Ex vivo platelet activation with extended duration pulse electric fields for autologous platelet gel applications

A new, potential clinical standard for platelet activation and perspectives for a more widespread adoption and improved wound healing with platelet gels

INTRODUCTION
Suboptimal wound healing represents a major threat to public health and an enormous financial burden to the healthcare system. Tens of millions of patients worldwide present every year with difficult-to-heal wounds. The major causes of these chronic skin ulcers are pressure (decubitus ulcers), venous stasis disease, and diabetes mellitus. It is estimated that 1%–2% of the population in developed countries will suffer from chronic wounds during their lifetime. More than 25% of the 29 million individuals with diabetes in the United States will develop a diabetic foot ulcer within their lifetime, resulting in about 200 amputations daily.

There is a pressing need to significantly improve outcomes for patients with chronic wounds, with enhanced safety and much lower costs. The use of autologous growth factors derived from platelets has emerged in the last decade as a promising wound-healing approach and has been explored for a variety of clinical applications, including diabetic foot ulcers, dentistry, cardiac surgery, sports medicine, and cosmetic surgery. Upon activation, platelets release multiple growth factors with crucial roles in tissue regeneration, angiogenesis, and epithelialisation. Activated platelet-rich plasma (PRP), also known as platelet gel, is a delivery vehicle for autologous growth factors involved in the wound-healing cascade. Platelet gel is the only known approach for delivering physiological combinations of growth factors than can act synergistically to repair wounds.

The workflow for the preparation of autologous platelet gel involves several steps: 1) blood draw from the patient; 2) PRP separation from whole blood, typically via centrifugation; 3) PRP activation using calcium chloride and thrombin (typically bovine thrombin), which results in clotting and growth fac-
tor release; and 4) application of platelet gel to the wound site.

Bovine thrombin is the most widely used platelet activator in the clinic. However, bovine thrombin is expensive, needs specific storage conditions and logistics, and may trigger side effects. A previous study states that “30% of the patients exposed to bovine thrombin develop cross-reacting antibodies”\(^\text{12}\). Bovine thrombin-associated immune-mediated coagulopathy costs from $16,584 to $163,072 per patient\(^\text{13}\). Some countries - Europe, Japan etc. - do not allow the use of bovine thrombin in the clinic\(^\text{14}\). Other types of thrombin (recombinant thrombin, human thrombin from donor plasma) have not overcome the cost and side effect challenges\(^\text{15}\), and autologous thrombin does not seem to be an effective platelet activator\(^\text{16}\). Additionally, the use of thrombin for platelet activation has not been standardised in clinical practice. The lack of an activation standard has been one of major challenges for the clinical application of platelet gel.

A promising platelet activation method that offers several advantages over bovine thrombin (cost, ease of use, no known side effects, potential for standardisation) has emerged recently. This method uses electric field pulses for ex vivo platelet activation\(^\text{17,18}\).

2. AN INSTRUMENT-BASED PLATELET ACTIVATION AS A POTENTIAL NEW CLINICAL STANDARD

Research in the last few years has generated encouraging results for platelet activation with pulse electric fields (PEF)\(^\text{17-20}\). Clotting and growth factors are released using nanosecond-duration electric field pulses (typically hundreds of nanoseconds). Although preliminary studies reported lower levels of growth factor release from pooled platelets exposed to nanosecond PEF compared to those exposed to bovine thrombin\(^\text{17}\), further research from GE Global Research showed growth factor release at the same level or higher than that of bovine thrombin activation\(^\text{18-20}\). It has been assumed that nanosecond PEF is needed for platelet activation, since these short pulses may produce specific intracellular effects involved in the activation cascade (e.g., release of intracellular calcium)\(^\text{17}\). However, pulse duration at the nanosecond level typically requires expensive and at times unreliable power electronics.

We have discovered that electric pulses longer than 1 microsecond can also activate platelets. The power electronics architecture required to generate these extended-duration pulses is simpler, more reliable, and easier to implement in clinical-grade instruments. We have built the first instrument prototype for platelet activation using extended-duration pulses (see Figure 1). This instrument has the compactness, reliability, ease of use, and ergonomics needed for in vitro biology and research-level wound-healing studies. The experimental workflow is user-friendly, and instrument packaging and high-voltage safety measures have been considered during the design phase of this platelet-activation platform.

For this study, we used whole blood donated by healthy human donors. PRP was separated from whole blood via centrifugation (see Fig. 2) using an approved clinical centrifuge. The PRP was then placed in a commercially available low-cost electroporation cuvette that was sterile and disposable (see Fig. 3) and exposed to extended-duration electric field pulses. After activation via electrical stimulation, the activated PRP/platelet gel may be used for wound-healing studies or in vitro analysis, as performed here. Although the power electronics topology of the platelet activation instrument will be described elsewhere, it should be mentioned that the instrument design properly addresses the unique requirements posed by the specific electrical properties of PRP (i.e., highly conductive, which is very different from the low-conductivity buffers used by other classes of instruments designed for electroporation). The instrument has electrical safety measures that enable its easy operation by a healthcare professional with minimal training.

