The effects of PRGF on bone regeneration and on titanium implant osseointegration in goats: A histologic and histomorphometric study

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Abstract: The effect of local application of scaffold-like preparation rich in growth factors (PRGF) on bone regeneration in artificial defects and the potential effect of humidifying titanium dental implants with liquid PRGF on their osseointegration were investigated. The PRGF formulations were obtained from venous blood of three goats and applied either as a 3D fibrin scaffold (scaffold-like PRGF) in the regeneration of artificial defects or as liquid PRGF via humidifying the implants before their insertion. Initially, 12 defects were filled with scaffold-like PRGF and another 12 were used as controls. The histological analysis at 8 weeks revealed mature bone trabeculae when PRGF was used, whereas the control samples showed mainly connective tissue with incipient signs of bone formation.

For the second set of experiments, 26 implants (13 humidified with liquid PRGF) were placed in the tibiae of goats. Histological and histomorphometric results demonstrated that application of liquid PRGF increased the percentage of bone-implant contact in 84.7%. The whole surface of the PRGF-treated implants was covered by newly formed bone, whereas only the upper half was surrounded in control implants. In summary, PRGF can accelerate bone regeneration in artificial defects and improve the osseointegration of titanium dental implants. © 2008 Wiley Periodicals, Inc. J Biomed Mater Res 91A: 158–165, 2009

Key words: bone regeneration; dental implant; growth factors; osseointegration; platelet activation; histomorphometry

INTRODUCTION

In the last few decades, the potential effects of growth factors in the repair and regeneration of tissues and specially of bone have been well documented.¹² Platelets contain a large list of proteins and growth factors including platelet-derived growth factor (PDGF), transforming growth factor-beta (TGF-β), insulin-like growth factor (IGF), vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and hepatocyte growth factors (HGF) among others, which have key roles on bone regeneration.³⁴

Since 1990, several platelet-rich preparations have been reported.⁵⁻⁸ In general, these products consist on a limited volume of plasma enriched in platelets that once activated release of a myriad of growth factors and proteins to the local environment, contributing to the accelerated bone regeneration. Some potential therapeutic applications of platelet-rich products include bone regeneration and faster titanium implant osseointegration. The latter is defined as a dynamic equilibrium at the site of the bone-implant contact, which ensures the stability and maintenance of dental implants.⁹

However, there are some controversies in the literature regarding the potential benefits of this procedure.¹⁰ In fact, although some authors have reported significant improvements in tissue healing and bone formation using platelet-rich plasma,⁵⁻⁶ others did not observe any improvement.¹¹,¹² These discrepancies are probably related to the lack of a suitable definition and standardization for the different PRP preparations currently being tested that differ from a qualitative and quantitative point of view.

In the last few years, we have developed the technology of preparation rich in growth factors (PRGF) by which it is possible to obtain different autologous preparations rich in growth factors from the same
patient’s blood depending on the coagulation and activation degree of the samples. One of these preparations is the scaffold-like PRGF composed of fibrillar and cellular components, which may be used to induce bone regeneration in postextraction sockets. Another interesting formulation is the liquid PRGF, which may be used to humidify and bioactivate dental implant surfaces to improve their osseointegration.

The present article aims to prove the potential of these two formulations, the scaffold-like PRGF and liquid PRGF, to accelerate and promote bone regeneration and faster osseointegration of dental implants. To address these issues, we have initially evaluated the bone regeneration effects of scaffold-like PRGF in artificial defects created in an experimental model in the tibiae of goats. In a second set of experiments, the potential effects of humidifying and bioactivating titanium dental implants with liquid PRGF on implant osseointegration in the goat model were also explored using histological and histomorphometric methods.

MATERIALS AND METHODS

Animals

Three adult goats, each weighting between 40 and 45 kg, were used in this study. The present protocol was approved by the ethical board of animal investigations (Royal Decree 223/88) for the approval of animal experiments. The animals were kept on high-calorie feed and allowed water ad lib.

Evaluation of titanium surface morphology

The surface of the titanium implants used throughout the study was modified by using an acid-etched treatment. Surface topography was examined by scanning electron microscopy (SEM) using a JSM 6400, JEOL LTD (Tokyo, Japan). The adsorption of PRGF proteins on the implant surface was visualized by environmental electron microscopy (ESEM) using an Electrosan 2020, Wilmington MC. No preparation was performed on the samples before analysis.

