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THE CO-INFUSION OF REHYDRATED, LYOPHILIZED PLATELETS WITH HBOC-201 FOR HEMOSTASIS IN DILUTIONAL THROMBOCYTOPENIA

ABSTRACT

Pilot studies were performed in pigs to ascertain if rehydrated, lyophilized (RL) platelets were capable of providing hemostasis after extensive exchange with Biopure hemoglobin-based oxygen carrier (HBOC) 201. Thrombocytopenia can occur as a result of the dilution of endogenous platelets that accompanies extensive replacement fluid infusion and is accompanied by a corresponding reduction in the concentration of coagulation factors. Dilutional (washout) thrombocytopenia can also be accentuated by the consumption of platelets and coagulation factors at wound sites and/or through disseminated intravascular coagulation. Dilutional thrombocytopenia can be particularly pronounced with the extensive exchange that can be achieved with HBOCs. RL platelets are being developed as a sterile infusion product for providing immediate hemostasis in coagulopathic conditions, including those related to hemorrhagic shock. Pigs were anesthetized, and then several sensors were placed to follow hemodynamic and vasoactive processes. Blood and HBOC-201 were respectively withdrawn and infused from contralateral femoral veins at rates of approximately 40 ml/min while maintaining hemodynamic parameters in the normal range. Red blood cell and platelet levels fell in an approximately exponential manner with rates of 5% per minute ($t_{1/2} = 20$ min). Bleeding times from ear vein lacerations and 23 gauge needle punch sites in the jugular vein were measured before exchange and during the exchange time course. Ear vein bleeding times increased during the exchange period from initial values of 2 to 3 minutes to >10 minutes at the 60 minute time point, when platelet and RBC levels were less than 15% of initial values. Similarly, jugular vein wound bleeding, which initially ceased in less than 2 minutes, was indefinite (uncontrollable hemorrhage) after RBC and platelet counts were lowered to less than 30% of initial values. Fluorescent-labeled porcine RL platelets, equivalent to 34% of initial platelet mass, were infused over a 5-minute period after 70 minutes of exchange when endogenous platelet levels were less than 15% of initial values. Uncontrolled hemorrhage from the jugular vein wounds ceased after the RL platelet infusion and bleeding times from ear vein lacerations shortened to more normal values between 5 to 7 minutes. Analysis of wound sites with fluorescent microscopy demonstrated that RL platelets adhered to damaged vascular tissue to form a platelet-rich, but fibrin-depleted plug. These results indicate that hemostasis can be obtained in dilutional thrombocytopenia when RL platelets are co-infused with HBOC-201.

INTRODUCTION

A widely recognized complication from extensive fluid replacement is the induction of coagulopathies related to the "washout" dilution of components of hemostatic systems [e.g., 1-6]. The coagulopathies can be related to the extent of dilution of platelets and coagulation factors [e.g., 6] and to the consumption of platelets and coagulation proteins in response to massive tissue injury and/or disseminated intravascular coagulation [6]. The generation of NO, PGI₂ and PGE₂, as well as multiple cycles of reversible activation during shock, can render platelets unresponsive. NO and inhibitory prostaglandin activates intracellular cyclases for the generation of cGMP and cAMP and then the activation of inhibitory protein kinase pathways. Multiple cycles of reversible activation, due to local and systemic elevation activating agents (e.g., epinephrine) and inflammatory mediators can result in platelets that are refractory to further activation through mechanisms that are similar to those operant in storage lesion [7].

Platelets do not exhibit native hemostatic function *in vivo* when RBC counts are reduced [e.g., 8]. Apparent platelet dysfunctions occur when RBCs do not rheologically "direct" platelets towards the endothelium [9,10], do not release ATP when damaged [11,12] and do not generate thromboxanes [9]. As HBOC-201 preparations come into wider use, the extensive degrees of exchange (e.g. < 1% initial RBC count) that can be achieved with acellular oxygen carriers are expected to result in more severe bleeding disorders, potentially resulting in spontaneous hemorrhage and neurological disorders.

