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Blood-Banking Techniques for Plateletpheresis in Swine

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During the past several years, trauma resuscitation in human patients has evolved from decreased use of crystalloids to increased use of blood products. Of high interest is the role of platelets in trauma resuscitation. Because conducting prehospital resuscitation in human trauma patients is very difficult, swine are often the animal model of choice for such studies because their coagulation and hemodynamic systems are similar to those in humans. However, consistent production of sufficient swine platelets for such studies has not previously been achieved. We developed a method for producing swine platelets by using standard human techniques and equipment. We assessed pH, pO_2, pCO_2, lactate, thromboelastography, and platelet aggregation over 5 d of storage to determine whether the swine platelet product met the American Association of Blood Banks (AABB) standards for transfusion. Swine platelets met AABB standards at 24 h but not at later time points. In addition, we fluorescently labeled nonautologous platelets and then measured their percentage recovery over 5 h (the time used in subsequent experimental studies) when transfused into a recipient pig. We showed that 80% of the platelets stored for 24 h remained in the circulation and increased the recipient pigs' thromboelastographic responses, indicating that the platelets were viable and active. Therefore, swine platelets stored for 24 h by using standard human products met the AABB criteria and were functional.

Abbreviations: AABB, American Association of Blood Banks; MTAB, modified Tyrode albumin buffer; PRP, platelet-rich plasma.

Pigs have coagulation and hemodynamic systems similar to those of humans,[30,39] with the caveat that pigs are hypercoagulable [21,25,41] and more resistant to fibrinolysis than humans.[12,20,25] Pigs are used extensively to model hemorrhagic shock and responses to various resuscitation strategies.[9,13,15,22,23] Blood products including fresh frozen plasma, packed RBC, platelets, and cryoprecipitate are used in Damage Control Resuscitation protocols to treat severely injured trauma patients.[6,10,29] Research of the efficacy of prehospital resuscitation treatments using human trauma patients is very difficult to conduct due to the difficulty to get informed consent.[33] In addition, it is not possible to measure the effect on blood loss because of inherent difficulty to estimate blood loss in trauma patients. It is of interest to develop swine models of noncompressible torso hemorrhage with which to optimize hemostatic resuscitation methods.[1,36] We therefore developed and validated swine blood banking techniques that meet standards comparable to the AABB standards[19] established for human components.

For the development of swine blood products, it is desirable to use the same commercially available blood component collection procedures and preservatives that are used in humans, but there are some challenges. Adult swine RBC do not use glucose as a major metabolic substrate and platelets may not use glucose, because swine RBC lose the membrane glucose transporter shortly after birth.[32,42,44] Therefore, commercially available preservatives with dextrose as the energy substrate likely will not support swine RBC for the storage times considered standard for human platelets. For example, human RBC in standard citrate–phosphate–dextrose solution (that is, CPD-AS3) are approved for storage for a maximum of 42 d at 4 °C and for 5 d at 22 °C.[19]

We therefore conducted these validation procedures to ascertain whether porcine platelets collected using standard human plateletpheresis methods could meet AABB standards at any time during their storage. In addition, we conducted functional tests for the platelets. We measured the clot strength (thromboelastogram) of platelet-rich plasma (PRP) and its aggregation to collagen as well as the percentage survival of fluorescently labeled platelets in the circulation for our period of interest (5 h). We found that swine platelets stored for 24 h by using standard human storage products met the AABB criteria (that is, units contain greater than or equal to 3×10^{11} platelets and have a pH greater than or equal to 6.2),[19] were functional and remained circulating for the duration of our 5-h experimental period.

