Linköping University Post Print

Variation of pH in lysed platelet concentrates influence proliferation and alkaline phosphatase activity in human osteoblast-like cells.

Ola Wahlström, Cecilia Linder, Anders Kalén and Per Magnusson

N.B.: When citing this work, cite the original article.

This is an electronic version of an article published in:

Ola Wahlström, Cecilia Linder, Anders Kalén and Per Magnusson, Variation of pH in lysed platelet concentrates influence proliferation and alkaline phosphatase activity in human osteoblast-like cells., 2007, Platelets, (18), 2, 113-118.

Platelets is available online at informaworldTM:

<http://dx.doi.org/10.1080/09537100600800537>

Copyright: Taylor & Francis

<http://www.tandf.co.uk/journals/default.asp>

Postprint available at: Linköping University Electronic Press

<http://urn.kb.se/resolve?urn=urn:nbn:se:liu:diva-28264>

Variation of pH in lysed platelet concentrates influence proliferation and alkaline phosphatase activity in human osteoblast-like cells

**Ola Wahlström, MD, PhD¹, Cecilia Linder, MSc²,
Anders Kalén, MD, PhD¹, Per Magnusson, PhD^{2,3}**

¹ Division of Orthopaedics, Department of Neuroscience and Locomotion, Faculty of Health Sciences, SE-581 85 Linköping, Sweden

² Division of Clinical Chemistry, Department of Laboratory Medicine, Linköping University Hospital, SE-581 85 Linköping, Sweden

³ Bone and Mineral Metabolic Unit, Division of Clinical Chemistry, Department of Biomedicine and Surgery, Faculty of Health Sciences, SE-581 85 Linköping, Sweden

Running title: pH of platelet concentrate and growth stimulation

Keywords: *Growth factors, cell proliferation, differentiation, growth stimulation*

Corresponding author: Ola Wahlström, MD, PhD¹
Tel: +46-13-22 41 81
Fax: +46-13-22 43 05
E-mail: olawa@inr.liu.se

ABBREVIATIONS

ALP	Alkaline phosphatase
ELISA	Enzyme-linked immunosorbent assay
LPB	Lysed platelets in buffer
PRP	Platelet-rich plasma
PBS	Phosphate-buffered saline
PDGF	Platelet-derived growth factor
TGF-β	Transforming growth factor- β
BMP-2	Bone morphogenetic protein 2

ABSTRACT

Activated platelets release a multifaceted blend of growth factors that has stimulatory effects on mesenchymal cells, both in vitro and in vivo, which imply beneficial effects on wound repair and tissue regeneration. Previous studies on fibroblast cultures have revealed that more potent growth factors, with respect to cell proliferation, are released in acidic preparations of lysed platelet concentrates in comparison with neutral and alkaline preparations. The current study was intended to investigate the influence of pH on lysed platelet concentrates with respect to release of growth factors, cell proliferation and alkaline phosphatase (ALP) activity in human osteoblast-like cells (hFOB 1.19). Cell proliferation was assessed with the MTT kit, ALP activity by conventional enzymatic reaction kinetics and growth factors platelet-derived growth factor (PDGF), transforming growth factor- β (TGF- β) and BMP-2 by enzyme-linked immunosorbent assays. Osteoblast-like cells were stimulated with lysed platelet concentrates preincubated at pH 4.4, 5.4, 7.4, and 7.6. A 3- to 13-fold increase of cell proliferation was found in comparison with controls and the most evident increase was observed with platelets activated at pH 5.4. The highest ALP activity was observed in preparations at pH 7.6. Platelets incubated in an acidic environment (pH 5.4) induced a higher proliferation compared with preincubation at neutral or alkaline pH and the level of PDGF was also found to be higher in acidic preincubations. The level of TGF- β was, in contrast, lowest at pH 4.4.

Interestingly, we also found BMP-2 in platelet lysate. However BMP-2 was only present in LPB pH 4.0. We suggest, based on these experimental findings, that acidic milieu influence platelets to release growth factors more potent to stimulate osteoblast proliferation than neutral and alkaline platelet preparations. Lysed platelet concentrates prepared at an alkaline pH might release additional components with stimulating effects

resulting in other features than cell proliferation. This is the first report, to our knowledge, about the presence of BMP-2 in platelet lysate and about a pH dependent stimulatory effect of lysed platelet concentrates on human osteoblast-like cell proliferation. Lysed platelet concentrates, preincubated in acidic or alkaline buffers, may benefit fracture healing, implant fixation and might also be advantageous in the treatment of wounds with platelet constituents; however, this has to be investigated in extended experimental and clinical settings.