We have discovered and refined a method for lyophilizing platelets for long-term storage that circumvents many limitations related to current methods of platelet storage [13,14]. Our method for preparing lyophilized platelets is based on the covalent cross-linking of surface membrane proteins and lipids to stabilize cellular structures during freezing, lyophilization and rehydration. We report here the results of two pilot studies which address the hypothesis that rehydrated, lyophilized (RL) platelets can provide hemostasis in hemorrhagic shock-related dilutional thrombocytopenia.

There are two bases for this hypothesis. First, RL platelets retain many aspects of native platelet function. RL platelets retain near-normal ultrastructure by electron microscopy and have many of the molecular functions of fresh platelets; glycoprotein IIb-IIIa and Ib-IX complexes respectively bind fibrinogen [15] and von Willebrand factor [16]. The cells also spread on foreign surfaces [14] and adhere to denuded subendothelium [14,17]. RL platelets are capable of a degree of intracellular stimulus response coupling; intracellular protein kinases such as protein kinase C and myosin light chain kinase are stimulated by platelet agonists [18]. The result of activation-dependent intracellular signaling is that *RL platelets provide positive feedback amplification of coagulation reactions*. RL platelets degranulate (for the secretion of coagulation factors and recruitment of additional platelets), generate thromboxanes and provide a procoagulative surface for the catalysis of prothrombin to thrombin conversion [15].

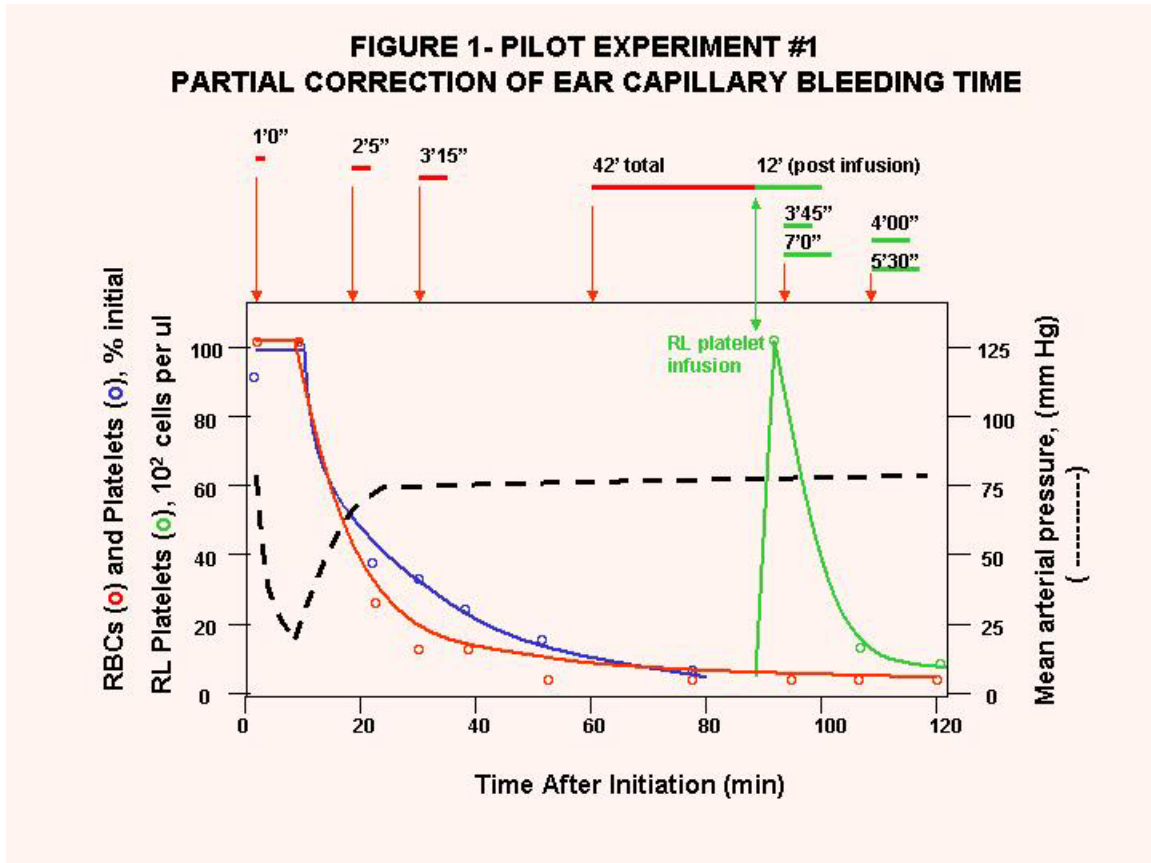
Secondly, RL platelets have been shown to provide immediate hemostasis in several animal model studies. The ability to provide immediate hemostasis is demonstrated in canine experiments in which endogenous platelets are rendered dysfunctional with extracorporealization during cardiopulmonary bypass; bleeding times from carotid artery punctures are restored to normal values immediately after infusion of the lyophilized cells [19]. In contrast, stored platelets require several hours in the circulation to recover from storage lesion and play a role in hemostasis [7, 20, 21]. RL platelets incorporate into sites of vascular injury *immediately* upon infusion [14], and are thus well suited for controlling hemorrhagic trauma.

