Chitosan-platelet-rich plasma implants actions *in vitro* and *in vivo*

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PhD candidate

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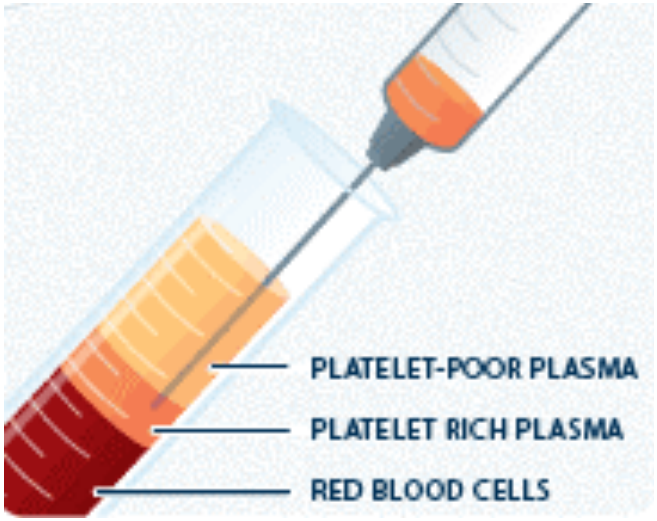
Disclosure

- AC, NTK and MDB are shareholders and MDB is a founder of Orthoregenerative Technologies Inc

Platelet-rich plasma

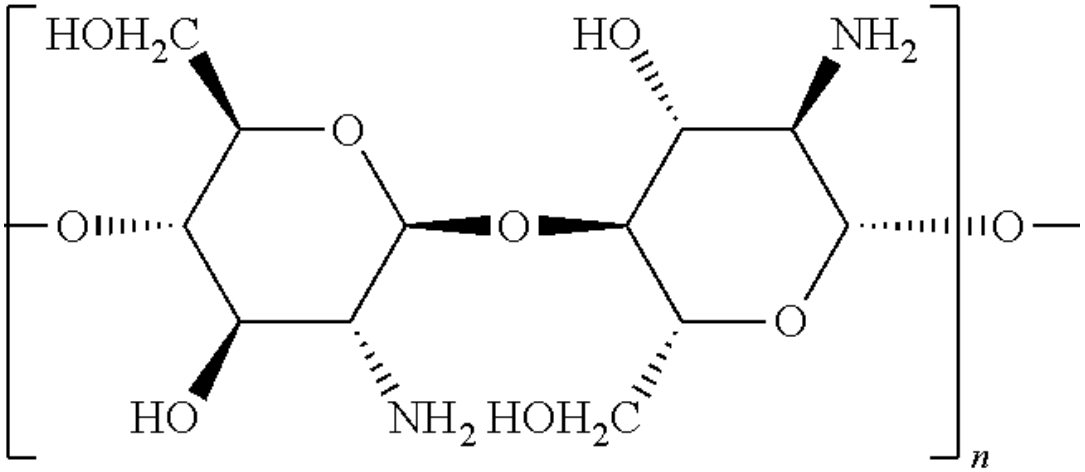
- Platelet-rich plasma (PRP), a product of whole blood is currently used in tissue regeneration.
- PRP has a high concentration of platelets and platelet-derived factors.



http://www.holisticveterinaryhealing.com/wp-content/uploads/2015/01/prp-3.png

Chitosan

Polysaccharide obtained from partial deacetylation of chitin



chitosan

DDA= degree of deacetylation

$$DDA = nD\text{-}GlcN \,/\, (nD\text{-}GlcN + nD\text{-}GlcNAc) \times 100$$

N-D-Glucosamine unit (GlcN)
N-acetyl-D-Glucosamine unit (GlcNAC)

Chitosan

- High DDA chitosan (>95% DDA) is slowly degraded.
- Lower DDAs (80-85%) degrade quickly and recruit local host cells to effect tissue repair (Hoemann et al. 2010).
- Our laboratory has worked previously with chitosan-glycerol phosphate (GP)/blood implants for cartilage repair applications (BST-CarGel).
- BST-CarGel has been approved for clinical use in 17 countries.

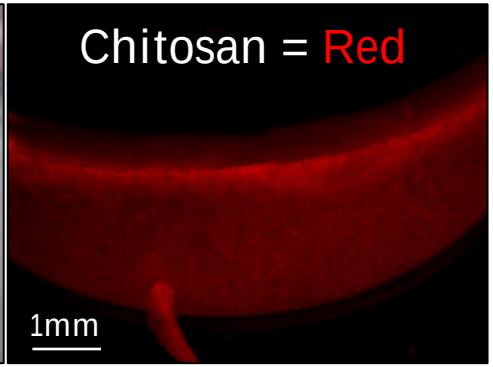
Previous work

- Freeze-dried Cakes contain chitosan, lyoprotectants and PRP activator, CaCl2
- Freeze-dried Cakes dissolve readily in PRP and coagulate rapidly to make homogenous chitosan-PRP hybrids that

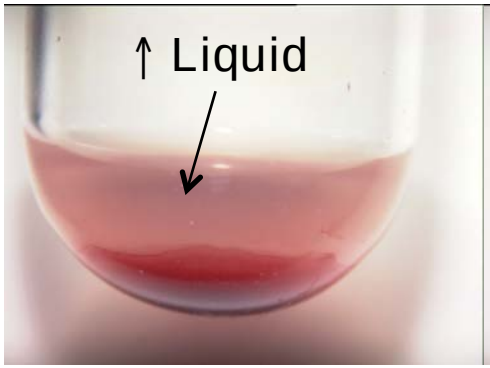
1) do not shrink, versus up to 90% volume loss in PRP
2) produce sustained biological activity
3) have in situ tissue building capacity

Freeze-dried cake

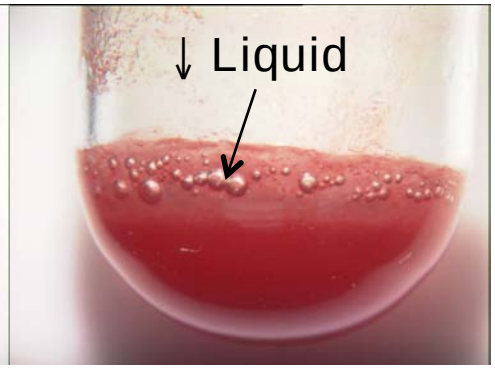
Hybrid clot



PRP only clot



Hybrid clot



(Chevrier et al BMM 2016 submitted)

Objectives

- Objective 1→ investigate chitosan-PRP hybrid clot retraction
- Objective 2→ investigate the levels of growth factors released from chitosan-PRP in culture medium.
- Objective 3 → characterize the effect of chitosan and lyoprotectant on platelet activation *in vitro*
- Objective 4 → inject freeze-dried chitosan/PRP implants subcutaneously in rabbit to assess the effect of DDA on cell recruitment.