INTRODUCTION

Growth factors, released from activated platelets, have a significant impact on the regulation and proliferation of mesenchymal cells during the wound healing process (1-3). Platelets, platelet-rich plasma (PRP), platelet lysates, platelet-derived growth factor (PDGF), and supernatants from activated platelets all have well-known stimulatory effects on mesenchymal cells in vitro (4-10) and in vivo (11). The clinical potential of platelet constituents is interesting and several clinical trials and observations on wound healing and bone formation have been published (12-18). Previously, it has also been described that mineralization and bone repair is pH dependent. The early acidity within fracture hematomas is later followed by a change in pH towards alkalinity (19-24). A low pH has also been reported during the early phase of wound healing, hence designated the acid tide (25). Previously, Liu et al. (26) reported that the media pH of platelet lysates influence the release of biologically active substance from platelets. Lysed platelet concentrates derived from an acidic environment had higher levels of PDGF in comparison with lysates prepared in neutral or alkaline pH buffers. Moreover, if the platelet lysate was buffered in acidic pH (pH 5.0) and added to fibroblast cell cultures, the fibroblast proliferation was significantly increased compared to lysates at a higher pH (pH 7.1 and 7.6).

The impact of lysed platelet concentrates prepared in buffers at different pH on osteoblasts has previously not been reported. The aim of this study was to evaluate cell proliferation and alkaline phosphatase (ALP) activity of human osteoblast-like cells, hFOB 1.19, stimulated with platelet concentrates lysed at pH 4.4, 5.4, 7.4 and 7.6. Furthermore, the concentrations of PDGF and transforming growth factor- β (TGF- β) were determined in platelet lysates incubated at different pH (pH 4.4, 5.4 and 7.4).

Furthermore, the concentration of PDGF, TGF- β , VEGF and BMP-2 were determined in platelet lysate incubated at different pH (pH 4.0, pH 5.0, pH 6.0, pH 7.0, pH 8.0, pH 9.0)

Platelets contain hundreds of proteins and peptides with potential to participate in wound healing/fracture healing process. Some of them are identified as important contributors in healing processes, others are still to be recognized.

The release of growth factors from the platelets may vary with the mode of activation, degree of platelet lysis and the pH of the media.

The pH of wound fluid and fracture hematoma is acidic during the initial inflammatory stage of healing, and become neutral and alkaline as the process continues to a more mature stage of healing.

The release of PDGF and TGF- β from platelets lysed by freezing and thawing have been studied previously and PDGF was higher in acidic lysate than in neutral and the proliferation of fibroblasts were increased when stimulated with platelet lysate buffered in acidic media.

Figure staplar från arbete 1. Dels proliferation, dels PDGF.

In this study we focus on the proliferation and ALP secretion of osteoblast-like cells stimulated with platelet lysate buffered in acidic or neutral pH.

The questions were:

- 1. Does pH of the platelet lysate (LPB) change the stimulatory impact on the proliferation of osteoblastlike cells ?**
- 2. Will the cells keep their differentiation, measured as secretion of ALP?**

Methods: PRP from blood donors were freezed to -70 and then thawed in water bath 37 ° C. **Incubation over night in acidic or neutral/alkaline buffer.** (Platelets were rapidly thawed in a water bath at 37°C and pH was adjusted to 4.4, 5.4, 7.4 and 7.6 by dilution 1:1 with four different buffers: 1) 0.2 M sodium acetate, pH 4.0; 2) 0.2 M sodium acetate, pH 5.0; 3) phosphate-buffered saline (PBS), pH 7.4; and 4) PBS, pH 8.0. The lysed platelets were incubated overnight at 37°C and subsequently diluted 1:1 with 4% bovine serum albumin in PBS. The incubated lysed platelets were termed lysed platelets in buffer (LPB). All LPBs were sterilized through 0.22 μ m syringe filters (Sartorius AG, Goettingen, Germany). Sparsely growing human osteoblast-like cells (hFOB 1.19) were exposed to LPBs at final concentrations of 9% and 18% in serum-free conditions based on previous work (26).

