



ORIGINAL ARTICLE

Use of autologous platelet-rich clots for the prevention of local injury bleeding in patients with severe inherited mucocutaneous bleeding disorders

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Summary. Stopping or preventing local bleeding in patients with inherited bleeding disorders linked to abnormal platelet function is traditionally treated by transfusion of blood cell products or recombinant factor VIIa. We now report the use in such patients of autologous platelet-rich clots as an aid to preventing bleeding and to facilitating tissue regeneration at superficial sites. Two patients with von Willebrand's disease (VWD) type 2B and one patient with type I Glanzmann thrombasthenia were treated after tooth extraction and dental surgery. A fourth patient with platelet-type VWD underwent a skin biopsy. Whereas

all four patients had a lifelong history of bleeding complications, the application of an autologous platelet-rich clot immediately after surgery combined with tranexamic acid intake to slow fibrinolysis prevented blood loss and resulted in rapid and normal healing. This new procedure is simple, safe and inexpensive; it provides extra security for patients with a bleeding risk undergoing dentistry or superficial surgery.

Keywords: bleeding disorder, Glanzmann thrombasthenia, autologous platelet-rich plasma, prevention of bleeding, tissue healing, von Willebrand's disease

Introduction

Patients with inherited bleeding syndromes associated with defective platelet function or a low platelet number are frequently transfused with platelet or red blood cell concentrates in the event of major haemorrhage [1–3]. It is the case for patients with Glanzmann thrombasthenia (GT) where the bleeding risk is substantial in the absence of platelet aggregation and a deficiency or non-functioning of the α IIb β 3 integrin [4]. For patients with von Willebrand's disease type 2B (VWD2B) or platelet-type VWD, up-regulated binding of von Willebrand factor (VWF) multimers to platelet GPIIb α prevents the normal adhesion of platelets at sites of vessel injury [5,6]. For VWD2B and platelet-type VWD, the situation

is complex as the haemorrhagic risk can result from an abnormal platelet production in addition to blocked platelet function and frequent loss of the highest molecular weight VWF multimers [7]. While infusion of blood products (platelets and/or VWF) has been a standard procedure for stopping bleeding in these patients, receivers can be at risk from viral or other contaminants. Newer approaches such as the use of recombinant factor VIIa (rFVIIa) (NovoSeven; Novo-Nordisk, Copenhagen, Denmark) promoting fibrin formation represent an alternative for inherited platelet diseases, particularly when inhibitors are present, but are expensive and require specialist care [8].

Use of autologous platelet-rich clots has been reported to favour tissue repair in many situations including dentistry and oral implantology, orthopaedics, skin ulcer treatment and cosmetic surgery [9,10]. Platelet-rich clots constitute a relatively new biotechnology for the stimulation and acceleration of tissue healing and bone regeneration. Their preparation from autologous blood offers advantages in terms of cost, rapidity and security with no possible exogenous viral or prion contamination. It is a novel therapeutic strategy for the acceleration of wound healing in a

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Ethical authorization was obtained by the CRPP under the promotion of INSERM.

Accepted after revision 15 November 2010

wide range of tissues due to the release of multiple growth factors from platelets, including PDGF-AB, TGF-beta1, IGF-1, HGF, VEGF-A, and EGF [9].

Here we report the use of platelet-rich clots as an aid to preventing bleeding and for tissue regeneration in patients with severe inherited mucocutaneous bleeding disorders. An antifibrinolytic agent, tranexamic acid, was also given orally. The clots were prepared from the patient's own platelet-rich plasma (PRP) after the addition of Ca^{2+} and placed on the injured site immediately after surgery. Their successful use without bleeding and without transfusions during dental extraction or skin biopsy for patients with VWD2B, platelet-type-VWD and GT is now reported.

Patients and methods

Patients

A total of four patients with a moderate-to-severe inherited bleeding syndrome were enrolled in this study after informed consent was obtained in accordance with the declaration of Helsinki. In all cases, treatment was in Bordeaux, France. All patients also received 50 mg kg^{-1} tranexamic acid (Exacyl, Sanofi-Aventis, Paris, France) for four consecutive days starting the day before surgery.

The initial patients had VWD2B. The first (a 60-year-old woman) has a heterozygous V1316M substitution in the VWF A1 domain [7]; at the time of study, her platelet count was $47\,000 \text{ platelets } \mu\text{L}^{-1}$ with giant platelets and some small aggregates. For VWF, the values were: VWF:RCO: 39%, VWF:Ag: 62%, FVIIIc: 50% with a VWF:RCO/VWF:Ag ratio of 0.62. The phenotype given by this mutation was previously considered the Montreal Platelet syndrome [11]. Extraction of a single tooth at position 36 was performed with alveolectomy under local anaesthesia (the cartridge also contained epinephrine). The second patient (a 22-year-old woman) had a heterozygous R1341Q substitution in the VWF A1 domain [7]. Her platelet count was $98\,000 \text{ platelets } \mu\text{L}^{-1}$ with giant platelets; VWF values were VWF:RCO: 70%, VWF:Ag: 92% and FVIIIc: 69% with a VWF:RCO/VWF:Ag ratio of 0.76. Extraction of three wisdom teeth (positions 18, 28 and 38) was performed under general anaesthesia. It should be noted that clot retraction remains unaffected in VWD2B.

