and allowed to attach overnight. Subsequently, the cells were grown for 24 and 72 h following the addition of 50 μl of seven different preparations: (i) DMEM only (negative control), (ii) DMEM + 10% FCS (positive control), (iii) non-activated plasma (NAP), (iv) activated plasma (PRP-1 ×), (v) PRP-2.5 ×, (vi) PRP-3.5 × and (vii) PRP-max (4.5–5.5 ×).

PRP was activated before insertion into the wells by adding 1 ml of each preparation to 50 μl of calcium gluconate and 50 μl autologous thrombin. Thereafter, 50 μl of each preparation was added to six different wells. The experiment was repeated for each of the three PRP donors.

Cell proliferation analysis was performed with the MTT™ cell proliferation kit (Roche GmbH, Mannheim, Germany), a colorimetric assay that measures the reduction of a tetrazolium component (MTT) into an insoluble formazan product by the mitochondria of viable cells. The amount of colour produced is directly proportional to the number of viable cells.

OB function

OBs were plated in 96-well plates as outlined above and were stimulated with NAP, activated plasma (PRP-1), PRP-2.5 ×, PRP-3.5 × and PRP-max. Following stimulation for 24 and 72 h, 70 μl of media was removed from the wells. The quantities of osteocalcin (OCN), osteopro-

tegerin (OPG) and TGF-β1 were then assessed.

OCN was measured using a competitive immunoassay kit (Metra™ Osteocalcin, Quidel Corporation, Santa Clara, CA, USA) according to the manufacturer's protocol and as described by Zhang et al. (2003). The antibody is conformationally dependent and recognizes only intact (*de novo*) OCN and not degraded fragments. OPG assessment was performed using a highly sensitive sandwich enzyme-linked immunosorbent assay (ELISA, Biomedica Gruppe, Wien, Austria) according to the manufacturer's protocol and as previously described (Buzi et al. 2004). The assay detects both monomeric and dimeric forms of OPG, including OPG bound to its ligand. TGF-β1 levels were also determined by a sandwich ELISA method (BioSource International, Camarillo, CA, USA) according to the manufacturer's instructions.

Statistical analysis

One-way ANOVA test was used to assess the effect of the various PRP preparations on OB and FB proliferation, as well as production of OCN, OPG and TGF-β1 by OBs. In order to identify differences between the various treatments, *post hoc* analysis was carried out using the Fisher LSD test and the Bonferroni test. Statistical differences between groups were accepted for *P*-values lower than 0.05.