Materials and Methods

All of the protocols were conducted in a facility accredited by AAALAC. Each study was approved by the IACUC of the US Army Institute of Surgical Research (Joint Base San Antonio, Fort Sam Houston, TX) and was conducted in compliance with the Animal Welfare Act, applicable Animal Welfare Regulations, and the principles of the *Guide for the Care and Use of Laboratory Animals*.[2,3,18]

Animal procedures. Three validation procedures were performed in this study: 1) development of the plateletpheresis technique in swine; 2) measurement of platelet stability during 5 d of storage; and 3) measurement of the percentage recovery of labeled plateletpheresis platelets infused into normal swine. A total of 14 pigs were used in this study. For the first procedure, plateletpheresis was performed on a total of 9 pigs (*Sus scrofa domestica*; Yorkshire–Duroc crossbred; weight, 71 ± 5 kg;

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Midwest Research Swine, Gibbon, MN). These 9 pigs were used as platelet donors for 1 to 6 times each. For those pigs used more than once, data are reported as the average for each animal (for example, Table 1). For 4 of these 9 pigs, the platelets were used in the second procedure; those data are reported in Tables 2 and 3 and Figures 1 through 4. In addition, 2 pigs (weight, 65 ± 3 kg) were platelet donors for 3 recipients; their data are shown in Figure 5. An additional 3 pigs (weight, 55 ± 2 kg) were used as recipients in the third procedure, and those data are reported in Figures 6 and 7.

Anesthesia. Pigs were food-fasted for 12 to 18 h before the procedure; water was available ad libitum. On the day of the procedure, the pigs were injected with glycopyrrolate (0.01 mg/kg IM; Robinul, Baxter Healthcare, Deerfield, IL) and tiletamine–zolazepam (8 mg/kg IM; Telazol, Wyeth, Fort Dodge, IA) for control of saliva secretion and for sedation, respectively. Anesthesia was induced via a facemask by using approximately 5% isofluorane (Forane, Baxter Healthcare) in 100% oxygen. The pigs then were intubated with a cuffed endotracheal tube (7.5 mm; Rusch, Teleflex Medical, Research Triangle Park, NC). During the procedure, anesthesia was maintained with 1% to 3% isofluorane in 100% oxygen in air by using a ventilator and monitor (Apollo Gas Anesthesia System and Infinity Explorer Monitoring System, Draeger Medical, Telford, PA). Pigs were placed in the supine position; the femoral area was clipped; and an electrocardiogram monitor (Draeger Medical) for measuring heart rate was secured and continuous monitoring started. Tidal volume initially was set at 7 mL/kg with a rate of 25 breaths per minute. Ventilation was adjusted to obtain an end-tidal pCO_2 of approximately 40 mm Hg.

Animal preparation. The pigs were anesthetized as described. The skin over the femoral vein or the jugular vein area was clipped and scrubbed. Using sterile technique, we inserted a percutaneous catheter (8.5 French; length, 10 cm; model SI-09880, Percutaneous Sheath Introducer Set, Arrow International, Reading, PA) into the vessel under ultrasound (model UMT-200, Mindray NA, Mahwah, NJ) guidance. A blood sample for CBC analysis was collected and sent to the laboratory for immediate determination of the platelet count, which was required to set up the collection parameters for the automatic blood-processing machine.

After the collection procedure (see following section), the catheter was removed; pressure was applied until hemostasis was assured; and the pig was weaned from the ventilator, allowed to recover under observation, and returned to the home cage.

Swine plateletpheresis procedure. An automatic blood-processing machine (model MCS+ LN9000, Haemonetics, Braintree, MA) with the standard disposable plateletpheresis set (model 994CF-CPP, Platelet Collection Kit, Haemonetics) was used. In preliminary studies, we measured that a 450-mL unit of swine whole blood contains approximately 1.6×10^{11} platelets per unit of blood; this platelet count compares well with that of humans, for which a unit of whole blood contains at least 5.5×10^{10} platelets per unit.[4] Several settings of the blood-processing machine were adjusted for its use with pigs, but otherwise the collection process was similar to that for humans. The first adjustment involved blood volume. The pig's total blood volume in milliliters was calculated manually by using an estimated blood volume of 70 mL/kg [7,17] multiplied by the pig's body weight in kilograms. The pig's height and weight characteristics were not applicable for entering into the setup screen, so arbitrary values were chosen that resulted in the calculated blood volume of the pig when the values entered into the machine. The Hemo Calculator Menu of the blood-processing machine enables the user to input the height and weight of a human patient and automatically calculates the blood volume for that patient according to an internal formula. However, to circumvent the human-based algorithm, for a 60-kg pig (132 lb entered on the machine) with a calculated blood volume of 4200 mL, the user must input an arbitrary height value of 12 feet to set the blood-volume value to 4200 mL in the machine.

