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Using extracellular calcium concentration and electric pulse conditions to tune platelet-rich plasma growth factor release and clotting

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A B S T R A C T

Platelet-rich plasma (PRP) is an emerging autologous biologic method for wound healing. Clinicians apply PRP either topically (where it is activated ex-vivo before treatment by adding an external agent to trigger clotting and the release of growth factors that facilitate wound healing) or through injection (where it is activated in vivo at the injury site with no prior activation before injection). Because topical PRP activation typically utilizes bovine thrombin, which has significant potential side effects and high costs, recent studies have assessed the efficacy of combining extracellular calcium (EC) and electric pulses (EPs) to activate PRP. The potential to apply this novel technique to PRP both topically and internally via injection raises the question about the ability to tune the clotting time and growth factor release for a given application. While previous studies have assessed the impact of applying EPs of various durations either directly (conductive coupling) or indirectly (capacitive coupling) to PRP containing EC, no studies have assessed the tunability of this activation based on modifying EP parameters, EP delivery method (conductive or capacitive coupling), and the EC concentration. We hypothesize that tuning these parameters will modify intracellular calcium uptake to permit the control of growth factor release and clotting time, which are critical for optimizing PRP for either topical or internal clinical applications. A pilot study for a single donor demonstrates the potential for tunability as a function of the intensity of membrane manipulation and calcium concentration, which facilitate the increase of cytosolic calcium. This motivates future studies assessing EC and EP optimization and in vivo studies to determine the overall efficacy of this tunability for wound healing.

Introduction

Platelet-rich plasma (PRP) is an emerging autologous biologic method for wound healing in clinical workflows [1,2]. PRP is obtained by drawing a patient's blood and centrifuging it to separate the PRP [3]. Clinical applications entail topically applying activated PRP or injecting non-activated PRP [2–4]. PRP activation includes platelet growth factor release and clotting. There are numerous applications for activated PRP, or platelet gel, including oral surgery, orthopedic surgery, recalcitrant ulcers (such as diabetic foot ulcers), and knee osteoarthritis [5]. Injected non-activated PRP has various uses in sports medicine (knee and elbow injuries) [6,7], with platelet activation occurring at the injury site through collagen activation pathways [4,8]. Clinicians inject only non-activated PRP for knee or elbow type injuries since they cannot inject clotted PRP (platelet gel).

For topical applications, clinicians typically activate PRP ex-vivo using a mix of bovine thrombin (BT), the state of the art activator in the clinic, and $CaCl_2$. Increasing cytosolic calcium concentration, either by releasing intracellular stores or facilitating extracellular calcium transport into the platelets, can activate platelets [9–11]. Upon activation, platelets release growth factors that play pivotal roles in the wound healing cascade [12–13], such as platelet-derived growth factor (PDGF), epidermal growth factor (EGF), and vascular endothelial growth factor (VEGF), while the PRP clots into a platelet gel [5,13,14]. Both thrombin and calcium induce growth factor release in a dose-dependent manner, although high levels of calcium seemed to prevent maximum growth factor release [15]. Another study considering growth factor release as a function of thrombin and calcium concentrations noted that lower concentrations of these substances tended to delay and reduce growth factor release, while higher concentrations immediately and significantly increased basic fibroblast growth factor (bFGF), transforming growth factor β1 (TGF-β1), and PDGF [16]. PDGF, VEGF, and TGF-β1 levels in platelet concentrate supernatants generally remained constant for six days after treatment while bFGF

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concentration decreased after 24 h [16]. Another study showed that adding 10% of $CaCl_2$, 10% of autologous thrombin, a 10% mixture of $CaCl_2$ and thrombin, or 10% of collagen type I caused different growth factor release characteristics [17]. For instance, $CaCl_2$ induced a progressive release of growth factors from 15 min up to 24 h after treatment [17]. On the other hand, collagen type I reduced the overall growth factor release compared to $CaCl_2$, autologous thrombin, or the mixture of $CaCl_2$ and thrombin [17]. Taken together, these studies demonstrate that different activation agents and different concentrations of these agents may tune platelet activation and growth factor release. However, workflow complexity and poor process standardization severely limit the clinical potential of tuning platelet activation and growth factor release using the above mentioned agents. A much simpler method to tune platelet activation and growth factor release, preferably instrument based and easily standardized, may open up new opportunities in PRP based wound healing and tissue regeneration.

