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British Journal of Oral and Maxillofacial Surgery 51 (2013) 438–443

BRITISH Journal of Oral and Maxillofacial Surgery

www.bjoms.com

In vivo evaluation of titanium-prepared platelet-rich fibrin (T-PRF): a new platelet concentrate

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Accepted 7 August 2012
Available online 28 August 2012

Abstract

We have developed a new, titanium-prepared, platelet-rich fibrin (T-PRF) together with the protocol for forming it, which is based on the hypothesis that titanium tubes may be more effective at activating platelets than the glass tubes used by Chouckroun in his platelet-rich fibrin (PRF) method. The aim of this study was to find a suitable animal model in which to evaluate the method and to investigate the efficacy of T-PRF for wound healing. Blood samples from 6 rabbits were used to confirm the protocol for formation of T-PRF. We evaluated T-PRF or T-PRF-like clots morphologically using scanning electron microscopy (EM). Blood samples from 5 rabbits were used to develop an experiment in which to evaluate the effects of T-PRF on wound healing. The mucoperiosteal flaps were filled with autologous T-PRF membranes from the vestibule in the anterior mandibular regions. Samples collected from the surgical sites were stained with haematoxylin and eosin. We found a mature fibrin network in T-PRF clots that had been centrifuged for 15 min at 3500 rpm and, 15 days after placement of the membrane, we found newly-forming connective tissue and islets of bony tissue in the T-PRF membrane. These results show that T-PRF could induce the formation of new bone with new connective tissue in a rabbit model of wound healing within 30 days of treatment.
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Keywords: Tissue engineering; Biomaterial(s); Scanning electron microscopy (EM); In vivo; Biocompatibility; Regenerative medicine

Introduction

Blood is a mixture of plasma, various types of cell, and platelets (biologically-active cellular fragments). Thrombocytes have an important role in coagulation, and prevent excessive blood loss in venous injuries. They contain numerous cytokines and growth factors that influence bony regeneration and maturation of soft tissue.[1] Platelet-derived growth factor AB (PDGF-AB), transforming growth factor β-1 (TGFβ-1), and vascular endothelial growth factor (VEGF) are key growth factors that are present in thrombocytes.

Platelet-rich fibrin (PRF) was first developed in France by Choukroun et al. in 2001 as an autologous biomaterial that contains leucocytes and platelet-rich fibrin (PRF).[2] Unlike other platelet-rich products, the technique requires neither anticoagulant nor bovine thrombin (or any other gelling agent).[3–7] Successful clinical results have been reported with PRF,[3–17] but some physicians[18] worry about a possible health hazard as it uses glass-evacuated collection tubes for the blood with silica activators. O'Connell[18] described the unavoidable contact with silica. The particles of silica in the tube, although they are dense enough to sediment with the red blood cells, are small enough for a fraction to remain suspended colloidally in the buffy coat, fibrin, and platelet-poor layers of plasma. The particles might therefore reach the patient when the product is used for treatment.

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0266-4356/$ – see front matter. Published by Elsevier Ltd. on behalf of The British Association of Oral and Maxillofacial Surgeons.
http://dx.doi.org/10.1016/j.bjoms.2012.08.003

Titanium-prepared, platelet-rich fibrin (T-PRF) is a new platelet concentrate, the method of preparation of which is based on the hypothesis that titanium tubes may be more effective at activating platelets than the glass tubes used in Chouckroun's method.[2–5] This material is used to avoid any adverse effects in the short or long term, or both, of dry glass or glass-coated plastic tubes and to eliminate any speculations about silica. In our initial trials, we found titanium- induced platelet aggregation similar to that in glass tubes, and the clot produced in titanium tubes was clinically identical to that produced in glass tubes. Activation of platelets with titanium compared with activation with silica particles provides the distinctive characteristics of T-PRF, including its increased biocompatibility.

