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Multiple platelet-rich plasma preparations can solubilize freeze-dried chitosan formulations to form injectable implants for orthopedic indications

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Abstract.
BACKGROUND: Platelet-rich plasma (PRP) has been used to solubilize freeze-dried chitosan (CS) formulations to form injectable implants for tissue repair.
OBJECTIVE: To determine whether the *in vitro* performance of the formulations depends on the type of PRP preparation used to solubilize CS.
METHODS: Formulations containing 1% (w/v) CS with varying degrees of deacetylation (DDA 80.5–84.8%) and number average molar mass (M_n 32–55 kDa), 1% (w/v) trehalose and 42.2 mM calcium chloride were freeze-dried. Seven different PRP preparations were used to solubilize the formulations. Controls were recalcified PRP.
RESULTS: CS solubilization was achieved with all PRP preparations. CS-PRP formulations were less runny than their corresponding PRP controls. All CS-PRP formulations had a clotting time below 9 minutes, assessed by thromboelastography, while the leukocyte-rich PRP controls took longer to coagulate (>32 min), and the leukocyte-reduced PRP controls did not coagulate in this dynamic assay. In glass culture tubes, all PRP controls clotted, expressed serum and retracted (43–82% clot mass lost) significantly more than CS-PRP clots (no mass lost). CS dispersion was homogenous within CS-PRP clots.
CONCLUSIONS: *In vitro* performance of the CS-PRP formulations was comparable for all types of PRPs assessed.

Keywords: Chitosan, platelet-rich plasma, injectable implants

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1. Introduction

Platelet-rich plasma (PRP) is an autologous blood-derived product that contains platelet concentrations above physiological levels. PRP is thought to modulate the tissue healing process by supplying growth factors, cytokines, and other bioactive compounds which play fundamental roles in hemostasis as well as tissue repair and remodeling. PRP safety profile, ease of preparation and application, low potential for disease transmission and minimal tissue rejection are some advantages. Initially, PRP's first clinical applications were limited to dentistry, oral and maxillofacial surgery to improve bone healing with platelet gels [1], but PRP is now used to treat several musculoskeletal and orthopaedic conditions, including osteoarthritis, meniscus tears, tendinopathy, rotator cuff tears and ligament tears [2].

To date, numerous PRP preparation systems are available, which produce PRP that contain varying concentrations of platelets, leukocytes and erythrocytes. On the basis of the platelet concentration, inclusion or exclusion of leukocytes, and fibrin architecture, Dohan Ehrenfest et al classified 4 families of PRP preparations [3]: (1) Leukocyte-rich PRP; and (2) Leukocyte-poor (or leukocyte-reduced) PRP, both of which are liquid suspensions that form solid clots upon activation of the coagulation cascade; and (3) Leukocyte-rich platelet-rich fibrin (PRF); and (4) Leukocyte-poor (or leukocyte-reduced) PRF, both of which are in solid format. Regarding the therapeutic effects of the different types of PRP preparations, platelet content has been a primary focus, since platelet-derived growth factors contribute to tissue repair, however, it is well established that concentration of erythrocytes and leukocytes are also important factors to consider. The exact role of these cells in the PRP-mediated reparative process has not been completely elucidated yet. Leukocytes are thought to induce a proinflammatory response by releasing some inflammatory mediators and catabolic enzymes such as interleukin (IL-1β), tumor necrosis factor (TNF-α) and IL-6. This has clearly been shown *in vitro* [4–6], which led to the notion that leukocyte-reduced PRP preparations would be superior to leukocyte-rich PRP preparations. However, increased levels of platelet-derived growth factor (PDGF)-AB, PDGF-BB, vascular endothelial growth factor (VEGF) and transforming growth factor (TGF)-β from leukocyte-rich PRP compared to leukocyte-poor PRP have been reported *in vitro* [7,8], which could potentially contribute to the improved tissue repair. In addition, the inflammatory response induced by leukocyte-rich PRP preparations appears to be limited to the early post-treatment period *in vivo* [9]. Furthermore, there is clinical evidence that a single ultrasound-guided injection of leukocyte-rich PRP is beneficial for treating tendinopathy [10]. As of now, it is still unclear what type of PRP preparation should be used to treat specific conditions.

Chitosan (CS) is a nontoxic, biodegradable and biocompatible polysaccharide composed of glucosamine and N-acetyl-glucosamine units, which is derived by alkaline deacetylation of chitin [11]. Our laboratory has developed freeze-dried formulations of CS that can be solubilized in PRP to form injectable CS-PRP implants that coagulate *in situ* and can be used for different tissue repair applications [12]. Previous studies showed that CS-PRP implants resist platelet-mediated retraction post-clotting, release increased amounts of platelet-derived growth factors and have prolonged residency *in vivo* compared to PRP alone [13]. CS-PRP implants have also shown potential to improve repair of rotator cuff, meniscus and cartilage in small and large animal models [14–17].

The goal of the current study was to answer multiple questions regarding this technology. First, in all of our previous studies, we used a leukocyte-rich PRP preparation that was prepared manually by double centrifugation, and not PRP preparations isolated with commercially available kits. Second, acceptable CS specifications for combination with PRP need to be defined. Therefore, the purpose of the current study was to (1) assess compatibility of this technology with the various types of PRP preparations that can be isolated with commercially available systems and (2) define a range of CS degree of deacetylation

Table 1
Properties of the CS used in the study

Degree of deacetylation (DDA)	Number average molar mass (M_n)	Polydispersity index (PDI)
82.5%	45 kDa	1.83
80.7%	35 kDa	1.81
80.5%	49 kDa	1.97
84.8%	32 kDa	1.90
84.6%	55 kDa	1.95

(DDA) and number average molar mass (M_n) that would yield freeze-dried formulations with acceptable performance characteristics. Our starting hypothesis was that although the different PRP preparation systems would yield PRPs with varying properties, all PRP preparations would be compatible with this technology.