Osteoblast-like cells med referens(hFOB 1.19)

MATERIALS AND METHODS

Preparation of lysed platelets in buffer (LPB)

Platelet concentrates ($0.8 - 1 \times 10^{12}$ cells/l), free of leucocyte components, were prepared from healthy blood donors from the Blood Center at Linköping University Hospital, Sweden, by standardised and certified procedures, and stored at -70°C prior to use. Platelets were rapidly thawed in a water bath at 37°C and pH was adjusted to 4.4, 5.4, 7.4 and 7.6 by dilution 1:1 with four different buffers: 1) 0.2 M sodium acetate, pH 4.0; 2) 0.2 M sodium acetate, pH 5.0; 3) phosphate-buffered saline (PBS), pH 7.4; and 4) PBS, pH 8.0. The lysed platelets were incubated overnight at 37°C and subsequently diluted 1:1 with 4% bovine serum albumin in PBS. The incubated lysed platelets were termed lysed platelets in buffer (LPB). All LPBs were sterilized through $0.22\ \mu\text{m}$ syringe filters (Sartorius AG, Goettingen, Germany). Sparsely growing human osteoblast-like cells (hFOB 1.19) were exposed to LPBs at final concentrations of 9% and 18% in serum-free conditions based on previous work (26).

Cell culture

Human osteoblast-like cells, hFOB 1.19, from the American Type Culture Collection (Manassas, VA, USA) were used throughout the current study. These cells provide a homogeneous and rapidly proliferating model system for studying normal human osteoblast differentiation, osteoblast physiology, and hormonal, growth factor, and other cytokine effects on osteoblast function and differentiation (27). The hFOB cells were grown in a 1:1 mixture of Ham's F12 medium and Dulbecco's modified Eagle's medium (without phenol red) supplemented with 10% fetal bovine serum, 2.5 mM L-glutamine, 0.3 mg/ml of neomycin G418 (Sigma Chemical Co., St. Louis, MO, USA)

and incubated at 34°C with 95% humidity and 5% CO₂. Cells were plated out in 96-well microtiter plates at an initial density of 12×10^4 cells/ml (that is, approximately 35% confluence), in a total volume of 100 µl/well and medium was changed after 24 hours to serum-free conditions. Medium was removed after an additional incubation of 24 hours and replaced with medium including 9% or 18% of the four different preparations of LPB (i.e., at pH 4.4, 5.4, 7.4 and 7.6). The hFOB cells were grown for 24 hours before assessment of cell proliferation and ALP activity. Identical culture conditions (including 4% bovine serum albumin) was used for the hFOB control cells except that the platelet concentrate was replaced by 0.9% NaCl.

Assessment of cell proliferation

Analysis of cell proliferation was performed with the MTT cell proliferation kit (Roche Diagnostics GmbH, Mannheim, Germany) that measures the metabolic activity of viable cells. The number of cells was determined from a standard curve in which the absorbance measured by the MTT assay is directly correlated to the number of hFOB cells in each well. The increased number of cells was verified by phase contrast microscopy. The option of using [³H]-thymidine incorporation as a measurement of cell proliferation could be considered, however, our previous study (26) demonstrated that it was unsuitable in this experimental setting.

Measurement of ALP activity

ALP activities were determined in 96-well microtiter plates. In brief, 50 µl serum-free cell-conditioned culture medium was added per well and mixed with substrate to a total volume of 300 µl, containing 1.0 M diethanolamine buffer (pH 9.8), 10 mM p-nitrophenyl phosphate (PNPP) (Sigma), and 1.0 mM MgCl₂. The time-dependent

increase in absorbance at 405 nm (reflecting p-nitrophenol production) was determined on a kinetic microplate reader (Model VMax, Molecular Devices Corp., Sunnyvale, CA, USA). The relation between the enzymatic activity units kat and U are 1.0 μ kat/l corresponds to 60 U/l.

Assessment of PDGF and TGF- β

LPB preparations at different pH were centrifuged for 5 min at 800 g and the content of PDGF (PDGF-AB) and TGF- β (TGF- β 1) were quantified in the supernatant by ELISA (R&D Systems, Minneapolis, MN) with a Multiscan® Spectrum (Thermo Electron Corporation, Vantaa, Finland). Both active and latent forms of TGF- β are detected with this ELISA assay according to the manufacturer.