Alternative methods of platelet activation are under investigation for both topical and injectable PRP. Injecting thrombin activated PRP is not possible since PRP clots too rapidly to reach the injury site for treatment. In this case, clinicians inject non-activated PRP since it may be activated by collagen activation pathways at the site of injury. Recent research has identified a physical method using light stimulation to release growth factors from platelets without clotting. Current investigations are assessing this new biological matrix comprised of non-clotted, light-stimulated PRP as an alternative to injecting non-activated PRP [18]. For topical applications of activated PRP, bovine thrombin may induce multiple adverse side effects, including immune responses, motivating research into alternative activation methods. Plateletx-Act is a commercial alternative that uses calcium gluconate and batroxobin [19,20], which is a thrombin-like enzyme derived from *Bothrops atrox* snake venom that cleaves fibrinogen to generate fibrin and allow clot formation without directly activating platelets [21]. The growth factor release induced by PRP generated using batroxobin is much slower than from thrombin [19,22], leading to delayed batroxobin-induced clot retraction [21]. Calcium gluconate has also been applied for platelet activation in various clinical conditions [23–25].

One of the emerging areas for activating PRP involves electrically stimulating platelets ex-vivo. Applying specific electric pulses (EPs) to PRP treated with $CaCl_2$ triggers clotting and growth factor release [26–30]. EPs offer a low cost, easy to use, instrument based [29] method for platelet activation, with great potential for standardization, while providing an animal byproduct-free alternative to bovine thrombin activation without the ensuing potential side effects. While electrical stimulation of PRP with $CaCl_2$ induces clotting and growth factor release, preliminary studies have demonstrated that treating PRP with EPs without added $CaCl_2$ induces growth factor release without clotting [32]. This discovery may open up promising clinical applications for PRP injection. For instance, instead of injecting non-activated PRP and relying on in vivo activation at the injury site, one could inject non-clotted PRP with the growth factors already released. Alternatively, we have shown that we can add very different $CaCl_2$ concentrations (2.5 mM and 20 mM) to tune PRP activation and growth factor release [33]. Taken together, these two studies directly motivate the present study to more broadly consider the parameter space for EPs and $CaCl_2$ concentration for tuning PRP activation and growth factor release.

This paper proposes a hypothesis concerning the use of $CaCl_2$ concentration and EP parameters for controlling PRP clotting and growth factor release, outlines the methods to test this hypothesis, reports results of an ex-vivo pilot study supporting the hypothesis, and discusses the implications of the results.

Hypothesis. *Previous studies have considered the two limiting cases [32] of combining EPs with PRP containing either no $CaCl_2$ or sufficient $CaCl_2$ to activate platelets. We hypothesize that $CaCl_2$ concentrations and EP conditions may be tuned to specifically control the clotting time and growth factor release depending upon the desired application. This hypothesis arises from the importance of cytosolic calcium concentration in platelet activation [9–11] and the use of EPs to permeabilize cell membranes by electroporation to facilitate the transportation of calcium ions into the cell by either electrophoresis during the EP or diffusion through longer-lived pores after the EP [34]. Moreover, since electroporation is driven by membrane voltage [35] and we have previously shown that capacitive coupling, where the electrodes do not make direct contact with sample under treatment, induces lower membrane voltages [36], we further hypothesize that adjusting the EP delivery method may also tune platelet activation and growth factor release. This flexibility will ultimately allow the clinician to tune the clotting behavior and growth factor release depending upon the clinical setting and whether one wants to deliver PRP topically or via injection. As a first step toward this goal, this paper explores the implications of combining EPs with various levels of $CaCl_2$ to support the hypothesis of controllable clotting and growth factor release in a single patient pilot study. This study drew blood and prepared PRP using clinical protocols.*

Methods

Healthy volunteers were qualified for enrollment in this study if they were aged ≥ 18 years, free of aspirin or other antiplatelet medication (≥10 days), and free of all other non-steroidal anti-inflammatory drugs (≥3 days). For this pilot study, 120 mL of blood was collected from one volunteer into 1/10th volume of acid-citrate-dextrose solution A (ACD-A). PRP was prepared according to the manufacturer's recommendation using the Harvest SmartPreP2 System (Harvest Technologies, Plymouth, MA, USA) with two 60 mL cartridges. The resultant PRP was pooled prior to further treatment. Complete blood cell counts were performed on the ACD-anticoagulated whole blood and the concentrated PRP in a Sysmex XN Hematology Analyzer.