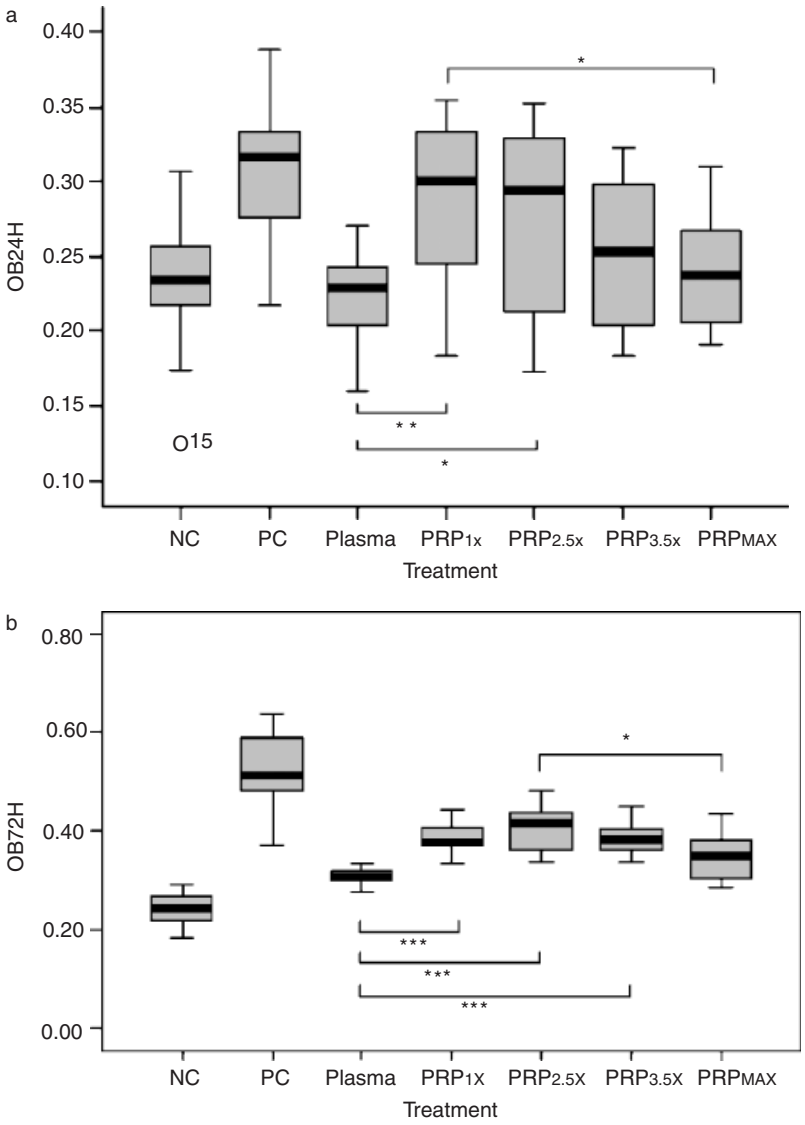

Fig. 1. (a, b) Box and whiskers plots showing the effect of PRP concentrations on osteoblasts at 24 and 72 h. Boxes refer to the 25th (bottom) and 75th (up) percentiles and the median is the horizontal line inside. Open circles represent outliers. Significant multiple comparison differences were observed between groups (*$P<0.05$, **$P<0.01$, ***$P<0.001$, Fisher LSD *adjustment*). Only differences between various platelet-rich plasma (PRP) concentration and non-activated plasma group are reported. Significant differences between positive (PC) and negative controls (NC) and the plasma/PRP groups are not reported in the graphs and explained in the text.

Results

Cell proliferation

OBs

The effects of PRP concentrations on OBs at 24 and 72 h are shown in Figs 1a and b, respectively. After 24 h, NAP did not induce a statistically significant proliferative response compared with the negative control. However, both activated plasma (PRP-1 ×) and PRP-2.5 × stimulated OB proliferation compared with NAP ($P<0.05$). The maximum OB proliferation was obtained with activated plasma (PRP-1 ×), with increasing concentrations of PRP resulting in decreased proliferation. The difference between PRP-1 × and PRP-max was statistically significant ($P<0.05$).

At 72 h, there was a statistically significant increase in OB proliferation by all preparations containing plasma (including NAP) compared with the negative control. Furthermore, PRP-1 × ($P<0.001$), PRP-2.5 × ($P<0.001$) and PRP-3.5 × ($P<0.001$) induced a statistically significant increase in proliferation compared with the NAP. The highest cell proliferation was obtained with PRP-2.5 ×, with a 70% increase in cell numbers at this concentration compared with the negative control. Furthermore, the increase of proliferation induced by PRP-2.5 × was statistically higher than PRP-max ($P<0.001$).

FBs

The effects of PRP concentrations on FBs at 24 and 72 h are shown in Figs 2a and b, respectively.

After 24 h, there was no statistical difference in viable cell numbers between NAP and the negative control. Compared with NAP, statistically significant induction of proliferation was noted with PRP-1 × ($P<0.001$) and PRP-2.5 × ($P<0.05$). Maximum FBs proliferation was obtained with PRP-1 × (Fig. 2a), but no statistical differences were noted

