Effect of platelet concentration in platelet-rich plasma on peri-implant bone regeneration

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Abstract

This study analyzed the effect of the platelet count in platelet-rich plasma (PRP) on bone regeneration in vivo.

Twenty male New Zealand white rabbits were used. PRP was produced using the Platelet Concentrate Collection System (PCCS) (3i, Miami, FL, USA). After inducing ketamine–xylazine anaesthesia, a self-tapping titanium screw (Branemark MK III TiUnite, 3.75×7 mm) was inserted in each distal femur; the femurs were randomized so that one side was treated with PRP while the other (control) was not. Intravital fluorochrome staining was performed on days 1, 7 (1.5 ml of 2% doxycycline/kg bodyweight), 14 (6% xylenol orange, 1.5 ml/kg), and 21 (1% calcein green, 5 ml/kg). Animals were euthanized on day 28 ($n = 20$). Specimens were prepared for histomorphological evaluation according to Donath and Breuner [J. Oral Pathol. 11 (1982) 318].

Comparing the bone regeneration (fluorochrome staining) in the 4-week implants ($n = 19$), the only significant difference (sign test, $P = 0.004$) was seen with intermediate platelet concentrations ($n = 9,503,000–1,729,000$ platelets/μl PRP).

There were no differences in the bone/implant contact rates between the test and the control side among the three groups.

The platelet concentration required for a positive PRP effect on bone regeneration seems to span a very limited range. Advantageous biological effects seem to occur when PRP with a platelet concentration of approximately 1,000,000/μl is used. At lower concentrations, the effect is suboptimal, while higher concentrations might have a paradoxically inhibitory effect. On the other hand, the effect of this type of platelet concentrate was not beneficial to accelerate the osseointegration of enosseous dental implants.
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Introduction

Platelets contain a variety of autologous growth factors, including platelet-derived growth factor (PDGF), transforming growth factors β1 and β2 (TGF-β1 and TGF-β2), insulin-like growth factor (IGF), epidermal growth factor (EGF), and epithelial cell growth factor (ECGF), as well as a growth factor for hepatocytes [5]. In 1998, Marx et al. [7,8] found that platelet-rich plasma (PRP) had a positive effect on bone regeneration, since it was a source of autologous growth factors. In 44 patients who underwent reconstruction of the mandible following resection, they demonstrated that the use of PRP in combination with autologous bone transplant led to increased bone regeneration and bone density. The use of PRP to support the osseointegration of endosseous dental implants also resulted in significantly increased bone regeneration in animal experiments [4], although there are some contradictory results [12].

This study analyzed the effect of PRP on peri-implant bone regeneration in a rabbit model and focused on the possible influence of the platelet concentration in the PRP.

Material and methods

Animal model

After receiving approval from our animal care committee, 20 male New Zealand white rabbits, weighing 3293 ±

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259 g, aged 9–12 months, were used for this study. The animals were kept in individual cages in the institution's animal care center and fed water and standard diet ad libitum.

PRP production

For the self-production of PRP, the Platelet Concentrate Collection System (PCCS System, 3i®, West Palm Beach, FL, USA) was used. After collecting 30 ± 2 ml of whole blood from the marginal auricular vein or the central auricular artery using a 60-ml syringe containing 3 ml of citrate–dextrose solution, 2.1 ± 0.4 ml of PRP were produced using the PCCS system. To determine the concentration of platelets and leukocytes in the whole blood, an additional 0.5–1.0 ml of whole blood were collected in an EDTA monovette (hemogram-monovette, Ref. Nr. 05.1167, Sarstedt).

The method of producing PRP was modified (by reduction of the utilized amount of platelet poor plasma) to take into account the reduced amount of whole blood collected (30 ml vs. 54 ml). The concentrations of platelets and leucocytes in whole blood and the PRP were analyzed automatically at the transfusion center (Cell Dyn 3500, Abbott, Wiesbaden–Erbenheim, Germany), using modified software for animal probes. Double measurements were used as controls, and only limited scattering (<10%) was seen. One milliliter of PRP was stored in Eppendorf tubes at −78°C for later analysis of the growth factor content.