METHODS

We have developed a porcine model system for investigating coagulopathies that are associated with hemorrhagic shock. This model is a refinement of a system established by Dr. Manning in collaboration with the Biopure Corporation for examining patho-physiological processes in shock [22, 23]. 40 to 50 kg mixed breed pigs are anesthetized with isoflurane and then several sensors are placed to follow hemodynamic and vasoactive processes: a pulmonary artery thermo dilution catheter is inserted via the external jugular vein into a pulmonary artery; micromanometer-tipped catheters are positioned via the left femoral vessels into the right atrium and thoracic aorta; a 22 gauge catheter is inserted into the left femoral artery and connected to a withdrawal pump; catheters are positioned via the left femoral vessels into the right atrium and thoracic aorta; a 5 Fr pigtail infusion catheter is inserted into the left ventricle.

The experiment is carried out in three stages. First, the experiment is initiated by withdrawal of blood from the left femoral artery. Blood is withdrawn over a 10 to 20 minute period until mean arterial pressure is reduced to between 20 to 25 mm Hg. At this point, the animal is tachycardic and in compensated shock. Secondly, mean arterial pressure is restored to normal values by infusing HBOC-201 to resuscitate the animal. Blood withdrawal and exchange with the HBOC-201 is continued for 60 to 80 minutes to further dilute endogenous red blood cells, platelets and coagulation cascade components. Finally, RL platelets are infused in 500 ml of saline over a 5-minute period. Several hematological endpoints were measured during the three stages of the experiment. The time of cessation of bleeding from a laceration of ear capillary beds (Merck saline bleed) and from a venipuncture of the external jugular vein (23g) were measured before and during the experiment. Red blood cell and endogenous platelet levels were measured before and during the experiment by withdrawing samples of blood and then performing differential cell counts. RL platelets were prepared with standard methods [14] and labeled with the fluorophore CMFDA for the flow cytometric quantification of infused cell levels as we have detailed elsewhere [14].