Objective 1:
Clot retraction

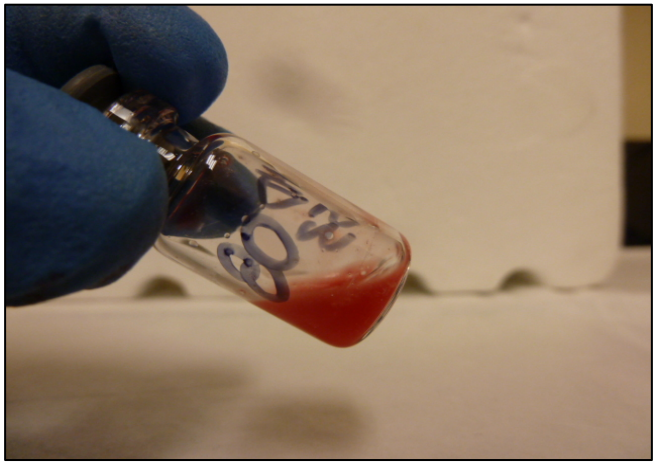
- Hypotheses:

- Chitosan binds to platelets →inhibits platelet aggregation which is required for strong clot retraction.

- The fibrin network formed in the presence of chitosan in chitosan-PRP hybrid clots is similar to the network formed PRP-only clots.

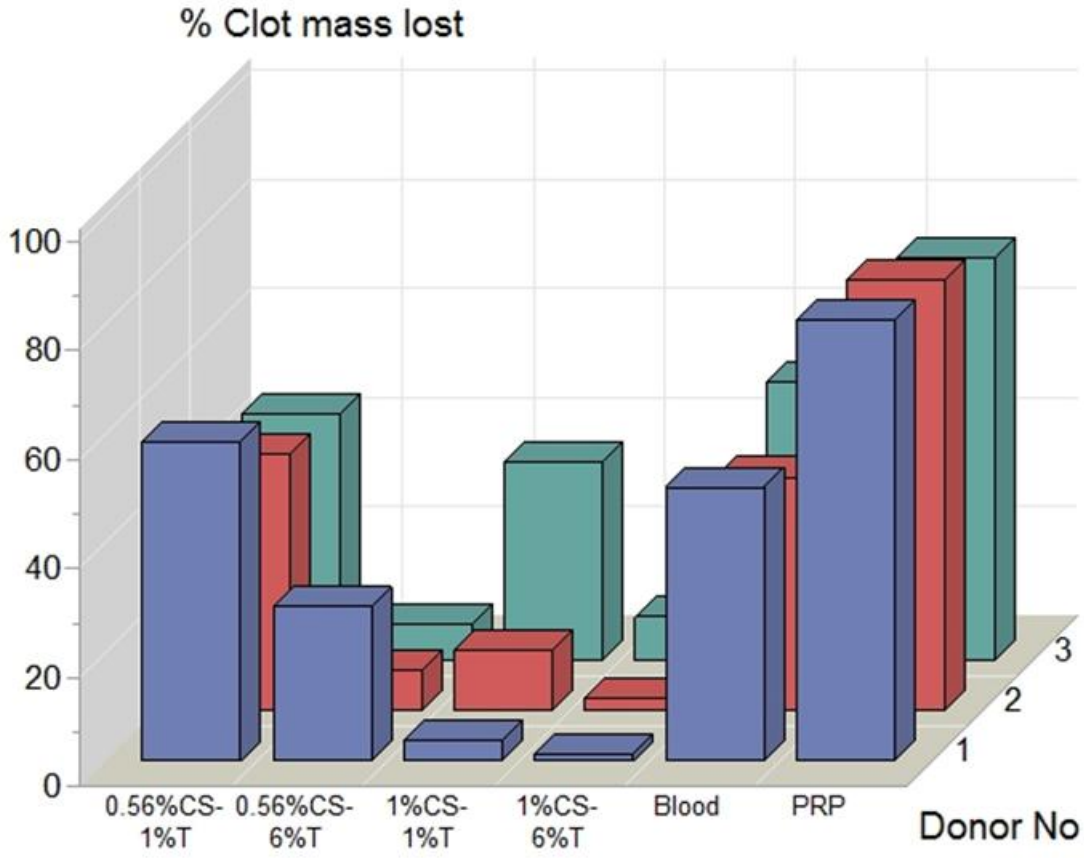
Clot retraction

- Methods:
- Four freeze-dried formulations solubilized in PRP
- Gravimetric measurements and Imaging on clots



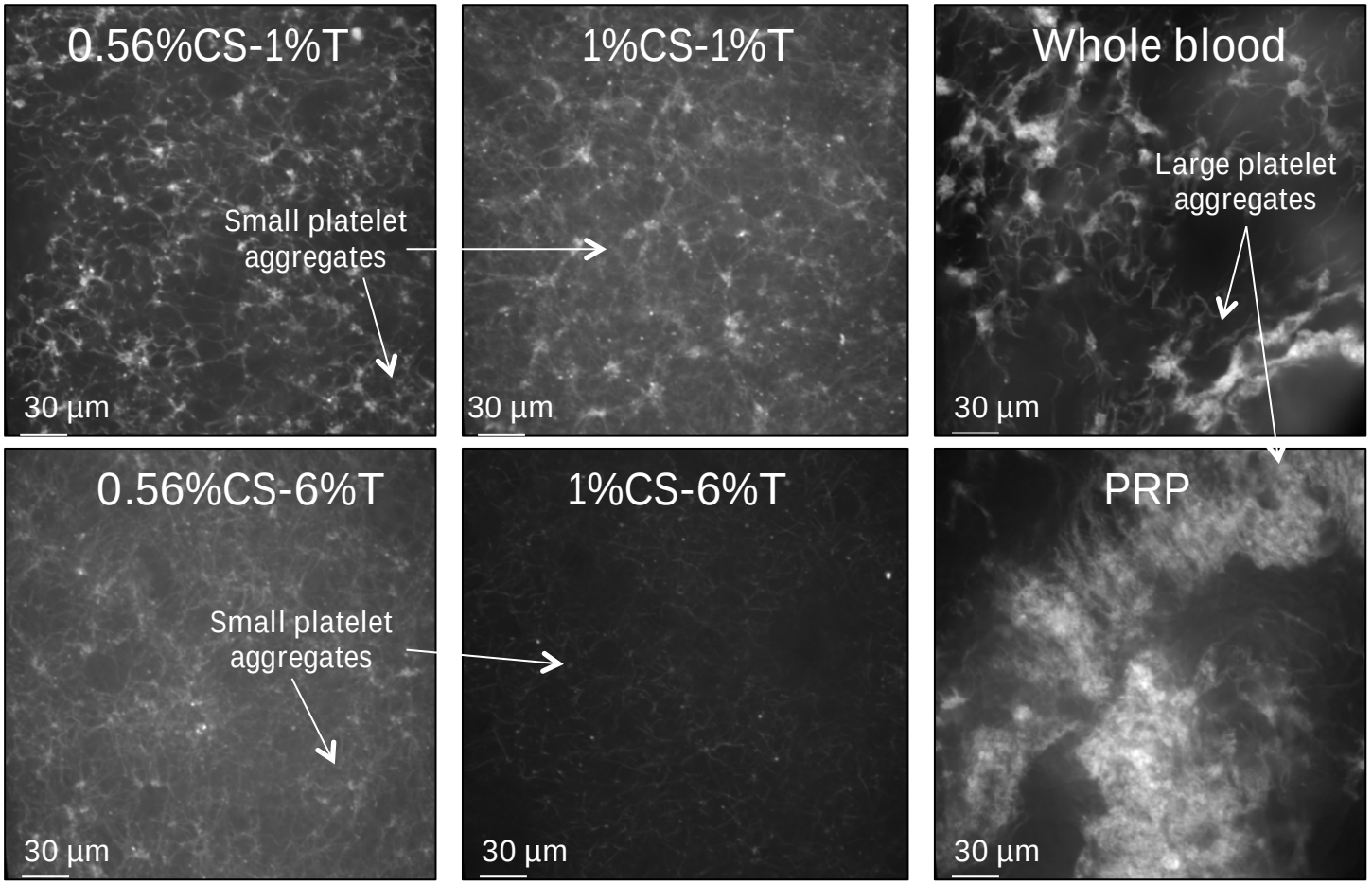
CS	Sol	Formulation	Chitosan (w/vol)	HCl for 60% protonated (mM)	Trehalose (mM)	$CaCl_2$ (mM)	Aliquot into	Rehydrated in (volume) of PRP
	1	0.56% CS-1% Trehalose	0.56%	16mM	26mM	42.2mM	1mL	1mL
	2	0.56% CS-6% Trehalose	0.56%	16mM	159mM-	42.2mM	1mL	1mL
	3	1% CS-1% Trehalose	1%	29mM	26mM	42.2mM	1mL	1mL
	4	1% CS-6% Trehalose	1%	29mM	159mM-	42.2mM	1mL	1mL