Surgical procedure

After inducing anaesthesia with an intramuscular injection of ketamine (30 mg/kg, Ketavet, Fa. Pharmacia Diagnostics GmbH, Freiburg, Germany, 100 mg/ml solvent) and xylazine (4 mg/kg, Rompun 2%, Fa. Bayer Vital GmbH, Leverkusen, Germany), a self-tapping Branemark titanium screw (MKIII, 7-mm length, ϕ = 3.75 mm, TiUnite surface, Nobel Biocare Deutschland GmbH, Wankelstrasse 9, D-50996 Köln, Germany) was implanted in the left and right distal femur of each animal. Implantation followed the manufacturer's recommendations. On one side, PRP was used during the insertion, while the other side served as a control. The side on which PRP was applied was determined randomly by flipping a coin.

On the test side, 0.5 ml of PRP was slowly injected into the prepared cavity at low pressure using an insulin syringe. In addition, the surface of the implant was moistened with PRP immediately before screwing in the implant. The wound was closed in multiple layers.

Fluorochrome staining sequence

For antibiotic coverage and simultaneous sequential intravital staining of the regenerating bone, the animals were injected with 1.5 ml/kg of 2% doxycycline ip, immediately after the operation. On days 7, 14, and 21 post-surgery, the animals were given 2% doxycycline (1.5 ml/kg), 6% xylenol orange (1.5 ml/kg), and 1% calcein green (5 ml/kg), respectively.

Specimen preparation

The 20 animals were examined histologically 4 weeks after the operation. One animal from the 4-week group could not be used for the subsequent analysis because of artifacts generated when counting thrombocytes in the PRP (due to coagulation of the PRP specimen).

The specimens were prepared using a technique to produce thinly ground layers of tissue as described by Donath and Breuner [1]. The specimens were cut along the axis of the implant, parallel to the axis of the femur. Then, thinly ground layers 40- to 60-μm thick were produced. After the histomorphometrical analysis of the fluorochrome staining, the specimens were colored with toluidine blue and examined histologically. In addition, the bone/implant contact rate was measured histomorphometrically.

Histomorphometrical evaluation

The implants were inserted in the transitional zone of the epiphyseal region of the femur directed from the metaphysis toward the diaphysis. Since significant differences between the reaction in the spongy zone of the metaphyseal bone and the medullary cavity on the diaphyseal side were expected (especially because the PRP tended to flow away from the medullary bone), only the metaphyseal side (which is representing the clinical situation of implantation in the spongy jaw) was evaluated for the fluorochrome labeling.

To analyze peri-implant bone regeneration, the fluorochrome staining of the spongy bone was quantified at three locations for each implant: at implant threads numbers 1 and 3 and at the caudolateral metaphyseal edge of the implant. Four photographs were taken of the same area for each of the three positions (Leica DMRX, Fa. Leica, CCD Color Video Camera, Fa. Sony, magnification ×100): one using transmission light microscopy without a specific filter and three fluorescence microscopy images to analyze the fluorochromes tetracycline, xylenol orange, and calcein green. The four microscope images were stored digitally and then evaluated histomorphometrically using a picture-analysis system (Image Tool for Windows, The University of Texas Health Science Center, San Antonio, TX, USA). Using this system, the number of pixels labeled with each fluorochrome was determined as a percentage of the total bone-regeneration surface in the pictures of the three implant areas. This was done separately for tetracycline, xylenol orange, and calcein green. The mean amount of tetracycline staining
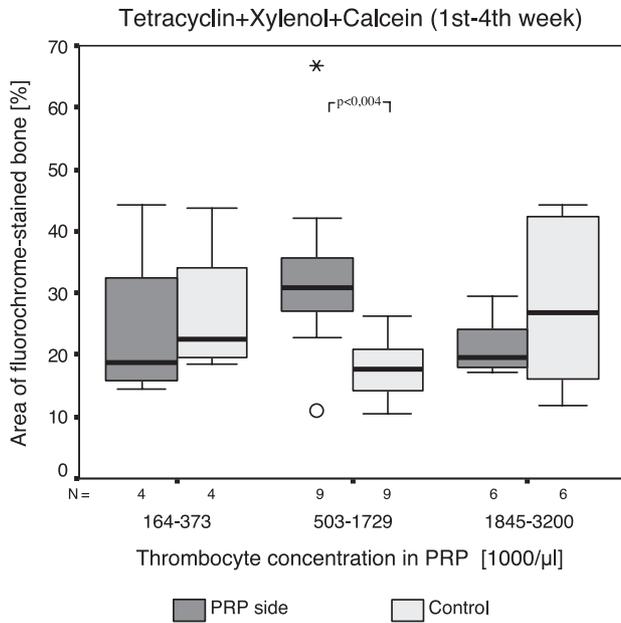
Fig. 1. Fluorochrome-stained bone surfaces after 4 weeks for the three groups based on the platelet concentrations in the platelet concentrate used (P values from the sign test for the intermediate platelet concentration group).

