Ex-vivo Platelet Activation using Electric Pulse Stimulation

Nicole LaPlante, V. Bogdan Neculaes, Brian D. Lee, Andrew S. Torres, Kenneth Conway, Steve Klopman and Antonio Caiafa
GE Global Research Center, Niskayuna, NY, U.S.A.

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Abstract: Activated platelet rich plasma (PRP), also known as platelet gel, is an encouraging autologous cell therapy with numerous applications in areas including: wound healing, haemostasis and wound infection control. Activation of PRP using electric pulse stimulation is a promising alternative to activation via biologics such as bovine thrombin. By removing the need for biologics, it is possible to deliver a cost-effective, fast, truly autologous platelet gel option. In this position paper, we describe parameters for effective ex-vivo release of several growth factors from human platelets in PRP using electric field pulses with the duration of hundreds of nanoseconds. Growth factor release levels with nanosecond pulse electric fields seem at the same level or higher compared to bovine thrombin, the standard platelet activator used in clinical practice. These findings suggest that electric pulse stimulation has the potential to become not only a viable alternative to biochemical platelet activators, but to actually enhance the desired in vivo biological effects, such as wound healing.

1 INTRODUCTION

Whole blood contains several components, including red blood cells, white blood cells, plasma and platelets. Platelets have a typical lifespan of about seven to ten days and will concentrate and aggregate at the site of injury as part of the body's response to promote haemostasis, tissue regeneration and revascularization (Tate and Crane 1999). Platelets are formed in the bone marrow and contain populations of granules – such as alpha granules and dense granules. Normal platelet count in whole blood is about 200,000 platelets/ul (Tate and Crane, 1999).

Platelet cell therapy is an approach to harvest the natural ability of the body to stop the bleeding and promote wound healing. By collecting one's platelets, activating them ex-vivo, and placing them back on the wound, a novel therapeutic approach has been developed, that dramatically enhances what the body has been programmed to accomplish naturally.

Platelet gel is a substance containing a concentrated amount of platelets which are activated to release proteins found within the alpha granules. These proteins, which include numerous growth factors, are released upon platelet activation and include platelet-derived growth factor (PDGF), transforming growth factor-beta (TGF- β), vascular endothelial growth factor (VEGF) and epidermal growth factor (EGF). Activated platelets, platelet gel, have been shown to enhance wound healing (Driver, 2006,, Lacci, 2010), induce hemostasis (Gunyadin, 2008), and provide antibacterial protection for the wound as it heals. The application landscape is quite broad for platelet gel. Among the many potential clinical applications, effective therapy has been shown for diabetic foot ulcers, dentistry, cardiac surgery, cosmetic surgery, orthopaedic surgery, sports medicine.

Typical workflow for generating platelet gel performed at the bed side includes a blood draw from the patient, platelet separation/concentration (centrifugation is the state of the art method), and, finally, ex-vivo platelet activation using a combination of thrombin and calcium chloride. After activation, platelet concentrates have a gel-like consistency. The final step is the application of this gel on the pre-determined wound. Currently, platelet activation is performed using bovine thrombin (state of the art in US) or other types of thrombin in Canada and Europe (recombinant thrombin, autologous thrombin or human thrombin isolated from donor plasma). Various types of thrombin currently used are rather expensive and can have

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significant side effects. For example, bovine thrombin–associated immune-mediated coagulopathy will incur a cost per patient from $ 16,584 to $ 163,072 (Alexander, 2009). The workflow of generating autologous thrombin can be complex and lengthy, about 40 minutes (http://www.biomet.com/biologics/international/print/BBI0004.1_081508.pdf). Human thrombin from donor plasma carries a risk for transmitting of infection diseases (Cada, 2008). A truly autologous, fast and significantly less expensive platelet activation method would eliminate potential side effects and will lower the cost for the platelet gel treatment, opening opportunities for increased access of this therapeutic approach.

This autologous, non-animal derived, non-biochemical activation method would allow a fast and completely autologous platelet gel solution in the clinic, for difficult to heal wounds. Autologous platelet gel could become the standard of care for difficult to heal wounds, such as diabetic foot ulcers.

2 PLATELET ACTIVATION USING ELECTRIC PULSE STIMULATION

Short duration pulse electric fields, in the nanosecond range, have been shown to have significant effects on the intracellular structures (Beebe, 2005). Typically, long pulse electric fields, larger than 0.1 ms, have been efficiently utilized for cell membrane permeabilization termed "electroporation", with a variety of applications such as exogenous molecule delivery, transfection/gene delivery, and tumor cell death using irreversible electroporation. The use of modelling tools in the context of an electrical model for biological cells, predicts that pulses with duration shorter than the charging time of the outer cellular membrane, can affect the intracellular organelles (Beebe, 2005).

