DRIED PLATELETS IN A SWINE MODEL OF LIVER INJURY

Kenji Inaba,* Galinos Barmparas,* Peter Rheë,† Bernardino C. Branco,‡
Michael Fitzpatrick,§ Obi T. Okoye,* and Demetrios Demetriades*

*Division of Trauma and Surgical Critical Care, University of Southern California, Los Angeles, California;†Division of Trauma and Surgical Critical Care and ‡Department of Surgery, University of Arizona, Tucson, Arizona; and §Cellphire Inc, Rockville, Maryland

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ABSTRACT—Introduction: Lyophilization may facilitate production of a safe, portable, easily storable, and transportable source of platelets for bleeding patients. The objective of this study was to examine the impact of lyophilized human and porcine platelets in a swine liver injury model of nonsurgical hemorrhage. Methods: Anesthetized pigs (40 kg) had a controlled 35% total blood volume bleed from the right jugular vein followed by cooling to 35°C and resuscitation with Ringer’s lactate to achieve a 3:1 blood withdrawal resuscitation. Through a midline laparotomy, the liver was injured with two standardized 5 × 5-cm grids with lacerations 1 cm apart and 0.5 cm deep. After 2 min of uncontrolled hemorrhage, the animals were treated with placebo (n = 5), lyophilized human (n = 5, HP), or swine platelets (n = 5, SP). At 15 min, shed blood was calculated. The animals then underwent abdominal closure. At 48 h, the animals were killed for histopathologic evaluation of the lung, kidney, and heart.

Results: Intraoperative blood loss at 15 min was significantly higher in the HP arm (SP: 4.9 ± 2.9 mL/kg, HP: 12.3 ± 4.7 mL/kg, and control: 6.1 ± 2.5 mL/kg; P = 0.013). Mortality at 48 h was 20% in all three arms, due to uncontrolled intra-abdominal bleeding. At the time the animals were killed, SP animals had a significantly higher hematocrit (SP: 22.0% ± 3.0%, HP: 15.1% ± 4.9%, and control: 13.9% ± 0.6%; P = 0.026). No significant difference was found in platelet count (SP: 319.3 ± 62.1 × 10^3/μL, HP: 361.5 ± 133.6 × 10^3/μL, and control: 242.7 ± 42.5 × 10^3/μL; P = 0.259). Histopathology of kidneys, lungs, and heart demonstrated no evidence of thromboembolic complications.

Conclusion: In this swine model of liver injury, human lyophilized platelets increased intraoperative blood loss. With the use of species-specific lyophilized platelets, however, this effect was abolished, with a decrease in blood loss at 48 h after injury.

KEYWORDS—Dried platelets, lyophilization, swine model of liver injury, hemorrhage, outcomes

INTRODUCTION

Hemorrhage remains the leading cause of preventable death after injury (1–3). As a direct consequence, strategies targeting hemorrhage control, both locally and systemically, are high-priority research areas in mitigating this potential loss of life (4–6). Over the last decade, different approaches have attempted to address the systemic coagulation defects, seen in upward of a quarter of critically injured patients at admission (7). Address the systemic coagulation defects, seen in upward of a quarter of critically injured patients at admission (7).

Over the last several decades (16, 17), the application of lyophilization techniques to platelets has also been pursued, resulting in a shelf stable product that can be administered systemically. When compared with liquid-stored platelets, there are numerous potential benefits including immediate availability, portability, rapid infusion, targeted procoagulant activity at the site of tissue and vessel disruption, and the elimination of transfusible disease. The lyophilized platelet preparation is structurally intact with retained functional procoagulant properties upon rehydration. Both von Willebrand factor (vWF)–mediated adhesion and surface thrombin generation have been demonstrated in both in vitro and in vivo testing to remain intact (20). In addition to cell surface interactions, intracellular functions such as the regulation of intracellular pH have been demonstrated to be retained in rehydrated lyophilized platelets developed by Tang et al. (21). In a recent study by Hawksworth et al. (22), infusion of rehydrated lyophilized human platelets in a swine model of grade III liver injury showed improved survival and decreased blood loss.