Chitosan-PRP hybrid clots inhibit retraction

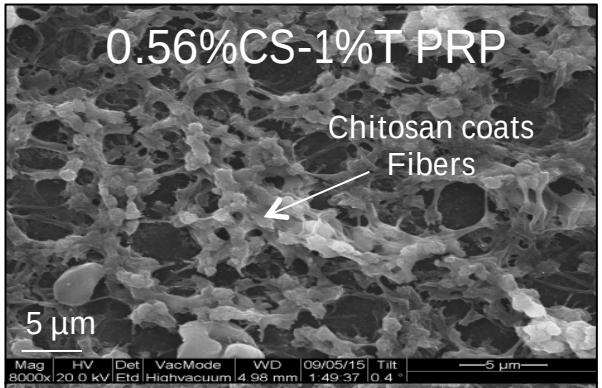


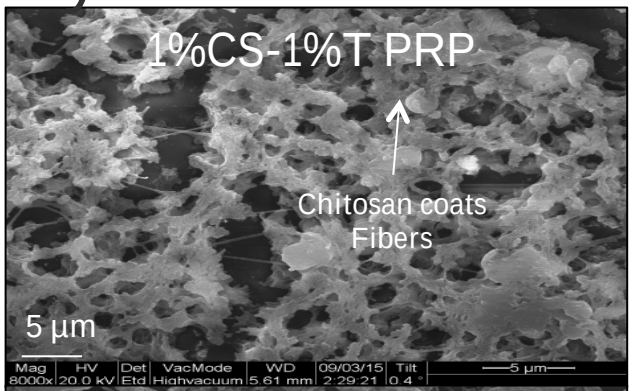
% Clot mass lost after clotting for 1 hour at 37°C for donor 1 (blue bars), donor 2 (red bars) and donor 3 (green bars) at 37°C. n = 2 clots for each measurement, with bars showing average of 2 clots.

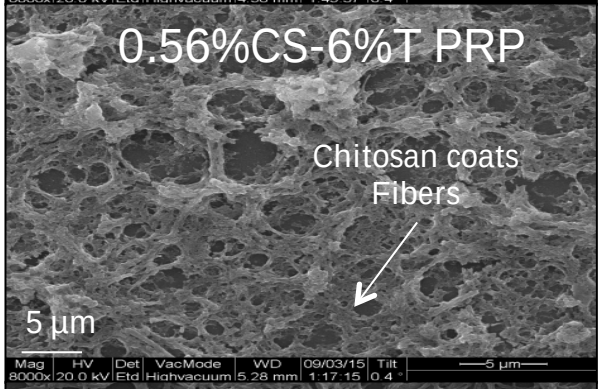
Platelet aggregates are smaller in chitosan-PRP hybrid clots and fibrin network is finer in presence of high trehalose

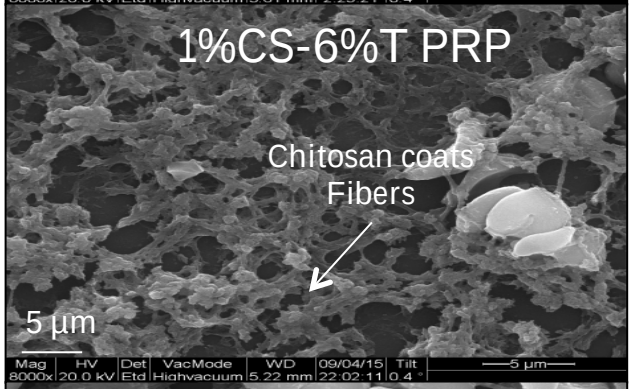


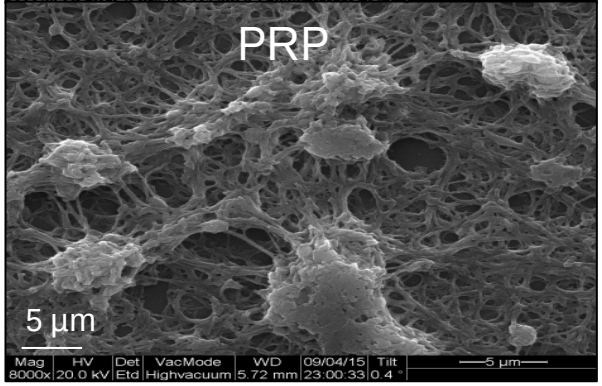
Cells and fibers are covered with chitosan in chitosan-PRP hybrid clots

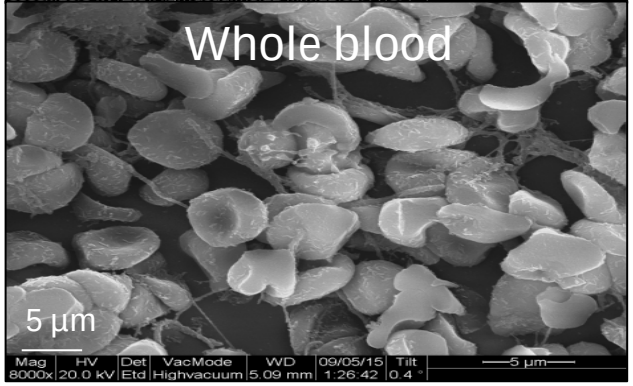












Conclusion: Objective 1

- Whole blood clots and PRP clots retracted significantly upon coagulation.
- Clot retraction was inhibited in chitosan-PRP hybrids.

Objective 2:
Growth Factor Release Profiles

Methods:

- One formulation (1% CS-1% Trehalose) solubilized in PRP from three donors → Duplicate clots from each donor cultured for 7 days, followed by ELISA for PDGF-AB, EGF and VEGF.