Experiments have indeed confirmed this theoretical prediction; numerous experimental demonstrations have pointed out biological effects of electric pulse stimulation in the nanosecond range: modulation of caspase activity (caspases modulate a variety of cell functions – proliferation, differentiation, cell cycle), apopotosis – programmed cell death and calcium mobilization (Beebe, 2005). These short, nanosecond electric field pulses are thought to create small pores – nanopores- in the organelle membranes.

Nanosecond pulse electric field effects on calcium transport have been recently introduced as a novel method for ex-vivo platelet activation (Zhang, 2008). It has been hypothesized that nanosecond pulse electric fields, nsPEF, cause calcium to leak out from the intracellular stores as of result of nanopores being created in organelles membrane, as well as an influx of extracellular calcium through plasma membrane nanopores (Zhang, 2008). This calcium transport with nsPEF has been correlated with platelet activation; initial experiments using newly outdated platelets from the American Red Cross showed platelet aggregation with nsPEF, with evaluation of one growth factor following activation, PDGF (Zhang, 2008). Measurements of PDGF release from washed platelets with nsPEF were compared to PDGF release using bovine thrombin; PDGF levels with nsPEF were generally close to the release measured using bovine thrombin (Zhang, 2008).

While these experiments have been very promising for the proof of concept platelet activation using nsPEF, there has been a need for a more complete characterization and benchmarking of this novel activation method. This paper presents a study of growth factor release from human platelets by looking at several growth factors, and reveals significant differences between thrombin-mediated activation and nsPEF-mediated activation of platelets.

2.1 Platelet Rich Plasma (PRP) Preparation

For each experiment, one unit of human whole blood from single donor was purchased from a commercial vendor (Bioreclamation) and shipped following lab testing for standard pathogens; the vendor used ACD as an anticoagulant. Blood was therefore 3 days old at time of PRP preparation. Haematological measurements are performed on the day of PRP preparation, including density of red blood cells (RBCs), platelets (PLTs) and haemoglobin.

Standard preparation of PRP was performed using a commercial kit and centrifuge (SmartPReP2 APC+, Harvest Technologies) per manufacturer's protocol. Briefly, 60mL of whole blood is placed in separation device and up to 7mL of PRP is recovered following centrifugation steps, which usually take about 15 minutes. Typical enrichment of PLTs is 3 times the amount of the starting density in whole blood. PRP is aliquoted (1mL per aliquot) into 4mm cuvettes (Molecular BioProducts catalog #212373) or 1.5mL Eppendorf tubes for activation

studies and allowed to sit at room temperature until used for experiment.

2.2 Activation of Platelets

2.2.1 Thrombin-mediated Activation

Reagents were prepared and stored on ice on the day of experiment. Bovine thrombin (BioPharm Laboratories catalog #91-010) was prepared in saline solution (0.9% NaCl) at a stock concentration to allow for 1:10 (vol/vol) standard dilution in all experiments.

Bovine thrombin preparation details are below: 124 mg/bottle = 10000 U/bottle in 1 mL 0.9% NaCl for injection = 10 U/uL. Do 1:10 dilution so that concentration = 1U/uL. For 50 U experiments, add 50 uL (we used 1, 5, 50, 500, 1000 U for our experiments of platelet activation with bovine thrombin). Unless stated – data presented in this paper will focus on results obtained with 1 U. CaCl2 (Sigma Aldrich) was prepared at stock concentration to allow for 1:100 (vol/vol) standard dilution in all experiments but is maintained in these studies at 10mM CaCl2.

We add bovine thrombin to 1 mL of PRP in 4mm cuvette (Molecular BioProducts catalog #212373) and allow sample to sit at room temperature; typically clotting with bovine thrombin occurs within roughly 30 seconds. The PRP is then centrifuged at 10,000 rpm for 10 minutes in an Eppendorf tube. The supernatant is pipetted from tube and either used in assay immediately or stored at ≤ -20 C.

2.2.2 Activation Studies: nsPEF-mediated Activation

For each experiment, we applied electric field pulses to 1 mL of freshly prepared PRP in 4mm cuvette (Molecular BioProducts catalog #212373) and allow sample to sit at room temperature for up to 30 minutes (clotting takes place within roughly 5 minutes). The PRP is then centrifuged at 10,000 rpm for 10 minutes in a Eppendorf tube. The supernatant is pipetted from tube and either used in assay immediately or at ≤ -20 C.