We evaluated PRP activation by EPs and bovine thrombin (1 U/mL final concentration, Biopharm Laboratories LLC, Bluffdale, UT, USA) with various calcium concentrations. Endpoints measured included 1) clot formation kinetics and strength by thromboelastography (TEG) and 2) growth factors released into the supernatant (EGF, PDGF). All endpoints except TEG were measured in samples taken 15 min after activation. Prior to activation with EP or BT, PRP samples were recalcified by adding 1/100th volume of CaCl2 (Bachem, Torrance, CA, USA).

Electrical stimulation of PRP was performed using a specially designed instrument prototype (GE Global Research, Niskayuna, NY, USA), described elsewhere [29]. The instrument takes into account the specific electrical properties of PRP, which is typically more conductive than the buffers used in electroporation [31]. Concentrated PRP (500 μL) was placed in a 2 mm electroporation cuvette (Molecular BioProducts, San Diego, CA, USA) and pre-loaded with 1/100th volume $CaCl_2$. We used a Tektronix DPO4104 oscilloscope and a Tektronix P6015A high voltage probe to measure the EPs applied to the cuvettes for PRP activation. Fig. 1a shows a representative capacitive coupling pulse with an amplitude of ~750 V, current of ~50 A, and pulse width of ~250 ns, and ~500 ns between each bipolar pulse. Fig. 1b and 1c show typical conductive coupling pulses of ~5 μs in pulse duration with peak voltages of 1.7 kV ("mild" conductive coupling) and 3.4 kV ("strong" conductive coupling), respectively, and peak currents of ~160 A and 300 A, respectively.

Immediately following exposure of PRP to activating conditions, 360 μL of activated PRP was quickly transferred to the thromboelastograph (TEG) cup and immediately analyzed by a TEG 5000 Hemostasis Analyzer System (Haemonetics Corporation, Braintree, MA, USA). We followed the clotting kinetics and clot characteristics for 30 min. Data collected included the time required to initiate clot formation (R time, minutes) and the clot strength (elastic modulus, derived from TEG maximum amplitude, MA, millimeters). Parallel samples activated