Release profiles

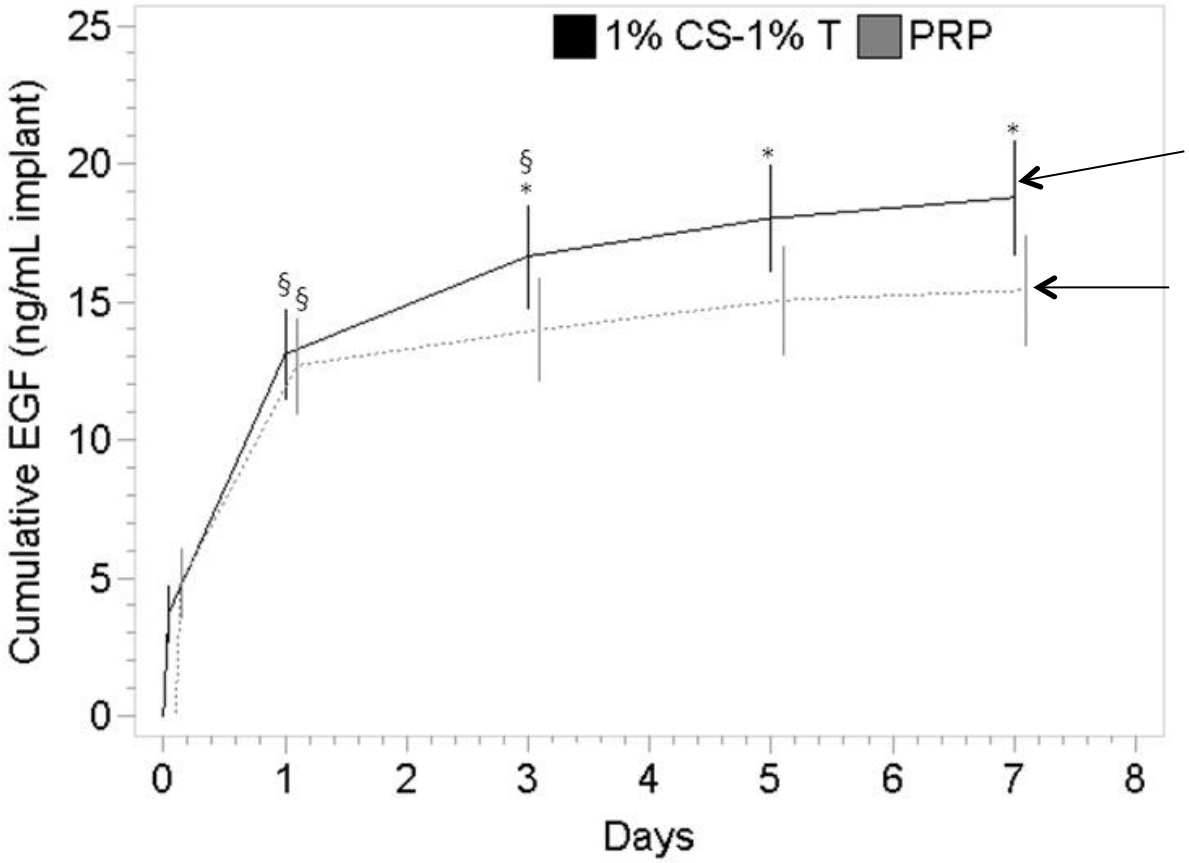
- Hypotheses:

- Negatively charged growth factors with low isoelectric points, would bind to positive CS to slow release.

- Positively charged growth factors with high isoelectric points would burst release, due to ionic repulsion.

Growth factors	PDGF-AB	VEGF	EGF
Isoelectric point	9.8	8.5	4.6
Charge	Positive	Positive	Negative

Continuous EGF release from hybrid and control clots and higher cumulative release from CS-PRP



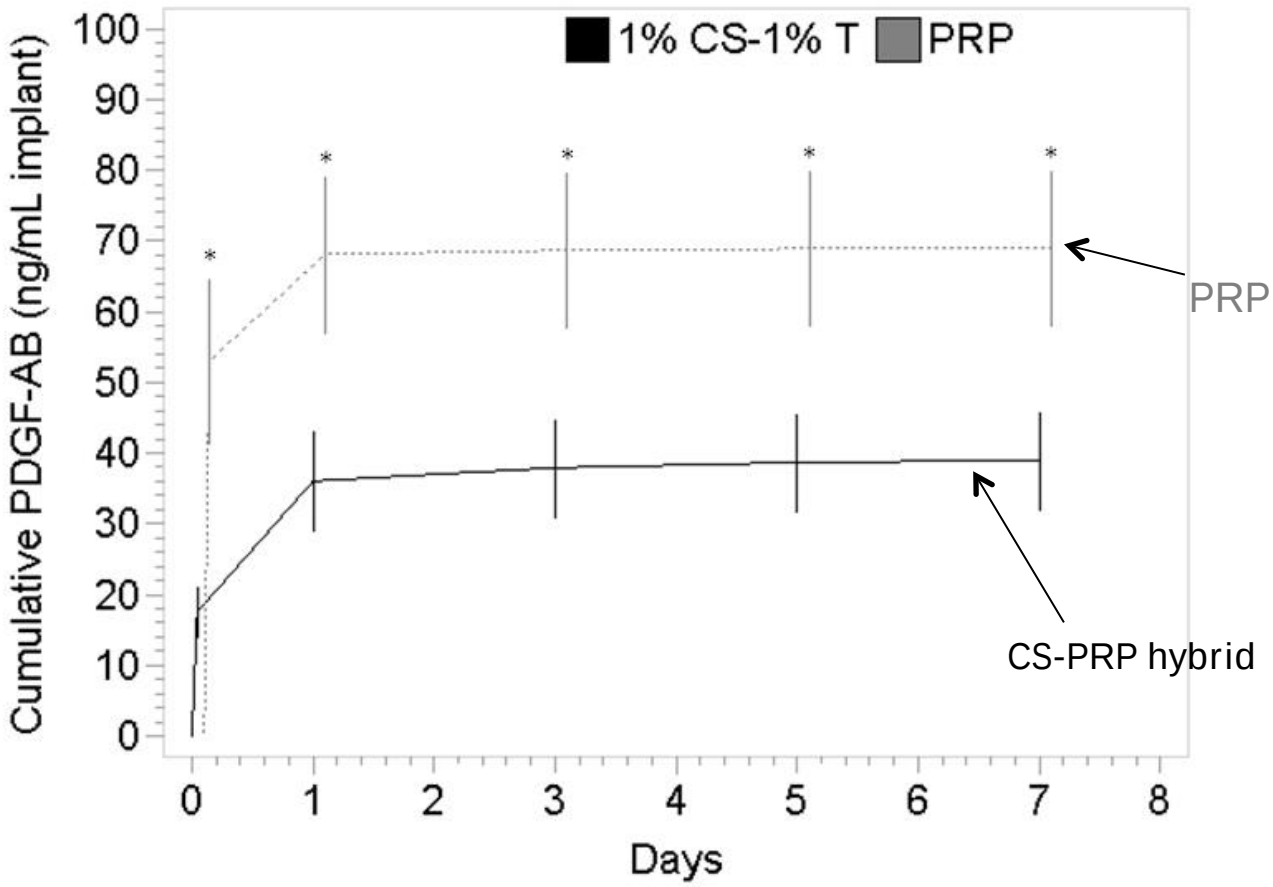
CS-PRP hybrid

PRP

-Implant 250µL
-Culture medium 500µL
-Medium collected at 0, 1, 3, 5, & 7 days

Results are presented as Mean ± SE. n = 6 clots. § $p < 0.05$ for time point immediately prior.