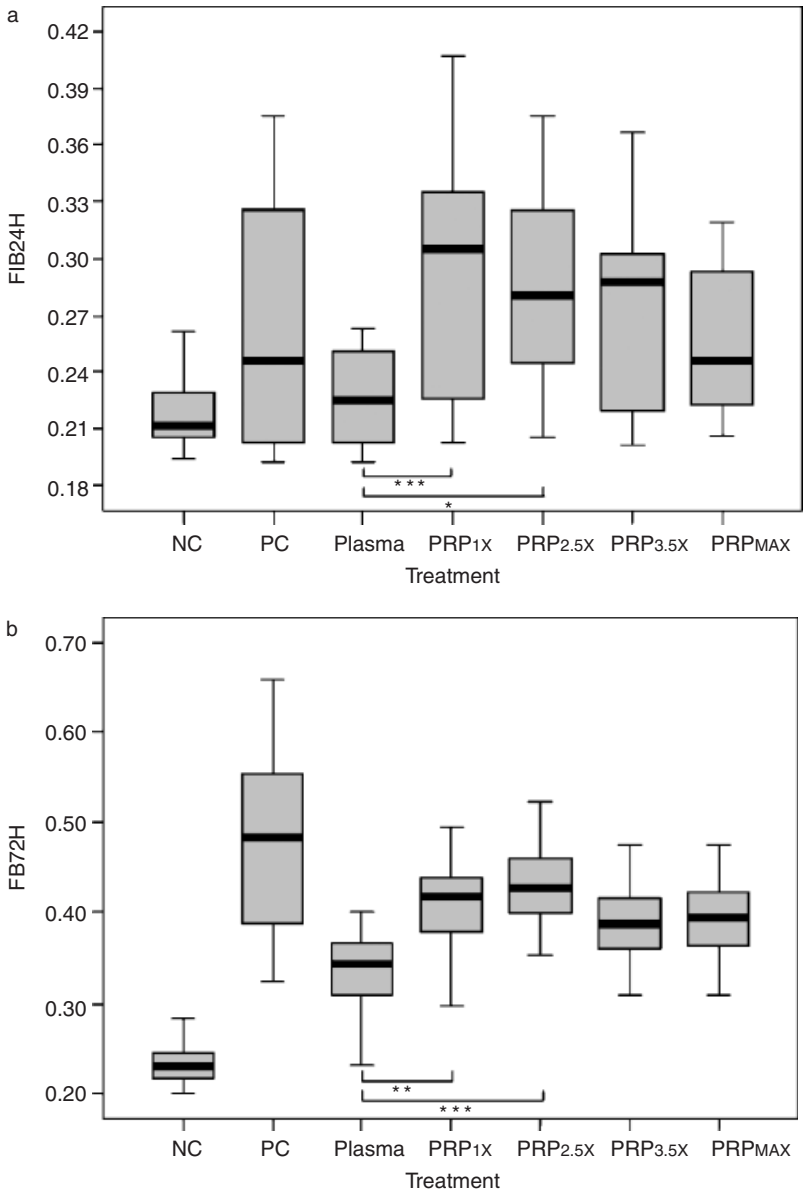

Fig. 2. (a, b) Box and whiskers plots showing the effect of platelet-rich plasma (PRP) concentrations on fibroblasts at 24 and 72 h. Significant multiple comparison differences were observed between groups (*$P<0.05$, **$P<0.01$, ***$P<0.001$, Fisher LSD *adjustment*). Only differences between various PRP concentrations and non-activated plasma group are reported. Positive (PC) and negative (NC) control that showed the significant differences are reported in the text.

between the various concentrations of PRP (Fig. 2a).

At 72 h, there was a significant increase in FB cell proliferation in NAP compared with the negative control ($P<0.001$). Furthermore, both PRP-1 × ($P<0.05$) and PRP-2.5 × ($P<0.001$) demonstrated statistically significant increases in proliferation compared with the NAP. The highest induction in proliferation was noted with PRP-2.5 × with an 85% increase in viable cell numbers compared with the negative control. However, no statistical significantly differences were detected between the various PRP concentrations.

OB function

Functional aspects of OB activity were assessed by measuring the production of three secreted proteins that are important to the regulatory role of OBs during regeneration – the extracellular matrix molecule OCN, the soluble receptor osteoprotegrin and the growth factor TGF-β1. OB function was evaluated following the addition of non-activated PRP, PRP-1 × , PRP-2.5 × , PRP-3.5 × and PRP-max.

The effect of the different concentrations of PRP on OCN and OPG levels is shown in Figs 3 and 4, respectively. At 24 h, there was a statistically significant ($P<0.05$) increase in OCN levels following treatment with PRP-max compared with both inactivated and activated (PRP-1 ×) plasma. Following 72 h of exposure to the various concentrations of PRP, statistically significant upregulation of OCN was observed with PRP-3.5 × and PRP-max compared with NAP, PRP-1 × and PRP-2.5 × ($P<0.05$). There was a generalized decrease in OCN levels between 24 and 72 h, especially following exposure to the lower concentrations of PRP (Figs 3a and b).