2. Materials and methods

2.1. Preparation of freeze-dried CS formulations

Five CS (raw material purchased from Primex) were deacetylated by alkaline treatment, depolymerized with nitrous acid, characterized by NMR spectroscopy [18] and analytical size exclusion chromatography/multi-angle laser light scattering [19]. DDA, M_n and polydispersity index (PDI) of the CS were: (1) 82.5% DDA M_n 45 kDa PDI 1.83, (2) 80.7% DDA M_n 35 kDa PDI 1.81, (3) 80.5% DDA M_n 49 kDa PDI 1.97, (4) 84.8% DDA M_n 32 kDa PDI 1.90 and (5) 84.6% DDA M_n 55 kDa PDI 1.95 (Table 1). These ranges of CS DDA and M_n were chosen based on the precision with which the deacetylation and depolymerization processes can be controlled. CS were dissolved in nuclease-free water and acid overnight at room temperature with sufficient HCl (Fluka, Product N° 1135328) added to protonate 60% of glucosamine groups. Trehalose (Life Sciences, Product N° TDH033) was added as lyoprotectant and calcium chloride ($CaCl_2$, Spectrum, Product N° CA95032) was added as a clot activator to final concentrations of 1% (w/v) and 42.2 mM respectively. The solutions were sterilized with a 0.22 µm polyvinylidene difluoride filter (Millipore) and dispensed into individual 3 mL sterile glass vials (1-mL per vial) for freeze-drying using a MillRock stoppering freeze-drier (LAB series). The freeze-drying process was divided into 3 phases: (1) ramped freezing to −40 °C in 1 hour, isothermal for 2 hours at −40 °C (without applying vacuum), (2) −40 °C for 48 hours, at 100 millitorrs, (3) ramped heating to 30 °C in 12 hours, isothermal for 6 hours at 30 °C, at 100 millitorrs.

2.2. Isolation of PRP preparations

The male subject enrolled in this research responded positively to an Informed Consent Form (Certificate #CÉR-15/16-17 dated Jan 28th, 2016) which was approved by the Polytechnique Montreal institutional committee (Comité d'éthique à la recherche avec des êtres humains). Blood was drawn from the same donor twice; once to isolate PRP using the ACE EZ-PRP system, and once to isolate PRP using the following systems: (1) Arthrex Angel set at 2% hematocrit, (2) Arthrex Angel set at 7% hematocrit,

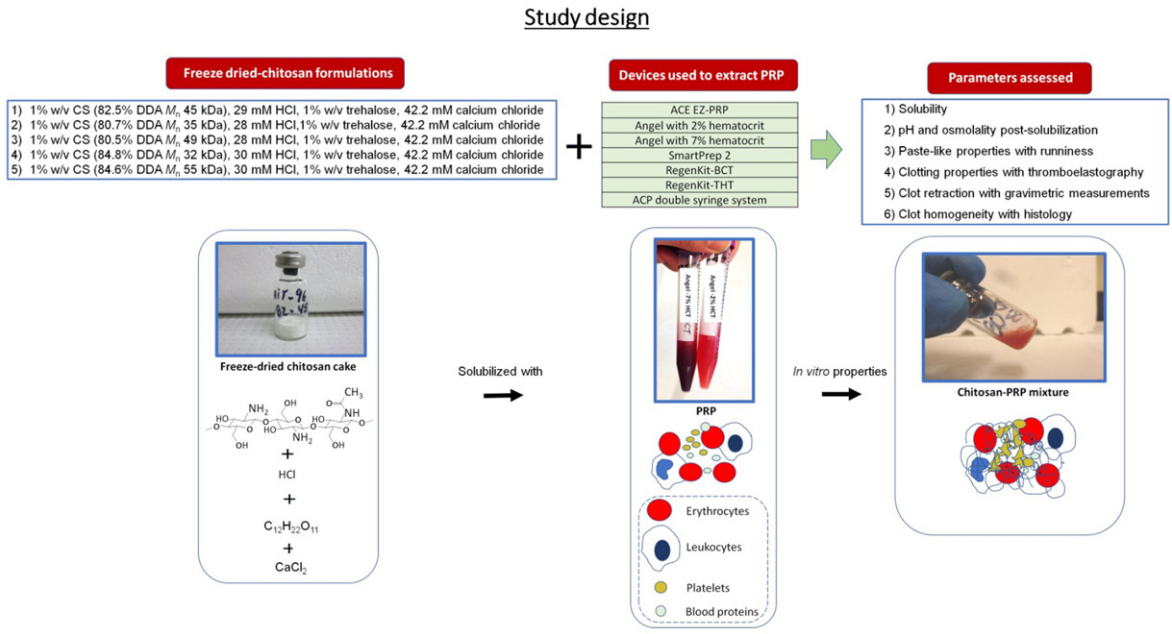

Fig. 1. Study design. Five different CSs (with M_n ranging from 32 to 55 kDa and DDA ranging from 80.5 to 84.8%) were used to prepare freeze-dried formulations that also contained trehalose as lyoprotectant and calcium chloride as clot activator. Freeze-dried cakes were solubilized with 7 different PRP preparations (Angel with 2% hematocrit and Angel with 7% hematocrit are pictured here). Performance characteristics of the solubilized CS-PRP mixtures were assessed *in vitro*.

(3) Harvest SmartPrep 2, (4) RegenLab RegenKit-BCT, (5) RegenLab RegenKit-THT, (6) Arthrex ACP double syringe system. Isolation protocols are further described in Table 2. Complete blood count analysis was performed on whole blood and PRP preparations using the Advia 120 hematology system (Siemens).

2.3. *Solubilization of freeze-dried CS formulations*

Each 1 mL cake was solubilized with 1 mL PRP, hand-shaken vigorously for 10 seconds and different performance characteristics were immediately assessed (Fig. 1). All five different CS formulations were solubilized with each PRP preparation, producing $n = 5$ CS-PRP samples per PRP preparation. Controls were prepared by recalcifying PRP to a final concentration of 42.2 mM $CaCl_2$, yielding $n = 1$ PRP control per PRP preparation.