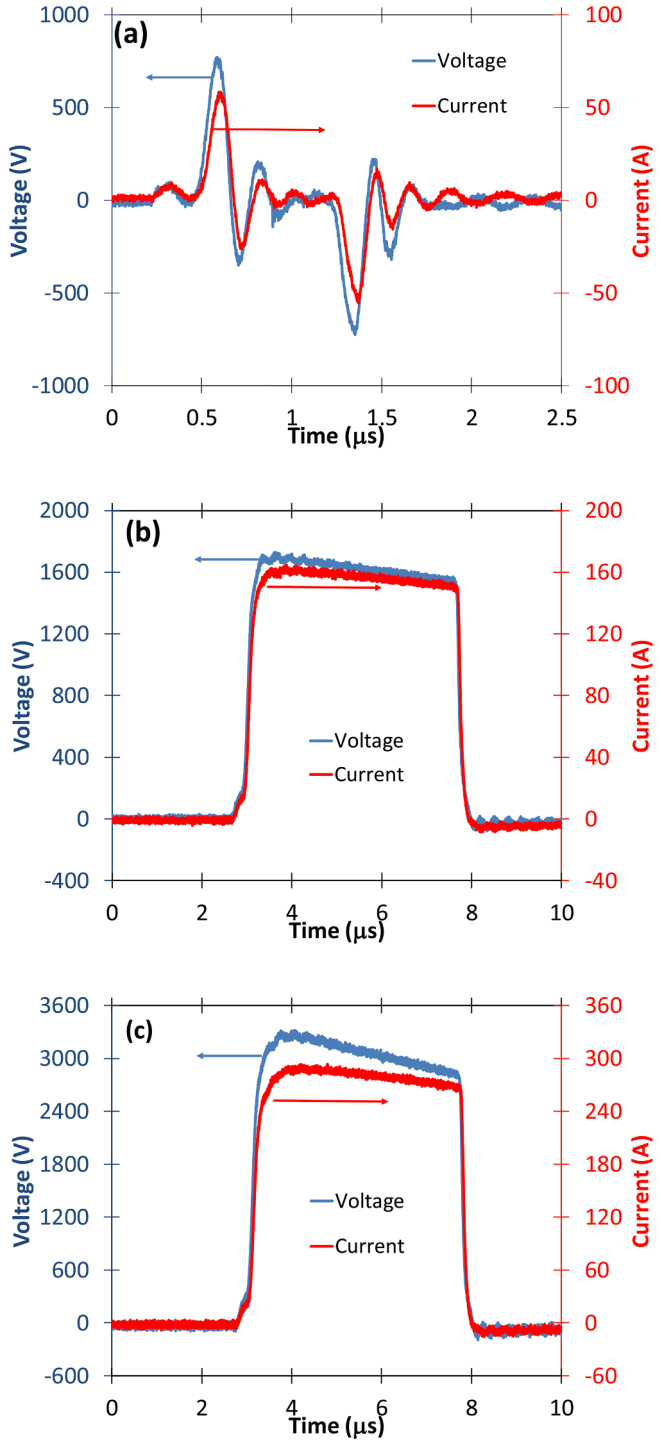

Fig. 1. Representative pulse waveforms for (a) capacitive coupling bipolar pulses with amplitude of ~750 V (3.75 kV/cm), ~50 A, ~250 ns pulse width with ~500 ns between a bipolar pulses, (b) "mild" conductive coupling with amplitude of ~1.7 kV (8.5 kV/cm), ~160 A, ~5 μs pulse width, and (c) "strong" conductive coupling with amplitude ~3.4 kV (17 kV/cm), ~300 A, ~5 μs pulse width.

under identical conditions stood for 15 min at room temperature following activation, after which we removed clots using the wooden handle of a cotton swab and froze the resulting serum at −80 °C for later evaluation of released growth factors. The EGF and PDGF levels in the supernatants of the treated PRP were measured using commercially available ELISA kits (R&D Systems, Minneapolis, MN, USA).

Table 1
Cell counts determined by Sysmex XE.

Parameter	Description	Units	Whole Blood	PRP
WBC	White blood cell	[10^3/μL]	6.62	19.44
RBC	Red blood cell	[10^6/μL]	5.09	2.11
HGB	Hemoglobin	[g/dL]	14.6	6
HCT	Hematocrit	[%]	43.2	19.1
MCV	Mean cell volume	[fL]	84.9	90.5
MCH	Mean cell hemoglobin	[pg]	28.7	28.4
MCHC	Mean cell hemoglobin concentration	[g/dL]	33.8	31.4
RDW-SD	RBC distribution width-size distribution	[fL]	36.7	41.1
RDW-CV	RBC distribution width-calculated	%	12.1	12.6
PLT &F	Platelet count (fluorescent staining)	[10^3/μL]	201	809
MPV	Mean platelet volume	[fL]	8.9	11.2
NEUT	Neutrophils	[10^3/μL]	3.96	5.3
LYMPH	Lymphocytes	[10^3/μL]	2.07	11.19
MONO	Monocytes	[10^3/μL]	0.45	2.75
EO	Eosinophils	[10^3/μL]	0.11	0.1
BASO	Basophils	[10^3/μL]	0.02	0.05
IG	Immature granulocytes	[10^3/μL]	0.01	0.05
NRBC	Nucleated RBC count	[10^3/μL]	0	0
RET	% Reticulocytes	[%]	1.92	4.3
IRF	Immature RET%	[%]	8.9	14.3
RET-He	Reticulocyte hemoglobin equivalent	[pg]	32.8	31.4
IPF	Immature platelet fraction	[%]	3.3	5.4

Results

To evaluate this hypothesis, we performed a proof-of-principle study of ex-vivo PRP activation using various concentrations of $CaCl_2$ combined with typical conductive or capacitive EPs shown in Fig. 1. Table 1 shows complete blood cell counts for the collected whole blood and prepared PRP. In particular, the platelet count increased from 242 × 10^3/μL in whole blood to 977 × 10^3/μL in PRP. Table 2 summarizes the estimated free Ca^{2+} based on the known concentration of citrate, an estimated concentration of plasma calcium of 1.5 mM, the amount of $CaCl_2$ added, and an estimated binding constant between citrate and calcium of $10^{3.5}$ M^{-1}.