PILOT EXPERIMENT #1

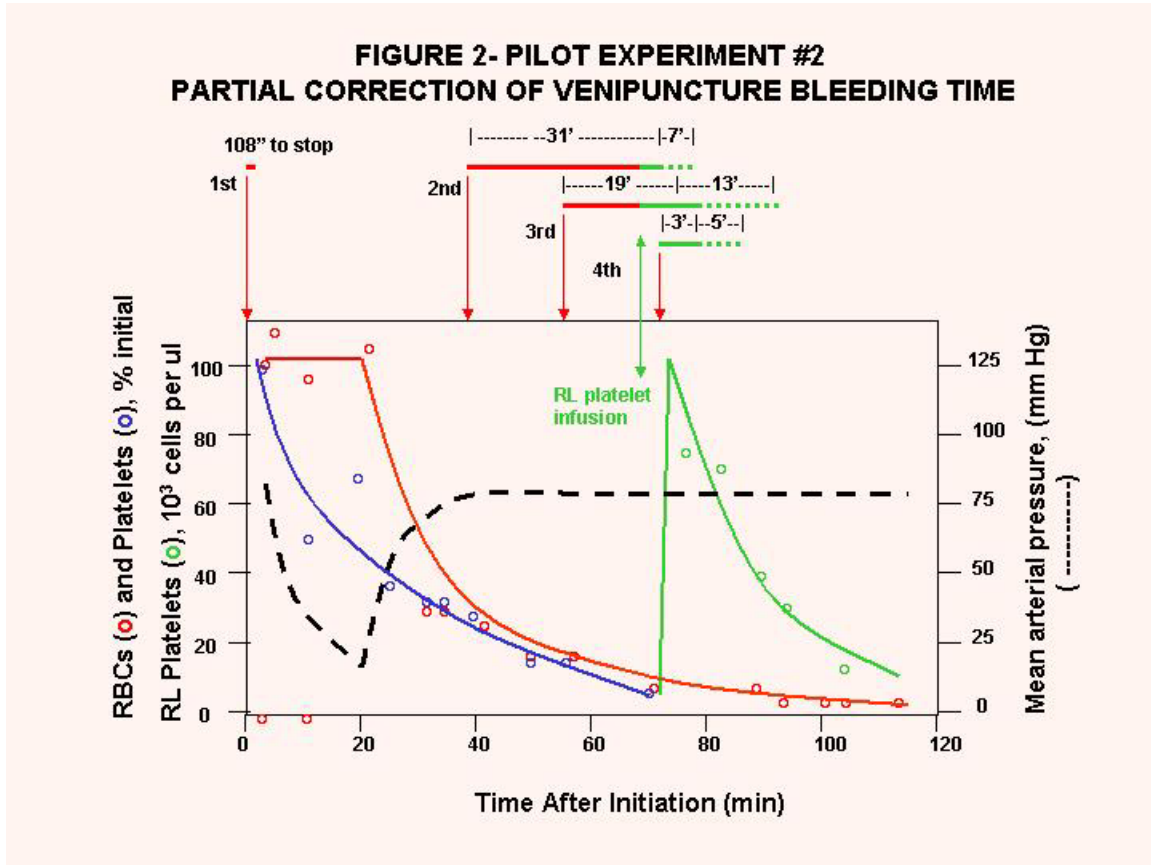


a) The blood withdrawal and subsequent resuscitation and exchange resulted in a reduction of red blood cell and platelet levels to under 10% of initial values at 90 minutes.

b) Ear bleeding times were indefinite after red blood cell and platelet levels were reduced to under ~20% of initial levels.

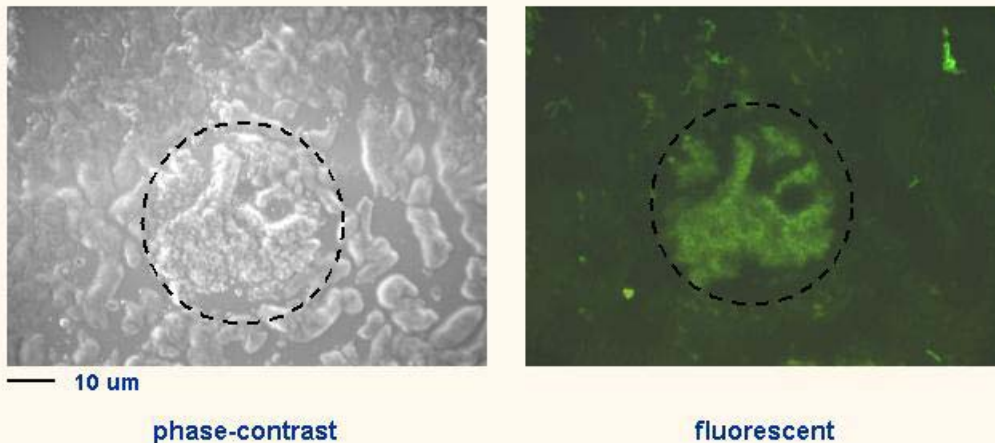
c) The infusion of RL platelets (equivalent to 100% of the endogenous platelet mass) provided partial hemostasis; capillary bleeding times were reduced from > 40 min to 3 - 7 min.

PILOT EXPERIMENT #2



- a) Red blood cell and platelet levels were reduced to under 10% of initial levels after blood withdraw and exchange at the 70 minute point.
- b) Hemorrhage from venipuncture sites were indefinite after red blood cell and platelet levels were reduced to under ~25% of initial levels.
- c) RL platelet infusion resulted in partial correction of the coagulopathy; the two hemorrhages that were ongoing at the time of infusion stopped, as did a third venipuncture after the infusion.
- d) The fluorescent-labeled RL platelets localized to the wound site as judged by fluorescent microscopy of the injured vessel.