Preparation of PRGF

Peripheral blood (total volume of 50 mL) from each goat was taken by venipuncture before surgery and placed directly into tubes (BTI blood collecting tubes®), which contain 3.8% (wt/vol) sodium citrate as anticoagulant. PRGF is prepared by centrifugation (PRGF System®, Vitoria, Spain) at 1400 rpm for 7 min at room temperature and the 0.5 mL plasma fraction located just above the red cell fraction, but not including the buffy coat, is collected and deposited in a glass dish. To initiate clotting and activate the PRGF formulation, PRGF activator® (calcium chloride solution 10% (w/v)) was added to the plasma preparation (50 μL PRGF activator per mL of preparation) and liquid PRGF was obtained. This liquid PRGF was used to humidify and bioactivate dental implants before their insertion in goats. The PRGF-scaffold was prepared using the same approach but waiting 5–8 min until the final clot or scaffold-like PRGF was formed. Both formulations were used immediately after their preparation.

Surgical procedure

Two different experimental procedures were performed for this study. In both cases, ketamine (20 mg/kg body weight) was used for induction of anesthesia and an endotracheal tube was inserted in the animals. Anesthesia was maintained with isoflurane (2.5%) by inhalation. Surgery was performed with due attention to aseptic precautions and under continuous ECG monitoring.

In the first experiment, flaps were elevated in every case and 24 artificial defects were created in the tibiae of the animals using with a trephine drill. The length of each artificial socket was 8.5 mm whereas the diameter was 3 mm. Distance among the artificial defects was constant. To evaluate the potential effect of scaffold-like PRGF on bone regeneration, 12 defects were carefully filled with scaffold-like PRGF, whereas the other 12 defects were filled with blood (control group).

In the second experiment aimed to evaluate the effect of liquid PRGF on implant osseointegration, 26 implants (3.75 mm width and 8.5 mm length) (Biotechnology Institute (BTI® implants, Vitoria, Spain) were inserted in the tibiae of the goats. Before installation, 13 implants were carefully humidified with liquid PRGF with the aim of bioactivating the implant surface, whereas the other 13 implants were placed without PRGF treatment.

Postoperative medication and follow-up

To prevent infections, all animals were medicated with amoxicillin (1.5 mg/kg body weight) by i.m. injection and cefazoline (30 mg/kg body weight) by i.v. injection. Buprenorphine (10 mg) was administered for pain relief. Animals were sacrificed at 8 weeks postsurgery by i.v. injection of a lethal anesthetic dose (sodium pentobarbital 65 mg/kg body weight).

Histology

Processing and staining of the bone samples obtained 8-weeks postsurgery were carried out using a standardized protocol. Briefly, the samples were fixed in 4% formaldehyde, dehydrated in a graded series of alcohols, and embedded in methylmethacrylate resin at 4°C. Photopolymerization of the samples was done by sample exposition to blue light during 48 h. Samples were orientated accordingly to visualize both sides. For the first experiment, 5-μm-thick histological sections were obtained using a high-speed rotational microtome (Micromet, Remet) and
stained with hematoxylin-eosin. For the second experiment, 40-μm-thick histological sections were obtained and stained with toluidine blue and fuchsins. The latter was used to differentiate the soft tissue layer. Photomicrographs were taken by means of a digital camera. New bone and osteoid formations were identified visually.

**Histomorphometry**

For histomorphometric analysis, histological samples of each treatment option (with and without PRGF) were digitized by means of a digital camera JVC TK-C1380 (JVC, London, UK). To analyze the effect of PRGF on bone formation, the newly formed bone in the periphery of the implant was evaluated. The digitized images were analyzed by independent experts in the field using the software program IAS2000. Bone-implant-contact (BIC) of each sample was evaluated to determine the potential effects of PRGF on bone regeneration. BIC was considered as the percentage of implant length at which there is direct bone-implant contact without intervening soft tissue layer.

**Statistical analysis**

Results are expressed as means ± the standard deviation. The Student t-test was used to determine statistical significance. Differences were considered statistically significant when p was < 0.05.

**RESULTS AND DISCUSSION**

In the last few decades, researchers are trying to develop new technologies and methodologies to repair soft and bone tissues at a faster rate. These advancements will have major implications in many fields and especially in orthopedics, maxillofacial surgery, and oral implantology. Particularly, one typical problem in oral implantology is the insufficient quantity and quality of alveolar bone in localized defects of the alveolar ridge. It has been observed that tooth loss might lead to the destruction of nearly half of the original tooth-supporting alveolar bone, which is an essential requirement for the installation of oral implants and their successful long-term prognosis.15,16

In this article, we have investigated the potential effects of PRGF technology on stimulating bone regeneration and accelerating osseointegration of dental implants. Using this technology, different growth factor-enriched formulations can be easily prepared from the same patient’s blood.14 In particular, a scaffold-like PRGF characterized by its 3D configuration and from which a wide range of growth factors and biologically active proteins are released. And on the other hand, a liquid PRGF formulation, obtained before the scaffold-life PRGF is formed, which may be used to humidify dental implants and improve their osseointegration.