2.4. *Assessment of formulation paste-like properties*

A previously developed runniness test was used to assess paste-like properties [12]. Briefly, a 40 µl drop of each CS-PRP formulation was pipetted onto a rigid piece of plastic fixed at 38 degrees to horizontal, pictures were acquired after 10 minutes and the drop mobility was measured using Image J 1.47v. Note that in this assay water runs off the plate and has a runniness exceeding 310 mm.

Table 2
Characteristics of the different PRP preparations used for the study

Device name	Company	Type of PRP	Hematocrit setting	Blood drawn (mL)	Number of centrifuge times	Centrifugal force (rpm) & time (min)	Open/Closed	Features and method of preparation	Final volume (mL)
ACE EZ-PRP	ACE Surgical Supply	Leucocyte-rich	Unavailable	10 mL	2	1. 1300 rpm for 10 min 2. 2000 rpm for 10 min	Open system	Manual	1.5 mL per tube; 4 tubes processed
Angel	Arthrex	Leukocyte-reduced	2% hematocrit	120 mL	1	Centrifugation for 22 min	Closed system	Automated, Cellular photo-spectrometry and fractionation	6 mL
Angel	Arthrex	Leucocyte-rich	7% hematocrit	60 mL	1	Centrifugation for 18 min	Closed system	Automated, Cellular photo-spectrometry and fractionation	6 mL
SmartPrep 2	Harvest Technologies	Leucocyte-rich	Unavailable	60 mL	2	1. 2500 rpm for 4 min 2. 2300 rpm for 10 min	Closed system	Automated	6 mL
RegenKit-BCT	RegenLab	Leukocyte-reduced	Unavailable	8 mL	1	3500 rpm for 5 min	Closed system	Automated, Thixotropic gel	2.5 mL per tube; 3 tubes processed
RegenKit-THT	RegenLab	Leucocyte-rich	Unavailable	8 mL	1	3500 rpm for 9 min	Closed system	Automated, Thixotropic gel	4 mL per tube; 2 tubes processed
ACP double-syringe system	Arthrex	Leukocyte-reduced	Unavailable	16 mL	1	1500 rpm for 5 min	Closed system	Automated	6 mL

2.5. Assessment of formulation coagulation

360 µl of each CS-PRP formulation was loaded into a standard specimen thromboelastography (TEG) cup pre-warmed to 37 °C. TEG measurements were carried out for 60 min using a TEG Model 5000 hemostasis analyzer system (Haemoscope Corp).

2.6. Assessment of clot retraction

250 µl of each CS-PRP formulation was dispensed into glass tubes and placed in a heat block set at 37 °C and left to clot for 60 min. Serum expressed from the clots was removed and % mass lost was measured.

2.7. Assessment of clot homogeneity

CS-PRP clots were fixed in 10% neutral buffered formalin (NBF), paraffin-embedded, sectioned at 5 µm using a RM2155 microtome (Leica) and stained with Fast Green/Iron Hematoxylin. Stained slides were scanned at 40X with a Nanozoomer RS scanner NDPView (Hamamatsu, Japan) and NDPView (Hamamatsu) was used to export images.

2.8. Statistical analysis

All analyses were performed with SAS Enterprise Guide 7.1 and SAS 9.4. For each PRP preparation, data obtained with the 5 different CS were averaged ($n = 5$) and compared to its corresponding recalcified PRP control ($n = 1$). Data are presented as either dots (for the PRP controls) or boxes where median (line); Box: 25th and 75th percentile; Whisker: Box to the most extreme point within 1.5 interquartile range. Correlations between the different performance criteria assessed and CS M_n, CS DDA, PRP platelet content, PRP leukocyte content and PRP erythrocyte content were analyzed by calculating the Pearson correlation coefficients. p-values < 0.05 were considered significant.

3. Results

3.1. Differences in the properties of the different PRP preparations

The lowest platelet concentration was obtained with the RegenLab BCT system (207 X 10E9/L, 1.0X that of whole blood), and the highest platelet concentration was obtained with the Harvest SmartPrep 2 system (905 X 10E9/L, 4.3X that of whole blood) (Table 3 and Fig. 2). Similarly, the lowest leukocyte concentration was obtained with the RegenLab BCT system (0.3 X 10E9/L, 0.1X that of whole blood), and the highest leukocyte concentration was obtained with the Harvest SmartPrep 2 system (11.5 X 10E9/L, 2.4X that of whole blood) (Table 3 and Fig. 2). Again, the lowest erythrocyte concentration was obtained with the RegenLab BCT system (0.02 X 10E12/L, 0.002X that of whole blood), and the highest erythrocyte concentration was obtained with the Harvest SmartPrep 2 system (2.0 X 10E12/L, 0.4X that of whole blood) (Table 3 and Fig. 2). Erythrocyte concentration determined the color of the different PRP preparations, ranging from pale yellow to dark red (Panel d in Fig. 2).