The T-PRF collection protocol in human subjects is similar to the conventional PRF protocol as follows.[3] A blood sample is collected without anticoagulant in 10 ml titanium tubes, which are immediately centrifuged at 2800 rpm for 12 min. The absence of anticoagulant implies that most platelets in the blood sample will be activated within a few minutes after contact with the wall of the titanium tube, which initiates the coagulation cascade. Fibrinogen is initially concentrated in the upper part of the tube before the circulating thrombin transforms it into fibrin. A fibrin clot is then formed in the middle of the tube between the red corpuscles at the bottom and the acellular plasma at the top.[3]

Numerous studies are necessary to evaluate a newly developed T-PRF product thoroughly. In vivo animal experiments are needed before its use in humans, so that we can fully understand the potential role that it may have in wound healing. However, conventional clinical studies on PRF have usually been done in humans.[6–17] There have been several recent studies using cells in vitro[9,19] and even fewer animal studies reported.[20] Serious criticisms have been brought to light regarding the studies of PRF in animals.[21] The objective of this study was to evaluate an appropriate animal model for testing the newly-described T-PRF platelet product, and to investigate the effectiveness of T-PRF in vivo on wound healing in connective tissue.

Material and methods

A total of 11 New Zealand white rabbits 5 months old, mean (range) weight 3 (2.8–3.2) kg, were used in the study. All experimental procedures were approved by the Institutional Review Board and Animal Use Committee of the Cumhuriyet University School of Medicine (Process No. 210, July 1 2010). The study was conducted according to the principles of the 2010 Basel Declaration.

Preparation of T-PRF or T-PRF-like clots for evaluation by scanning EM

Blood samples from 6 rabbits were used to show the structure of T-PRF using scanning EM. Titanium tubes were produced from grade IV titanium. A total of 10 ml blood from each rabbit was drawn with a syringe from the marginal vein on the right or left ear in one attempt and transferred to titanium tubes. The blood samples were collected rapidly, and the tubes were immediately centrifuged at 3000 rpm for 10 min, 3000 rpm for 15 min, 3500 rpm for 10 min, 3500 rpm for 15 min, 4000 rpm for 10 min, or 4000 rpm for 15 min using a specific table centrifuge (EBA 20, Andreas Hettich GmbH & Co. KG, Tuttlingen Germany) at room temperature. After centrifugation, the clots were removed from the tubes using sterile tweezers, separated from the red cell base, and placed on sterile woven gauze. Each clot was left to release its serum on to the sterile woven gauze slowly over a 20-min period. After each clot had been cut into two parts along the long axis, parts were processed for evaluation by scanning EM.

Histological processing for evaluation by scanning EM

Morphological evaluation of T-PRF or T-PRF-like clots was made using a scanning EM (LEO 440, Leica & Zeiss Co., Cambridge, England). Each clot was fixed in 2.5% glutaraldehyde for 1 h and treated for desiccation. Specimens were sputter-coated with 20 nm gold/palladium and subsequently examined in a scanning EM. Photographs were taken at 20 kV at magnifications ranging from ×750 to ×10 000.

Preparation of T-PRF membranes and operations

After the T-PRF clot that was most suitable for use was clinically established (centrifugation at 3500 rpm for 15 min) and scanning EM images had been obtained, blood samples from 5 rabbits were used for the T-PRF operations. Blood was rapidly collected and immediately centrifuged at 3500 rpm for 15 min. After centrifugation, clots were removed from the tubes using sterile tweezers, separated from the base of red blood cells (RBC), and pressed between two pieces of gauze. All animals were operated on under general anaesthesia induced by xylazine 10 mg/kg (Rompun 2%; Bayer, Istanbul, Turkey) intramuscularly and ketamine hydrochloride 50 mg/kg (Ketalar; Eczacibasi, Istanbul, Turkey). Researchers also gave ceftriaxone 50 mg/kg (Rocephin; Roche, Basel, Switzerland) intramuscularly every 24 h for 4 days starting 1day before the operation, and carprofen 4 mg/kg (Rimadyl; Pfizer, New York, IL) intracutaneously postoperatively for 3 days immediately after the operation. After the local anaesthetic had been given, buccal sulcular incisions were made, and the mucoperiosteal flap was raised from the vestibule in the anterior mandibular region using a sharp periosteal elevator. Care was taken to preserve as much mucoperiosteal flap as possible. Autologous T-PRF membranes were placed into the mucoperiosteal flap. The flaps were positioned coronally to cover the graft completely, and resorbable 5/0 polyglactin 910 sutures (Vicryl, Ethicon, USA) were placed directly over the graft. After 3, 5, 10, 15, and 30 days, the