in each of the three implant areas was considered as measure of the bone regeneration in the first two post-operative weeks, the mean xylenol staining that for the third postoperative week, and calcein green for the fourth week.

After staining the specimens with toluidine blue, a digital image of the entire implant together with the adjacent osseous tissue was made at $16\times$ magnification. The bone/implant contact rate was determined as a percent of the implant surface using the picture-analysis system (Image Tool for Windows).

Statistical methods

All the quantitative measurements were characterized using descriptive statistics (n, mean, standard deviation, median, minimum, maximum, and other quartiles).

To determine whether the platelet concentration in the PRP preparations influenced bone regeneration, the 4-week animals were divided into three groups according to the platelet concentrations in the self-produced PRP:

1. Low platelet concentrations ($0.5-1.5\times$ the concentration in whole blood, i.e., 164,000–373,000 platelets/µl PRP),
2. Intermediate platelet concentrations ($2-6\times$ concentration, 503,000–1,729,000 platelets/µl PRP), and
3. High platelet concentrations ($9-11\times$ concentration, 1,845,000–3,200,000 platelets/µl PRP). Such high concentrations are usually only achieved using modified methods for PRP production.

The fluorochrome-stained specimens from each group were analyzed morphometrically as described above. First, the total fluorochrome-labeled bone surface for each of the dyes was calculated for the three areas of each implant (bottom, middle, and top). The mean of the three totals per implant (bottom, middle, and top) was used as a combined parameter of bone regeneration in the peri-implant region. The paired median values for bone regeneration were displayed graphically for each of the three platelet concentration groups using box plots. In addition, the test and control sides were compared using a sign test (for non-normally distributed linked data) for the medium platelet concentration group ($n = 9$).

To analyze the timing of the possible PRP effect on bone regeneration, each of the fluorochrome stains was analyzed separately for the three groups. The tetracycline data represented bone formation during weeks 1 and 2, the xylenol orange represented week 3, and the calcein green week 4.
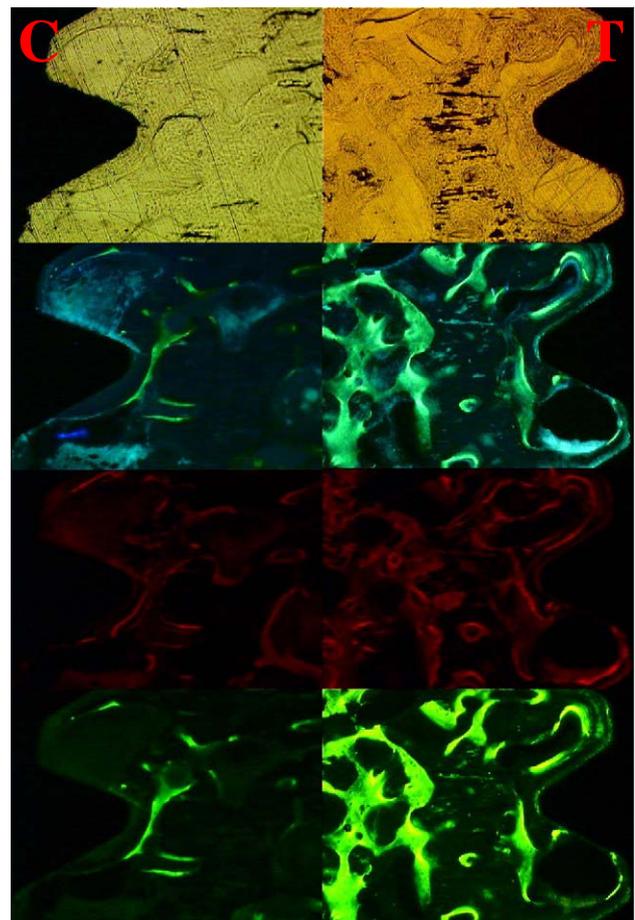