OPG levels were downregulated following exposure to increasing concentrations of PRP. At 24 h, both PRP-max and PRP-3.5 × induced statistically significant ($P<0.05$) decreases in OPG levels compared with non-activated and activated plasma (PRP-1). At 72 h, there was a statistically significant decrease in OPG levels for PRP-max compared with NAP, PRP-1 × and PRP-2.5 × ($P<0.05$). Interestingly, a large increase in OPG levels was noted between 24 and 72 h (Figs 4a and b).

TGF-β1 levels were not greatly affected following exposure to the various PRP concentrations (Fig. 5). There was a general trend towards increased levels of TGF-β1 following exposure to increased concentrations of PRP. Indeed, there was a statistically significant increase of TGF-β1 levels in PRP-max compared with PRP-1 × at 24 h. No statistical differences between the groups were noted following 72 h.

Discussion

In recent years, the use of PRP has gained considerable popularity for the purpose of delivering growth factors to the wound-healing site in order to promote regeneration. Because PRP is prepared in the dental surgery from autogenous blood, it confers several advantages over other products and techniques, most notably in terms of simplicity, safety and cost-effectiveness. However, because of these favourable characteristics, the use of PRP has been perceived as a therapy, which, at the very

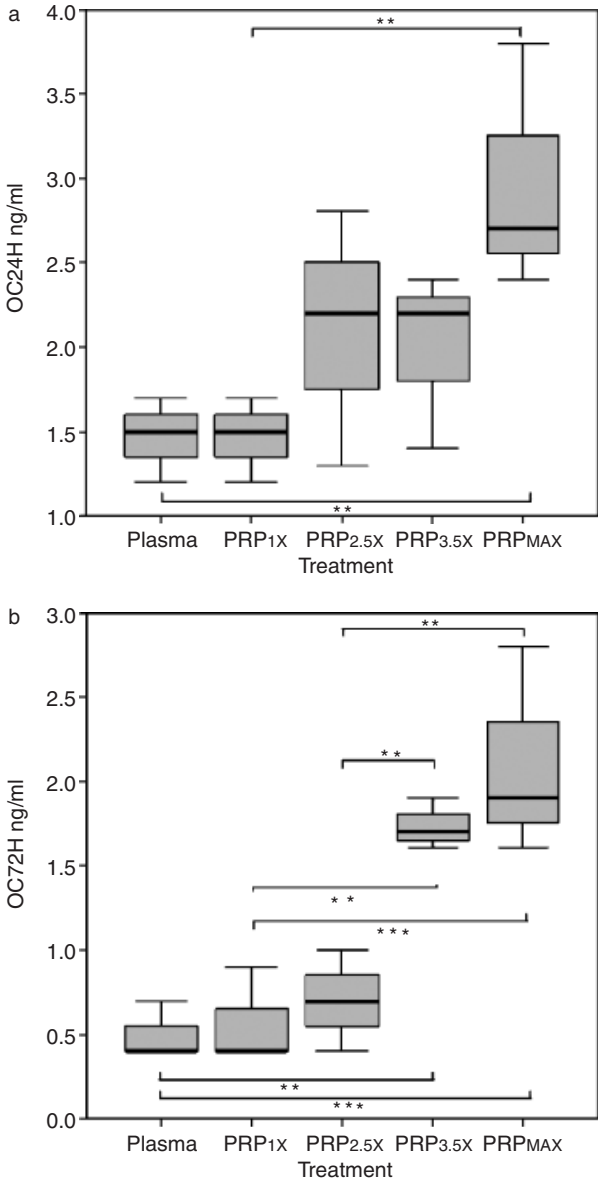

Fig. 3. (a, b) Effect of the different concentrations of platelet-rich plasma (PRP) on osteocalcin (OCN) levels produced by osteoblasts at 24 and 72 h, respectively.

