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To cite this article: Gabrielle Déprés-Tremblay et al 2018 Biomed. Mater. 13 015005

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Chitosan inhibits platelet-mediated clot retraction, increases platelet-derived growth factor release, and increases residence time and bioactivity of platelet-rich plasma in vivo

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Keywords: chitosan, platelet-rich plasma, clot retraction, platelet activation, implant residency

Abstract
Platelet-rich plasma (PRP) has been used to treat different orthopedic conditions, however, the clinical benefits of using PRP remain uncertain. Chitosan (CS)–PRP implants have been shown to improve meniscus, rotator cuff and cartilage repair in pre-clinical models. The purpose of this current study was to investigate in vitro and in vivo mechanisms of action of CS–PRP implants. Freeze-dried formulations containing 1% (w/v) CS (80% degree of deacetylation and number average molar mass 38 kDa), 1% (w/v) trehalose as a lyoprotectant and 42.2 mM calcium chloride as a clot activator were solubilized in PRP. Gravimetric measurements and molecular/cellular imaging studies revealed that clot retraction is inhibited in CS–PRP hybrid clots through physical coating of platelets, blood cells and fibrin strands by chitosan, which interferes with platelet aggregation and platelet-mediated clot retraction. Flow cytometry and ELISA assays revealed that platelets are activated and granules secreted in CS–PRP hybrid clots and that cumulative release of platelet-derived growth factor (PDGF-AB) and epidermal growth factor is higher from CS–PRP hybrid clots compared to PRP clots in vitro. Finally, CS–PRP implants resided for up to 6 weeks in a subcutaneous implantation model and induced cell recruitment and granulation tissue synthesis, confirming greater residency and bioactivity compared to PRP in vivo.

1. Introduction
Platelets are blood cell components that have recently been implicated in the regulation of immune responses, cancer metastasis, vascular development, and angiogenesis, but are primarily responsible for hemostasis in the wound response, while simultaneously playing an essential role in healing by initiating specific cell responses through the release of several growth factors [1]. Platelet-derived growth factors (PDGFs) have received attention for tissue engineering and regeneration purposes in orthopedics and in other regenerative medicine fields. Unfortunately, growth factors are costly to produce and often inefficient in delivery to specific tissues [2]. A more direct approach to produce and deliver growth factors is through injection of platelet-rich plasma (PRP). PRP is an autologous blood-derived product that has an increased concentration of platelets compared to physiological levels. Once the platelets in PRP are activated they release their alpha-granules containing multiple growth factors, among them PDGF, transforming growth factor (TGF-β), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF) and insulin-like growth factor (IGF-1). PRP has been used clinically to treat different orthopedic conditions [3], since it is believed to enhance not only cell proliferation, but also extracellular matrix deposition, remodeling, angiogenesis, and collagen synthesis. However, delivery of PRP to specific sites is problematic since its physical stability is low resulting in rapid dispersion and low residence time [4]. In addition, growth factors have a very short half-life and are released rapidly from PRP. Currently, the clinical benefits of using PRP to improve tissue repair and regeneration remain uncertain [3].
Chitosan (CS), a polysaccharide obtained by chitin deacetylation, has been used in several tissue engineering and regenerative medicine applications [3]. CS is known to promote wound healing by enhancing migration of inflammatory cells, cell proliferation and matrix formation. This polysaccharide is non-toxic, biocompatible and biodegradable, making it suitable for pre-clinical and clinical use. We have worked extensively with CS for many years, beginning with the initial discovery that CS can be mixed with glycerol phosphate (GP) and still remain soluble in near-neutral conditions of pH and osmolality [6]. CS-GP solutions were mixed with whole blood to form voluminous stable clots that are applied to cartilage defects in order to improve repair induced by marrow stimulation procedures such as microfracture [7–10]. We subsequently developed a method to produce lyophilized formulations of CS, trehalose (as a lyoprotectant) and calcium chloride (as a clot activator) that are soluble in PRP and form injectable CS-PRP implants that coagulate rapidly in situ [4]. We identified some formulation properties that control implant performance and showed that CS–PRP implants have the potential to improve meniscus, rotator cuff and cartilage repair [1–3]. Combinations of CS and PRP have been used in other pre-clinical injury models as well, however, studies are scarce and report varying levels of success. CS films (degree of deacetylation or DDA of 85% and 400 kDa) were used in conjunction with PRP in a rat excision model, and found to improve wound healing when compared to sham control, PRP or CS film alone [14]. In a rabbit cranial defect model, an 86% DDA CS sponge used alone or in combination with PRP failed to improve repair compared to recalcified PRP alone [15]. A composite of CS (DDA 94% and 680 kDa) and tricalcium phosphate (TP) was mixed with PRP and injected into osseous defects in the goat, where it improved repair compared to CS-TP by itself or untreated controls [16].