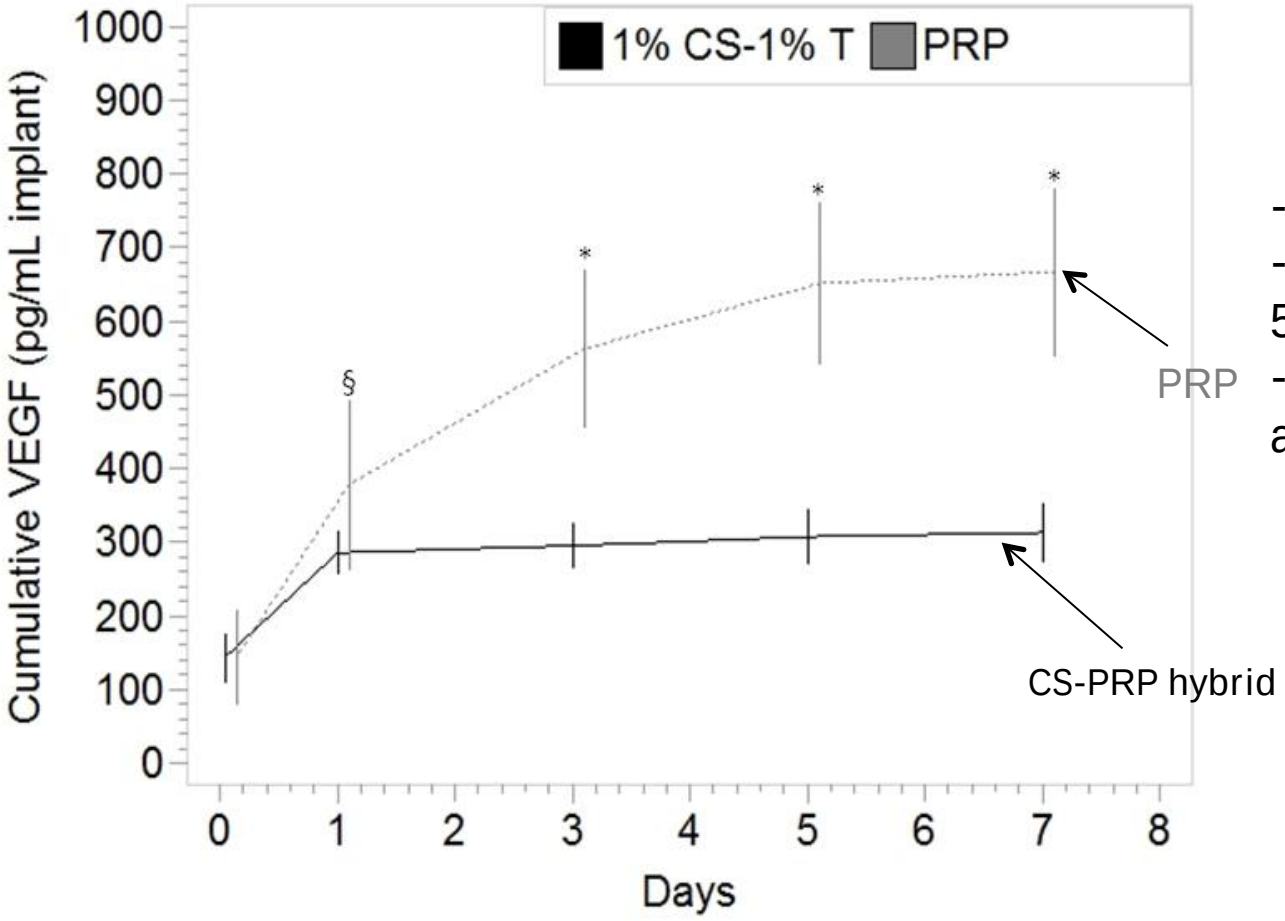
Burst PDGF-AB release from hybrid and control clots and lower cumulative release from CS-PRP



-Implant 250µL
-Culture medium 500µL
-Medium collected at 0, 1, 3, 5, & 7 days

Results are presented as Mean ± SE n = 6 clots. § $p < 0.05$ for time point immediately prior.

Burst VEGF release from hybrid clots and continuous release from control clots



-Implant 250µL
-Culture medium 500µL
-Medium collected at 0, 1, 3, 5, & 7 days

Results are presented as Mean ± SE. n = 6 clots. § $p < 0.05$ for time point immediately prior.

Conclusions of Objective 2

- High inter-individual variation between the amount of growth factor released by each donor, but the patterns of release were similar for all three donors.

- Release profiles did not support our charge-dependent hypotheses. Investigations into how growth factors bind to chitosan in our hybrids are still ongoing .

Objective 3:
Characterize the effect of chitosan and lyoprotectant on platelet activation *in vitro*

Hypothesis:

- Chitosan and trehalose will induce platelet activation.

Platelet activation

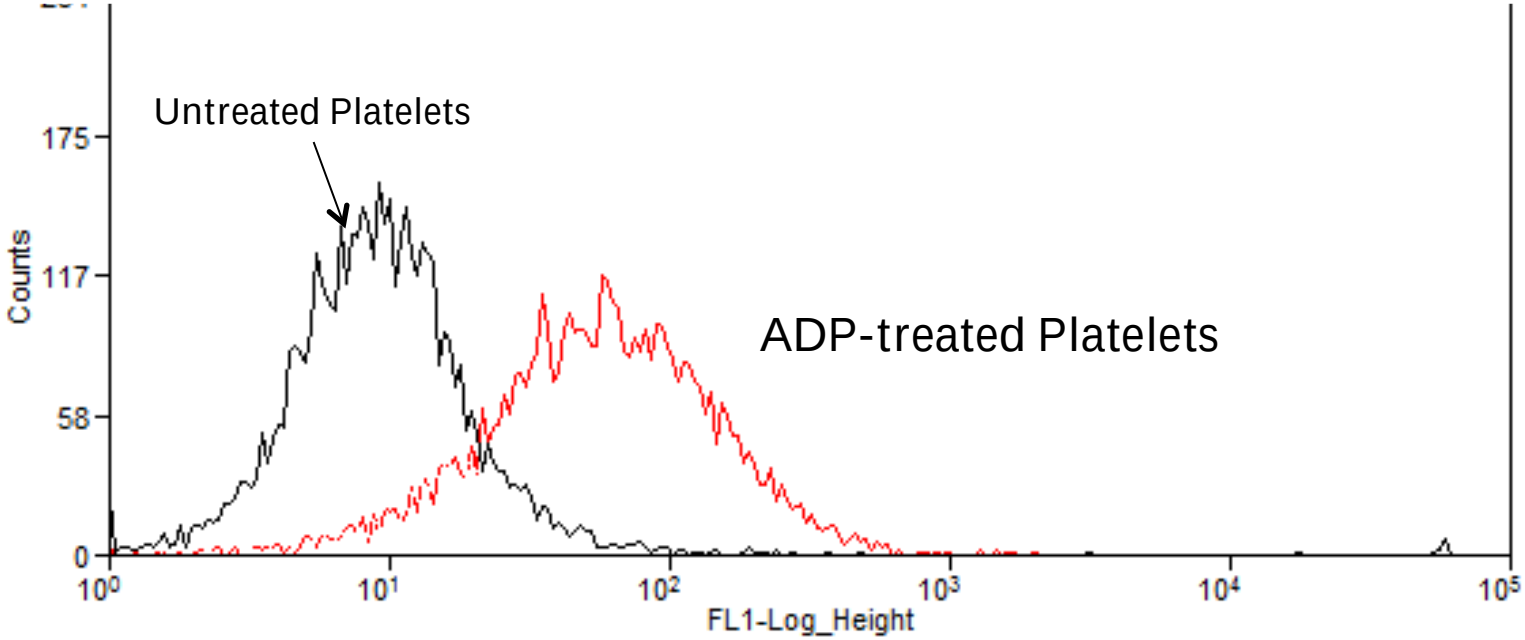
Methods proposed:

- Isolated platelets flow cytometry → assess platelet function after contact with chitosan and/or lyoprotectant.