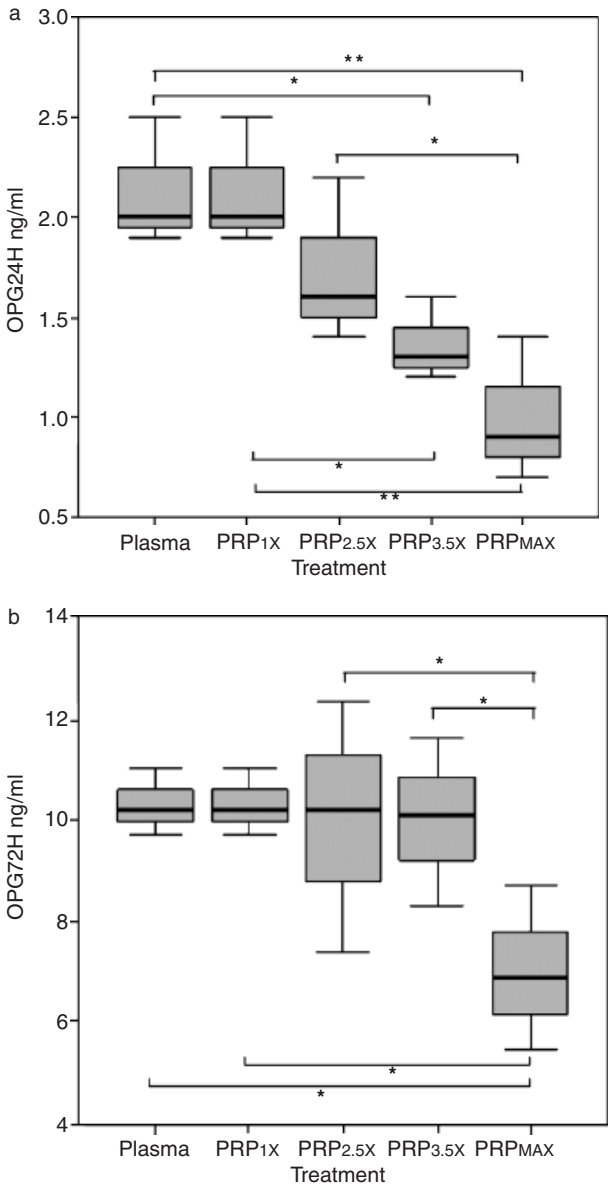

Fig. 4. (a, b) Effect of the different concentrations of platelet-rich plasma (PRP) on osteoprotegerin (OPG) levels produced by osteoblasts at 24 and 72 h, respectively.

worst, will not negatively affect the clinical outcome. Consequently, the use of PRP has not been placed under as much scrutiny as other techniques, especially those utilizing exogenous growth factors. Although the biological rationale for the use of PRP is theoretically sound, the precise methodology that will result in the optimal benefit from this therapy is yet to be elucidated. In particular, the precise PRP concentrations that would be ideal are yet to be determined. This study was designed to test the effect of varying the concentrations of PRP on the function of primary human OBs and FBs during the early phases of the healing process (days 1–3).

This study investigated the effect of PRP on the proliferation of OBs and FBs, the two cell types critical for wound healing of soft tissues and bone. Furthermore, as PRP has been used extensively for bone regeneration, we investigated the effect of different PRP concentrations on osteoblast function. Although a variety of different animal- and human-derived cell lines have been used to investigate responses to PRP *in vitro*, this study utilized primary OBs and FBs derived from oral sites, as these cells would normally be involved in wound healing in the oral region.

The findings of this study indicate that PRP stimulates OB and FB proliferation, which is consistent with findings reported by a number of other studies investigating the effect of PRP on the proliferation of a variety of cell types, including AG1518 FBs (Liu et al. 2002), rat calvarial bone cells (Soffer et al. 2004), human mesenchymal progenitor cells (Lucarelli et al. 2003; Gruber et al. 2004), endothelial cells (Kilian et al. 2004), primary periodontal ligament cells (Okuda et al. 2003), com-