The purpose of the current study was to (1) investigate possible mechanisms by which CS inhibits retraction of CS–PRP hybrid clots in vitro, (2) characterize the effect of CS, trehalose and a combination of both on platelet activation and granule secretion in vitro, (3) characterize the release profile of PDGF-AB and EGF from CS–PRP hybrid clots in vitro, and (4) histologically assess the residency, bioactivity and biodegradability of CS–PRP implants in vivo. Our starting hypotheses were that (1) CS would bind to platelets in a non-specific fashion to inhibit platelet aggregation in hybrid clots and platelet-mediated clot retraction; (2) CS would activate platelets and induce granule secretion; (3) the release of growth factors with low isoelectric point (negatively charged at neutral pH), such as EGF, would be more sustained from CS–PRP hybrids than the release of growth factors with high isoelectric points (positively charged at neutral pH), such as PDGF-AB, due to electrostatic interactions with cationic CS; and (4) CS–PRP implants would reside longer than PRP in vivo, where they would induce cell recruitment and angiogenesis, but be degraded within 6 weeks.

2. Materials and methods

2.1. Preparation of freeze-dried chitosan (CS) formulations

Raw CS was purchased from Marinard, processed in-house and characterized by nuclear magnetic resonance spectroscopy [17] for degree of deacetylation (DDA) and size-exclusion chromatography/multi-angle laser light scattering [18] for number average molar mass (M_n). CS (80% DDA; M_n 38 kDa) was dissolved in deionized water and 28 mM hydrochloric acid (Sigma-Aldrich) for 16 h on a rotator at room temperature. Filter-sterilized CaCl_2 (270 mM) and trehalose (15% w/v) solutions (both from Sigma-Aldrich) were then added to reach final concentrations of 1% (w/v) CS, 42.2 mM CaCl_2 and 1% (w/v) trehalose prior to filtration through 0.2 μm filters (Millipore). The solution was dispensed in 1 ml aliquots in 3 ml glass vials for freeze-drying with the following cycle: (1) Ramped freezing to −40 °C in 1 h then isothermal 2 h at −40 °C, (2) −40 °C for 48 h at 100 millitorrs and (3) ramped heating to 30 °C in 12 h then isothermal 6 h at 30 °C, at 100 millitorrs. 10 μl rhodamine–CS tracer [19] of corresponding DDA and M_n was added to the vials that were subsequently used for fluorescent microscopy.