+assessment of BMP-2

Statistical analysis

Data are presented as the median of replicates (10 per LPB and control group) and expressed as median $\pm$ SD. Student's *t*-test was used to test for differences between LPB preparations and controls, and a difference was considered significant at $p < 0.05$.

RESULTS

Cell culture medium pH was determined over a period of 12 h after addition of the various LPB preparations (data not shown). The pHs were in the range of the buffering capacity of the medium within 10 minutes except for the 9% and 18% LPB preparations at pH 4.4, where the medium remained acidic.

LPBs (9% and 18%, both at pH 4.4, 5.4, 7.4, and 7.6) were added to osteoblasts, which clearly demonstrated a pH-dependent pattern of growth stimulation. Osteoblast proliferation ceased almost completely for both the LPB preparation and for the control at pH 4.4. The 9% and 18% LPBs at pH 5.4 induced a 6- and 13-fold proliferation increase, LPBs at pH 7.4 a 3- and 7-fold proliferation increase, and LPBs at pH 7.6 a 5- and 7-fold proliferation increase, respectively, in comparison with controls. The 18% LPB preparations had higher stimulatory effects than the 9% LPBs (Figure 1).

Total ALP activity increased with LPBs prepared at pH 5.4, 7.4, and 7.6; however, the 18% LPB preparations did not significantly increase the ALP levels more than the 9% LPB preparations (Figure 2). ALP increased more in the LPB preparations at pH 7.4 and 7.6 in comparison with LPB pH 5.4. Statistically significant differences were observed, except between LPB 9% pH 5.4 versus pH 7.4. The ALP activity was low and irregular for the LPB at pH 4.4 due to decreased cell proliferation, which probably was an effect of acidification of the culture media. The ALP activities per cell demonstrated a significant increase in the cultures stimulated with LPBs at pH 7.6 and pH 7.4, in comparison with LPB at pH 5.4.

Growth factors PDGF and TGF- β were measured in the supernatants of LPB preparations at pH 4.4, 5.4 and 7.4. LPB preparations pH 4.4 and 5.4 contained the highest PDGF levels while the lowest TGF- β concentration was found in LPB pH 4.4 (Figure 3).

The determination of growth factors in pH 4.0 showed low activity of VEGF, TGF- β and high concentration of PDGF.

BMP-2 was detectable , but only in supernatant from LPB of pH 4.0.

Figure 1. LPBs in various pHs induced cell proliferation differently. Human osteoblast-like cells, hFOB 1.19, were exposed to 9% and 18% LPBs at pH 4.4, 5.4, 7.4, and 7.6. Control cells were only exposed to buffer. Values are medians $\pm$ SD, $n = 10$ for each LPB and control. Cells treated with LPBs prepared at pH 5.4, 7.4, and 7.6 showed significantly higher proliferation in comparison with control cells. Cells exposed to LPBs at pH 5.4 also showed significantly higher cell proliferation than cells exposed to LPBs at pH 7.4 and 7.6 ($p < 0.001$).

Figure 2. LPBs in various pHs induced ALP activity in osteoblast-like cells in culture. LPBs at pH 7.6 increased ALP activity more than LPBs at pH 5.4 ($p < 0.001$). Values are medians $\pm$ SD, $n = 10$ for each LPB and control. The ALP activity with LPB pH 4.4 was low and irregular due to low osteoblast proliferation.

Figure 3. PDGF and TGF- β in different LPB preparations. PDGF and TGF- β were determined in LPB preparations at pH 4.4, 5.4 and 7.4. The LPB preparation at pH 4.4 contained significantly higher amounts of PDGF than both pH 5.4 and 7.4. PDGF was also significantly higher in LPB pH 5.4 in comparison with LPB pH 7.4. TGF- β was significantly lower in LPB pH 4.4 in comparison with LPB preparations at pH 5.4 and 7.4. LPB pH 5.4 contained significantly higher amount of TGF- β than LPB pH 7.4 ($p < 0.001$). Values are medians $\pm$ SD, $n = 10$.

DISCUSSION

Tissue repair processes such as wound and fracture healing include complex but well-regulated cascades of events. The acid tide of wound healing, i.e., the pH within wounds and fractures, change from acidic to neutral and alkaline pH as the healing process progress (18-24). The local environment is acidic during the initial stage of healing. Platelets release components that contribute to improved proliferation of mesenchymal cells. The local environment will become more neutral as the healing progress and, ultimately, alkaline which results in the release of other platelet components that catalyse more differentiated osteoblast events such as ALP expression.