Fig. 2. Fluorochrome-stained bone surface after 4 weeks for the intermediate platelet concentration group (C = control, T = test side, from top to bottom: transmission light microscopy, tetracycline, xylenol orange, calcein green).
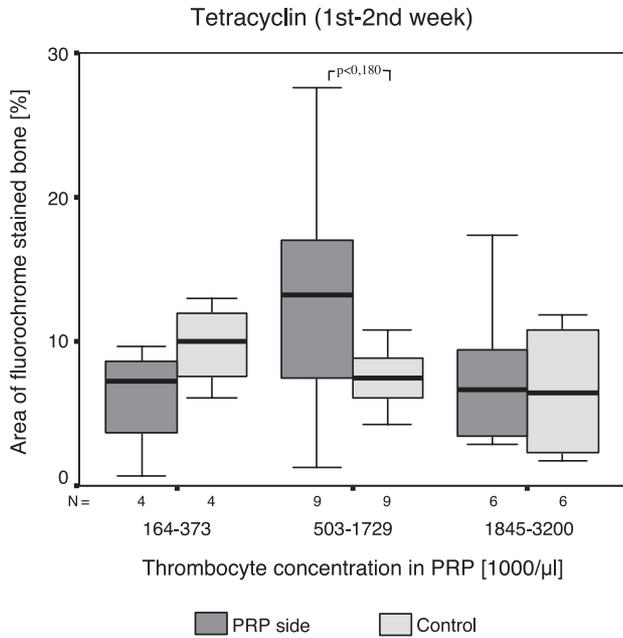
Fig. 3. Tetracycline-stained bone surfaces after 4 weeks for the three groups based on the platelet concentrations in the platelet concentrate used (P values from the sign test for the intermediate platelet concentration group).

Fig. 5. Calcein-green-stained bone surfaces after 4 weeks for the three groups based on the platelet concentrations in the platelet concentrate used (P values from the sign test for the intermediate platelet concentration group).

To evaluate the bone/implant contact rates, the results were displayed for each of the three platelet concentration groups. In addition, the intermediate group was analyzed using a sign test for non-normally distributed linked data.

Box plots of bone regeneration and the bone/implant contact rate for the low platelet concentration group (n = 4) were also drawn, although the median for this group was used, which has only limited statistical validity.
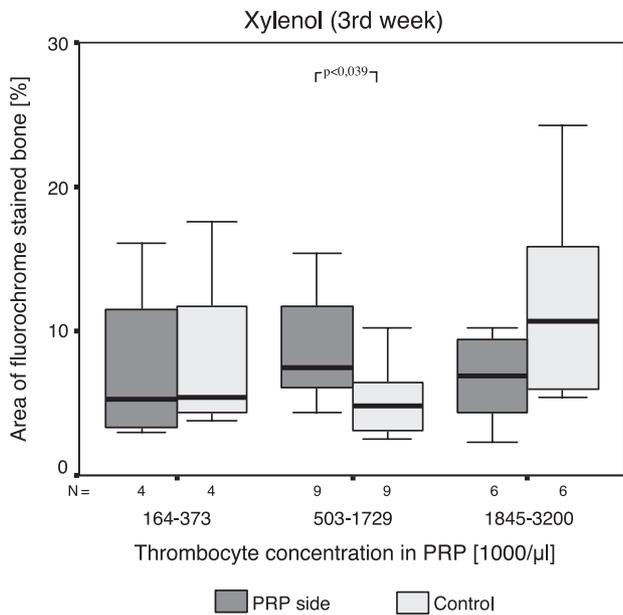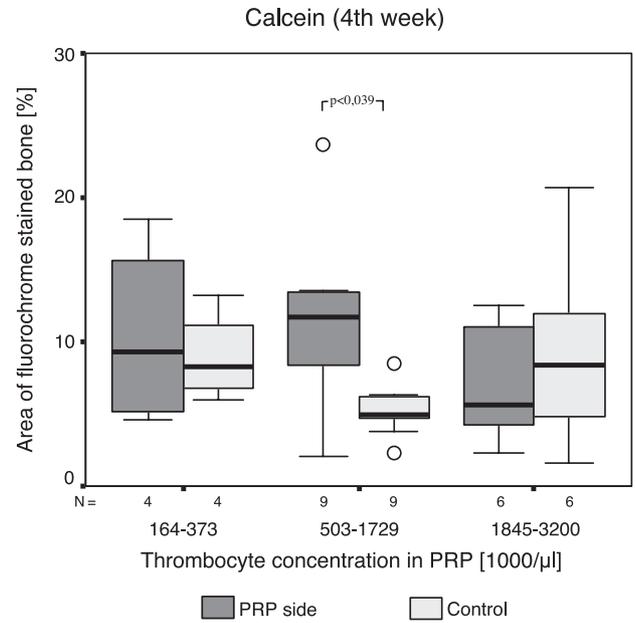