2.2. Preparation of PRP

The Polytechnique Montreal institutional ethics committee approved the project and all subjects enrolled in this research (n = 3 males and n = 3 females, with some donors sampled more than once) responded positively to an Informed Consent Form. For each donor, blood was extracted and anticoagulated with 12.9 mM sodium citrate. The blood was then centrifuged using an ACE E-Z PRPTM centrifuge at 160 g for 12.9 mM sodium citrate. The blood was then centrifuged again at 400 g for 10 min at room temperature. The supernatant and first 1–2 mm of erythrocyte sediment was removed and then centrifuged again at 400 g for 10 min at room temperature. Only the bottom ~1.5 ml of each tube was retained and resuspended to make PRP. This isolation method yields a leukocyte-rich PRP (L-PRP) which contained on average 9.31 ± 3.48 × 10^{11}/L platelets (3.5× the concentration of whole blood), 5.4 ± 1.1 × 10^9/L leukocytes (0.9× the concentration of whole blood) and 3.6 ± 1.1 × 10^{12}/L erythrocytes (0.8× the concentration of whole blood). The monocyte content in PRP was on average 0.42 ± 0.12 × 10^9/L (1.5× that of whole blood). The PRPs from the different donors were kept as individual patients PRPs for further use. For confocal microscopy purposes, to allow visualization of blood-derived components, an Alexa-647 fibrinogen (Invitrogen) imaging tracer was prepared as a stock solution at 1.5 mg ml \(^{-1}\) in 0.1 M
sodium bicarbonate (pH 8.3) and added to the PRP (0.5 ml of Alexa-647 fibrinogen added to 4.5 ml of PRP). It should be noted that additional fibrinogen is not necessary for clot formation in this system.

2.3. Solubilization of freeze-dried CS formulations in PRP
Each freeze-dried cake (1 ml freeze-dried formulation in each vial) was solubilized with 1 ml of PRP and mixed vigorously for 10 s. The freeze-dried cakes were completely dissolved in PRP formulation. The solubilized formulations were dispensed either into glass tubes to assess clot retraction and for imaging or into 48-well culture plates for characterization of release profiles. Controls were PRP recalcified with 42.2 mM CaCl₂. The same mixing method was applied to the PRP controls during the recalcification step, as it is expected that vigorous mixing will contribute to the activation of platelets and initiation of coagulation.

2.4. Assessment of clot retraction
CS–PRP formulations (~250 μl) were dispensed in glass tubes placed on a heat block at 37 °C and allowed to clot for 1 h. Serum was removed and % clot mass lost was quantified by gravimetric measurements. Clots were fixed with 0.5% (v/v) glutaraldehyde (EMS)/0.3% (w/v) paraformaldehyde (Sigma-Aldrich)/0.3% (v/v) Triton X-100 (Sigma-Aldrich). Duplicate clots were prepared for each donor, except for one donor, where one clot was prepared.

2.5. Confocal fluorescent and spinning disk microscopy imaging
Fixed clots were sectioned with a razor blade at ~1 mm thickness and mounted with Mowiol 4-88 (Fluka)/glycerol (Sigma-Aldrich)/n-propyl gallate (Sigma-Aldrich) mounting medium (prepared in-house) on MatTek glass bottom dishes (Cedarlane). High resolution two- and three-dimensional (3D) images were captured with an Olympus FV1000 spectral confocal laser scanning microscope (Olympus Canada), using a PLAPON Apochromat oil objective (60X, NA 1.42). The excitation/emission wavelengths were 635/644–755 nm for the fibrin network (Alexa-647 fibrinogen tracer), and 543/555–625 nm for the CS (rhodamine tracer). Erythrocytes were also imaged for some samples (autofluorescence using 488/500–540 nm). The acquisition parameters were adjusted to avoid signal saturation. 3D images were reconstructed with Imaris software (Bitplane). The confocal microscope is also adapted for a Yokogawa spinning disk module (Quorum), controlled with the MetaMorph software (Molecular Devices). Given the high dynamic range of the EM-CCD digital camera (Hamamatsu), the spinning disk module was used to capture images with fixed acquisition settings for all the samples without reaching any signal saturation. Only the fibrin network was imaged with this module, using a UPLSAPO Super Apochromat objective (40X, NA 0.95) and excitation/emission wavelengths of 642/662–738 nm.