- Monoclonal antibody :
  - Pac-1 fluorescein-conjugated antibodies (Activated $\alpha IIb\beta 3$).

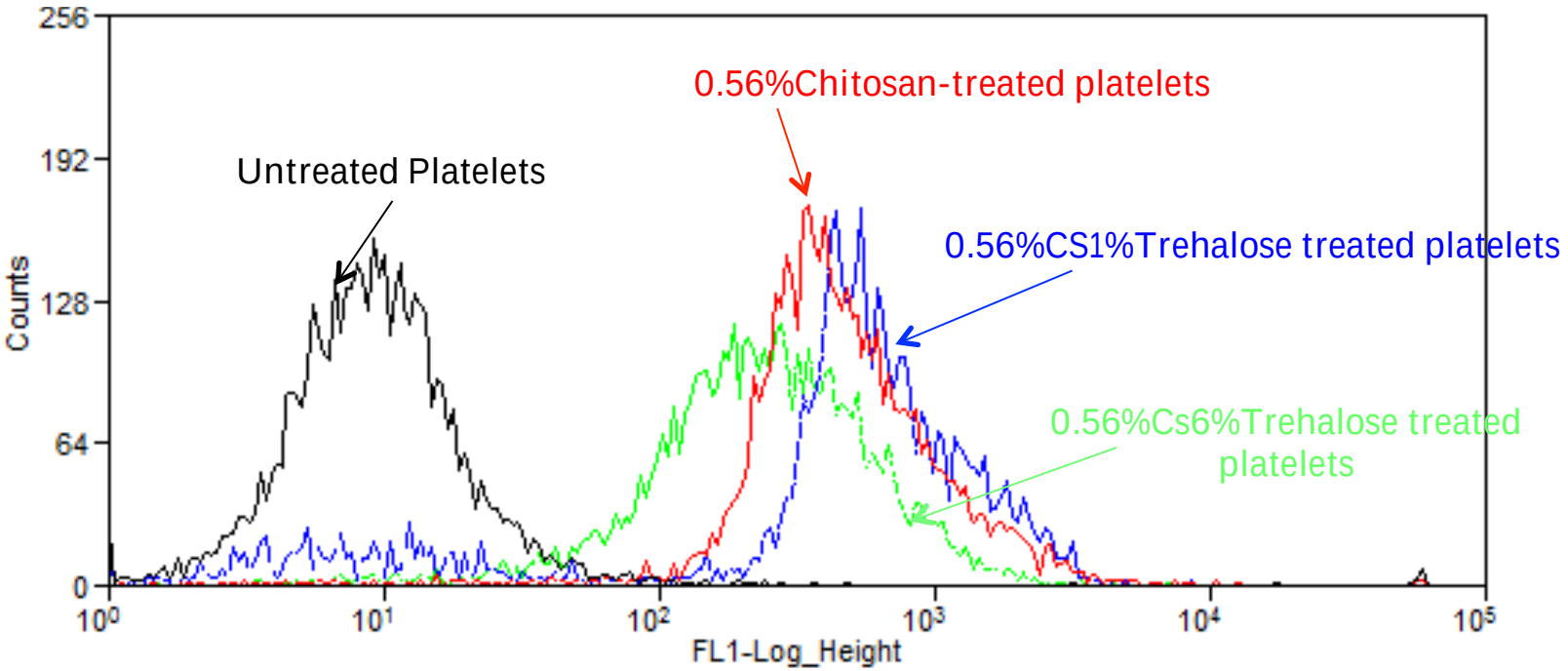
Platelet activation

PAC-1 Flow cytometry



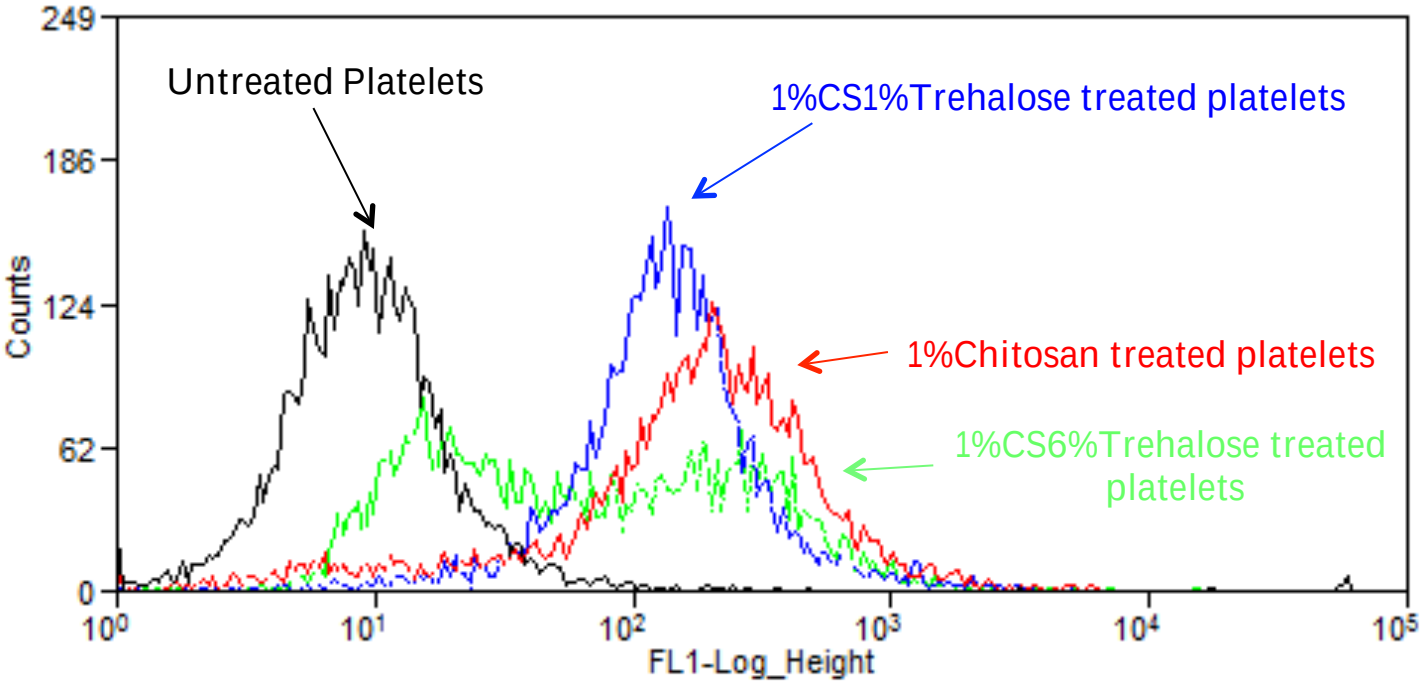
Platelets activated in contact with 0.56% chitosan