The third patient is a 61-year-old woman with platelet-type VWD with a heterozygous G233S substitution in GPIIb α [12]. Her platelet count was $186\,000 \text{ platelets } \mu\text{L}^{-1}$; VWF values were VWF:RCO: 70%, VWF:Ag: 120% with a VWF:RCO/VWF:Ag ratio of 0.58. Clot retraction was unaffected by the presence of this mutation. This patient has a metal aortic valve and for this reason receives warfarin; at the time of

surgery, this treatment was stopped and replaced by unfractionated heparin. She underwent a skin biopsy on her leg for a lesion suspected to be a malignant tumour.

The final patient is a previously unreported 60-year-old man with type 1 GT [4] with no platelet aggregation and less than 5% expression of the $\alpha\text{IIb}\beta 3$ integrin on his platelets. His platelet count was normal, but platelet aggregation was absent. The lesion was due to compound heterozygosity for two missense mutations within the *ITGA2B* gene (unpublished data). No plasma isoantibodies to $\alpha\text{IIb}\beta 3$ were detected despite previous transfusions. Clot retraction was absent for this patient. Extraction of two premolars and one molar (positions 34, 35 and 42) was performed under local anaesthesia (as for patient 1) during his first visit and two molars (positions 46 and 47) were extracted during a second visit 2 weeks later.

For all patients, paracetamol was given as a pain killer and non-steroidal anti-inflammatory drugs such as aspirin were avoided.

Preparation of platelet-rich clots

For each patient, 20–30 mL blood was taken by venipuncture into 5 mL siliconized glass tubes containing 3.8% citrate [Becton-Dickinson (BD), Pont de Claix, France]. Approximately 1 h before surgery, the blood was centrifuged at 100 g for 10 min. Platelet-rich plasma (PRP) was taken into a syringe and transferred to non-siliconized and sterile vacutainer tubes (BD Ref 367624) to a volume of 4 mL PRP per tube. Then, $50 \mu\text{L mL}^{-1}$ of sterile calcium chloride (Biotechnology Institute, Vitoria, Spain) was added [13]. The tubes were maintained at 37°C for 20–30 min to obtain the clot. The latter were then placed to fill each extraction socket, or to cover the biopsy injury, and afterwards openings were closed by suturing.

Study of the morphology of platelet-rich clots by electron microscopy

Samples were taken 3, 10, 30 and 60 min after recalcification of the PRP of control donors as described above and fixed in 1.25% glutaraldehyde (Fluka Chemie, Buchs, Switzerland) diluted in 0.1 M phosphate buffer pH 7.2 for 1 h at room temperature. Samples were processed for electron microscopy (EM) as previously described by us [7]. Sections were observed with a Jeol JEM-1010 transmission EM (Jeol) at 80 kV.

Results

Preparation of the platelet-rich clots

Our strategy was to avoid the use of potentially immunogenic bovine thrombin by progressive Ca^{2+} -induced

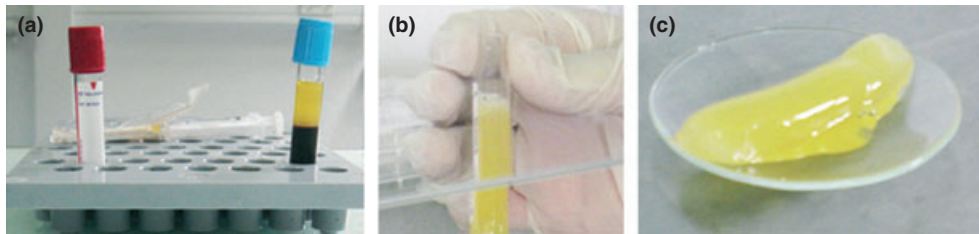


Fig. 1. Preparation of the platelet-rich clot. (a) Anticoagulated blood is taken into a sterile tube (5 mL) and centrifuged at 100 g for 10 min (a, right). The PRP (upper layer) is transferred under sterile conditions to non-silicized and sterile vacutainer tubes (a, left) containing CaCl_2 to promote clotting and maintained at 37°C (b). After 20–30 min, the gelatinous clot is removed (c) and applied to the wound site prior to clot retraction.

generation of endogenous thrombin to transform autologous PRP into a clot. Clots formed after 20–30 min incubation without agitation had a gel-like appearance (Fig. 1). Clot retraction did not begin at this time. The clots are taken from the tubes with tweezers by the surgeon. The morphology of platelets during the clotting process was assessed by EM for a normal donor and is illustrated in Fig. 2; progressively, platelets were activated and incorporated into aggregates. Fibrin fibres formed in the presence of Ca^{2+} are progressively visualized in close proximity to the platelets. After 30 min, fibrin and platelet aggregates form a joint mass and many platelets are de-granulated. After 1 h (long after the recommended time of clinical use), the clot is compacted and platelets show signs of apoptosis. As well as having lost their granules, the platelets were round and vacuolar and often smaller. A fibrin network with a well-defined architecture is now present.