2.6. Scanning electron microscope (SEM) imaging
Fixed clots were embedded in paraffin (Fisher), sectioned at 3 μm thickness with a Leica RM2155 microtome and collected on SuperFrost Plus glass slides (Fisher). The sections were then deparaffinized, post-fixed in 2% (v/v) glutaraldehyde (EMS)/0.1 M sodium cacodylate (Sigma) pH 7.2 and washed in water. The post-fixed sections were removed from slides using a super fine point tweezers and placed onto a conductive carbon adhesive tape (EMS). The sections were immobilized by blowing compressed air and then gold sputter coated for 25 s using an Agar manual gold sputter-coater (Marivac Inc.). SEM images were acquired with a Quanta FEG 200 ESEM (FEI Company) in high vacuum mode with working distance 5.6–5.7 mm and accelerating voltage 20 kV.

2.7. Transmission electron microscope (TEM) imaging
Fixed clots were post-fixed in 1% (v/v) osmium tetroxide (Sigma-Aldrich), washed in deionized water, incubated with 2% (v/v) uranyl acetate (EMS) for 1 h, washed, dehydrated in a graded ethanol series, cleared in xylene and embedded in Embed-812 (EMS) medium at 60 °C. 100 nm sections were collected using a diamond blade and an RMC MT-7 ultramicrotome and mounted on copper grids. The images were acquired with a JEM 2000FXII TEM (JEOL; Tokyo, Japan) operated at 80 kV.

2.8. Preparation of cell suspension and flow cytometry
Whole blood was anticoagulated with 10.9 mM sodium citrate, centrifuged at 190 g for 15 min and the supernatant collected. Supernatant was centrifuged at 2500 g for 5 min to pellet the cells. Cell pellet was resuspended in HEPES/Tyrode’s buffer (10 mM HEPES, 137 mM NaCl, 2.8 mM KCl, 1 mM MgCl₂, 12 mM NaHCO₃, 0.4 mM Na₂HPO₄, 5.5 mM glucose) with 1.35% (w/v) bovine serum albumin (all from Sigma-Aldrich) and left to incubate for 1 h. The cell suspension was then mixed with, either adenosine diphosphate (ADP) (Sigma, final concentration 20 μM), solubilized CS 80% DDA M₉, 38 kDa (final concentration 1% w/v), trehalose (final concentration 1% w/v) or 1% (w/v) CS and (1% w/v) trehalose simultaneously. Fluorescein isothiocyanate labeled anti-Pac-1 and anti-CD62P antibodies (Biolegend), which recognize activated platelet GPIIb/IIIa complex and granule membrane protein P-selectin, respectively, were added to the tubes as per manufacturer’s instructions and the tubes were incubated at room temperature for 20 min. The cells were fixed with 1%
(w/v) paraformaldehyde/10 mM HEPES/0.15 mM NaCl pH 7.4 for 15 min at room temperature. The samples were then kept on ice until analysis. The fluorescence intensity of 10 000 events per sample were analyzed using a MoFlo flow cytometer (Beckman Coulter Life Sciences).

2.9. Quantification of growth factors released from clots
Freeze-dried CS cakes were solubilized in PRP as described above and 250 μl of the solubilized mixture was dispensed into each well of a 48-well plate culture plate and allowed to clot for 1 h in air atmosphere at 37 °C with 5%CO₂. Wells were washed with 500 μl α-MEM (minimum essential medium) cell culture medium (Invitrogen), the culture medium was immediately removed and replaced with fresh α-MEM. The cell culture medium was then removed and replenished at day 1, 3, 5 and 7. Culture medium was centrifuged at 400 g for 10 min and frozen at −80 °C prior to ELISA quantification using PDGF-AB or EGF Quantikine kits (Product N° DHD00C and DEG00 from RandD systems). Controls were PRP recalcified with 42.2 mM CaCl₂. Duplicate clots were prepared for each donor, except for one donor, where one clot was prepared.