2.2.3 Growth Factor Measurements

All measurements are performed using commercial enzyme-linked immunosorbent assays (ELISAs) using manufacturer's protocols: PDGF (R&D Systems, #DAA00B), IGF-1 (R&D Systems, #DG100), EGF (R&D Systems, DEG00), VEGF (R&D Systems, DVE00).

3 PULSE GENERATOR FOR PLATELET ACTIVATION AND EXPERIMENTAL RESULTS

Electric pulse generation relies on a few approaches, the most common being capacitive energy discharge and pulse forming networks. Capacitive energy discharge methods are the simplest, but they provide pulses that can be very difficult to regulate in amplitude and duration. Pulse forming networks organized as lines or an ensemble of passive elements such as inductors and capacitors are by far the most common topology to generate short square pulse with specified pulse width.

The pulse generator for ex-vivo activation of platelets has been designed and built at Old Dominion University (ODU) and delivers 300 ns pulses to the load; the load is a 4-mm cuvette containing platelet rich plasma. The output voltage of the instrument is 12 kV and creates an electric field of 30 kV/cm in the 4 mm cuvettes. The instrument was designed so that the impedance of the 4 mm cuvette with platelet rich plasma matches its output impedance. Typically, the 4 mm cuvette with platelet rich plasma will behave like a resistive load, with an impedance of roughly 15 ohm.

The nanosecond pulse generator is powered by standard 110 V AC and can deliver 1 – 9 nanosecond electric field pulses in a single sequence. The device also provides a cuvette holder that is designed for standard electroporation cuvettes that are commercially available for in vitro workflows.

The nanosecond pulse generator uses a pulse forming network to generate the 300 ns pulses. The pulse forming network consists of a combination of capacitors and inductors arranged in a Blumlein-line configuration, as shown in Figure 1. The generator uses an AC-DC rectifier and a DC-DC converter to step up the voltage from 110 V AC to about 12 kV DC. A spark gap switch is used to determine the output voltage of the pulse forming network.

The cuvette holder is easily accessed through an opening that is placed on the top of the device.

A picture of the internal components of the nanosecond pulse electric field generator is shown in Figure 1.

Generally, experiments for platelet activation using the instrument described here used electric

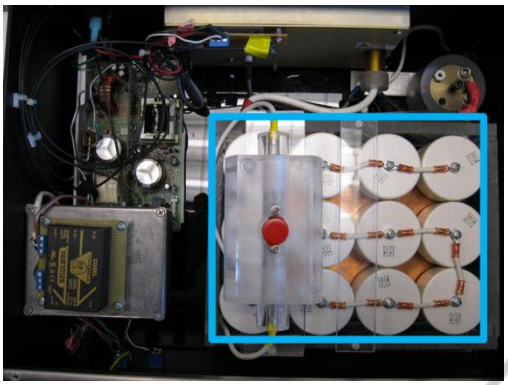

Figure 1: Picture of internal architecture of the nanosecond pulse electric field generator; the pulse forming network is highlighted in blue.

fields on the order or 30 kV/cm. Five pulses at 5 Hz, were used for platelet activation here. Previous experiments at Old Dominion University have identified the optimal number of pulses for activation as five.

Figure 2 shows an example of activated platelets using nanosecond pulse electric fields. The gel like consistency is easily observed – as a result of platelet aggregation during activation. There are red blood cells in the platelet rich plasma typically separated by the Harvest Technologies instrument – therefore the platelet gel is red. Other platelet separation technologies can leave out the red blood cells – the platelet gel will be yellowish in color.

Figure 2: Example of platelet gel created at GE Global Research using nanosecond pulse electric fields.

Finally, Figure 3 gives a simple visual representation of PRP activation in cuvettes: nsPEF versus negative control (no pulsing). Platelet aggregation and clot formation prevent the PRP to flow to the bottom of the cuvette after nanosecond pulsing; in the control cuvette PRP flows to the bottom, as no activation or clotting occurs.