Some specific properties of PRGF technology make this approach and the resulting preparations different from other reported platelet rich plasmas (PRPs). Initially, calcium chloride instead of bovine thrombin is used as activating agent. The former provides a more controlled release of the growth factors and avoids potential risks associated with the bovine thrombin.17,18 As a consequence, both the scaffold-like PRGF and the liquid PRGF are 100% autologous. Other potential advantages are the versatility, cost-efficiency, and biosafety of these products. In fact, none of both formulations contain neutrophils, which may express matrix-degrading enzymes, such as matrix metalloproteinases-8 (MMP-8) and MMP-9, and release reactive oxygen species that destroy surrounding injured or healthy cells.4,19

To address all these issues, we initially prepared the scaffold-like PRGF from goats’ blood, and we studied its potential bone regeneration effects on empty artificial defects made in the tibiae of goats, which simulate postextractions defects (Fig. 1). The PRGF elaborated as described earlier resulted in a significant enrichment in platelet number, 3.17-fold increase comparing with peripheral blood. On the contrary, leukocyte content was below the detection limit of the coulter, confirming the absence of leukocytes in the PRGF preparations, which will improve the homogeneity of the product and reduce donor-to-donor variability.10,14 None of the goats showed signs of weakness after retrieval of blood before surgery. All goats had an uneventful postoperative recovery, and their weight remained stable during the experimental period. At sacrifice, no clinical signs of inflammation or adverse tissue reactions were observed. Histological analysis of all artificial defects was performed 8-weeks postsurgery. The damage caused by the drilling procedure appeared to be limited and no signs of immune response were observed around the defects.

The light microscopic evaluation of the defects revealed some important differences between both treatment groups. Histological examination on the defects loaded with PRGF showed well-formed trabeculae with osteocytes inside, flanked by marrow cavities with the presence of blood vascular channels [Fig 2(A)]. In fact, a dense connective tissue and newly formed bone tissue, which represented approximately the 50% of the total volume of the biopsy, were detected. No sign of fibrotic tissue layer was observed. In the case of the control defects, a fibrous matrix with high cell density and small areas of incipient spicule formation surrounded by the osteoid material becoming mineralized was found [Fig. 2(B)]. These results suggest that PRGF had formed more bone than the control, which stands for
accelerating the regeneration of bone and soft tissues. For example, the addition of PRP to Bio-Oss® significantly increased bone density in noncritical size defects in rabbits. In another approach, PRP added to β-tricalcium phosphate significantly increased the bone area percentage 6 weeks after grafting compared with the biomaterial alone. These differences were still significant 12 weeks after grafting (52.5% vs. 49.4%) but not after 24 weeks, reinforcing the idea that the main function of platelet rich products is to accelerate bone regeneration.

Our experimental approach might have implications for the treatment of postextraction defects in dentistry, especially when a complete regeneration of the alveolar bone and surrounding soft tissues are totally necessary to ensure the future success of the implant. Referring to other clinical applications, reconstruction of the anterior cruciate ligament for

Figure 1. A: Macroscopic view of scaffold-like PRGF. B: Experimental design of artificial sockets created in the tibia of goats; cavities were prepared using a 3 mm diameter trephine bar; two of them are fully occupied by PRGF clots. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Figure 2. Photographs of the histological sections seen by light microscopy at 8 weeks. Slides were stained with hematoxylin and eosin. Original magnification ×330. (A) In the PRGF group, well-formed trabeculae surrounded by vascularized dense connective tissue occupied the whole area. (B) In the control group, connective tissue with high cell density and small areas of incipient intramembranous ossification were observed. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]
example typically involves creating a tunnel through the tibia, drilling a closed tunnel (socket) into the femur, and inserting a substitute graft from one tunnel to the other. By providing PRGF within the tibial tunnel and applying it into the femoral socket, it is possible to accelerate bone growth and enhance fixation of the graft. In all of these situations in which patients are obligated to wait long periods of time, the use of PRGF might be very useful to accelerate bone regeneration and function.