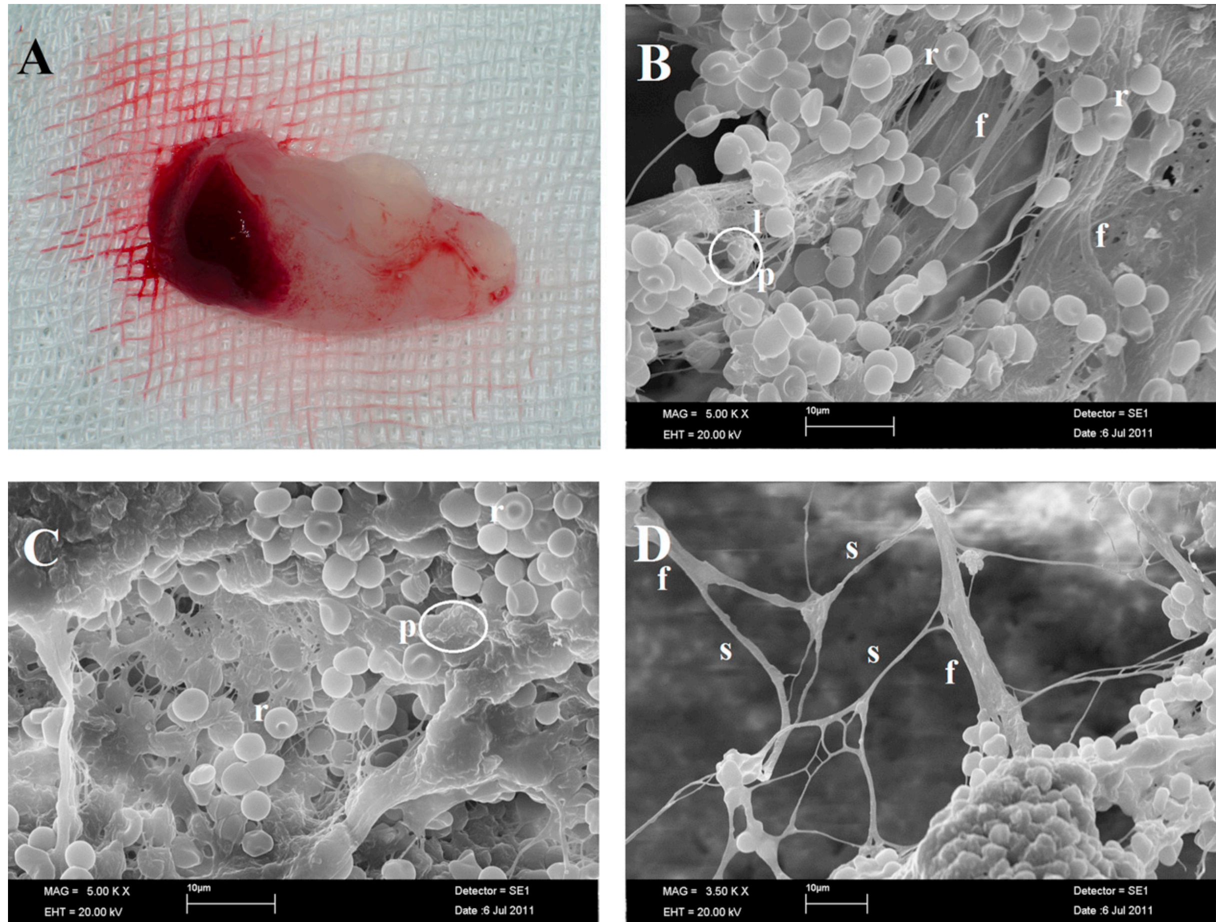

Fig. 1. The titanium-prepared platelet-rich fibrin (T-PRF) clot (centrifuged for 15 min at 3500 rpm). (A) Clinical view. A mature T-PRF clot. (B) Scanning electron micrograph (EM). T-PRF fibrin matrix (f) and cellular components. RBC (r) trapped within the fibrin matrix, leucocytes (l) appeared as spherical structures, platelets were often enmeshed in the fibrin network, but sometimes appeared as aggregates (p) (white circle) (scanning EM; original magnification ×5000). (C) Fen-like T-PRF fibrin matrix, RBC (r), and platelets embedded in the fibrin matrix (p) (white circle) (scanning EM, original magnification ×5000). (D) Secondary fibrils (s) in addition to main fibrillary structures (f) (scanning EM, original magnification ×3500).

full-thickness flap was raised with an ablating sulcular incision of the lower incisor teeth and 1 cm vertical incisions from the distal site of the mandibular incisors. Full-thickness tissue samples 7 mm × 7 mm were excised. The rabbits were killed with an overdose of pentobarbital (Pentothal, Abbott, USA) 200 mg/kg given intravenously. The specimens were preserved in a 10% formaldehyde solution 24–72 h after extraction.