The objective of this study was to compare the impact of infusion of rehydrated lyophilized human and porcine platelets...
in a less severe swine liver hemorrhagic injury model (damage control swine liver injury model of nonsurgical bleeding).

METHODS

This is a randomized controlled animal trial that was conducted within the facilities of the Department of Animal Resources of the University of Southern California after approval by the local Institutional Animal Care and Use Committee. All animals were cared for according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Female Yorkshire-Hampshire swine (n = 15) weighing approximately 40 kg were purchased from IPFS Inc (Norco, Calif) and were housed in quarantine for 7 days before the experiment. Food was withheld the night before operation with free access to water.

On the day of the experiment, the animals were premedicated with an intramuscular injection of tiletamine/zolazepam (4 mg/kg each) and xylazine (300 mg), followed by 0.01 mg/kg of glycopyrrolate. A 20-gauge catheter was placed in the marginal ear vein for peripheral venous access. The animals were then intubated using a 7F endotracheal tube. Anesthesia was maintained with sevoflurane 1% to 5% in 100% oxygen (DragerNarkomed 4 Anesthesia System; Drager Medical, Inc, Telford, Pa). An esophageal thermistor probe was utilized for continuous core body temperature monitoring.

The right carotid artery was cannulated with a 20-gauge angiocatheter and used for continuous invasive arterial blood pressure monitoring and blood draws. The right external jugular vein was cannulated with a 9F introducer sheath for administration of resuscitative fluids.

Each animal underwent a standardized 35% blood volume (25 mL/kg, based on a 7% of body weight blood volume) withdrawal through the external jugular vein sheath. Following the blood withdrawal, the animals were resuscitated with room temperature lactated Ringer's solution (LRS) to achieve a standard 3:1 fluid-to-blood withdrawal resuscitation.

The lyophilized platelets were tested in a previously developed swine model of nonsurgical liver injury (23) designed to replicate a nonsurgical liver hemorrhagic injury with systemic abnormalities due to blood loss, hypothermia, and crystalloid dilution, similar to many injuries seen in clinical practice. Briefly, all animals underwent a midline celiotomy, and two frozen 500-mL LRS bags were placed in the marginal ear vein for peripheral venous access. The animals were then intubated with room temperature lactated Ringer's solution (LRS) to achieve a standard 3:1 fluid-to-blood withdrawal resuscitation.

After 15 min of bleeding, all free intraperitoneal blood was collected with preweighed gauze. The abdomen was then closed in two layers after infiltrating the incision with 12 mL of 0.5% bupivacaine. At the conclusion of the experiment, the animals were resuscitated with warm LRS to maintain a minimum invasive mean arterial pressure (iMAP) of 60 mmHg.

At 48 h after initial operation, animals were killed. Euthanasia was induced with an overdose of 120 mg/kg sodium pentobarbital administered intraveously. The left kidney, left lung, and left anterior descending coronary artery were sent for pathological evaluation. Each specimen was examined by a veterinary pathologist, blinded to the treatment arm for any evidence of thrombus or emboli.

Blood samples were obtained at four different time points: at baseline, after blood withdrawal and induction of hypothermia, at closure of the abdomen, and at the time the animals were killed at 48 h. Arterial and venous blood samples were sent for analysis to the USC Clinical Reference Laboratory. Blood assays included hematocrit (Hct), platelet count, pH, lactate, base excess, and prothrombin time (PT). In addition, an ACT-10 Hematology Blood Analyzer (Beckman Coulter Inc, Fullerton, Calif) was used to measure the real-time platelet count.

The primary outcome measure of the experiment was the safety of dried platelets. Secondary outcomes included shed intraperitoneal blood at 15 min after treatment during the initial procedure, 48-h survival, hemoglobin and platelet count at the time the animals were killed, and evidence of thromboembolic complications in the kidney, lungs, or heart. Specimens were sent to the laboratory fixed in formaldehyde as per laboratory protocol and on average; each organ was subjected to a minimum of 10 sections. For animals dying before the end of the 48-h study period, the causes of death were also documented.