In the second set of experiments, the effect of humidifying dental implant surface with liquid PRGF on implant osseointegration was evaluated. We hypothesized that by applying liquid-state PRGF to titanium dental implants, we could generate a biologically active surface which will facilitate implant osseointegration, enhancing bone to implant contact (BIC). Titanium, a biofunctional and biocompatible metal, is widely used in the manufacturing of surgical implants. Independently of the metal option, it is possible to improve the osteogenic properties using different approaches such as introducing topographic surface modifications and/or specific protein coatings. Acid treatment provides reactive rough surfaces prone to trigger thrombotic responses and superior osseointegration of titanium. As it is shown in Figure 3, the activated liquid PRGF coagulates upon contact with the acid-etched titanium implant and the resulting fibrin polymer adheres to the surface, modifying the interfacial properties of titanium.

To address the above hypothesis, 13 implants humidified with liquid-PRGF and another 13 implants without the autologous preparation (control group) were inserted in the tibiae of goats (Fig. 4). BIC of the samples was determined 8 weeks postimplant fixation. The biopsies of the implant samples revealed that those humidified with liquid PRGF exhibited significantly higher bone-to-implant contact than non-PRGF treated implants. In fact, BIC percentages after the histomorphometric analysis were 50.8 ± 13.5 for the PRGF group and 27.5 ± 6.3 for the control group (p < 0.001) (Fig. 5). Therefore, the application of liquid PRGF increases the percentage of bone in contact with the implant in 84.7%.

The histological sections of the implants showed also some interesting differences between both groups. In fact, the whole surface of the PRGF-treated implants was covered by newly formed bone whereas in the case of nontreated implants only the upper half was surrounded by bone (Fig. 6). A similar result was obtained when we retired the implants from the goats. As it is illustrated in Figure 7, the PRGF-treated implants appeared totally surrounded by a solid and dense bone cylinder while the nontreated implants were only partially covered. These

Figure 3. Environment scanning electron micrograph showing the interface between the titanium acid-etched surface of the implant and the activated liquid PRGF. Magnification, ×6000.

Figure 4. A: Macroscopic view of a dental implant humidified with liquid PRGF. B: Experimental design of the artificial sockets with the inserted implants in the goat tibia. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]
results demonstrate that liquid-PRGF favored and enhanced bone-implant contact of roughened titanium implants.

These results may be explained in part by the mitogenic, quimiotactic, and proliferative effects induced by some of the growth factors released by the PRGF on osteoprogenitor cells. In fact, it has been fully demonstrated that the growth factors derived from platelets can stimulate the proliferation of different cells including human trabecular bone cells, human osteoblast-like cells, human stromal stem cells, and human mesenchymal stem cells. Additionally, because of the polarity of the titanium surface, the negatively charged proteins present in the liquid PRGF-like vitronectin and fibronectin may be adsorbed on the implant’s surface. The latter is particularly important as these proteins may provide specific sites for cell adhesion. Fibronectin is a well-known adhesive protein, which will enhance the formation of focal adhesions by osteoblasts and improve the adhesion and spreading of gingival fibroblasts on the implant surface.

Our results come along with those published by Fuerst et al. Using a similar approach, these authors reported a 55.3% BIC when implants were coated with PRGF versus 38.91% BIC of the control group after 4 weeks of healing time. At 8 weeks, the percentages were 70.36% with and 48.2% without PRGF. In another study, Nikolidakis et al. observed that PRP improved BIC for roughened implants inserted in goats. The authors reported that the percentage of bone contact for implants humidified

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**Figure 5.** Histomorphometrical evaluation. Mean percentage of bone-implant contact between the two groups. *p < 0.05.

**Figure 6.** Photographs of the histological sections seen by light microscopy at 8 weeks. Sections were stained with fuchsin and toluidine blue. Original magnification, ×20. (A) The regenerated bone covered nearly all the threads in the PRGF group, while many were exposed in the control group (B). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

**Figure 7.** Representative macroscopic view of a PRGF (A) and control (B) implant obtained with an 8 mm trephine drill at 8 weeks. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]
with liquid PRP was 79% ± 11% whereas in the case of noncoated implants it was only 60% ± 9%.

CONCLUSIONS

The results of this study confirmed that scaffold-like PRGF contributes to accelerate bone regeneration in artificial defects, which might be relevant to obtain bone tissue earlier in postextraction defects and thus reduce the time elapse from tooth extraction to implant insertion. Furthermore, by humidifying titanium implants with liquid PRGF, it is possible to improve the integration of oral implants in cortical bone. The latter have major implications in obtaining a suitable implant osseointegration and consequently high implant stability. The potential therapeutic effects of this approach might be extrapolated to other prosthetic devices.

References