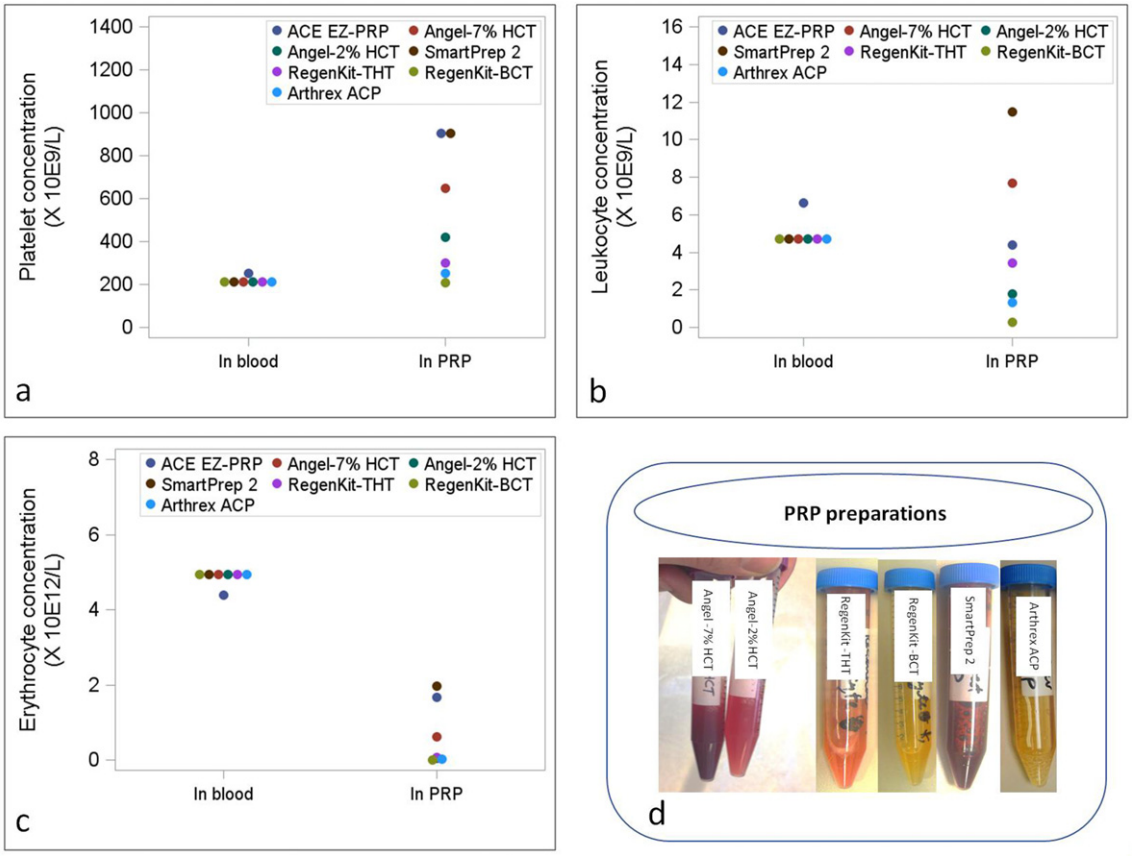

Fig. 2. Complete blood count (CBC) analysis of whole blood and resultant PRP from each system is shown in panels a (platelet concentration), b (leukocyte concentration) & c (erythrocyte concentration). Macroscopic appearance of different PRP preparations is shown in panel d. Erythrocyte concentration (panel c) influenced the colour of PRP preparations (panel d).

Table 3
Complete blood count (CBC) analysis in whole blood and different PRP preparations

Device name	Platelets (X 10E9/L)			Leukocytes (X 10E9/L)			Erythrocytes (X 10E12/L)		
	Whole blood	PRP	Fold change	Whole blood	PRP	Fold change	Whole blood	PRP	Fold change
ACE EZ-PRP	254	905	3.6	6.7	4.4	0.7	4.4	1.7	0.380
Angel-2% HCT	212	419	2.0	4.7	1.8	0.4	5.0	0.05	0.010
Angel-7% HCT	212	650	3.1	4.7	7.7	1.6	5.0	0.6	0.124
SmartPrep 2	212	903	4.3	4.7	11.5	2.4	5.0	2.0	0.402
RegenKit-BCT	212	207	1.0	4.7	0.3	0.1	5.0	0.01	0.002
RegenKit-THT	212	299	1.4	4.7	3.4	0.7	5.0	0.08	0.016
Arthrex ACP	212	253	1.2	4.7	1.4	0.3	5.0	0.02	0.004

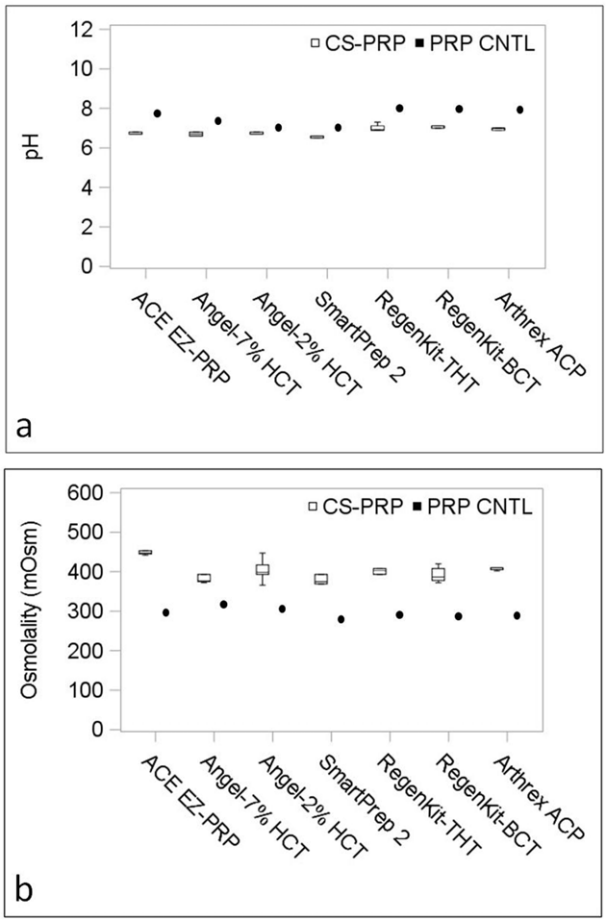

Fig. 3. pH (panel a) and osmolality (panel b) of formulations post-solubilization with PRPs isolated with the different devices. pH of CS-PRP formulations was lower than recalcified PRP controls (a). Osmolality of CS-PRP formulations was higher than recalcified PRP controls (b). Data are presented as box plots where median box indicates the 25th and 75th percentile; Whisker extends to the most extreme data point within 1.5 times the interquartile range of data. $n = 5$ samples for each type of CS-PRP formulation and $n = 1$ for each recalcified PRP control.