3. MATERIALS AND METHODS

A. Platelet-rich plasma preparation

We handled whole blood and samples derived from whole blood, such as platelet-poor plasma and PRP, using universal precautions. Single units of human whole blood from individual donors were purchased and shipped overnight at room temperature from a commercial vendor\(^\text{14}\). All studies used acid citrate dextrose as an anticoagulant. We report here experimental results using the blood from a single donor.

For a single preparation of PRP, 60 mL of whole blood was drawn from the original unit of blood using the syringe and needle components of the preparation kit\(^\text{15}\). The whole blood was injected into the separation consumable (Fig. 2) and centrifuged using the clinically approved centrifuge\(^\text{16}\). Complete separation by centrifugation typically takes 15 minutes (see Fig. 2). We then collected PRP, with
a yield of -10 mL per 60 mL whole blood used. The PRP was maintained at room temperature for all experiments. The proportion of platelets in the prepared PRP was threefold higher than in the original whole blood sample.

**B. Platelet activation**

1) Reagents
All biochemical reagents were prepared and stored on ice on the day of experiment. A stock solution of bovine thrombin was prepared in saline solution (0.9% NaCl) at a concentration of 10 U/μL to allow for 1:10 (vol/vol) standard dilution in all experiments. Final concentration after addition to PRP was 1 U/μL. A stock solution of CaCl₂ was prepared at 1 M concentration to allow for 1:100 (vol/vol) standard dilution in all experiments. The final concentration after addition to PRP was 10 mM CaCl₂.

2) Thrombin-mediated activation of platelet-rich plasma
Bovine thrombin (100 U/μL) was added to 0.5 mL PRP with 5 μL PRPCaCl₂ (1 M) in 1.5-mL sterile tubes, diluted 1:100 (final concentration 1 U/μL), and then incubated at room temperature. Tubes were then centrifuged at 10,000 rpm for 10 minutes. The resulting supernatant was pipetted from the tube and either used immediately or stored at or below -20°C.

3) Pulse electric field-mediated activation of platelet-rich plasma
For each experiment, 5 μL CaCl₂ (1 M) was added to 0.5 mL freshly prepared PRP in a 2-mm cuvette (see Figure 3). The sample was subsequently exposed to one electric pulse of extended duration, transferred to a 1.5-mL sterile tube, and incubated at room temperature for approximately 15 minutes. The samples were then centrifuged at 10,000 rpm for 10 minutes. The supernatant was pipetted from the tube immediately after the pulse and either used immediately or stored at or below -20°C.

**C. Growth factor measurements**
We measured the release of platelet-derived growth factor-AA (PDGF-AA), epidermal growth factor (EGF), and vascular endothelial growth factor (VEGF) using the following three commercial kits: Human/Mouse PDGF-AA Immunoassay, Human EGF Immunoassay, and Human VEGF Immunoassay. The measurement of PDGF-AA and EGF required supernatant dilutions of 1:10 and 1:20, respectively; for VEGF, no dilution was required. We followed the manufacturer’s protocol for each assay without deviation.

**D. Cell proliferation assays**
MCF-10A cells were maintained in media supplemented with 10% foetal bovine serum. These cells are a non-tumorigenic epithelial line that should respond to growth factors. Human dermal fibroblasts (HDF) and human dermal microvascular cells (HMDVECs) were each maintained in MCDB-131 medium supplemented with foetal bovine serum and a culture medium. The HDFs model epithelialisation, with increased growth of these cells indicating wound healing. The HDMVECs model vascularisation, with increased growth of these cells indicating the formation of new blood vessels, also important to wound healing.

Cells were seeded at a density of 200,000 cells/cm² and incubated for 24 hours. The cells were then washed twice with a balanced salt solution, and the medium was replaced with serum-free medium and incubated for 24 hours. Supernatant from PRP was diluted to 10% vol/vol with serum-free medium and added to the serum-starved cells (total volume 100 μl), which were then incubated 24 hours. This dilution was determined experimentally with a dose-response characterisation study. ATP was measured as a function of cell proliferation using a luminescence ATP

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a. GE Global Research
b. Bioreclamation, Westbury, NY, USA
c. SmartPrep2 APC + PRP, Harvest Technologies, Lakewood, CO, USA
d. Human Growth Technologies

e. Catalog #91-010, BioPharm Laboratories, Buffalo, UT, USA
f. Sigma Aldrich, St. Louis, MO, USA
g. Eppendorf, Hamburg, DE
h. Molecular BioProducts, catalog #21-237-2
i. Eppendorf
j. Catalog #DAA008, R&D Systems, Minneapolis, MN, USA
k. Catalog #DEG00, SEG00, PDEG00, R&D Systems
l. Catalog # DVE00, R&D Systems
m. American Type Culture Collection, Manassas, VA, USA
n. McCoy’s, In Vitro, Grand Island, NY, USA
o. VEC Technologies, Rensselaer, NY, USA
p. Life Technologies, Grand Island, NY, USA
q. EndoGro, VEC Technologies
r. Hank’s Balanced Salt Solution (HBSS), In Vitro
s. ATPlite 1step Single Addition Luminescence ATP Detection Assay, Perkin Elmer, Waltham. MA, USA
detection assay system according to the manufacturer’s recommendations. The experimental conditions used for all cell types were serum starvation, addition of unactivated PRP, addition of bovine thrombin-activated PRP, and addition of PEF-activated PRP. Serum-starved cells were given no additional growth factors; this is considered a basal level of growth. All other conditions were compared to serum starvation as the baseline.