Application of autologous clots to patients with a bleeding syndrome

For all patients, the platelet-rich clot formed normally. For patients 1, 2 and 4, autologous clots formed after 20–30 min were carefully placed into the cavity immediately after tooth extraction and held in place by closing and suturing the lesion.

For patient 1 with the VWD2B V1316M mutation, a lifelong and severe bleeding syndrome has required many transfusions of VWF concentrates and/or platelet concentrates. Signs of easy bruising are constantly present. Despite this history of bleeding and her moderate thrombocytopenia, no bleeding was observed at the site of her lesion following the application of her platelet-rich clot. This was despite the additional removal of bone tissue. Wound healing and bone formation occurred normally in the following weeks.

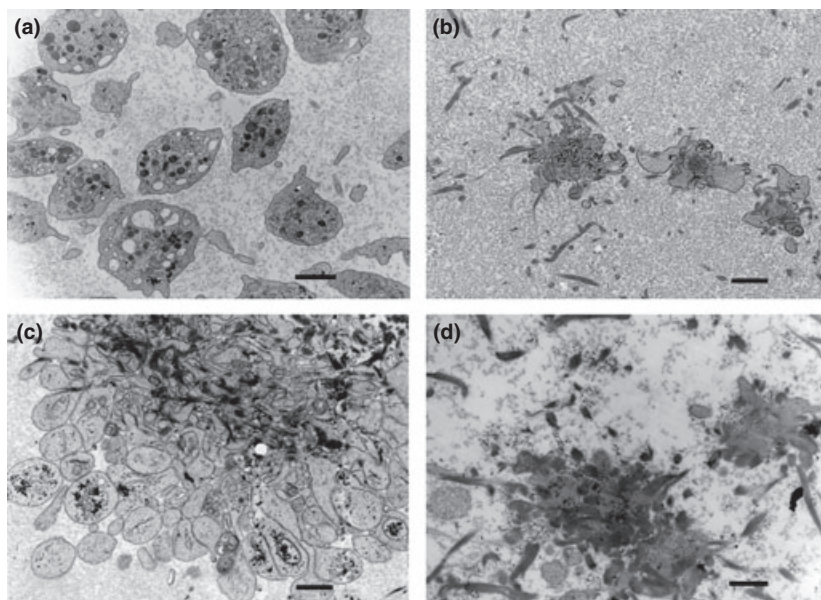


Fig. 2. Time-dependent changes in platelet morphology during clotting. Samples were taken at 3 (a), 10 (b), 30 (c) and 60 min (d) after the addition of an excess of Ca^{2+} to citrated PRP from a normal donor and fixed in glutaraldehyde prior to being processed for EM. As shown in (a), after 3 min, platelets remained isolated and show few signs of activation. After 10 min (b), many platelets are in small aggregates and secretion has started. Fibrin fibres start to be seen at 30 min (c) the aggregates are substantial and many platelets have secreted their granule contents. After 1 h (d), the morphology of the aggregate is less clear and platelets show signs of apoptosis. All bars = 1 μm .

For patient 2 with the VWD2B R1341Q mutation, mucocutaneous bleeding and menorrhagia have accompanied a lifelong moderate bleeding syndrome. The use of platelet-rich clots after the extraction of three wisdom teeth prevented untoward bleeding and no VWF or platelet transfusion was required.

For patient 3 with platelet-type VWD, a lifelong syndrome of mucocutaneous bleeding included a previous history of prolonged bleeding after tooth extraction and menorrhagia. Here, the autologous platelet-rich clot was used to cover the wound left following a skin biopsy. No bleeding was observed and wound healing was rapid and of good quality.

For patient 4 with type I GT, a lifelong history of moderate-to-severe mucocutaneous bleeding has necessitated several transfusions of platelets or red blood cell concentrates. Multiple tooth extraction was performed during two visits to the surgery. After tooth removal with local anaesthesia, some bleeding occurred at the points of needle puncture and during extraction, but as soon as the clots were placed in the dental cavity, bleeding stopped and did not restart during the following days.