3.2. Solubility of CS cakes

Macroscopically, the cakes were white, homogenous and were slightly retracted from the vial walls following lyophilisation. Cake solubility was rated as excellent in most cases, except for the cakes prepared with CS M_n 55 kDa, which had to be shaken for a few extra seconds to become completely homogenous. pH of the CS formulations was between 6.27 and 6.47 before freeze-drying. Osmolality of the CS formulations was between 147 and 192 mOsm prior to freeze-drying. Post-solubilization, pH of the CS-PRP formulations was lower than physiological (from average 6.56 to average 7.12), and always lower than that of the recalcified PRP preparations (from 7.10 to 8.10) (Fig. 3a). Post-solubilization, osmolality of the CS-PRP formulations was higher than physiological (from average 389 mOsm to average 448 mOsm) and higher than that of the recalcified PRP preparations (from 282 mOsm to 320 mOsm) (Fig. 3b).

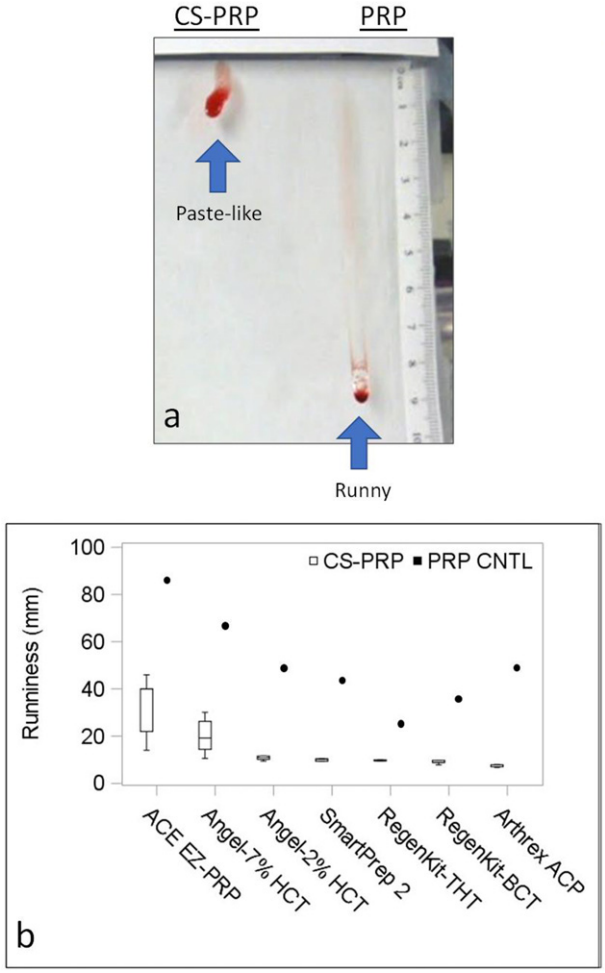

Fig. 4. Runniness of the CS-PRP and PRP formulations was assessed on an inclined plate. Panel a shows runniness of CS-PRP and a recalcified PRP control prepared with CS 84.6% DDA M_n 55 kDa and ACE EZ-PRP as an example. CS-PRP formulations were paste-like and less runny than the recalcified PRP controls panel (b). Note that in this assay water runs off the plate and has a runniness exceeding 310 mm. Data are presented as box plots where median box indicates the 25th and 75th percentile; Whisker extends to the most extreme data point within 1.5 times the interquartile range of data. n = 5 samples for each type of CS-PRP formulation and n = 1 for each recalcified PRP control.

3.3. Runniness of CS-PRP formulations

A representative example illustrating runniness of CS-PRP (prepared with CS 84.6% DDA M_n 55 kDa and ACE EZ-PRP) and its recalcified PRP control is shown in Fig. 4a. Runniness of the CS-PRP formulations (from average 7 mm to average 32 mm) was always lower than that of the recalcified PRP controls (from 25 mm to 86 mm).