2.10. Subcutaneous implantation
The protocol for this study was approved by the University of Montreal ethics committee and was consistent with the Canadian Council on Animal Care guidelines for the care and use of laboratory animals. Five New Zealand White male rabbits (±2.5 kg) were used for the study. Rabbits were anesthetized with a ketamine/xylazine cocktail. From each animal, 18 ml blood was collected from the car artery and anticoagulated with sodium citrate (final citrate concentration 12.9 mM). Blood was centrifuged in an ACE EZ-PRP™ centrifuge for 10 min at 160 g and then for 10 min at 400 g to extract PRP, as described above. The back of the rabbit was shaved and the skin disinfected with three passages of Baxedin, then with three alternating passages of providine and isopropanol 70%. Freeze-dried CS formulations (300 μl) containing 1% (w/v) CS (80–85% DDA and M₆₈–85 kDa), 1% (w/v) trehalose and 42.2 mM CaCl₂ were solubilised with 300 μl of PRP and shaken vigorously for 10 s. A 1 cm² syringe equipped with a 26 gauge needle was used to deliver 150 μl of each implant under the skin of the back of the rabbits (n = 4 CS–PRP implants injected in each rabbit). Controls consisted of 150 μl PRP recalcified with 42.2 mM CaCl₂ prior to injection (n = 2 control PRP implants injected in each rabbit). Animals were sacrificed at 2 weeks (n = 2), 4 weeks (n = 2) or 6 weeks (n = 1) post-implantation. At the time of sacrifice, rabbits were anesthetized with ketamine/xylazine cocktail and euthanized with an overdose of sodium pentobarbital. The skin with attached implants was carefully removed and fixed in 10% neutral buffered formalin (Fisher) for 3 d. Each implant was excised using razor blades and a horizontal band was collected from the central area for paraffin embedding. Paraffin sections (5 μm) were collected and stained with Fast Green/Iron Hematoxylin and Mason Trichrome (all reagents from Sigma-Aldrich). The stained sections were then scanned for histological evaluation using a Nanozoomer RS system and images exported using NDPView software (both from Hamamatsu).

2.11. Statistical analysis
All statistical analyses were performed with SAS Enterprise Guide 7.1 and SAS 9.4. Data in the text are presented as mean ± standard deviation. Data in the figures are presented as median (line); box: 25th and 75th percentile; whisker: box to the most extreme point within 1.5 interquartile range. The mixed model task in SAS Enterprise Guide was used to compare the different groups with post-hoc analysis to look at pairwise differences. Fixed effects were type of sample (CS–PRP versus PRP control) and time. Donor identity was used as a random effect in the model to account for inter-individual variability and because some donors were sampled more than once.

3. Results

3.1. Clot retraction and platelet aggregation are inhibited in CS–PRP hybrid clots
Gravimetric measurements showed that PRP clots lost 78 ± 4% of their initial mass after clotting for 1 h at 37 °C (figure 1(a)). Although there was some variability between donors, % mass loss was significantly less for CS–PRP hybrid clots at average 21 ± 18% (figure 1(a)). Spinning disk microscopy and confocal microscopy images of clots fixed after clotting for 1 h showed that platelet aggregates were smaller in CS–PRP hybrid clots (figures 1(b), (d) and (f)) compared to PRP clots (figures 1(c), (e) and (g)).

3.2. Chitosan coats clot components in CS–PRP hybrid clots
SEM images demonstrated that blood components were coated with CS in CS–PRP hybrid clots (figures 2(a), (b)). Both cells and fibers were covered by a coating of what is presumed to be CS (figures 2(a), (b)). In contrast, the fibrin network was readily visible in PRP clots (figures 2(c), (d)). In TEM images of CS–PRP hybrid clots, CS was observed in the space between cellular elements, and also at the surface of fibrin strands, erythrocytes and platelets (figures 2(e), (f)). Erythrocytes were closely packed and platelet aggregates were large in PRP clots (figures 2(g), (h)).
Figure 1. After clotting for 1 h at 37 °C, clot retraction and serum expression (expressed as % clot mass lost) was greater from PRP clots compared to CS–PRP hybrid clots (a). Data are presented as mean (diamond) and median (line) of $n = 7$ clots from three different donors; box: 25th and 75th percentile; whisker: box to the most extreme point within 1.5 interquartile range. The mixed model task in SAS Enterprise Guide 7.1 and SAS 9.4 was used to compare the different groups using sample (CS–PRP versus PRP) as a fixed effect and donor as a random effect. *$p < 0.05$ compared to PRP. Platelet aggregates were smaller in CS–PRP hybrid clots (b), (d) and (f) compared to PRP clots (c), (e) and (g), as shown by spinning disk microscopy images (b) and (c), confocal microscopy images (d) and (e) and 3D stacks of confocal microscopy images (f) and (g). An Alexa-647 fibrinogen tracer was added to allow imaging of fibrin-covered platelets and fibrin in white. A rhodamine-chitosan tracer was added to allow imaging of chitosan in red.
3.3. Chitosan induces platelet activation and granule secretion in cell suspension