Standard statistical analysis was performed using the Statistical Package for Social Sciences version 18 (SPSS Inc, Chicago, Ill) for Mac. Values are reported as means ± SEM. Proportions were compared using the Fisher exact test, and means were compared using the nonparametric Wilcoxon rank test. One-way analysis of variance (ANOVA) was used to compare proportions and means between the three different arms. Bonferroni adjustments were used for post hoc analyses.

RESULTS

A total of 15 pigs were included in the study (five SP, five HP, and five controls). The average weight of the animals was 40.1 ± 3.0 kg, and the average blood withdrawn for hemodilution was 1,002 ± 74.7 mL per animal. The average volume of crystalloid given ranged from 2,775 to 3,225 mL. The hemodilution resulted in a significant reduction of iMAP (from 86.3 ± 15.8 mmHg to 70.0 ± 14.4 mmHg; P < 0.001) and core body temperature (from 36.5°C ± 0.9°C to 34.4°C ± 0.3°C; P < 0.001). Figure 2

<table>
<thead>
<tr>
<th>TABLE 1. Hematocrit and platelet count at different phases of the experiment, stratified by treatment arm</th>
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<td>Hct, %</td>
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<td>End of blood draw</td>
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<td>Closure</td>
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<td>PLT, ×10^9/L</td>
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<td>Baseline</td>
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<td>End of blood draw</td>
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<td>PT, s</td>
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<td>Closure</td>
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</table>

P values were derived from one-way ANOVA. Values are reported as mean ± SD. HP indicates human platelet; PLT, platelet; SP, swine platelet.
Lactate, mmol/L

Count (from 369.7 to 0.7 was noted in arterial pH (from 7.49 to 0.001). No significant increase in the PT was noted (from 11.6 to 0.6 mmol/L, HP: 12.3 \pm 4.7 mL/kg, and control: 6.1 \pm 2.5 mL/kg; P = 0.013) (Fig. 4). Post hoc analysis did not show a significant difference between the SP and control arms (P = 1.000).

After injury, the iMAP continued to drop in all arms. The lowest recorded iMAP after injury was in the SP arm (SP: 40.8 \pm 8.2 mmHg, HP: 43.0 \pm 10.1 mmHg, and control: 61.3 \pm 12.5 mmHg; P = 0.024) (Fig. 2). There was also a decrease in the pH, Hct, and platelet count after injury (Tables 1 and 2).

A total of three animals (Table 3) died before 48 h (one in each arm), all due to uncontrolled intra-abdominal bleeding. At the time the animals were killed, SP animals had a significantly higher Hct than the HP or control arms (SP: 22.0 \pm 3.0%, HP: 15.1 \pm 4.1%, and control: 13.9 \pm 0.6%; P = 0.026). No significant difference was found in platelet count (SP: 213.3 \pm 166.8 \times 10^{3} \muL, HP: 252.6 \pm 63.5 \times 10^{3} \muL, and control: 261.3 \pm 59.7 \times 10^{3} \muL; P = 0.259). Histopathology of kidneys, lungs, and coronaries demonstrated no evidence of thrombi or emboli to distant organs (Table 3).

**DISCUSSION**

Platelets are an essential component of the balanced resuscitation of injured patients who are bleeding (11, 12, 26). Traditional banked platelets have logistic limitations that include a rigorous set of storage conditions, short half-life, the propensity to develop infectious complications, and the potential for graft-versus-host interactions. For the civilian sector, maintaining an adequate supply, especially at high-volume centers, can usually be accomplished with minimal wastage. For less well-developed blood banking systems, in developing countries without a stable regional supply, or for the military, the current system for platelet storage and dispensing is not ideal. A lyophilized product would offer numerous logistic advantages including ease of storage

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**TABLE 2. Arterial blood gas values stratified by arm and phase of the experiment**