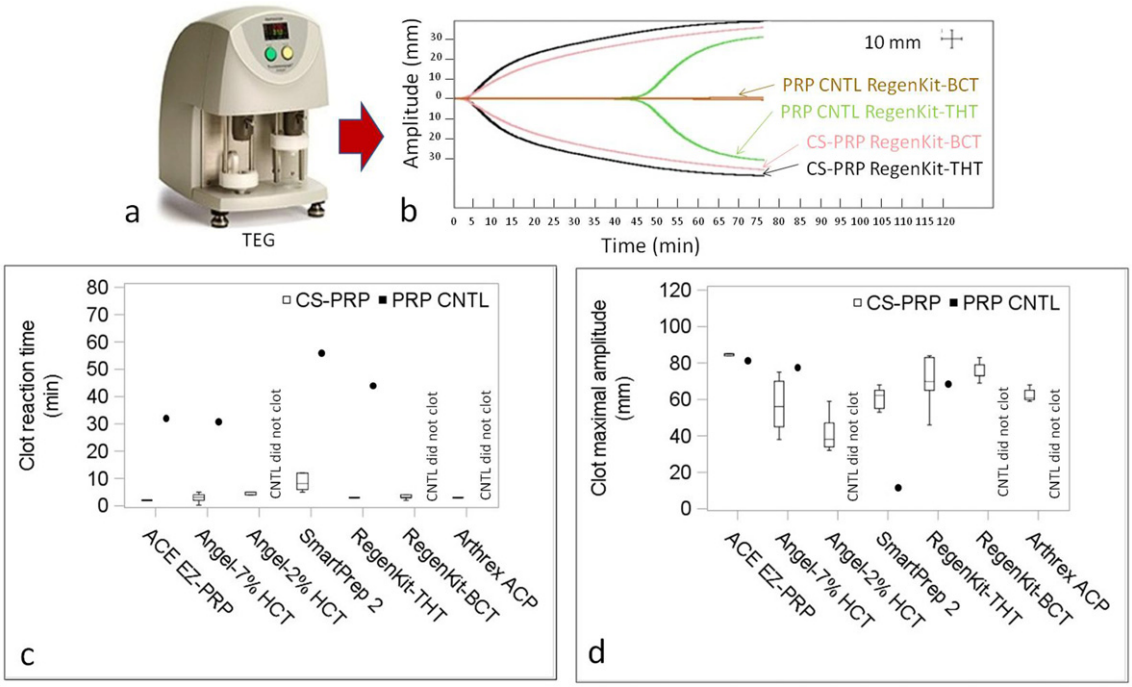

Fig. 5. A thrombelastograph (panel a) was used to assess clotting properties of formulations *in vitro*. Panel b shows TEG traces obtained for formulations prepared with CS 84.6% DDA M_n 55 kDa and RegenKit-BCT or RegenKit-THT as an example. Clot reaction time (R) is the time in minutes from initiation of the tracing to the point where branches have diverged by 2 mm. Maximal amplitude (MA) is the maximal distance in mm between the two diverging branches and corresponds to clot strength. All CS-PRP formulations clotted and had average clot reaction times between 2 and 9 minutes (panel c). Clot reaction times of the recalcified leukocyte-rich PRP controls were greater, between 32 and 57 minutes (panel c). CS-PRP formulations had average clot maximal amplitude above 42 mm (d). Recalcification of the leukocyte-reduced PRP controls with 42.2 mM was insufficient to induce clotting in this dynamic system (c & d). Recalcified SmartPrep 2 PRP control had barely started to clot when the assay was terminated so that its clot reaction time was high (57 minutes) (c) and its clot maximal amplitude was low (11 mm) (d). Data are presented as box plots where median box indicates the 25th and 75th percentile; Whisker extends to the most extreme data point within 1.5 times the interquartile range of data. $n = 5$ samples for each type of CS-PRP formulation and $n = 1$ for each recalcified PRP control.

3.4. *Coagulation of CS-PRP formulations*

Representative TEG tracings obtained for formulations prepared with CS 84.6% DDA M_n 55 kDa and RegenLab RegenKit-BCT or RegenKit-THT and their recalcified PRP controls are shown in Fig. 5b. Clot reaction time of the CS-PRP formulations (from average 2 min to average 9 min) was lower than that of the recalcified PRP preparations (from 32 min to 57 min) (Fig. 5c). Clot maximal amplitude of the CS-PRP formulations was between 42 mm and 84 mm (Fig. 5d). Recalcification of the leukocyte-reduced PRP preparations was insufficient to induce coagulation in this dynamic system, while the leukocyte-rich PRP preparations clotted (Fig. 5c&d). Of note, the Harvest SmartPrep 2 control had barely started to clot when the assay was terminated so that its clot reaction time was high (57 minutes) (Fig. 5c) and its clot maximal amplitude was low (11 mm) (Fig. 5d).

Table 4
Pearson correlation coefficients r and corresponding p values between the performance characteristics of the CS-PRP formulations and the properties of the CS and PRP preparations used to prepare the formulations. * % clot mass lost was 0 for all CS-PRP formulations assessed; N/A = Non applicable

Performance characteristic assessed	Chitosan M_n	Chitosan DDA	Platelet concentration (X 10E9/L)	Leukocyte concentration (X 10E9/L)	Erythrocyte concentration (X 10E9/L)
Runniness (mm)	−0.161 *(0.357)*	−0.138 *(0.430)*	0.354 *(0.059)*	0.330 *(0.070)*	0.139 *(0.457)*
Clot reaction time (min)	−0.173 *(0.319)*	0.235 *(0.174)*	0.008 *(0.965)*	0.218 *(0.230)*	−0.007 *(0.969)*
Clot maximal amplitude (mm)	0.289 *(0.093)*	−0.105 *(0.550)*	0.115 *(0.510)*	−0.095 *(0.586)*	0.240 *(0.165)*
Clot retraction (% clot mass lost) *	N/A	N/A	N/A	N/A	N/A

3.5. Serum expression from CS-PRP hybrids

Images of a CS-PRP hybrid clot prepared with CS 84.8% DDA M_n 32 kDa and Harvest SmartPrep 2 and its recalcified PRP control are shown in Fig. 6a. Images of a CS-PRP hybrid clot prepared with CS 82.5% DDA M_n 45 kDa and Arthrex ACP and its recalcified PRP control are shown in Fig. 6b. The absence/presence of erythrocytes in the two PRP preparations is easily observed by the yellow/red hue of the preparations. None of the CS-PRP formulations expressed serum and the hybrid clots remained voluminous after clotting for 1 hour at 37 °C (Figs 6a, b & c). Recalcification with 42.2 mM $CaCl_2$ was sufficient to induce coagulation of all PRP preparations in this static assay in glass tubes, and PRP clots lost up to 82% of their original mass through serum exudation upon clotting (Figs 6a, b & c).

3.6. Dispersion of CS within hybrid clots

Histological sections of a CS-PRP hybrid clot prepared with CS 84.8% DDA M_n 32 kDa and Arthrex Angel with 7% hematocrit and its recalcified PRP control are shown in Fig. 7a–d. Histological sections of a CS-PRP hybrid clot prepared with CS 84.8% DDA M_n 32 kDa and Arthrex Angel with 2% hematocrit and its recalcified PRP control are shown in Fig. 7e–h. As expected, erythrocytes were more abundant and densely packed in clots prepared with Arthrex Angel with 7% hematocrit compared to Arthrex Angel with 2% hematocrit. CS dispersion was homogenous within most CS-PRP clots (Figs 7a, b, e & f), while a minority of clots contained some larger CS aggregates.

3.7. In vitro performance characteristics of the CS-PRP implants

There were no significant correlations between runniness, clot reaction, and clot maximal amplitude of the CS-PRP hybrids and CS M_n, CS DDA, platelet concentration, leukocyte concentration and erythrocyte concentration (Table 4). In addition, there were no significant correlations between runniness, clot reaction, and clot maximal amplitude of the recalcified PRP controls and platelet concentration, leukocyte concentration and erythrocyte concentration (Table 5).