As expected, incubation with ADP (in green), a known platelet agonist, caused increased Pac-1 and p-selectin expression in a cell suspension (unactivated cells in red) (figure 3). Incubation with 1% (w/v) CS (in dark blue) also led to platelet activation (figure 3(a)) and granule secretion (figure 3(b)). In contrast, incubation with 1% (w/v) trehalose alone (in yellow) did not induce platelet activation and granule secretion (figure 3). Finally, incubating the cell suspension with 1% (w/v) CS simultaneously with 1% (w/v) trehalose...
(in pale blue) induced platelet activation and granule secretion, albeit less than incubation with 1% (w/v) CS by itself (figure 3).

3.4. CS–PRP hybrids provide a greater and sustained release of PDGF-AB and EGF over time

PDGF-AB release into culture medium was ~100 times greater than that of EGF for both CS–PRP hybrid clots and PRP clots (figure 4). For both growth factors, a phase of fast release was observed in the first 24 h, followed by a more controlled release from day 1 to day 7 (figure 4). In addition, for both growth factors, release was still ongoing at day 7 and no plateau had been reached (figure 4). Both time (p < 0.0001 and p = 0.0003) had an effect on the release of PDGF-AB and EGF, respectively (figure 4). The cumulative release of PDGF-AB from CS–PRP hybrid clots was significantly greater than the cumulative release from PRP clots from day 1 to day 7 (figure 4(a)). Similarly, the cumulative release of EGF was significantly greater from CS–PRP hybrid clots than from PRP clots from day 3 to day 7 (figure 4(b)).

3.5. CS–PRP hybrids reside for at least 6 weeks in vivo and induce cell recruitment

No PRP implants could be recovered for histology at any time point tested. In contrast, CS–PRP implants were resident for up to 6 weeks post-implantation (figure 5). The area occupied by the implants decreased...
with time (Figure 5). Host-derived cells were recruited to the implants and were observed invading the implants from 2 weeks on (Figure 5). Neutrophils and mononuclear phagocytes were among these host-derived cells and most likely contributed to the implant biodegradation (Figure 5). A collagen-rich granulation tissue surrounded the implants and abundant new blood vessel formation was observed (Figure 5). None of the CS–PRP implants induced deleterious effects in rabbits. No sign of infection or rejection were noted macroscopically or histologically. Blood work was normal and rabbits did not develop anorexia post-surgery.

### 4. Discussion

One objective of this study was to investigate the mechanisms by which CS inhibits platelet-mediated clot retraction and liquid expression in CS–PRP hybrids, as shown in Figure 1(a). Under static or low shear stress conditions, which is the case here in our *in vitro* assay, platelet aggregation is mainly mediated by the interactions of GPIIb/IIIa on platelet surface with fibrinogen [20]. Stimulation of platelets with agonists induces cytoskeletal rearrangement, shape change, protein synthesis, granule secretion and increases the affinity of the GPIIb-IIIa platelet receptor for fibrinogen [21]. Platelet aggregation results from binding of multiple platelets to the same fibrinogen molecule [1]. Clot retraction, mediated by the platelet actin and myosin contractile system then follows, as long as platelet stimulation, comprising shape change and primary aggregation, are maintained [22, 23]. In the absence or non-functioning GPIIb/IIIa, clot retraction does not occur. Confocal, SEM and TEM images (figures 1 and 2) support our first hypothesis that CS physically coats platelets and other components of the blood clot to inhibit platelet aggregation, which is needed for clot retraction. In CS–PRP hybrids, CS physically interferes with the ability of the platelets to adhere to each other and the fibrin network and exert mechanical forces.