Histological processing for light microscopy

Soft tissue samples taken from the sites that had been treated with T-PRF membranes were dehydrated in increasing gradients of alcohol (70%, 95%, and 100%) and placed in toluene before paraffin inclusion. For each soft tissue sample, a series of 20 successive sections were obtained according to the long axis of the tissue. These sections were stained with haematoxylin and eosin.

Results

Evaluation by scanning EM

After centrifugation for 10 min at 3000 rpm, 15 min at 3000 rpm, or 10 min at 3500 rpm, the T-PRF-like clot seemed to be clinically immature. We could see no fibrin networks in the T-PRF-like structures. Pomegranate-like-looking structures were formed by unfractionated thrombocytes, and RBC seemed to be bound together. In addition, there were thin extensions in the form of short hairs. These thin, short extensions were interpreted as being primitive fibrin-like structures that were weak and labile. Platelets were generally enmeshed in the fibrin network but sometimes appeared as aggregates.

After centrifugation for 15 min at 3500 rpm, we found a clinically mature T-PRF clot (Fig. 1A). A mature fibrin network and platelets embedded in the fibrin network were also found in the clot. RBC were located outside the matrix or adhered to the matrix between the red area and the yellow

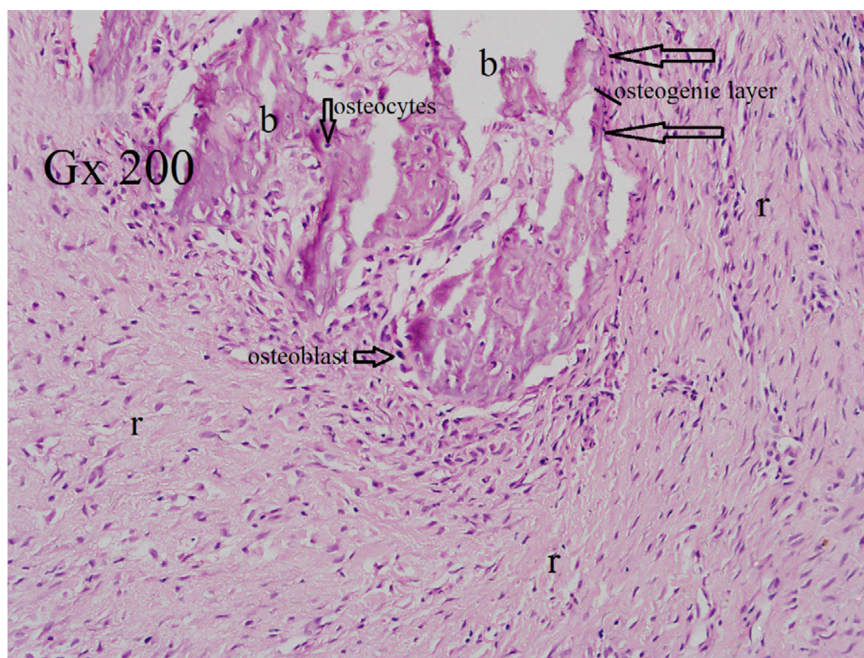

Fig. 2. Light microscopic analysis. Remodelled connective tissue (r), bone callus (b), osteocytes, osteoblasts, and osteogenic layer (haematoxylin and eosin).

clot. In addition to RBC, there were a small number of cellular components and different-shaped structures (Fig. 1B). A fen-like matrix was the primary image generated by scanning EM. Although it was not possible to calculate the shape and type of the various structures, the RBC and platelets were visible embedded in the fibrin matrix (Fig. 1C). There were primary and secondary fibrillary structures, and the fibrils formed a patch-like structure from numerous cruciate ligaments (Fig. 1D).