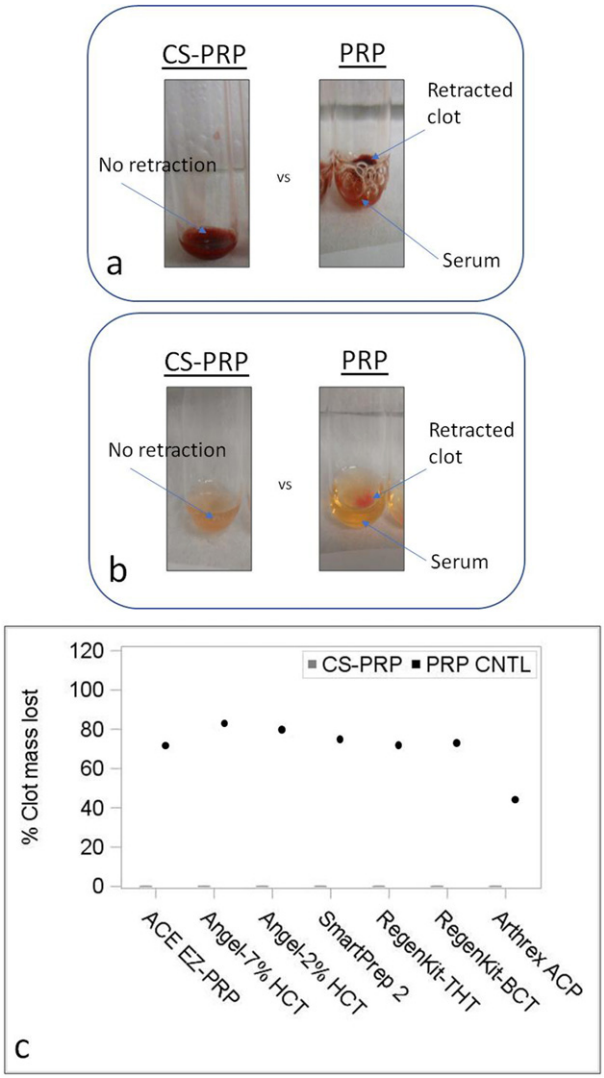

Fig. 6. Clot retraction was assessed by gravimetric measurements. Panel a shows a CS-PRP hybrid clot and a recalcified PRP control prepared with CS 84.8% DDA M_n 32 kDa and SmartPrep 2 as an example. Panel b shows a CS-PRP hybrid clot and a recalcified PRP control prepared with CS 82.5% DDA M_n 45 kDa and Arthrex ACP as an example. All CS-PRP hybrid clots remained voluminous after clotting for 1 h at 37 °C and did not express any serum (panel c). Recalcification with 42.2 mM $CaCl_2$ was sufficient to induce coagulation of all PRP controls in this static assay. Recalcified PRP controls expressed a lot of serum upon clotting and lost 43% to 82% of their original mass upon clotting (panel c). n = 5 samples for each type of CS-PRP formulation and n = 1 for each recalcified PRP control.

4. Discussion

The main objectives of the current study were to define CS specifications for the previously developed technology [12] and assess its compatibility with the various types of PRPs that can be isolated with commercial systems. We found that *in vitro* performance of CS-PRP implants was similar for all PRP preparations tested, so that our starting hypothesis was supported. We also found that freeze-dried cakes

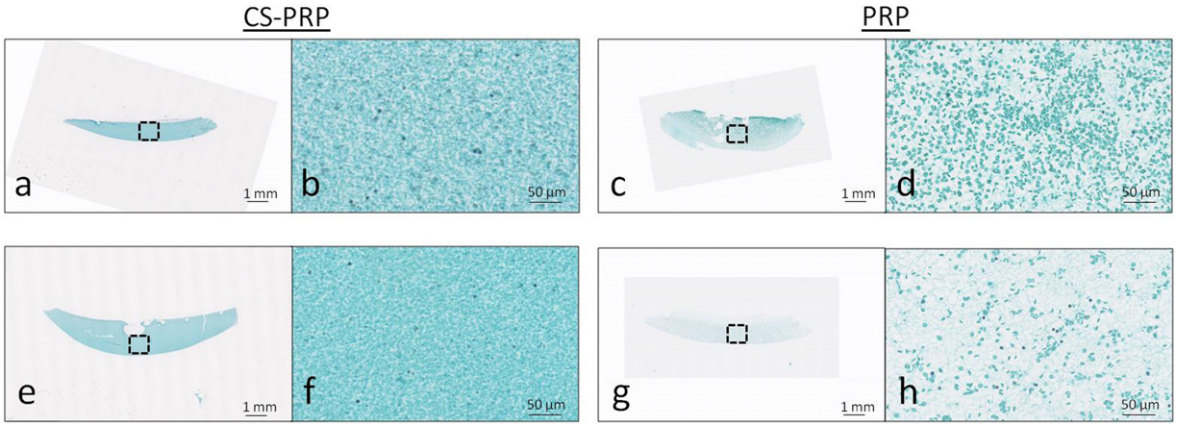

Fig. 7. Clot homogeneity was assessed with Fast Green and Iron Hematoxylin staining of paraffin sections. Panels a & b show a CS-PRP hybrid clot prepared with CS 84.8% DDA M_n 32 kDa and Angel with 7% hematocrit as an example and panels c & d show the recalcified PRP control. Panels e & f show a CS-PRP hybrid clot prepared with CS 84.8% DDA M_n 32 kDa and Angel with 2% hematocrit as an example and panels g & h show the recalcified PRP control. Dispersion of CS within the hybrid clots was usually homogenous (b & f). Erythrocytes were more abundant in clots prepared with Angel with 7% hematocrit compared to Angel with 2% hematocrit (compare panel d to h). Outlines in panels a, c, e & g show where higher magnification images b, d, f & h were acquired.