Our second and third aims were to investigate whether platelets are activated in CS–PRP hybrid clots and, if so, how PDGFs are released from CS–PRP implants. CS (DDA > 90% and 50 kDa) was previously shown to be a platelet agonist and stimulate platelet activation and GPIIb/IIIa expression *in vitro* [24]. In another study, stimulation of platelet suspensions with CS (DDA 84%) induced p-selectin and GPIIb/IIIa expression, in a process that was shown to be modulated by plasma and extracellular matrix proteins [25]. Consistent with these previously published data and our second hypothesis, we found that CS induces platelet activation and granule secretion in cell suspensions (Figure 3), even more so than ADP (20 μm), a known platelet agonist. Interestingly, incubation of cell suspension with trehalose along with CS slightly decreased expression of Pac-1 and p-selectin compared to incubation with CS alone. This is consistent with published reports that lyoprotectants impede hemostatic mechanisms [26, 27].

Even though test conditions in the flow cytometry assay are different than in the hybrid clot system, we
expected platelets within the CS–PRP hybrid clots to be activated and release their granule content, and this was ascertained by ELISA assays. In the case of physical adsorption of growth factors to CS, release is believed to be controlled by the electrostatic interactions that exist between the growth factors and the CS [28]. Therefore, our third starting hypothesis was that the isoelectric point of PDGFs would determine how growth factors would be released from our CS–PRP hybrids. The isoelectric point of PDGF is 9.8 [29] and, under physiological conditions, we expected ionic repulsion between positively charged PDGF-AB and cationic CS to result in burst release. Meanwhile, EGF, which has an isoelectric point of 4.6 [30] would be expected to bind to CS under physiological conditions and be released in a more sustained manner. In contrast to this, we found that CS–PRP hybrid clots sustained and increased release of both PDGF-AB and EGF for 1 week in vitro, which suggests that additional factors are controlling their release in this system (figure 4).

We found that the cumulative levels of PDGF-AB and EGF released in the culture medium were higher in the case of CS–PRP clots compared to PRP clots (figure 4). These results were not completely unexpected. Kutlu et al [31] previously prepared CS–PRP scaffolds by either adding PRP to a CS gel before freeze-drying or by delivering PRP to a lyophilized CS sponge. Sustained release of PDGF-BB was achieved in the first group, similarly to what we observed here, while a sharp burst release was observed in the second group. Interestingly, both of their CS–PRP scaffolds secreted higher cumulative levels of PDGF-BB when compared to unactivated PRP or PRP activated with type I collagen, similarly to what was found here for PDGF-AB. Hattori et al [32] showed that platelets in whole blood are activated when mixed with solutions of CS with different DDA and Mw. Of particular relevance to our study, the amount of PDGF-AB released was the highest when Cs of DDA 75%–85% and Mw 50–190 kDa were used in conjunction with calcium chloride than when calcium chloride was used by itself. Shen et al [33] showed that stimulation with CS of DDA > 90% and 450 kDa induced release of PDGF-AB and EGF from PRP for up to 60 min. Shimojo et al [34] prepared lyophilized scaffolds containing different concentrations of CS (DDA 83% and Mw 400 kDa), loaded the scaffolds with PRP activated with autologous serum and calcium chloride and showed that PDGF-AB cumulative release was higher from the scaffolds compared to activated PRP alone, provided that the scaffolds be lyophilized at low temperatures. In a subsequent study, Shimojo et al [35] showed that stabilizing the CS scaffolds by treating them with NaOH prior to loading them with PRP was another way to increase cumulative PDGF-AB release from scaffolds lyophilized at −20 °C.