RL PLATELETS IN WOUND SITE



CONCLUSIONS

The results reported here indicate that RL platelets can provide immediate hemostasis in washout thrombocytopenia. The ability of RL platelets to provide immediate hemostasis, in the results reported here as well as in the canine bypass thrombasthenia model, is related to two specific properties of the cell's surface membrane. First, the GPIIb/IIIa complex is prepared in a partially activated state with bound fibrinogen [15]. Secondly, phosphatidylserine is surface exposed for catalysis of thrombin formation. RL platelets are thus insensitive to many of the mechanisms that inhibit activation responses of endogenous platelets in shock. The partial activation of RL platelets distinguishes them from stored and cryopreserved platelets that are refractory to activation. Despite being primed for immediate hemostatic function, RL platelets have not proven hyper-thrombotic in animal model studies [19].

The ability of RL platelets to provide hemostasis with highly diluted coagulation factors might also be due to the ability of these cells to undergo stimulus response coupling for activation. RL platelets contrast with other platelet substitutes that are under development (see Blajchman [25] for a recent review) that provide an approximately static surface. Fibrinogen-coated albumin microcapsules [26], fibrinogen-coated erythrocytes [27] and RGD-modified RBCs [28] do not have mechanisms for increasing the surface density of the glycoprotein IIb-IIIa ligands

(fibrinogen or RGD) in an activation dependent manner. Also, it is not clear if infusible platelet membranes (derived from outdated heat-inactivated, sonicated platelets [29] undergo stimulus-response coupling.

RL platelets were found to only partially restore hemostasis and to yield clots that were judged qualitatively as having less than native structural integrity. Future research is focusing on co-lyophilizing RL platelets with coagulation factors to resolve this issue.

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REFERENCES

1. Sheldon, G., Lim, R. and Blaisdell, F. (1975) The use of fresh blood in the treatment of critically injured patients. *J. Trauma-Injury Infect. Crit. Care.* 15, 670-677.
2. Collins, J. (1975) Problems associated with the massive transfusion of stored blood. *Surgery* 75, 274-295.
3. Beck, E. (1975) Unexpected hemorrhage in surgery. *Schweiz. Mediz. Woch.* 105, 775-777.
4. Hehne, H., Nyman, D., Burri, H. and Wolff, G. (1976) Management of bleeding disorders in traumatic-haemorrhagic shock states with deep frozen fresh plasma. *Eur. J. Intensive Care Med.* 2, 157-161.
5. Wenz, B. (1993) Massive blood transfusion: the blood bank perspective. *Transfusion Sci.* 14, 353-359.
6. Lucas, C., Ledgerwood, A., Saxe, J., Bombi, G. and Lucas, W. (1996) Plasma Supplementation is beneficial for coagulation during hemorrhagic shock. *Am. J. Surg.* 171, 399-404.
7. Bode, A. (1990) Platelet activation may explain the storage lesion in platelet concentrates. *Blood Cells* 16, 109-126.
8. Valeri, C., Crowley, J. and Loscalzo, J. (1998) The red cell transfusion trigger: Has a sin of commission now become a sin of omission? *Transfusion* 38, 602-608.
9. Turitto, V. and Weiss, H. (1980) Red blood cells: their dual role in thrombosis formation. *Sci.* 207, 541-543.
10. Joist, J., Bauman, J. and Suter, S. (1998) Platelet adhesion and aggregation in pulsatile shear flows: effects of red blood cells. *Thromb. Res.* 92, S47-S52.
11. Alkhamis, T., Beissinger, R. and Chediak, J. (1988) red blood cell effect on platelet adhesion and aggregation in low shear flow. *Myth or fact? Transplantation* 34, 868-873.