4. EXPERIMENTAL RESULTS
This section presents data on growth factor release from clinical-grade human PRP and cell proliferation to demonstrate the potency of growth factors released upon activation by extended-duration PEF.

Figure 4 shows growth factor release results for unactivated PRP, bovine thrombin-activated PRP, and PRP activated by PEF for three growth factors: PDGF-AA, VEGF, and EGF. Compared with bovine thrombin activation, PEF activation triggered similar PDGF-AA release, higher VEGF release, and much higher EGF release.

Figure 5 illustrates the effects of growth factors released via bovine thrombin and PEF activation on cell proliferation. These experimental plots show cell proliferation after 24-hour stimulation with unactivated PRP supernatant, bovine thrombin-activated PRP supernatant, and PEF-activated PRP supernatant compared to serum-starved cells (baseline control). Using HDMVECs (Fig. 5c) as a surrogate for angiogenesis and HDFs (Fig. 5b) as a surrogate for epithelialisation, our results show that PEF creates a platelet gel with significant effects on epithelialisation and angiogenesis. It is interesting that trends in Figures 5a (MCF10A non-tumourigenic epithelial cell line) and Figure 5b (HDFs) appear similar, which are satisfying results since both of these cell lines are related to epithelialisation.

5. DISCUSSION AND NEXT STEPS
Our paper introduces the concept of ex vivo platelet activation via extended duration PEF and presents the first instrument prototype designed and validated experimentally for growth factor release from human PRP exposed to electrical stimulation. Our experimental results confirm the growth factor release obtained recently with nanosecond PEF18, showing that electric stimulation releases growth factors at the same level or higher than that produced by bovine thrombin. Cell proliferation assays were used as surrogate measures for wound healing. These assays demonstrate the potency of growth factors released by electrical stimulation of PRP with the instrument shown in Figure 1. Recent in vivo experiments showed improved wound healing with activated PRP via nanosecond PEF compared to PRP activated by bovine thrombin21, the...
platelet activator generally used in the clinic today. Additional encouraging findings also suggest that the PRP activated by nanosecond PEF exhibits other improvements over bovine thrombin activation (e.g., higher endogenous antioxidant levels, reduced formation of reactive oxygen species and matrix metalloproteinase-2), hinting towards improved healing outcomes compared to today’s platelet gels. Since a recent study suggests that intracellular effects may be possible not only with ultra-short, nanosecond PEF but also via pulses of extended duration (>1 microsecond), one may expect similar beneficial effects with extended-duration PEF activated PRP (e.g., improved healing, higher antioxidant levels, reduced reactive oxygen species). In addition, the use of extended-duration PEF makes possible a low-cost and reliable instrument for clinical use. More details about the instrument platform design will be presented elsewhere, but in terms of cost per activation, the consumed used in this study (Figure 3) is about 20 times less expensive than bovine thrombin.

This instrument-based method for PRP activation via electrical stimulation could soon become a standard in clinical workflows, since its activation performance is consistent, does not depend on the operator skill, is easy to implement and control, and the activated PRP does not appear to produce side effects.

Future work should focus on further exploring wound-healing benefits of platelet gels activated by extended-duration PEF in in pre-clinical and clinical applications. There may be additional considerations for wound sizes that can be addressed with autologous platelet gels when designing these studies. One recent publication describes diabetic wounds with an area of 185 cm² treated with autologous platelet gel, another recent work stated that about 45–50 mL whole blood would be needed to generate enough platelet gel to treat a 100 cm² wound. It is possible to obtain 130–150 mL whole blood from a patient in a single doctor visit to enable the treatment of a ~300 cm² wound. Nevertheless, trade-offs in terms of maximum wound size for a patient would need to be made in the clinic.

The new technique of activating PRP at a push of a button, in less than a second, may open the door to improved healing outcomes for patients worldwide suffering from chronic wounds. Recent trends point towards the use of platelet gels not only as a replacement for, but also in addition to more traditional wound-healing approaches (e.g., negative pressure therapy). Thus, improvements in wound healing may be enabled by the instrument-based platelet activation method introduced here.

ACKNOWLEDGEMENTS

The authors would like to thank John Burczak (GE Global Research) for supporting this research, and James Rothman (Yale University), Alan Michelson (Harvard Medical School and Center for Platelet Research Studies at Boston Children’s Hospital) and Andrew Frelinger (Harvard Medical School and Center for Platelet Research Studies at Boston Children’s Hospital) for valuable suggestions in this project.

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