<table>
<thead>
<tr>
<th></th>
<th>SP (n = 5)</th>
<th>HP (n = 5)</th>
<th>Control (n = 5)</th>
<th>P</th>
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</thead>
<tbody>
<tr>
<td><strong>pH</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>7.473 \pm 0.037</td>
<td>7.494 \pm 0.040</td>
<td>7.509 \pm 0.031</td>
<td>0.297</td>
</tr>
<tr>
<td>End of blood draw</td>
<td>7.446 \pm 0.026</td>
<td>7.464 \pm 0.049</td>
<td>7.501 \pm 0.032</td>
<td>0.100</td>
</tr>
<tr>
<td>Closure</td>
<td>7.397 \pm 0.029</td>
<td>7.416 \pm 0.016</td>
<td>7.450 \pm 0.031</td>
<td>0.025*</td>
</tr>
<tr>
<td><strong>Lactate, mmol/L</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>1.3 \pm 0.3</td>
<td>1.2 \pm 0.3</td>
<td>1.3 \pm 0.6</td>
<td>0.948</td>
</tr>
<tr>
<td>End of blood draw</td>
<td>3.2 \pm 0.8</td>
<td>3.0 \pm 0.6</td>
<td>2.9 \pm 0.5</td>
<td>0.759</td>
</tr>
<tr>
<td>Closure</td>
<td>4.5 \pm 1.3</td>
<td>4.2 \pm 1.2</td>
<td>4.0 \pm 1.0</td>
<td>0.755</td>
</tr>
<tr>
<td><strong>Base excess, mEq/L</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>10.6 \pm 3.5</td>
<td>7.8 \pm 2.9</td>
<td>8.4 \pm 1.9</td>
<td>0.291</td>
</tr>
<tr>
<td>End of blood draw</td>
<td>6.8 \pm 1.3</td>
<td>4.2 \pm 2.1</td>
<td>4.8 \pm 1.8</td>
<td>0.085</td>
</tr>
<tr>
<td>Closure</td>
<td>4.0 \pm 3.4</td>
<td>2.2 \pm 1.6</td>
<td>2.6 \pm 3.2</td>
<td>0.592</td>
</tr>
</tbody>
</table>

P values were derived from one-way ANOVA. Values are reported as mean \pm SD.

HP indicates human platelet; SP, swine platelet.

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**FIG. 2. Invasive MAP at selected time points during the experiment according to study arm.**

The p-values were derived from analysis of variance. *p-values < 0.05 are statistically significant.

SEM, standard error of the mean.
and facilitated transportation. Especially for the combat care setting, platelets that are shelf stable in a wide range of ambient temperatures, easily portable, mixable, and injectable would allow much greater flexibility in where and by whom platelets could be administered. The potential for postprocessing manipulation with possible additives, viral inactivation, and ABO universality also exists.

For plasma, the concept of freeze drying dates back to the World War II era where lyophilized plasma was in widespread use. This was halted, however, because of the use of pooled donor plasma where viral contamination (hepatitis B and C) spread quickly through large batches of the product. Recently, however, there has been a resurgence of interest in lyophilized plasma with the German Red Cross fielding a product called LyoPlas (German Red Cross Blood Transfusion Service West). In Thailand and in South Africa, there has also been limited use for hemophiliac and burn patients, respectively. Although no US Food and Drug Administration-approved product is available, several recent preclinical studies have validated both the in vitro factor activity and the ability of this liquid plasma substitute to correct coagulation profile abnormalities in vivo (18, 19).

The concept of lyophilization for platelets is also not new, dating back to the 1950s (16, 17). With advances in lyophilization technology, retention of the structural integrity of the native platelet can be expected in addition to both in vitro and in vivo demonstration of intact procoagulant function. Both vWF-mediated adhesion and the ability to generate thrombin remain functional. The proposed mechanism by which lyophilized platelets act includes four functions: adhesion to the subendothelial collagen, recruitment of endogenous platelets, assembly of tenase complex with subsequent local thrombin generation, and platelet mediated fibrin clot formation for wound closure.