In this study, the use of lysed platelet concentrates preincubated in acidic, neutral and alkaline buffers demonstrated that it is possible to modulate the response of osteoblasts without changing the cultured cells pH environment. The results show that a decreased pH of the LPB significantly increase proliferation stimuli on osteoblasts in vitro. These results are in conjunction with our previous results on the effect of lysed platelet concentrates on fibroblast proliferation (26).

(Direct effects of low pH on osteoblasts in vitro have previously been reported (28, 29, 30). Acidification down to pH 7.0 decrease proliferation, collagen synthesis, and ALP activity in comparison with pH 7.4 (28, 29). We observed that LPB preparations at pH 4.4 contributed to a significant acidification of the culture media, which was not compensated by the media buffering capacity. The extremely low proliferation with LPBs at pH 4.4 could therefore be a direct effect of acidification of the culture media. (However, we also used acidic buffers at higher pH to give the platelet lysates a low pH

milieu. The factors released from the lysate were then utilised in osteoblast-like cells cultures, where the pH milieu was normal due to the culture media buffering capacity.)

The normalization of pH in LPB 4.0 should have been performed before use in the cell cultures.

Liu et al. (26) reported that the hydrogen concentration had an impact on the release of growth factors from the platelet lysate, e.g., PDGF. The increased release of PDGF at low pH could correspond to a higher demand of PDGF in the early part of the healing process. Our results are in conjunction with these findings since we also found higher concentrations of PDGF in LPBs prepared under acidic conditions. Further more we also found BMP-2 in the acidic preparation of LPB. However, there could be other platelet components responsible for the increased proliferation in both fibroblast and osteoblast cultures.

The high proliferation observed in acidic LPB preparations did not correspond to equally high ALP activities. The observed ALP increase from osteoblasts with LPB preparations at pH 7.4 and 7.6, might be explained by normalisation of proliferation and that the higher ALP expression represent ALP activities when osteoblasts are not stimulated to increase proliferation.

Liu et al.(26) reported also that the release of TGF- β was higher from LPBs at pH 7.1 and 7.6 than from LPB at pH 5.0. In our experimental setting, the lowest concentration of TGF- β was found in LPB at pH 4.4 in comparison with LPB pH 5.4 and 7.4. TGF- β has significant influence on osteoblasts and may contribute to the increased levels of ALP (31-32).

The results support the hypothesis that lysated platelet concentrates prepared at low pH release components that stimulate proliferation, while platelets prepared at a more alkaline pH stimulate osteoblasts to increased ALP expression.

BMP has been localized in megakaryocytes and platelets by Sipe et al 2004(33), but the release of measurable amounts of BMP-2 from PRP or platelet lysate has not been reported previously. It is specially interesting that BMP-2 could only be found in pH 4.0. The presence of BMP-2 in the platelet lysate encourages the use of this preparation of LPB in stimulation of osteoblasts in vitro, in vivo and in clinical settings.

Soffer et al. (10) used lysed platelets without different pH buffers in a study on fetal rat calvarial cells. They found increased cell proliferation, but decreased ALP production when lysed platelets were added to cell cultures. Though our results showed increased proliferation and ALP production with LPBs at pH 5.4, 7.4 and 7.6, there was a difference between the groups. The highest proliferation corresponds to relatively low ALP production, which is in accordance with Soffer et al. (10). This could be explained by that proliferating osteoblasts do not express high amounts of ALP (34).

Previous reports on the osteogenic differentiation of bone cells are contradictory (6, 10, 9). Gruber et al. (6) and Soffer et al. (10) prepared stimulatory agents from washed platelets while Doucet et al. (9) used platelets in original plasma without thrombin-activation. Supernatants from thrombin-activated washed platelets had stimulating effects on proliferation of mesenchymal cells but decreased osteogenic differentiation (6), while platelet lysates had stimulatory effects on both proliferation and osteogenic differentiation on mesenchymal cells (9). Platelet lysates in plasma seems to involve factors supporting expression of various proteins while supernatants from washed thrombin-activated platelets do not. The importance of the stimulatory factors of the

plasma fraction of PRP has not been studied specifically but warrants indeed future investigations. Our in vitro model, including plasma components, is more relevant to wound and fracture healing in vivo than LPB preparations of washed platelets.