Fig. 4. Xylenol-orange-stained bone surfaces after 4 weeks for the three groups based on the platelet concentrations in the platelet concentrate used (P values from the sign test for the intermediate platelet concentration group).
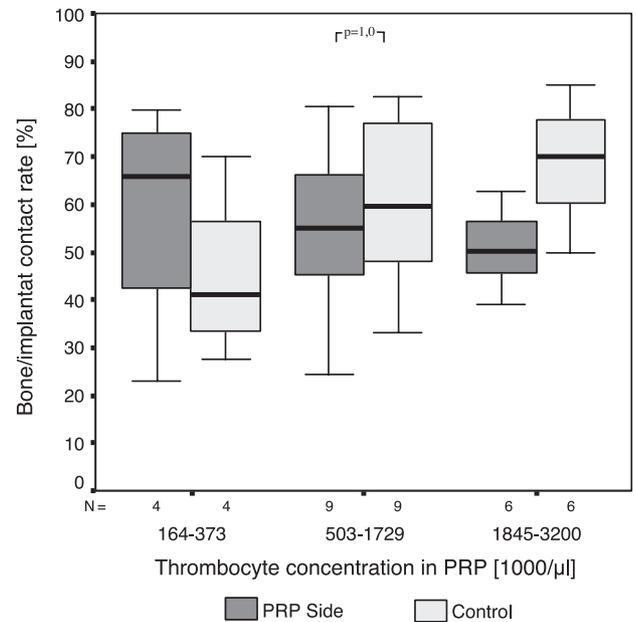

Fig. 6. Box plot of the bone/implant contact rate (whole implant surface in the ground specimens) after 4 weeks (P values from the sign test for the intermediate platelet concentration group).

Results

Intravital fluorochrome staining

Transmission light microscopy was used to compare the implant position in the spongy bone of the epiphysis with respect to the metaphysis and the medullary cavity of the diaphysis of the femur, which lacks spongy bone. For the most part, regeneration was limited to the part of the implant in contact with spongy bone for both the control and PRP-treated groups. Therefore, only the fluorochrome staining in the area of spongy bone was evaluated histomorphometrically.

In the 4-week specimens ($n = 19$), the difference in fluorochrome-labeled bone between the treated and control specimens was significant only for the intermediate platelet concentration group ($503,000-1,729,000$ platelets/μl PRP) (Fig. 1; sign test, $P = 0.004$) (Fig. 2). In the high platelet concentration group, the median amount of fluorochrome staining on the test side was smaller than that for the controls.

When the individual fluorochromes were analyzed, significant differences between the test and control groups were seen only for the intermediate platelet concentration group. Moreover, the differences within this group were significant only for xylenol orange (sign test, $P = 0.039$), calcein green ($P = 0.039$) (Figs. 3–5), and tetracycline plus xylenol orange ($P = 0.004$).