12. Bell, D., Spain, S., and Goldsmith, H. (1990) The effect of red blood cells on the ADP-induced aggregation of human platelets in flow through tubes. *Thromb. and Haemo.* 63, 112-121.
13. Read M.S., Reddick R.L., Nichols T.C., Bellinger D.A., Bode A.P., Taylor K.K., Brinkhous K.M., and Griggs T.R. (1993) Transfused rehydrated platelets support hemostasis and thrombosis. *Blood* 82 (Suppl): 159a.
14. Read M.S., Reddick R.L., Bode A.P., Bellinger D.A., Nichols T.C., Taylor K.K., Smith S.V., McMahon D.K., Griggs T.R., and Brinkhous K.M. (1995) Preservation of hemostatic and structural properties of rehydrated lyophilized platelets: Potential for long-term storage of dried platelets for transfusion. *Proc. Natl. Acad. Sci.* 92: 397-401.
15. Fischer, T., Khandelwal, G., Merricks, S., Raymer, R., Bode, A., Bellinger, D., Russell, K., Reddick, R., Sanders, W., Nichols, T. and Read, M. (2000b) Thrombus Formation and Lysis with Rehydrated, Lyophilized Platelets. *Vox Sang.* (Submitted).
16. Khandelwal, G., Sanders, W., Bode, A., Nichols, T., Erickson, G. and Read, M. (1997) von Willebrand factor binding to rehydrated lyophilized platelet surface GP1b and inhibition by monoclonal antibody to GP1b. *FASEB J.* 11, 1812.
17. Bode A.P., Read M.S., and Reddick R.L. (1999) Activation and adherence of lyophilized platelets on canine vessel strips in the Baumgartner perfusion chamber. *J. Lab. Clin. Med.* 133: 200-211.
18. Fischer, T., Merricks, E., Russell, K., Raymer, R., White, G., Bode, A., Nichols, T. and Read, M. (2000) Intracellular Signalling in Rehydrated, Lyophilized Platelets. *Brit. J. Haem.* 111, 167-175.
19. Bode, A. and Read, M. (2000) Lyophilized platelets: Continued development. *Transfusion Science* 22, 99-105.
20. Simon, T., Akl, B. and Murphy, W. (1984) Controlled trial of routine administration of platelet concentrates in cardiopulmonary bypass surgery. *Ann. Thor. Surg.* 37, 359-364.
21. Owen, M., Holme, S., Heaton, S., Sawyer, S. and Cardinali, S. (1992) Post-transfusion recovery of function of 5-day stored platelet concentrates. *Brit. J. Haem.* 80, 539-544.
22. Katz, L., Manning, J., Pearce, B., Wang, Y., Rockoff, S., Brown, C. and Keady, M. (2001) A model of severe hemorrhage and liver injury in swine. *Academic Emergency Medicine* 8, 535.
23. Katz, L., Manning, J., Pearce, B., Wang, Y., Rockoff, S., Brown, C. and Keady, M. (2001) Resuscitation with HBOC-201 allows 96 hour survival after severe hemorrhagic shock. *Academic Emergency Medicine* 8, 534-535.
24. Fischer, T., Merricks, E., Bellinger, D., Hayes, P. Smith, R., Raymer, R., Read, M., Nichols, T. and Bode, A. (2001) Splenic clearance mechanisms of rehydrated, lyophilized platelets. *Art. Cell. Blood Subs. Imm. Biotech.* (in press).

25. Blajchman, M. (2000) Platelet substitutes. *Vox Sang.* 78, 183-186.
26. Levi, M., Friederich, P., Middleton, S., de Groot, P., Wu, Y., Harris, R., Biemond, B., Heijnen, H., Levin, J. and Cate, J. (1999) Fibrinogen-coated albumin microcapsules reduce bleeding in severely thrombocytopenic rabbits. *Nature Med.* 5, 107-111.
27. Agam, G. and Livne, A. (1991) Erythrocytes with covalently bound fibrinogen as a cellular replacement for the treatment of thrombocytopenia. *Eur. J. Clin. Invest.* 22, 105-112.
28. Coller, B., Springer, K., Muthusamy, M., Scudder, L., Narla, M. and Beer, J. (1991) Further invitro characterization of thromboerythrocytes (TE), a potential autologous, semiartificial platelet substitute. *Throm. and Haem.* 65, 755.
29. Chao, F., Kim, B., Houranieh, A., Liang, F., Konrad, M., Swicher, S. and Tullis, J. (1996) Infusible platelet membrane microvesicles; a potential transfusion substitute for platelets. *Transfusion* 36, 536-542.