In summery, our data show that LPB at pH 5.4 stimulates osteoblast proliferation in vitro similar to previous results on fibroblasts (26). LPBs pH 7.4 and 7.6 stimulated osteoblasts to increase their ALP synthesis but also to a relatively decrease of proliferation. Hypothetically, this may reflect different stages of fracture healing with an acidic stage in immature matrix with high cell proliferation and relative insignificant ALP activities and a later stage with osteoblasts in neutral to alkaline pH environment with higher ALP activities and less significant cell proliferation. From a clinical point of view, considering the use of PRP and platelet lysate on wound healing and bone formation, acidification of the platelet preparation would be beneficial on proliferation of osteoblast-like cells (and osteoblastic differentiation).

Studier att göra:

- 1. normalisera pH i supernatanten av LPB4.0 . Använd för cellodling.**
- 2. blockera BMP-2 och PDGF i dessa cellodlingar.**
- 3. BMP-2 och andra BMP om det finns.**
- 4. Fundera på om andra tekniker än ELISA behövs.**

REFERENCES

1. Bennett NT, Schultz GS. Growth factors and wound healing: Biochemical properties of growth factors and their receptors. *Am J Surg* 1993;165:728-737.
2. He C, Hughes MA, Cherry GW, Arnold F. Effects of chronic wound fluid on the bioactivity of PDGF in serum-free medium and its direct effect on fibroblast growth. *Wound Rep Reg* 1999;7:97-105.
3. Guerne PA, Sublet A, Lotz M. Growth factor responsiveness of human articular chondrocytes: Distinct profiles in primary chondrocytes, subcultured chondrocytes and fibroblasts. *J Cell Physiol* 1994;158:476-484.
4. Slater M, Patava J, Kingham K, Mason RS. Involvement of platelets in stimulating osteogenic activity. *J Orthop Res* 1995;13:655-663.
5. Gruber R, Karreth F, Frommlet F, Fischer MB, Watzek G. Platelets are mitogenic for periosteum-derived cells. *J Orthop Res* 2003;21:941-948.
6. Gruber R, Karreth F, Kandler B, Fuerst G, Rot A, Fischer MB, Watzek G. Platelet-released supernatants increase migration and proliferation, and decrease osteogenic differentiation of bone marrow-derived mesenchymal progenitor cells under in vitro conditions. *Platelets* 2004;15: 29-35.
7. Kawase T, Okuda K, Wolff LF, Yoshie H. Platelet-rich plasma-derived fibrin clot formation stimulates collagen synthesis in periodontal ligament and osteoblastic cells in vitro. *J Periodontol* 2003;74:858-864.
8. Arpornmaeklong P, Kochel M, Depprich R, Kubler NR, Wurzler KK. Influence of platelet-rich plasma (PRP) on osteogenic differentiation of rat bone marrow stromal cells. An in vitro study. *Int J Oral Maxillofac Surg* 2004;33:60-70.

9. Doucet C, Ernou I, Zhang Y, Llense JR, Begot L, Holy X, Lataillade JJ. Platelet lysate promote mesenchymal stem cell expansion: A safety substitute for animal serum in cell-based therapy applications. *J Cell Physiol* 2005; 205:228-236
10. Soffer E, Ouhayoun JP, Dosquet C, Meunier A, Anagnostou F. Effect of platelet lysate on selected bone cell functions. *Clin Oral Impl Res* 2004;15:581-588.
11. Roldàn JC, Jepsen S, Miller J, Freitag S, Rueger DC, Acil Y, Terheyden H. Bone formation in the presence of platelet-rich plasma vs. bone morphogenic protein-7. *Bone* 2004;34:80-90.
12. Knighton DR, Fiegel VD, Austin LL, Ciresi KF, Butler EL. Classification and treatment of chronic nonhealing wounds. Successful treatment with autologous platelet-derived wound healing factors (PDWHF). *Ann Surg* 1986;204:322-330.
13. Knighton DR, Ciresi K, Fiegel VD, Schumerth S, Butler E, Cerra F. Stimulation of repair in chronic, nonhealing, cutaneous ulcers using platelet-derived wound healing formula. *Surg Gynecol Obstet* 1990;170:56-60.
14. Holloway GA, Steed DL, Demarco MJ, Masumoto T, Moosa HH, Webster MW, Bunt TJ, Polansky M. A randomized controlled multicenter dose response trial of activated platelet supernatant, topical CT-102 in chronic, nonhealing diabetic wounds. *Wounds* 1993;5:198-206.
15. Steed DL, Goslen JB, Holloway GA, Malone JM, Webster MW. Randomized prospective double-blind trial in healing chronic diabetic foot ulcers. CT-102 activated platelet supernatant, topical versus placebo. *Diabetes Care* 1992;15:1598-1604.
16. Marx RE, Carlson ER, Eichstedt RM, Schimmelse SR, Strauss JE, Georgeff KR. Platelet-rich plasma. Growth factor enhancement for bone grafts. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 1998;85:638-646.