PAC-1 Flow cytometry

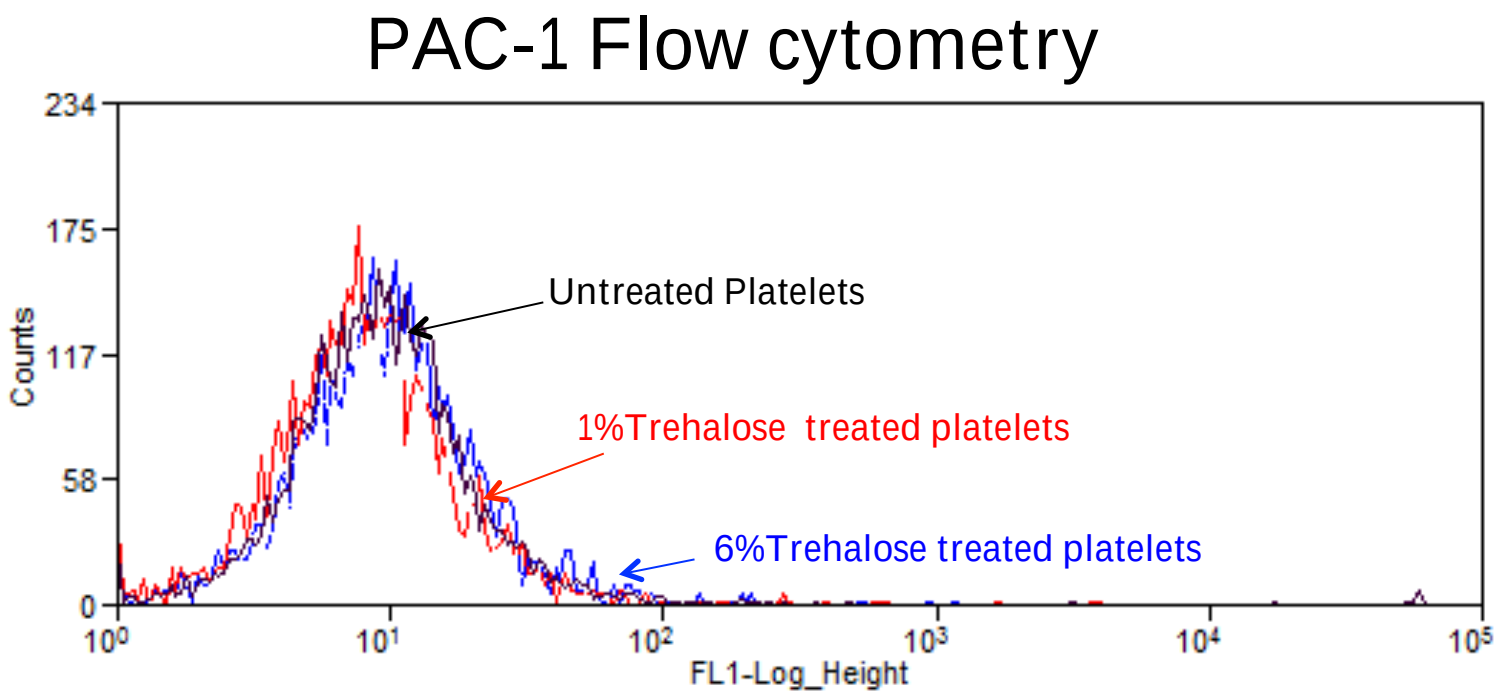


Platelets activated in contact with 1% chitosan

PAC-1 Flow cytometry



Lyoprotectant alone does not activate platelets



Conclusions of Objective 3

- Platelets are activated when placed in contact with chitosan (0.56% or 1%) with or without lyoprotectant.
- No activation with lyoprotectant only.
- Inhibition of clot retraction is not due to inhibited platelet activation.

Objective 4
Subcutaneous implants

- Methods:

- 4 different Chitosan/PRP formulations (*M_n 40kDa 1% (w/v) CS, 1% (w/v) trehalose and DDA 80%, 85%,90%, 95%*) were injected subcutaneously in the back of five rabbits.

Test Article	**Total Volume (mL)**	**# Animals / time points**		
Chitosan-PRP	150 µL per implant	n = 2 at day 14	n = 2 at day 28	n = 1 at day 42

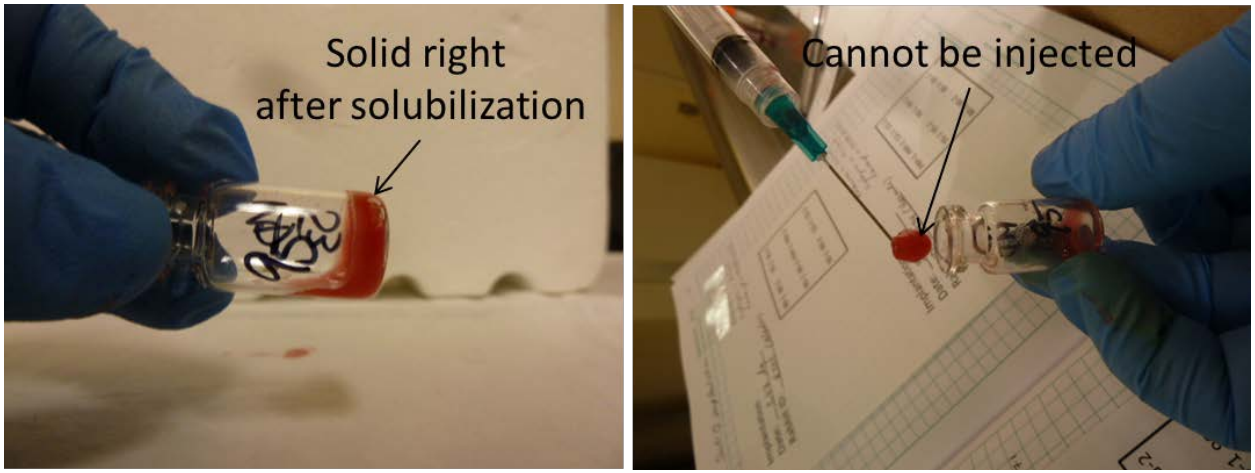
Subcutaneous implants

- Hypotheses:

- Chitosan-PRP formulations containing chitosan of higher DDA will reside longer subcutaneously *in vivo.*