The second setting adjustment involved the number of cycles. The number of cycles used by the blood-processing machine was based on the pig's initial platelet count and the target level (that is, 4×10^{11} platelets), which were entered into the machine's settings. The usual target level for a standard '6-pack' of human plateletpheresis platelets is 3×10^{11},[19] but according to preliminary experiments with pigs, we needed to increase the standard human target to obtain an equivalent number of pig platelets. The final adjustment addressed the amount of saline used. Given that our experiments were survival procedures, we chose for 100 mL of saline to be returned to the animal during each cycle to compensate for the volume removed in the platelets product. Alternatively, we returned fluid after the plateletpheresis procedure, because giving fluid during the procedure may reduce the hematocrit excessively, causing the instrument to abort the collection.

Table 1. Various experimental parameters (mean ± 1 SD)

Platelet-donor pigs (n = 9)		
	Body weight (kg)	71 ± 5
	Hematocrit (% RBC)	30 ± 2
	Platelet count ($\times 10^3$/μL)	324 ± 66
	Estimated total blood volume (mL)	4945 ± 309
Machine[a] readings during plateletpheresis		
	Number of cycles (range)	5 ± 1 (4–6)
	Total volume (mL) of blood processed per pig	2965 ± 584
	Volume (mL) of blood processed per cycle	614 ± 32
	Total volume (mL) of anticoagulant citrate dextrose used per pig	384 ± 53
	Volume (mL) of anticoagulant citrate dextrose infused per pig	338 ± 51
	Amount (g) of citrate infused per pig	9.9 ± 1.5
	Elapsed time (min [range])	82 ± 17 (54–108)
	Volume (mL) of saline infused per pig[b]	445 ± 134
Product characteristics		
	Target yield ($\times 10^{11}$ platelets per bag [range])	5 ± 1 (3.5–6.3)
	Estimated yield ($\times 10^{11}$ platelets per bag [range])	5 ± 1 (4.2–6.3)
	Actual yield ($\times 10^{11}$ platelets per bag [range])	2.3 ± 1.9 (0.5–5.9)
	Volume (mL) of platelets per bag (range)	240 ± 72 (184–399)
	Platelet count ($\times 10^3$/μL) per bag[c]	870 ± 523

[a]MCS LN-9000, Haemonetics, Braintree, MA.
[b]Approximately 100 mL per cycle.
[c]On day 0.

Table 2. Effects of plateletpheresis on venous blood samples collected before and after plateletpheresis and on day-0 platelet product (n = 4)