The difference between the test and control groups was greatest when the median values for calcein green staining of animals in the intermediate platelet concentration group were compared. The difference was less for tetracycline and was least for xylenol orange (difference in medians: calcein green 6.7%, tetracycline 5.7%, xylenol orange 2.6%, total 13.3%). The standard deviation was highest for the tetracy-
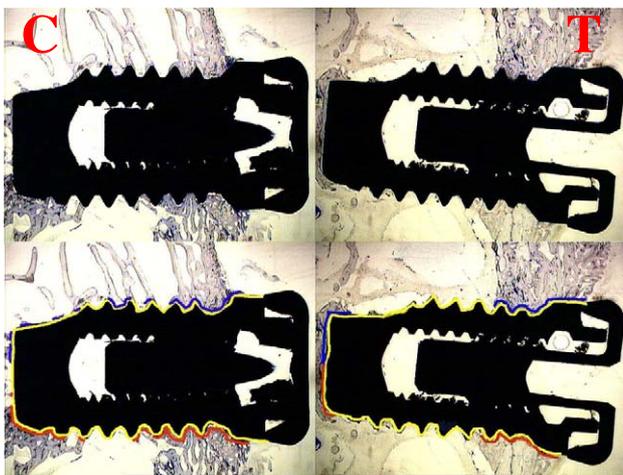

Fig. 7. Histology of the bone/implant contact rate (whole implant surface in the ground specimens) after 4 weeks (platelet concentration, 1.644.000/μl, C = control, T = test group).

cline coloring (initial phase of bone regeneration), implying that there was no statistical difference between the control and test groups during this time period.

Bone implant contact rate

In the 4-week specimens, there were no significant morphologic or histomorphometrical differences in the bone/implant contact rate between the test and control sides in the three platelet concentration groups (Figs. 6 and 7) (sign test group 2, $P = 1.0$). Moreover, no differences were seen in a separate evaluation of the juxta- and tele-articular sides. However, it seems remarkable that the median bone/implant contact rate for the high platelet concentration group was much smaller on the test side than on the control side (Fig. 6).

Discussion

The fluorochrome staining method used in this study is an established method. The morphometrically determined bone surface areas can be used as a measure of bone regeneration [6,11,14].

The extent of the difference in fluorochrome staining between the test and control groups at intermediate platelet concentrations suggests an increase in peri-implant bone regeneration of about 90% with the use of concentrated PRP, which might be a clinically relevant difference. In their miniature pig model of instant implantation, Zechner et al. [17] also found that the amount of bone regeneration was roughly double the control value at 3 and 6 weeks when using PRP with a ("intermediate") platelet concentration of 960,000 platelets/μl. In a human study, Marx et al. [8] demonstrated an increase in bone density from $55.1 \pm 8\%$ to $74 \pm 11\%$ six months after using platelet concentrate with platelet concentrations $595,000-1,100,000$ platelets/μl. Using a dog model, Kim et al. [4] found an increased bone density after 6 weeks when using PRP to accelerate bone regeneration in a bone defect in a peri-implantitis model (bone density: $43 \pm 15.4\%$ vs. $72 \pm 16.4\%$). Our results concerning the improved bone regeneration (an increase of approximately 90% vs. controls) lies between the results of these studies.

Centrifugation of the blood resulting in a low or reduced platelet concentration (group 1) did not substantially increase bone regeneration, which was expected (given the expected concentrations of platelet growth factors). Unlike the intermediate concentration group ($2-6\times$), the use of highly concentrated platelet preparations (group 3, $6-11\times$ concentration) appeared to have an inhibitory influence on osteoblast activity. Possible reasons could be unwanted inhibitory and cytotoxic effects of growth factors at such high concentrations. A concentration-regulated anti-mitogenic effect of TGF-β, a major growth factor in platelets, has been reported previously [2,9]. Moreover, given the

limited data for the high platelet concentration group ($n = 6$), a false negative result cannot be excluded.

Overall, the fluorochrome staining indicated that the application of platelet concentrate can have a positive effect on bone regeneration. However, this positive effect only occurs when the platelet preparation contains platelet concentrations within a certain range. This concentration-dependent effect of platelet preparations on cells of osseous origin has already been found in in vitro studies of the biological effect of PRP on human osteoblast-like cells. Moreover, only a small biological effect was found at low thrombocyte concentrations. Increasing the thrombocyte concentration increased the cell proliferation rate until a plateau was reached. Stimulation with greater platelet concentrations seemed to decrease the regeneration rate [13].