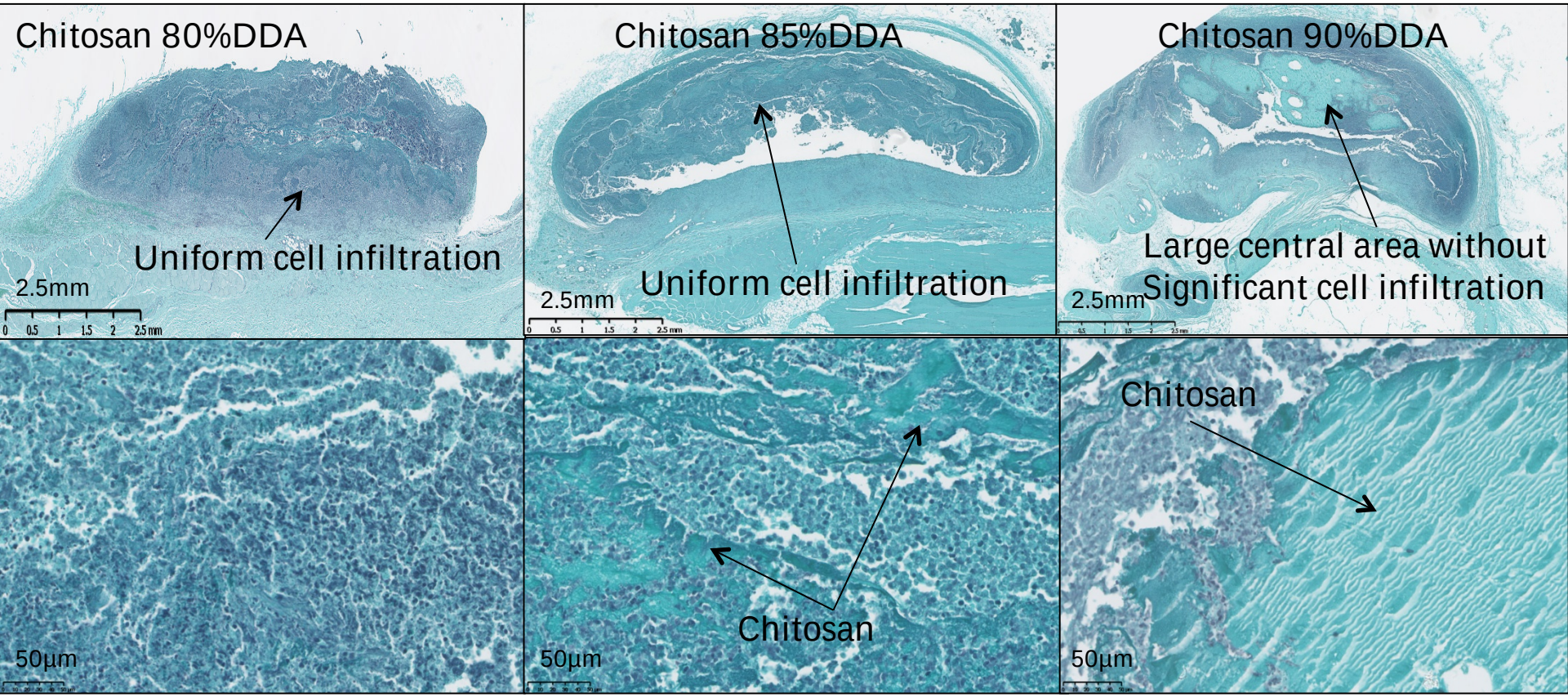
- Chitosan-PRP formulations containing chitosan of lower DDA will induce a greater acute inflammatory reaction (Lafantaisie-Favreau, C.-H., Hoemann, C. D. 2013).

95% DDA chitosan-PRP solidified immediately



Erythrocyte agglutination induced by chitosan?

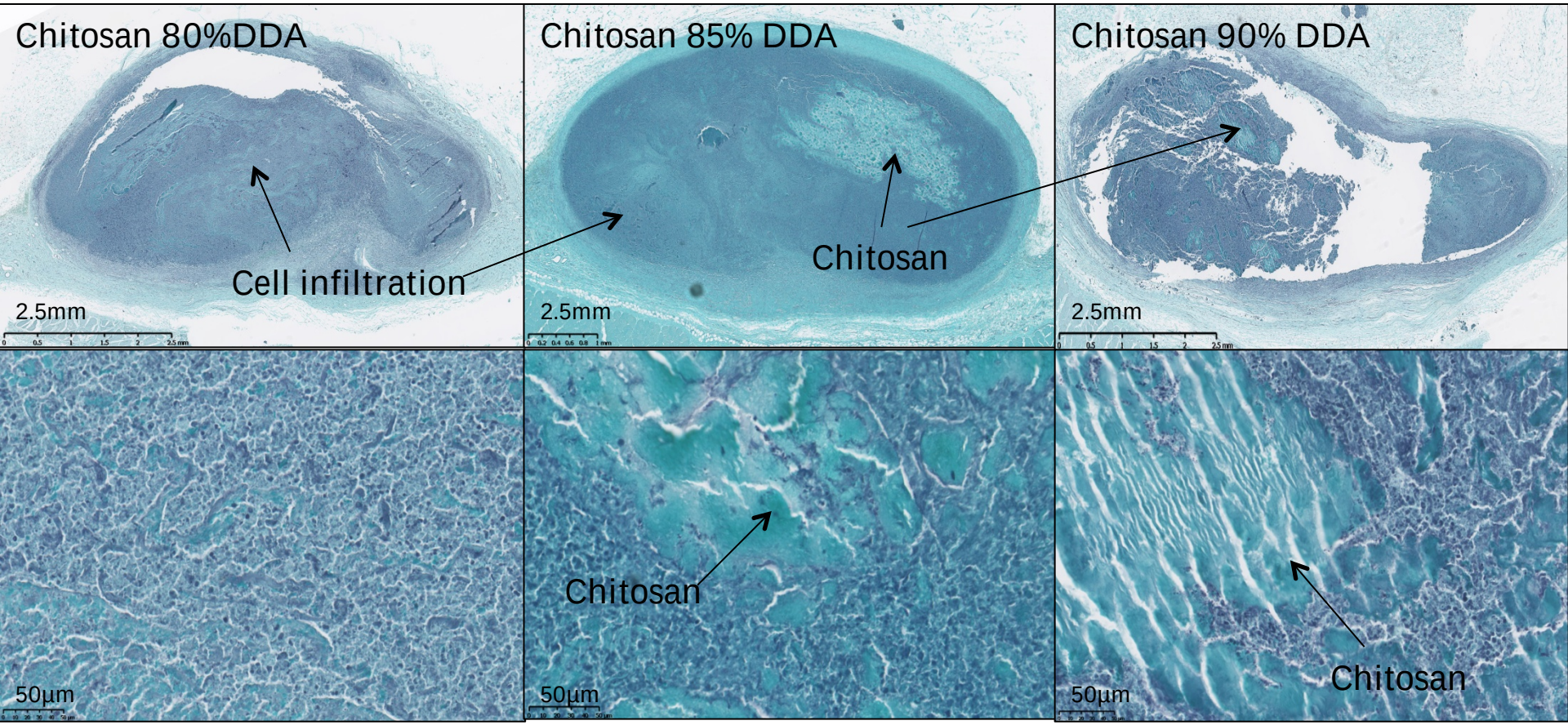
Cell infiltration is more uniform with chitosan of lower DDA at 2 weeks



Iron hematoxylin/Fast Green stained paraffin section of chitosan-PRP
Bottom pictures magnitude 40X

*PRP alone completely degraded at two weeks

Chitosan-PRP implants persist until 6 weeks in this model



Iron hematoxylin/Fast Green stained paraffin section of chitosan-PRP
Bottom pictures magnitude 40X

Conclusions of Objective 4

- Chitosan-PRP implants were resident until at least 6 weeks post-implantation → prolonged bioactivity *in vivo.*
- Invasion of the implants by host cells → influenced by the degree of deacetylation of the chitosan at early time points.
- Size of the implants decreased with time.

Overall Conclusions

- Platelet aggregation and clot retraction are inhibited in chitosan-PRP hybrid clots; platelets are activated.
- Residency of chitosan-PRP in vivo leads to increased biological activity in vivo compared to PRP alone.
- We are currently testing the efficacy of Chitosan-PRP in Rotator cuff tear repair in rabbit and sheep models.

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