Fig. 2a shows the clotting time as a function of the added extracellular calcium concentration (final concentration of $CaCl_2$ added in mM) for the three EP parameters shown in Fig. 1. No clotting occurred using electrical stimulation for added calcium concentrations below 8.49 mM. We have previously calculated that the membrane potential for cells exposed to EPs delivered using capacitive coupling is dramatically lower than for those delivered using conductive coupling [36]. The membrane potential is an important metric for inducing membrane permeabilization, with membrane voltages on the order of 250 mV–1 V typically taken as the threshold for electroporation [37]. Thus, we anticipate that cells exposed to capacitive coupling will exhibit less membrane perturbation than those exposed to conductive coupling and, logically, the membrane potential will be higher for cells exposed to higher voltages using conductive coupling. Fig. 2a shows that the clotting time is consistently higher for the EPs that induce the lower

Table 2
Estimated free concentration of Ca^{2+} as a function of $CaCl_2$ concentration added.

Final concentration of $CaCl_2$ added (mM)	Estimated free Ca^{2+} (mM)
0	≈0.03–0.06
5.35	0.20
8.49	0.40
11.61	0.80
17.04	3.00

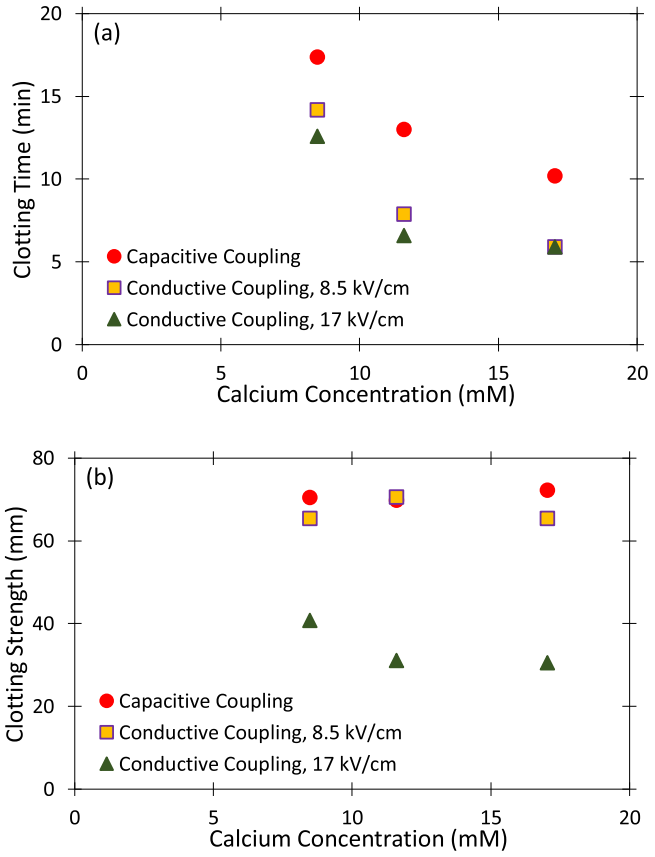

Fig. 2. (a) Clotting time and (b) clotting strength as a function of extracellular $CaCl_2$ concentration for capacitive coupling (Fig. 1a), "mild" conductive coupling (Fig. 1b), and "strong" conductive coupling (Fig. 1c). Clotting time and clot strength for bovine thrombin combined with 17.04 mM of $CaCl_2$ was 1 min and 72.5 mm, respectively.

membrane potential, or cause the lowest degree of electropermeabilization that would facilitate the transport of calcium into the cell. Furthermore, the clotting time decreases with increasing extracellular calcium concentration, as expected since one would expect greater diffusion of calcium into the cells under this condition. At the highest $CaCl_2$ concentration, clotting time for both conductive coupling stimulations is identical, and shorter than capacitive coupling stimulation. This indicates that both EP modality and $CaCl_2$ concentration can tune clotting time compared to BT, specifically that parameters that increase cytosolic calcium concentration will reduce the clotting time.

Fig. 2b shows the clotting strength as a function of extracellular calcium concentration for the three EP parameters shown in Fig. 1. The clotting strength is noticeably higher for the capacitive coupling and lower intensity conductive coupling EPs than for the higher intensity conductive coupling EP. Moreover, the clotting strength is independent of calcium concentration for the capacitive coupling and lower intensity conductive coupling EPs, while it initially decreases with increasing $CaCl_2$ concentration before reaching a constant for the higher intensity conductive coupling EP. The capacitive coupling EP yields very similar clotting strength to that of the positive control BT at 17.04 mM $CaCl_2$, while the conductive coupling EPs both have lower clotting strengths. This indicates that clotting strength is very sensitive to the membrane potential with higher membrane potentials dramatically reducing the clotting strength. This also shows that clotting strength is generally insensitive to cytosolic calcium concentration and more sensitive to membrane perturbation with EPs inducing higher membrane potentials more likely to induce lower clotting strength. In this case, using capacitive coupling or the lower intensity conductive coupling field can electrically mimic the clotting strength of BT.