For the intermediate platelet concentration group, the box plots of the individual fluorochromes after 4 weeks showed a slight difference between the test and control sides for the tetracycline labeling ($P = 0.180$), and significant differences for xylenol orange and calcein green ($P = 0.039$). This implies that the platelet concentration (intermediate concentration group) had a positive effect, especially during the third and fourth weeks. This coincides with the known temporal pattern of bone repair, as seen in the healing of fractures. In bone fractures, resorption occurs for the first 1 to 2 weeks, to deal with bone necrosis at the fracture site. Therefore, little osteoblastic regeneration is expected in the initial 2 weeks, as reflected in the weak tetracycline labeling. Subsequently, the side that was treated with PRP showed a significant increase in repair processes in the third (xylenol orange labeling) and fourth (calcein green labeling) weeks. Overall, our results indicate that the effect of PRP in our model persisted for at least 4 weeks.

Analyzing the bone/implant contact rate, no differences were found for the three platelet concentration groups. This is astonishing, because when measuring loco-regional peri-implant bone regeneration, the use of platelet concentrate should at least increase the ossification rate in the intermediate group.

One possible reason lies in the method of PRP application. There might be an effect of trauma caused by the application of PRP with a 1-ml syringe, because this syringe was forced into the implant cavity. This might have enlarged the cervical and middle part of the cavity, which would have a major impact on the bone/implant contact rate. A second possible reason might be the time of examination. Literature data shows for a later examination the benefits of PRP, for example, Zechner et al. [17] for the miniature pig and Kim et al. [4] for the dog model.

In this context, it is interesting that Terheyden et al. [12] failed to find a positive effect of PRP when using 15% autologous bone with bovine hydroxyapatite for sinus augmentation, while with 50% autologous bone ($n = 5$), the bone density increased from approximately $50–75\%$ (t test, $P = 0.019$) and the bone/implant contact rate decreased from approximately $18–10\%$ ($P = 0.041$).

The bone/implant contact rate in the group with the highest platelet concentration ($1,845,000–3,200,000$ platelets/μl) was much smaller on the test side than on the control side. This concurs with the quantification of fluorochrome-stained bone surfaces and seems to be another indication of an inhibitory effect of the use of PRP with the highest platelet concentration, as already discussed.

The data of this study implicates that the acceleration of bone formation by platelet-rich plasma (under certain conditions) may clinically be helpful for the augmentation of alveolar defects in the future. But when it comes to an enosseous implant, on which the most important aspect is the biomechanical strength of the direct interface, the application of PRP demonstrated no beneficial effect under the analyzed conditions.

The dependence of the biological effect of PRP on the platelet concentration in vivo (group 1: no difference; group 2: a possible increase of 90%; group 3: an apparently inhibitory effect) determined in this study might partly explain the different results found in other studies. Some authors found a significant positive effect of PRP at a platelet concentration of approximately $1,000,000/\mu$l [3,4,8,17], while others found little [16] or no effect using platelet concentrates with an extremely wide range of platelet concentrations [10,15].

It is not clear how far the results of this study can be extrapolated to different clinical situations or different species, especially the platelet concentration producing positive biological effects and the optimum concentration $1,000,000$ platelets/μl PRP. However, the studies of Zechner et al. [17], Kim et al. [4], and Marx et al. [8] support our results. They found that platelet concentrations of approximately $1,000,000/\mu$l had a positive effect when using PRP in three different species (miniature pig, dog, and human). Therefore, with our rabbit study, PRP with a platelet concentration of $1,000,000$ platelets/μl has been shown to have positive, and possibly clinically relevant, effects in four different species.

Conclusions

From the combined data on the biological effect of PRP, it can be concluded that PRP seems to be able to activate the osseous regeneration processes under optimized conditions. However, we do not fully understand the conditions necessary for it to stimulate osseous regeneration. The stimulatory effect of PRP in vitro on the proliferation of osteoblasts seems to start in vivo in the second week, can be evaluated statistically significant from the third week, and still exists in the fourth week. Our data support the findings that the platelet concentration of PRP is important in determining its resulting biological effect. The platelet concentration required for a positive PRP effect seems to span a small range of concentrations. Particularly advantageous biological effects seem to appear when using PRP with a platelet

concentration of approximately 1,000,000/μl. Below this range, the effect is suboptimal; beyond this range, there may be a paradoxically inhibitory effect.

On the other hand, this study demonstrates that the effect of this type of platelet concentrates is not beneficial to accelerate the osseointegration of enosseous dental implants and at extremely high concentrations may actually inhibit bone formation under the tested circumstances.