Several growth factors were evaluated in terms of release, using nanosecond pulse electric fields (nsPEF) and bovine thrombin: TGF – β1, PDGF –

Figure 3: Effects of nsPEF on platelet rich plasma: left hand side cuvette was used as control (no nsPEF), while the right hand side cuvette was pulsed with nsPEF. Pulsed PRP cuvette shows clotting (platelet activation) – not PRP flow. In the control cuvette the PRP flows to the bottom, as no clotting occurs. Here cuvettes are turned upside down.

aa, IGF, VEGF and EGF. The platelet enrichment obtained was around three times higher compared to whole blood. Generally platelet rich plasma has a platelet concentration about three to five times higher compared to whole blood (Whitlow, 2008). Platelet-derived growth factor (PDGF) is responsible with cell replication, stimulates angiogenesis, and regulates collagen synthesis (Tate and Crane, 1999). Transforming growth factor-beta (TGF- β) stimulates undifferentiated mesenchymal cell proliferation, stimulates angiogenesis and regulates mitogenic effects of other growth factors (Tate and Crane, 1999). Vascular endothelial growth factor (VEGF) stimulates angiogenesis and acts as mitogenetic factor for endothelial cells (Tate and Crane, 1999). Epidermal growth factor (EGF) stimulates angiogenesis and promotes growth and differentiation of chondrocytes and osteoblasts (Tate and Crane, 1999). Insulin-like growth factor 1 (IGF-1) has effects on differentiation, peripheral growth, and survival in various cells and tissues.

Blood from several human donors was used for these experiments. As pointed in literature, there are donor-to-donor variations with respect to amount of growth factor released upon platelet activation. As a general trend for the work presented here, we observed that differences between nsPEF and bovine thrombin for VEGF and EGF levels can be considerable.

As expected, nsPEF and bovine thrombin do not increase IGF-1 levels upon platelet activation (Everts, 2006). IGF-1 levels for nsPEF and bovine thrombin are roughly equivalent (data not shown). The fact that IGF-1 levels in activated platelets are at the same levels as in non-activated platelets is

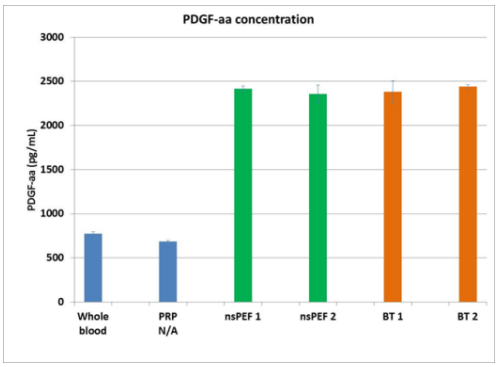

Figure 4: Example of data for PDGF-aa release for nsPEF compared to bovine thrombin (BT) for one donor; 1 and 2 designate the number of cuvettes tested; from each cuvette, three samples of supernatant were tested for growth factor release, and data averaged for each bar graph.

explained by considering that the plasma pool of IGF-1 is greater than the platelet pool; IGF-1 is mainly excreted by the liver in the plasma. Additionally, PDGF-aa and TGF – β1 levels have been observed as largely equivalent between nsPEF and bovine thrombin (TGF – β1 data not shown here).

Representative data from our studies are shown here. "nsPEF" indicates the samples activated with nanosecond pulse electric fields, while "BT" indicates samples activated with bovine thrombin. First, PDGF-aa levels nsPEF versus bovine thrombin are displayed here for a single donor (Figure 4).

We observed that growth factor levels are significantly increased compared to whole blood and platelet rich plasma (PRP) that was not activated. Unless specified, the bovine thrombin activation was performed with 1 U/ul. Each bar graph was obtained from a different cuvette – using PRP from the same donor.

Figure 5 shows that the use of nsPEF increases the levels of VEGF and EGF compared to negative controls, whole blood and non-activated platelet rich plasma. As pointed here, significantly higher levels of VEGF and EGF seem to be released with nsPEF compared to bovine thrombin. This was an unexpected result. Various measurements of growth factor release with several bovine thrombin concentrations – from 1 to 1000 units – did not trigger similar VEGF and EGF levels as obtained with nsPEF (data not shown here). Therefore the results in Figures 5 do not seem to be caused by an insufficient amount of added bovine thrombin.