With regard to growth factor release, it is important to consider the contribution of each cell type present in the PRP preparation. Platelets are the main contributors to growth factor release from PRP and positive correlations were previously found between platelet doses and the amount of released growth factors including PDGF-AB, TGF-β1, VEGF and EGF [36, 37]. While it appears that the inclusion of leukocytes in PRP increases the content of some pro-inflammatory cytokines [38–41], the effect of leukocytes on growth factor content and release is still not fully understood. Previous studies found that leukocyte-rich PRP contained higher concentrations of growth factors compared to leukocyte-poor PRP, but that may be due to the fact that systems that include theuffy coat layer are usually more efficient at capturing platelets [39, 42–46]. However, positive correlations and close associations were also found between PRP leukocyte counts and levels of PDGF-AB, VEGF and EGF [36, 37], which suggests that leukocytes contribute to the release of growth factors from PRP. Here, we found increased cumulative PDGF-AB and EGF release from CS–PRP clots compared to PRP clots (figure 4). One possible reason for this is that CS used in conjunction with calcium chloride stimulates platelet activation and granule secretion more than calcium chloride by itself. Another possibility would be that leukocytes, especially monocytes, present in CS–PRP hybrids are secreting higher amounts of growth factors than in PRP without CS. While it has been reported that M0 and polarized M2a macrophages secrete PDGF-BB [47, 48], and that biodegradable CS particles (DDA 81.5% and Mw 132 kDa) enhance release of PDGF-BB from M0 and M2a macrophages [49], we believe that the main contributors to growth factor release in the CS–PRP hybrid system are platelets and not monocytes, for the following reasons: (1) monocytes typically require specific stimulatory signals to become macrophages, (2) there is a low number of monocytes present in each CS–PRP clot, compared to the number of platelets (on average ~2200X more platelets than monocytes); (3) in light of previous reports on the amount of growth factors released by M0 and M2a macrophages, it seems unlikely that such a limited number of monocytes could secrete the amount of PDGF-AB and EGF that was measured here in the culture medium. Presence or absence of leukocytes in the PRP preparations would be expected to have an impact on the release of pro-inflammatory cytokines, but this was not assessed here.

Our fourth aim was to investigate the implants in vivo, and, as previously shown [4], CS–PRP hybrids exhibited longer residency and higher bioactivity than PRP controls (figure 5). CS–PRP implants were surrounded by a highly vascularized collagen-rich granulation tissue. In a subcutaneous implantation model in the mouse, porous CS scaffolds were found to elicit neutrophil migration into the implantation area along with angiogenic activity, as was shown here as well [50]. It is of interest to mention that the site of implantation along with implant volume influences
biodegradability. We have previously shown that CS–
PRP implants are degraded within 3 weeks in menis-
cus tears in the sheep [13], and between 2 and 8 weeks in
rotator cuff tears [11] and cartilage lesions in the
rabbit [12] compared to more than 6 weeks in the cur-
rent study. However, we expect that the chitosan
would eventually be completely cleared out in the sub-
cutaneous implantation model as well, like what was
seen in the tissue repair models. One limitation of this
study is the low number of animals used for the in vivo
subcutaneous implants study. Another limitation is
the limited number of growth factors studied for the
subcutaneous implantation model as well, like what was
recent study. However, we expect that the chitosan
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strands in CS–PRP hybrid clots, thus inhibiting platelet
aggregation, which is required for clot retrac-
tion. Platelets are activated, granules secreted and
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Finally, CS–PRP implants reside for at least 6 weeks
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5. Summary

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Acknowledgments

We acknowledge the technical contributions of Gene-
vieve Picard and the funding sources (Canadian
Institutes of Health Research, Canada Foundation for
Innovation, Groupe de Recherche en Sciences et
Technologies Biomédicales, Natural Sciences and
Engineering Research Council of Canada, Ortho
Regenerative Technologies Inc.).

Competing interests

AC and MDB hold shares and MDB is a Director of
Ortho Regenerative Technologies Inc.

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