17. Marx RE. Platelet-rich plasma: Evidence to support its use. *J Oral Maxillofac Surg* 2004;62:489-496.
18. Embil JM, Papp K, Sibbald G, Tousignant J, Smiell JM, Wong B, Lau CY. Recombinant human platelet-derived growth factor-BB(becaplermin) for healing chronic lower extremity diabetic ulcers: an open-label clinical evaluation of efficacy. *Wound Rep Reg* 2000;8:162-168.
19. Swenson O and Lloyd Claff C. Changes in the hydrogen ion concentration of healing fractures. *Proc Soc Exp Biol Med* 1946;61:151-154.
20. Edlow DE, Sheldon WH. The pH of inflammatory exudates. *Proc Soc Exp Biol Med* 1971;137:1328-1332.
21. Chakkalakal DA, Mashoof AA, Novak J, Strates BS, McGuire MH. Mineralization and pH relationship in healing skeletal defects grafted with demineralised bone matrix. *J Biomed Mat Res* 1994;28:1439-1443.
22. Heuwinkel R, Schneider HM, Störkel S. Der gewebs-pH als stimulans desmalen knochenneubildung. *Unfallheilkunde* 1980;83:577-585.
23. Newman RJ, Duthie RB, Martin JO. Nuclear magnetic resonance studies of fracture repair. *Clin Orthop* 1985;198:297-303.
24. Newman RJ, Francis MJ, Duthie RB. Nuclear magnetic resonance studies of experimentally induced delayed fracture union. *Clin Orthop* 1987;216:253-261.
25. Hollinger J, Wong MEK. The integrated process of hard tissue regeneration with special emphasis on fracture healing. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 1996;82:594-606.
26. Liu Y, Kalén A, Risto O, Wahlström O. Fibroblast proliferation due to exposure to a platelet concentrate in vitro is pH dependent. *Wound Rep Reg* 2002;10:336-340.

27. Harris SA, Enger RJ, Riggs BL, Spelsberg TC. Development and characterization of a conditionally immortalized human fetal osteoblastic cell line. *J Bone Miner Res* 1995;10:178-186.
28. Spector JA, Mehrara BJ, Greenwald JA, Saadeh PB, Steinbrech DS, Bouletreau PJ, Smith LP, Longaker MT. Osteoblast expression of vascular endothelial growth factor is modulated by the extracellular microenvironment. *Am J Physiol Cell Physiol* 2000;280:C72-C80
29. Kaysinger KK, Ramp WK. Extracellular pH modulates the activity of cultured human osteoblasts. *J Cell Biochem* 1998;68:83-89.
30. Frick KK, Bushinsky DA. Metabolic acidosis stimulates RANKL RNA expression in bone through a cyclo-oxygenase-dependent mechanism. *J Bone Miner Res.* 2003;18:1317-1325.
31. Centrella M, McCarty TL, Canalis E. Skeletal tissue and transforming growth factor β . *FASEB J* 1988;2:3066.
32. Rodan GA. Introduction to bone biology. *Bone* 1992;13:3-6.
33. Sipe JB, Zhang J, Waits C, Skikne B, Garimella R, Anderson HC. Localization of bone morphogenetic proteins(BMPs)-2,-4,and -6 within megakaryocytes and platelets. *Bone* 2004; 35:1316-1322.
34. Stein GS, Lian JB, Stein JL, Van Wijnen AJ. Transcriptional control of osteoblast growth and differentiation. *Physiol Rev* 1996;76:593-629.