Finally, if one looks at donor to donor variability for EGF and VEGF release – our data seem to indicate much higher variability for bovine thrombin

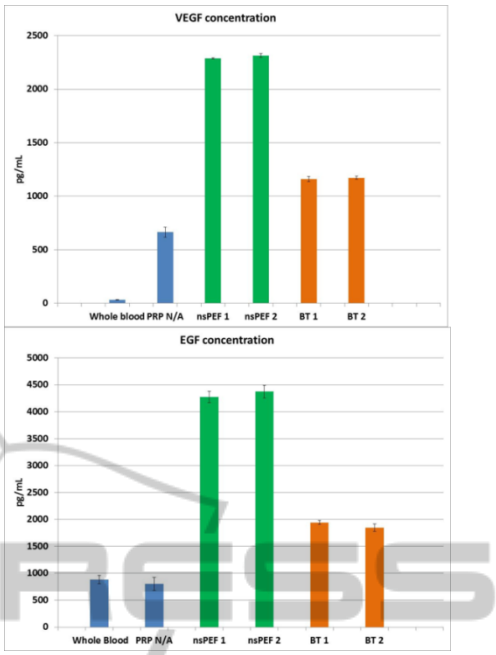

Figure 5: Example of data for VEGF and EGF release for nsPEF compared to bovine thrombin (BT) for one donor. 1 and 2 designate the number of cuvettes tested; from each cuvette, three samples of supernatant were tested for growth factor release, and data averaged for each bar graph.

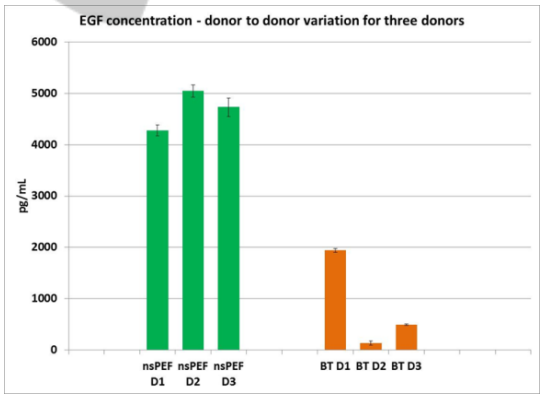

Figure 6: Example of data for EGF release for nsPEF compared to bovine thrombin (BT), for three donors – D1, D2 and D3.

compared to nsPEF. As an example, Figure 6 displays EGF release data for three donors, bovine thrombin (1 U) versus nsPEF.

What is striking about the data in Figure 6 is not necessarily the high donor to donor variability for EGF release with bovine thrombin – this has been noted previously in literature. The much lower variability of EGF release with nsPEF from donor to donor is intriguing and unexpected.

4 DISCUSSION OF RESULTS

Experimental data for growth factor release seem to indicate that using electric pulse stimulation in the nanosecond range for platelet activation would result in a different growth factor profile compared to bovine thrombin. For example, existing data in literature show that typical physiological platelet activators – thrombin, ADP, collagen – tend to have relatively similar growth release profiles for VEGF (Maloney, 1998). While the use of nsPEF can offer the advantage of platelet activation without the use animal based activators already on the market – bovine thrombin, bovine derived collagen – one would have to establish the in vivo wound healing effects of this different growth factor mix.

There are additional questions to be answered with in vitro experiments before considering a clinical path, such as the effects of nsPEF on other components in the platelet rich plasma (white blood cells - WBC, red blood cells - RBC). Additionally, one would need to understand how nsPEF act on platelet rich plasma compositions produced by the numerous platelet separation machines commercially available.

Different devices produce various versions of platelet rich plasmas – RBC count, WBC count, platelet enrichment, viability of PRP components can vary. These versions of PRP may not only be different from a biology point of view, but also they could exhibit different electrical behaviours, which may need to be accounted when one would design a commercial instrument. Finally, experiments described in this work use typical electroporation cuvettes that may need additional qualification for any human in vivo work.

5 CONCLUSIONS

The use of nanosecond electric field pulses for ex-vivo platelet activation is an exciting novel technology, which opens promising opportunities for a truly autologous solution in the platelet gel space, by accomplishing platelet aggregation and growth factor release without using animal derived activators. It should be noted that previous researchers demonstrated various means for platelet activation that do not include the use of thrombin or other bio-chemical vectors – based on the use of physical means such as ultrasound (Poliachik, 2001), light (Verhaar, 2008) and high speed centrifugation (Mazzucco, 2009). However the use of nsPEF for platelet activation has significant advantages over previous attempts to bypass the use of biochemical activators: speed (~ 1 s exposure to electric field pulses), process control, low cost, simplified workflow. The wide potential applicability of platelet gel therapy – healing of non-healing wounds such as diabetic foot ulcers, haemostasis, and reduction of wound infection – may be further fostered by the introduction of this rapid, low cost, easy access, truly autologous, non-animal derived platelet activation method.

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