After centrifugation at 4000 rpm for 10 min, or 4000 rpm for 15 min, we saw a torn fibrillary structure that lacked integrity. No fibrin network had formed. Erythrocytes were among the visible cellular structures. Platelets were fractionated after taking the form of gel, and had created a glue-like structure.

Light microscopy

On the third day after the operation, inflammatory cells that attempted to permeate into a well-organised fibrin network were apparent in the samples collected from the operative sites where the T-PRF membranes had been placed. The integrity of the membranous tissue was largely preserved within the cell. On the fifth day, half of the membrane had been resorbed, and we could see inflammatory tissue cells in the samples. Remnants of the membrane and new connective tissue that was attempting to replace the membrane could be seen in the samples on the tenth day, and there were fewer inflammatory cells surrounding the membranous tissue. Newly-forming islets of bony callus and connective tissue were noted in the samples on the fifteenth day. Islets of accumulations of bone and calcium in the bony callus were found in the samples on the thirtieth day, when 3 main aspects of the operative sites were apparent. The first was an area of inflammation that contained the remaining membrane; the second was an area of granulation tissue (proliferation area); and the third was a remodelling area. Most of the remodelling area consisted of connective tissue and a new bone-like callus. The osteogenic layer, osteocysts, and accumulation of calcium generated by osteoblasts, were obvious in the new bony area (Fig. 2).

Discussion

We have shown that rabbits could be used as the animal model for experimental studies of T-PRF. This is a new platelet product that was able to form new connective tissue during the early stages of an in vivo study in rabbits. T-PRF used alone as a membrane formed new bone with connective tissue in the model of connective tissue wound healing in which regeneration was not expected. The T-PRF membrane began to resorb in rabbit tissue on the fifth day and was able to remain in the tissues for at least 10 days, which was sufficient time for the initiation of formation of new bone.

In previous in vivo studies with Chouckroun's PRF in rabbits,[20,22] the classic PRF protocol used in human subjects was not changed, and the material obtained was accepted as PRF. However, because it was not possible to obtain a sufficient amount of blood in a short period of time from a rabbit, which Dohan Ehrenfest et al.[21] considered to be a rather small animal, it was necessary to attempt in vivo studies of PRF on larger animals. Dohan Ehrenfest et al.[21] had stated that the

PRF-like product obtained from rabbits using the human protocol was not actually PRF, and that studies in rabbits would not produce accurate results. In preliminary trials in rabbits using the human PRF protocol to produce T-PRF, we encountered the same product described by Dohan Ehrenfest et al.[21] Although we had adjusted the duration of the blood collection based on the human protocol, we found that the structure did not change and we were not able to obtain clinically complete T-PRF with the desired consistency. These results led us to experiment with protocols to produce platelet-rich products in rabbits based on the structural differences between human and rabbit blood.[23]

Based on this hypothesis, we gradually increased the duration and speed of centrifugation. After obtaining a clinical picture similar to T-PRF, centrifugation of rabbit blood for 15 min at 3500 rpm showed optimal characteristics of formation of fibrin based on observations using scanning EM. Clinically mature T-PRF clots were seen in all test subjects (6 rabbits) the blood of which was centrifuged for 15 min at 3500 rpm. T-PRF had an effect in rabbits at least 30 days after insertion and initiated bone formation after 15 days when used as a membrane.

We are aware of the limitations of this study. We had few subjects, even though 20 sections were obtained from the central parts of the clot and within the limits of the distal of two central teeth in all test subjects. We were not able to obtain quantitative results because the model could not be described as a bone wound healing model. However, our study comes to two important conclusions. First, a T-PRF formation protocol was established using rabbit blood based on centrifugation for 15 min at 3500 rpm. We think that this would be beneficial for the formation of conventional PRF in rabbits. Secondly, the first in vivo application of T-PRF established it as a new platelet product that induced new connective tissue to form in as little as 15 days with excellent regenerative potential. T-PRF used alone as a membrane formed new bone with connective tissue in the wound healing model of connective tissue, in which regeneration was not expected. These results could lead to additional T-PRF animal and human studies of T-PRF in oral and maxillofacial surgery, implantology, and periodontology, and contribute to the understanding of this new platelet-rich product.

Acknowledgement

This study was supported by the Scientific Research Project Coordination Center of the Gulhane Military Medical Academy.

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