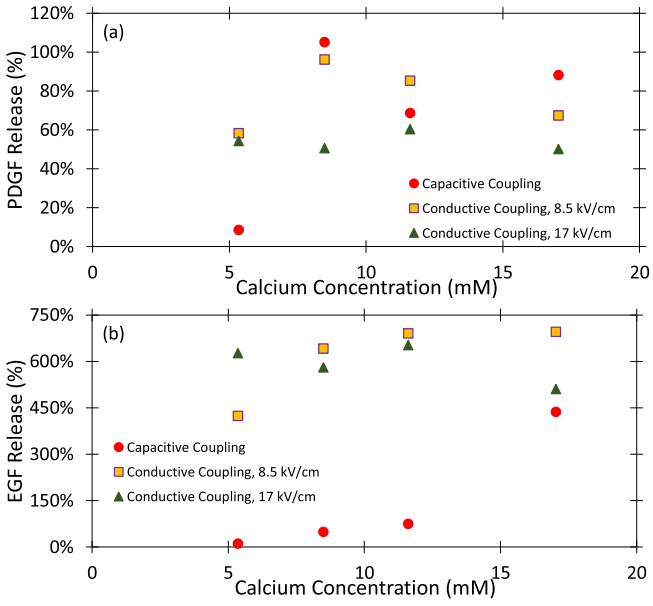

Fig. 3. Release of (a) Platelet derived growth factor (PDGF) and (b) endothelial growth factor (EGF) as a percentage of growth factor release induced by bovine thrombin as a function of as a function of extracellular $CaCl_2$ concentration for capacitive coupling (Fig. 1a), "mild" conductive coupling (Fig. 1b) and "strong" conductive coupling (Fig. 1c).

EP parameters and $CaCl_2$ concentration may also be used to tune growth factor release. Fig. 3a shows PDGF release as a percentage of that induced by BT at 17.04 mM $CaCl_2$, as a function of extracellular calcium concentration for the three EPs shown in Fig. 1. Initially, PDGF for capacitive coupling is much lower than either conductive coupling condition, while the lower intensity conductive coupling EP has a higher PDGF than the higher intensity EP. At higher $CaCl_2$ concentrations, the PDGF level following capacitive coupling is comparable to or higher than that induced by the lower intensity conductive coupling EP and generally higher than the higher intensity conductive coupling EP. At 8.49 mM $CaCl_2$, delivering the EP using capacitive coupling induced the same level of PDGF as BT and a higher level than either conductive coupling case. At all $CaCl_2$ concentrations, the lower intensity conductive coupling EP induced a higher PDGF level than the higher intensity EP, suggesting a threshold above which additional membrane perturbation does not benefit growth factor release. Taken together, these results suggest that capacitive coupling, with its slight membrane perturbation, is sufficient to induce PDGF release similar to BT at sufficiently high $CaCl_2$, while the additional membrane perturbation of conductive coupling at higher $CaCl_2$ is not beneficial. This indicates the potential to tune PDGF by using EP modality, EP intensity, and $CaCl_2$ concentration.

Fig. 3b shows EGF release as a percentage of that induced by BT at 17.04 mM $CaCl_2$, as a function of extracellular calcium concentration for the three EPs shown in Fig. 1. In this case, EGF release due to capacitive coupling remains depressed compared to both conductive coupling conditions for all added $CaCl_2$ concentrations. The conductive coupling modalities are several times higher than the capacitive coupling case for $CaCl_2$ concentrations up to 11.61 mM. Thus, the BT and capacitive coupling treatments induce the lowest EGF release while the lower voltage conductive coupling at 17.04 mM $CaCl_2$ causes the highest EGF release. This suggests that some degree of additional membrane perturbation facilitates EGF release, but a limit is reached where applying stronger electric fields does not give additional benefit. Again, this demonstrates the ability to tune the EGF release by controlling calcium concentration, field delivery modality, and field intensity.