Table 5
Pearson correlation coefficients r and corresponding p values between the performance characteristics of the recalcified PRP controls and the properties of the PRP preparations

Performance characteristic assessed	Platelet concentration (X 10E9/L)	Leukocyte concentration (X 10E9/L)	Erythrocyte concentration (X 10E9/L)
Runniness (mm)	0.662 *(0.105)*	0.206 *(0.657)*	0.513 *(0.239)*
Clot reaction time (min)	0.048 *(0.952)*	0.567 *(0.433)*	0.315 *(0.685)*
Clot maximal amplitude (mm)	−0.347 *(0.653)*	−0.819 *(0.181)*	−0.547 *(0.453)*
Clot retraction (% clot mass lost)	0.389 *(0.388)*	0.386 *(0.393)*	0.242 *(0.602)*

prepared with CS of DDA between 80.5–84.8% and M_n between 32–55 kDa had acceptable performance characteristics when solubilized with PRP.

In the current study, two families of PRPs were prepared according to the definition of Dohan Ehrenfest [3]: Leukocyte-rich PRPs (ACE EZ-PRP, Arthrex Angel with 7% hematocrit, Harvest SmartPrep 2 and RegenLab RegenKit-THT) and leukocyte-reduced PRPs (Arthrex Angel with 2% hematocrit, RegenLab RegenKit-BCT and Arthrex ACP). As expected, and earlier shown by others [7,8,20–23], the various PRP preparation devices varied in their abilities to concentrate platelets, leukocytes and erythrocytes (Table 3 and Fig. 2). This might explain the significant variability in the clinical effectiveness of PRP which has been published in the literature, and supports the notion that PRP should always be characterized when

used. Variability in donor blood parameters and PRP's poor stability *in vivo* [24] are two other possible reasons why results have been inconsistent so far. Although some authors have stated that PRP should possess a 3 to 5-fold increase over baseline in platelet concentration (~1 million platelet/µl) to be effective [25], the optimal PRP recipe has yet to identified, and could very well be different for different indications.

Interestingly, recalcified PRP controls performed differently from one another with respect to runniness, coagulation and clot retraction in the current study, and this could not be attributed to platelet, leukocyte and erythrocyte concentrations individually. Other properties that were not assessed here might control performance characteristics or a combination of several properties. One unexpected finding in this study is that recalcification with 42.2 mM was insufficient to induce clotting of the leukocyte-poor PRP preparations in the dynamic thromboelastography assay, which suggests that leukocytes also contribute to coagulation, possibly through release of coagulation factors or by contributing to platelet activation [26]. In addition, the leukocyte-poor PRP preparations were also the ones that had the lowest concentration of erythrocytes, which are believed to also participate in thrombin generation [27]. We should highlight that this finding was restricted to the thromboeastography assay, that higher concentrations of $CaCl_2$ or the addition of another platelet agonist might have induced coagulation, and that the thromboelastrography assay is not representative of the *in vivo* situation.

Combinations of freeze-dried CS and PRP have been described by other authors [28–30], but, to our knowledge, these have all been solid scaffolds intended to remain solid for implantation. In contrast, our aim here was to solubilize freeze-dried CS formulation with PRP so that they become liquid and coagulate post-injection. As expected, CS-PRP formulations had pH lower than their respective PRP controls and lower than physiological (Fig. 3), due to the presence of acid, but we do not expect that this would negatively affect tissues *in vivo*. In addition, osmolality of CS-PRP formulations was higher than physiological and their respective PRP controls (Fig. 3) since the cakes contained excipients (trehalose and $CaCl_2$), but not enough to impair coagulation and associated events as previously shown [12]. Regardless of the PRP preparation used to solubilize the freeze-dried cakes, CS-PRP formulations were all paste-like due to the presence of the polysaccharide (Fig. 4) and clotted rapidly since CS contributes to platelet activation in this system [13], and possibly through inducing red blood cell agglutination [31,32] (Fig. 5). Resulting CS-PRP clots did not retract post-coagulation (Fig. 6) due to CS ability to physically impair platelet-mediated clot retraction [13] and were for the most part homogenous (Fig. 7), since the CS selected to prepare the cakes had M_n close to 40 kDa, which has been shown to result in homogenous distribution of CS throughout the blood components [12].

Since rather narrow ranges of CS DDA and M_n were selected, it was not surprising to find that all CS-PRP formulations tested behaved similarly *in vitro*. However, although the *in vitro* performance characteristics assessed were similar for all PRP preparations tested, we expect that other assessments would most likely show differences between the various PRP preparations. For example, release of platelet-derived growth factors and inflammatory factors and cytokines would be expected to depend on the cellular profile of the PRP preparations used to solubilize the formulations [4–8], and this could very well modulate repair events *in vivo*. Further studies are required to determine the bioactivity level of the CS-PRP formulations and how this would influence the repair process. It is possible that some PRP preparations would be preferable for some indications, while others should be avoided.

This study had some limitations. First, although a single donor was used to generate all PRP preparations in order to avoid inter-individual variability and allow direct comparisons between the different separation systems, it would be useful to increase sample size and isolate PRP from additional donors. Second, we chose the commercial isolation systems based upon their availability and we are aware that other systems are also widely used in the clinic. Third, the release of platelet-derived growth factors and inflammatory

factors and cytokines was not measured in this study. Fourth, the runniness test is insufficient to fully characterize viscoelastic behavior of the CS-PRP formulations. Fifth, the study was limited to an *in vitro* assessment of product performance. The current study allowed us to establish the CS specifications (DDA and M_n) for the product and that it is compatible with different PRP preparations isolated with commercial systems. However, further *in vitro* and *in vivo* studies are necessary to understand how the properties of the PRP preparations used to solubilize the CS will affect bioactivity and healing potential of the implants.

5. Conclusion

PRP remains controversial in the orthopaedic field, and inconsistent results in the literature may have resulted from inter-individual variability in blood parameters, the heterogeneity of the PRP preparations used, or its poor stability *in vivo*. Freeze-dried CS formulations containing a lyoprotectant and a clot activator can be solubilized in different types of PRP preparations to yield injectable implants that are paste-like and coagulate rapidly to form homogenous CS-PRP hybrid clots that remain voluminous. These could possibly be used as implants to treat different orthopaedic conditions in the future.

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Conflict of interest

AC, MDB, CDH and ML hold shares and MDB and CDH are Directors of Ortho Regenerative Technologies Inc.

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