At this time, widespread clinical use of the available PRP techniques cannot be recommended, because of the variety of factors influencing the results as well as the limited knowledge of the background conditions required for its use.

Acknowledgments

References

[1] Donath K, Breuner G. A method for the study of undecalcified bones and teeth with attached soft tissues. The Sage-Schliff (sawing and grinding) technique. J Oral Pathol 1982;11:318–26.

[2] Floege J, Topley N, Resch K. Regulation of mesangial cell proliferation. Am J Kidney Dis 1991;17:673–6.

[3] Gruber R, Varga F, Fischer MB, Watzek G. Platelets stimulate proliferation of bone cells: involvement of platelet-derived growth factor, microparticles and membranes. Clin Oral Implants Res 2002;13: 529–35.

[4] Kim SG, Chung CH, Kim YK, Park JC, Lim SC. Use of particulate dentin–plaster of Paris combination with/without platelet-rich plasma in the treatment of bone defects around implants. Int J Oral Maxillofac Implants 2002;17:86–94.

[5] Kiuru J, Viinikka L, Myllyla G, Pesonen K, Perheentupa J. Cytoskeleton-dependent release of human platelet epidermal growth factor. Life Sci 1991;49:1997–2003.

[6] Knöfler W, Graf H, Gröschel T, Löwicke G. Zur Knochenreaktion auf Biomaterialien: II. Ergebnisse der fluoreszenzmikroskopischen Untersuchung zur Beobachtung der initialen Knochenbildung. Z Zahnärztl Implantol 1990;VI:145–52.

[7] Lynch SE, Genco RJ, Marx RE (eds.). Tissue engineering: Quintessence Publishing Co., Inc. Chicago, 1999.

[8] Marx RE, Carlson ER, Eichstaedt RM, Schimmele SR, Strauss JE, Georgeff KR. Platelet-rich plasma: growth factor enhancement for bone grafts. Oral Surg Oral Med Oral Pathol Oral Radiol Endo 1998;85:638–46.

[9] Pollard JW. Tumour–stromal interactions. Transforming growth factor-beta isoforms and hepatocyte growth factor/scatter factor in mammary gland ductal morphogenesis. Breast Cancer Res 2001;3:230–7.

[10] Schlegel KA, Kloss FR, Schultze-Mosgau S, Neukam FW, Wiltfang J. Healing patterns at the bone-implant-interface using different topical measurements. Dtsch Zahnaerztl Z 2002;57:194–9.

[11] Terheyden H, Jepsen S, Möller B, Tucker M, Rueger D. Sinus floor augmentation with simultaneous placement of dental implants using a combination of deproteinized bone xenografts and recombinant human osteogenic protein-1. Clin Oral Implants Res 1999;10:510–21.

[12] Terheyden H, Roldan-Ossa JC, Miller J, Jepsen S, Acil Y. Platelet-rich plasma in bone regeneration. Preliminary results of two experimental studies. Implantologie 2002;10:195–205.

[13] Weibrich G, Gnoth SH, Otto M, Reichert T, Wagner W. Stimulation of the proliferation rate of human osteoblast like cells by platelet concentrates in vitro. Mund-Kiefer-Gesichtschir 2002;6: 168–74.

[14] Wetzel A, Stich H, Caffesse RG. Bone apposition onto oral implants in the sinus area filled with different grafting materials. Clin Oral Implants Res 1995;6:155–63.

[15] Wiltfang J, Kloss F, Kessler P, Zimmermann R, Schulte-Mosgau S, Neukam F, et al. Tierexperimentelle Studie zum Einsatz von Knochenersatzmaterialien und thrombozytenreichem Plasma in klinisch relevanten Defekten. Dtsch Zahnaerztl Z 2002;57:307–11.

[16] Wiltfang J, Schlegel K, Zimmermann R, Merten H, Kloss F, Neukam F, et al. Evaluation of bony regeneration following sinuslift procedures with bone substitutes and platelet rich plasma. Dtsch Zahnaerztl Z 2002;57:38–42.

[17] Zechner W, Tangl S, Tepper G, Furst G, Bernhart T, Haas R, et al. Influence of platelet-rich plasma on osseous healing of dental implants: a histologic and histomorphometric study in minipigs. Int. J Oral Maxillofac Implants 2003;18:15–22.