Discussion

We hypothesized that EP parameters, EP delivery method, and extracellular $CaCl_2$ concentration can control platelet activation and growth factor release by manipulating the membrane potential and cytosolic calcium. In other words, one may leverage the ability of EPs to permeabilize membranes by inducing sufficient membrane potential, releasing intracellular calcium stores, and transporting calcium into the cytoplasm by electrophoresis (during the EP) and/or diffusion (after the EP). The results above indicate the importance of EP parameters and $CaCl_2$ on tuning platelet activation, specifically clotting time, clot strength, and growth factor release in accordance with this hypothesis.

We can describe these observations through the impact of the selected parameters on the resulting cytosolic calcium. For instance, capacitive coupling will induce the lowest membrane potential of the EP modalities considered here, meaning that it induces the least membrane perturbation. As discussed in the Results section, capacitive coupling induces a longer clotting time than BT and either conductive coupling condition, indicating that the clotting process speeds up for more intense membrane perturbation and higher $CaCl_2$ concentration. However, the clot strength due to capacitive coupling is the same as BT and is independent of $CaCl_2$ concentration. Therefore, simply tuning the $CaCl_2$ concentration with capacitive coupling controls *when* the PRP will clot, allowing one to induce slower clotting with the same clot strength as BT. This may be beneficial for complex wounds, where longer topical application times could be impactful for clinicians.

Growth factor release also varies with $CaCl_2$ concentration. For instance, at the $CaCl_2$ concentrations where capacitive coupling induces clotting, PDGF release is above ~ 68% of that by BT for capacitive coupling and from 49% to 436% of BT for EGF release. While the physiological significance of these differences in growth factor release remains unclear until assessed by in vivo experiments, the data suggest the potential for future tunability of growth factor release to be similar to, less than, or greater than BT.

Increasing the membrane potential by applying EPs using conductive coupling provides further tunability. Increasing the membrane potential reduces the clotting time, although neither conductive coupling case induces clotting as rapidly at BT, which again could be advantageous for complex wounds, where more time for topical application may be valued. As for capacitive coupling, clotting time decreases with increasing $CaCl_2$ concentration for conductive coupling. The lower intensity conductive coupling EPs induced similar clot strength as both BT and capacitive coupling, while the higher intensity conductive coupling EPs induced a lower clotting strength. This suggests a membrane potential threshold that may alter clot strength. Thus, one may be able to use the applied voltage of the conductive coupling EP in conjunction with $CaCl_2$ to control clot strength and duration.

Furthermore, this pilot study opens up opportunities to obtain growth factor release without clotting by controlling the concentration of the added $CaCl_2$; at 5.35 mM $CaCl_2$, Fig. 2 shows that all EP parameters studied induced no clotting. This gives the clinician the option of injecting non-clotted PRP with growth factor release for knee or elbow injuries, demonstrating unique tunability capabilities. For instance, one could use capacitive coupling to achieve minimal growth factor release (~10% for PDGF and EGF), mild conductive coupling to achieve ~50% PDGF and ~400% EGF, or stronger conductive coupling to achieve ~ 50% PDGF and ~600% EGF.

While the results reported in this pilot study are far from exhaustive, they strongly support the hypothesis of using EP parameters and $CaCl_2$ concentration to modify platelet activation and growth factor release that was suggested by previous results [32,33]. Future work will assess the theoretical and clinical aspects of this capability. Since cytosolic calcium, which is a function of membrane permeabilization and extracellular calcium, drives this behavior, future studies could mathematically model various combinations of EP parameters and Ca^{2+} motion and relate that to experimental behavior of clotting and growth factor release to tune future parameter ranges. In vivo experiments assessing PRP topical application under various EP and $CaCl_2$ conditions would elucidate the impact of controlling clotting time and growth factor release for wound healing. Additionally, future in vivo experiments of injectable PRP in non-clotted form, but with various growth factor release levels, will quantify clinical benefits compared to the state of art of injecting PRP with no growth factor release. In summary, the present hypothesis and data from this pilot study open up novel avenues for tuning PRP treatments for multiple clinical applications.

Conflicts of interest

There are no conflicts of interest to declare.

Role of the funding source

GE funded the work; however, the funders did not have any additional role in the study design, data collection analysis, or preparation of the manuscript.

Author contributions

All authors meet the criteria for authorship stated in the Uniform Requirements for Manuscripts Submitted to Biomedical Journals and agree with the presented findings. This work has not been published before nor is being considered for publication in another journal.

Conflicts of interest

There are no conflicts of interest to declare.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mehy.2019.02.036.

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