Recently, there have been several preclinical evaluations of lyophilized platelets performed in a variety of models. The use of lyophilized human platelets in a swine model of grade III liver injury with uncontrolled bleeding (22) demonstrated an improvement in mortality from 20% to 80% and a decrease in blood loss throughout the experimental run. In a rabbit model of thrombocytopenia (27), lyophilized platelets were able to decrease bleeding time. Similarly, in a canine model of cardiopulmonary bypass (28), bleeding time was decreased when compared with control (3 min 10 s vs. 6 min 59 s; \( P = 0.01 \)).
In our swine model of nonsurgical bleeding, the administration of porcine lyophilized platelets did not affect short-term blood loss or mortality. However, there was a significant improvement in the Hct measured at the 48-h take-back, due in part to a decrease in the postoperative bleeding in these animals. In those animals that received human platelets, however, there was an increase in blood loss seen intraoperatively. It is hypothesized that this may have been due to high-affinity nonphysiological interaction of porcine vWF with human glycoprotein Ib (GPIb) in the absence of high shear, resulting in the activation of human platelets leading to aggregation and subsequent thrombocytopenia (29). This can then lead to an increase in activated GPIb/IIIa receptors on the platelet membrane. Binding of porcine vWF or fibrinogen with GPIIb/IIIa receptors then amplifies the coagulation cascade (30). A study utilizing experimental baboons (31) undergoing orthotopic pulmonary xenotransplantation was designed to examine the interaction between porcine vWF and human platelets as a cause of xenograft-associated disseminated intravascular coagulation. Blocking the porcine vWF-GPIb interaction prevented in vitro agglutination of human and baboon platelets. The use of an anti-GPIb monoclonal antibody also prevented platelet deposition in vivo. Interestingly, the aggregation of human platelets can be triggered by swine vWF in the absence of an exogenously added agonist and high shear (32, 33). Swine plasma has been reported to induce human platelet aggregation resulting in severe thrombosis after xenotransplantation. With the infusion of lyophilized porcine platelets, however, a similar increase in bleeding tendency was not seen. Compared with control, the intraoperative blood loss was unchanged with an improvement in the hemoglobin at 48 h, likely due to decreased blood loss in the time period between the damage control closure and take-back surgery. However, in an earlier study by Hawkworth et al. (22), human platelets were utilized in a swine model with an improvement in blood loss parameters and significantly reduced mortality. This difference in response may have been due to critical differences in manufacturing process and inclusion of cryoprotectants and bulking agents during lyophilization or may have been due to the inherent differences between models and infusion volumes. In that study (22), platelets were washed and fixed with low amounts of paraformaldehyde before the addition of bulking agent (5% human serum albumin) and lyophilization as compared with no washing, and fixation steps were involved in the preparation of platelets for the present study, and lyophilization was performed in the presence of a cryoprotectant and different bulking agent (24, 25). In addition, a short (360 min) grade III liver injury was utilized without hypothermia or hemodilution, mimicking a prehospital infusion of platelets, with blood losses that were larger than the nonsurgical blood loss tested in our model (48 h). It was also noted in their analysis that even at these doses that are orders of magnitude lower than those used for fresh platelets, significant thrombotic complications can occur. When they utilized a dose of 6.25 × 10^10, all of the experimental animals died of thrombotic complications. Even at their therapeutic dose of 4.17 × 10^9, endocardial and pulmonary artery thrombi were detected. In contrast, the dose of 8 × 10^10 particles of SP or HP administered in this study did not induce thrombi or emboli. These safety data suggest that the different manufacturing method used for the SP and HP may have a safety profile, which will allow exploration of the efficacy of higher doses without inducing thrombi.

The small numbers and lack of dose modulation are a limitation of this study. The optimal dosing schedule for this model is not known, and the results may simply reflect an inadequate volume of the lyophilized platelets. One of the key findings of this study is the negative interaction between human platelets and the swine model. This has implications for further preclinical work where it will be important that any such interactions be minimized. As the final product will be human platelet based, despite the advantages of a swine model, non-human primate or canine models may be required for any further preclinical evaluation. In addition, banked platelets were unavailable for comparison as a separate treatment arm. They are currently the standard of care for critically ill trauma patients requiring platelet replacement and therefore the criteria on standard for comparison for future studies looking specifically at the performance of lyophilized platelets.

In this study, the use of human lyophilized platelets in a swine model of nonsurgical bleeding resulted in an increase in blood loss when compared with control. With the use of species-specific platelets, however, this effect was abolished, with a decrease in blood loss at 48 h after damage control packing.

### REFERENCES