Discussion

For patients with inherited bleeding disorders, surgery is always a source of difficulties due to the haemorrhagic risk even for minor events. For example, dental extractions are often not performed on time because patients and dentists are afraid of bleeding. Frequently, the surgery is performed in specialized centres after the transfusion of blood products combined or not with the use of antifibrinolytic therapy [1,2]. Furthermore, with transfusion, the risk of viral or prion transmission is an added worry. For some disorders such as GT, the presence of isoantibodies or anti-HLA antibodies will also render transfused platelets ineffective and/or restrict the use of blood transfusions for future bleeding events.

The use of autologous platelet-rich clots acting as both a physical barrier to blood loss and a source of growth factors is a new technology based on the observation that the local application of a freshly prepared clot containing platelets that have released their content of stored proteins favours wound healing and reduces inflammation [9,10]. Activated platelets provide a large variety of biologically active substances that stimulate cellular proliferation and differentiation [10,14]. Recently, evidence has been provided that pro- and antiangiogenic factors may be stored in separate populations of α -granules allowing their differential release [15,16]. During clot formation, platelets expose phosphatidylserine at their surface both promoting and amplifying thrombin generation. Some thrombin incorporated within clots remains bound to fibrin; this is important as thrombin also

has cell-stimulating properties [17]. Bleeding will be slowed and stopped by the physical contact of blood cells with the fibrin and the favouring of new fibrin formation. Secretion of platelet-derived proteins from the clot is facilitated by clot retraction and, as far as possible, we apply the clot before retraction has occurred. Secreted growth factors may bind to the fibrin fibres and set up chemotactic gradients facilitating cell recruitment to the injured sites and cell growth [reviewed in 9,10]. For example, among recruited cells are circulating mesenchymal stem cells that in dentistry promote osteoblast formation and bone formation [18]. The presence of fibrin gel constitutes a barrier largely used in surgery.

Using platelet-rich clots is ideal for minor surgery as described here such as tooth extraction or skin biopsy, while it can also be used for skin lesions such as leg ulcers [19]. The use of autologous platelet clots constitutes an inexpensive and safe treatment and allows the patient to be more confident in undergoing necessary and timely dental care. It should be noted that we have used Ca^{2+} to induce clot formation; Ca^{2+} is a safer alternative to commercial bovine thrombin preparations that have the risk of immunological side effects (discussed in [9]). The procedure is simple and although it should initially be performed under specialized supervision, training in the preparation of the clots can be easily given. For all of the patients treated by us, tranexamic acid was given to avoid premature fibrinolysis. Further studies will be required to assess whether this precautionary measure is necessary.

An important question is whether the nature of the genetic defects would interfere with the efficacy of the method. In neither VWD2B nor platelet-type VWD is clot retraction affected, whereas platelets of these patients have a normal α -granule content and secretion. Blocking of GPIIb-dependent adhesion does not interfere with the platelet functions required here, whereas the extent of the thrombocytopenia in these diseases would rarely reach the levels expected to cause a major deficiency of growth factors or secreted products. In GT, there is reason for further investigations. In type I disease, the most common form [4], clot retraction is affected and this was the case for our patient. The lack of platelet aggregation would prevent the formation of the platelet 'hubs' that characterize the organization of the normal clot [illustrated by immunofluorescence microscopy in 10] and facilitate its retraction. Nonetheless, while GT platelets fail to bind Fg when stimulated, they are able to bind fibrin and are readily incorporated within the clot [20,21]. It is relevant that rFVIIa has been used successfully in GT and works by consolidating fibrin deposition and structure at sites of vessel injury [21,22]. GT platelets have a normal number of α -granules, the contents of which are secreted on platelet activation. Thus it appears, at least under the conditions where the clot is enclosed within a

dental cavity, that clot retraction may not be a necessary process for PRP technology. Autologous fibrin gels made without platelets have also proved beneficial in GT in the past [23].

Although the rareness of the disorders is limiting, our reports on four patients need to be followed up by extended studies. A restriction for the use of platelet-rich clots is the accessibility of the wound, although injections of platelet releasates are now being widely used as an aid to muscle or tendon repair in sports injuries [reviewed in 24] For more critical surgery, platelet-rich clots can also be used as an adjunctive therapy to the transfusion of blood products to favour the arrest of bleeding and provide better and more rapid healing. In conclusion, we have described a simple method that can be used for minor surgery that can improve the quality of life of patients with inherited bleeding disorders. The benefits include not only decreased bleeding but also added security that will

encourage patients to seek early help for painful local procedures such as tooth extraction or skin ulcers.

Disclosures

PN, IY, FS, EL and ATN have no competing financial interests. EA is a director of BTI Biotechnology company and IA was an employee of the company at the time of the study.

Authorship

PN, IY, FS and EL participated in clot preparation and treating the patients. PN, EA, IA and ATN participated in study design and in writing the manuscript.

Funding

The study was funded by a subvention accorded to ATN by GIS-Maladies Rares.

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