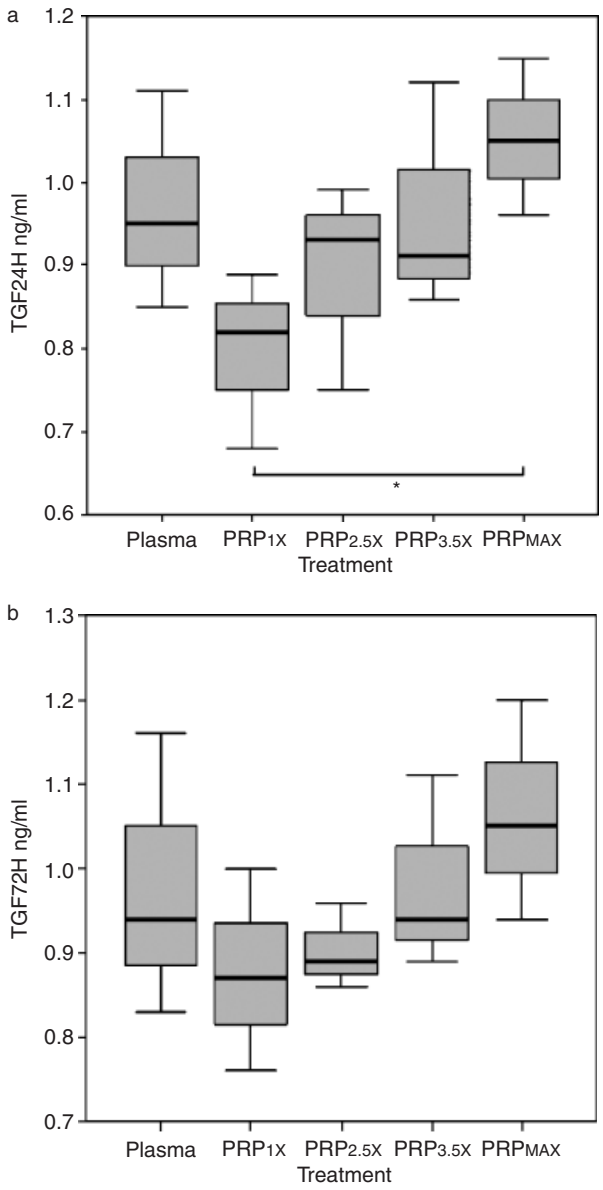

Fig. 5. (a, b) TGF-β1 levels after application of different platelet-rich plasma (PRP) concentrations were not greatly affected after 24 and 72 h.

mercially available human gingival FB (Gin-1), OB MG63 and rat OB UMR106 cell lines (Okuda et al. 2003) and human osteosarcoma cell lines HOS and SaOS-2 (Kanno et al. 2005). Indeed, some recent studies have shown a PRP dose-dependent proliferation increase in the cells under investigation (Lucarelli et al. 2003; Gruber et al. 2004; Kilian et al. 2004; Soffer et al. 2004), with increasing PRP concentrations resulting in enhanced cell proliferation. However, these studies varied greatly in the methodology that was utilized, including length of observation, method of PRP preparation, composition of the PRP preparations (co-incubated with 1–2% FCS) and the type of cells used (mesenchymal stem cells, rat OBs).

In the present study, although the effect of PRP on proliferation was dose dependent, increasing PRP concentrations did not result in increasing proliferation. As cell proliferation is a critical event during early wound healing, it appears that the maximal PRP concentrations used in this study, which varied between a 4.2- and 5.5-fold increase over the original platelet concentration, may not provide the optimal environment for the promotion of wound healing. Our results correlated with the findings of Liu et al. (2002), who investigated the effect of different platelet concentrations on FBs. They prepared maximally concentrated platelet preparations and diluted them in media to final concentrations of 8.8%, 17.5% and 35%. They found that superior proliferation was obtained with the 8.8% and 17.5% preparations compared with the 35% concentration. Correlation can be made with our study where the PRP-max proliferation was obtained by adding 50 μl of the maximal platelet concentrate to 100 μl of media, giving a final concentration of 33%. This concentration results in inferior proliferation compared with PRP-2.5 ×, which is approximately half of the PRP-max concentration (i.e. 16.5%). Liu et al. (2002) suggested that the inferior proliferation was pH dependent, and that high PRP concentrations resulted in pH changes that negatively affected proliferation.

Aside from proliferation, this study also examined OB function by assessing the production of OCN, OPG and TGF-β1 by OBs following exposure to different PRP concentrations. OCN is a bone-specific non-collagenous matrix protein that regulates mineralization (Boskey et al. 1998) and plays a significant role in the differentiation of osteoclast progenitors (Webber et al. 1990) and therefore enhances bone remodelling activity. OPG is a member of the TNF receptor superfamily and is unique in that it lacks transmembrane and cytoplasmic domains and is secreted by osteoblastic cells as a soluble protein (Simonet et al. 1997). OPG acts as a decoy receptor by binding the receptor activator of nuclear factors κB (RANKL) and preventing RANKL-induced osteoclastic bone resorption (Lacey et al. 1998). TGF-β1 is a growth factor that promotes osteogenic differentiation and osteoclastogenesis (Yan et al. 2001; Koseki et al. 2002; Wildemann et al. 2004). It has been suggested that the effect of PRPs on cells is cell type-specific and mediated via TGF-β1 (Okuda et al. 2003). Interestingly, there have been different reports regarding the osteogenic differentiation potential of PRPs, with some studies indicating stimulation (Kilian et al. 2004) and others showing inhibition (Arpornmaeklong et al. 2004; Gruber et al. 2004).

In our study, increasing PRP concentrations led to increased production of OCN and decreased production of OPG by

the OBs. The increase in OCN activity suggests that the OBs are undergoing differentiation. Additionally, the decrease in OPG and the increase in OCN are indicative of increased osteoclastic activity. Furthermore, it was noted that TGF-β1 was increased in PRP-max, which correlates with the OCN and OPG findings and suggests that, during the early phases of healing, PRP-max stimulates osteoclastogenesis and OB differentiation.

Although both proliferation and differentiation are important for bone regeneration, these two processes cannot occur in the cell at the same time. Our findings indicate that high concentrations of platelets may lead to an increase in OB differentiation at the cost of proliferation. This may lead to suboptimal clinical results because cell proliferation is essential in the early phases of regenerative wound healing and has to occur before differentiation is initiated. Our findings are supported by Weibrich et al. (2004), who histologically evaluated the *in vivo* effect of different concentrations of platelets on the bone regeneration around implants placed in femurs of rabbits. 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 basal platelet count) appeared to have an inhibitory effect on healing,

In summary, it can be concluded that PRP is an effective stimulator of OB and FB proliferation, as well as OB function. Furthermore, these data indicate that a moderate increase of the normal platelet count provided the most optimal environment for wound healing through a more ideal balance between proliferation and differentiation. These findings suggest that future clinical studies should be designed with the understanding that PRP concentrations may affect clinical outcome.

要旨

目的：本研究の目的は、様々な濃度の多血小板血漿（PRP）が骨芽細胞と線維芽細胞の機能に及ぼす影響を in vitro で測定し、PRP 使用の生物学的根拠を評価することであった。

材料と方法：標準プロトコールを用いてボランティア・ドナーから PRP を得た。ヒト初代培養の口腔線維芽細胞（FB）と骨芽細胞（OB）を、活性血漿と非活性血漿、及び様々な濃度のＰＲＰ（２．５Ｘ，３．５Ｘ、最大４．２－５．５Ｘ）に暴露した。ＭＴＴ増殖アッセイを用いて、細胞増殖を２４時間及び７２時間後に評価した。ＯＢ中でオステオカルシン（ＯＣＮ）、オステオプロテジェリン（ＯＰＧ）及びＴＧＦ－β１の産生を２４時間後と７２時間後に評価した。統計学的分析を片側ＡＮＯＶＡによって行った。

結果：ＰＲＰは骨芽細胞と線維芽細胞の両方で細胞増殖を刺激した。様々な濃度のＰＲＰは７２時間後に細胞増殖に最も顕著な影響を及ぼした。２．５倍の濃度で最大の効果が得られ、それ以上の高濃度では細胞増殖は減少した。ＰＲＰ濃度の増加に伴い、ＯＣＮレベルのアップレギュレーションとＯＰＧレベルのダウンレギュレーションが、２４時間、７２時間の両時点で顕著に認められた。ＴＧＦ－β１のレベルはＰＲＰの濃度増加によって刺激を受け、７２時間まで増加したレベルが続いた。

結論：ＰＲＰ製剤は用量特異的な作用を口腔の線維芽細胞と骨芽細胞に及ぼした。２．５倍の血小板濃度で最適な結果が得られたが、これは獲得可能な最大濃度のほぼ半分であった。濃度の増加は、増殖の減少と骨芽細胞の機能低下をもたらした。従って異なるＰＲＰの濃度は in vivo での結果に影響を及ぼすと思われる。

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