	Pigs		
	Prepheresis	Postpheresis	Platelet bag day 0
Total volume (mL)	4919 ± 257	4845 ± 238	331 ± 102
Platelet count ($\times 10^3/\mu L$)	341 ± 67	208 ± 57[a]	1259 ± 280[a,b]
No. of platelets ($\times 10^{11}$) per pig or bag	16.9 ± 4.0	10.1 ± 3.1	4.3 ± 1.9[a,b]
Platelet volume (fl)	9.1 ± 1.4	7.6 ± 0.7	9.6 ± 1.3
Hematocrit (%)	30 ± 1	28 ± 2	1.6 ± 0.4[a,b]
Hemoglobin (g/dL)	10.1 ± 0.4	9.3 ± 0.7	0.04 ± 0.07[a,b]
WBC count ($\times 10^3/\mu L$)	11.8 ± 2.9	10.2 ± 1.2	38.0 ± 11.4[a,b]
No. of WBC ($\times 10^{11}$) per pig or bag	0.6 ± 0.2	0.5 ± 0.08	0.1 ± 0.02[a]
Neutrophil count ($\times 10^3/\mu L$)	2.4 ± 0.8	4.4 ± 1.9	0.02 ± 0.03[a,b]
No. of neutrophils ($\times 10^{11}$) per pig or bag	0.1 ± 0.04	0.2 ± 0.09	0.00005 ± 0.00008[a,b]
Neutrophil percentage (%)	21.4 ± 8.6	45.5 ± 23.7	0.04 ± 0.07[b]
Lymphocyte count ($\times 10^3/\mu L$)	8.5 ± 5.3	5.3 ± 3.0	37.4 ± 11[a,b]
No. of lymphocytes ($\times 10^{11}/\mu L$) per pig or bag	0.4 ± 0.1	0.3 ± 0.2	0.1 ± 0.02[a,b]
Lymphocyte percentage (%)	71.1 ± 8.7	50.6 ± 23.7	98.5 ± 0.6[a,b]
Monocyte count ($\times 10^3/\mu L$)	0.4 ± 0.2	0.2 ± 0.05	0.4 ± 0.2
No. of monocytes ($\times 10^{11}/\mu L$) per pig or bag	0.02 ± 0.008	0.009 ± 0.002	0.001 ± 0.0003[a]
Monocyte percentage (%)	3.1 ± 0.5	1.8 ± 0.3	2.3 ± 2.8
Eosinophil count ($\times 10^3/\mu L$)	0.3 ± 0.1	0.08 ± 0.06[a]	0 ± 0[a]
No. of eosinophils ($\times 10^{11}$) per pig or bag	0.02 ± 0.005	0.004 ± 0.003[a]	0 ± 0[a]
Eosinophil percentage (%)	2.8 ± 0.7	0.8 ± 0.5[a]	0 ± 0[a]
Basophil count ($\times 10^3/\mu L$)	0.2 ± 0.07	0.1 ± 0.04	0.2 ± 0.2
No. of basophils ($\times 10^{11}$) per pig or bag	0.010 ± 0.003	0.006 ± 0.002	0.0006 ± 0.0004[a,b]
Eosinophil percentage (%)	1.6 ± 0.2	1.3 ± 0.4	0.6 ± 0.3[a]
Inorganic calcium (mmol/L)	1.3 ± 0.01	1.0 ± 0.08[a]	0
Lactate (mmol/L)	1.3 ± 0.6	0.8 ± 0.3	1.5 ± 0.15

Data are presented as mean ± 1 SD.
[a]Value is significantly ($P < 0.05$) different from that before plateletpheresis.
[b]Value is significantly ($P < 0.05$) different from that after plateletpheresis.

Anticoagulant citrate dextrose (CaridianBCT, Lakewood, CO) containing (per 100 mL) 2.45 g dextrose monohydrate, 2.2 g sodium citrate dihydrate, and 0.73 g citric acid anhydrous was used as the anticoagulant. The ratio of anticoagulant citrate dextrose administered to whole blood was 1:9. The platelets were stored in autologous plasma.

With the standard disposable set we used, the blood-processing machine withdrew a constant volume of blood at 90 mL/min into a custom-designed transparent cylindrical centrifuge bowl, which centrifuges the blood as it is collected. RBC fall to the outside of the bowl, and the WBC, platelets, and plasma form concentric rings inside the RBC ring. When enough volume has been collected, a sensor detects the plasma–platelets–RBC interface, and the plasma–platelet layer is diverted into a separate collection bag. Another sensor along the tubing to the plasma collection bag detects the refraction of the platelet-enriched portion of the plasma and diverts the platelets into the platelet collection bag. The machine then returns the RBC and plasma to the pig, along with the user-selected volume of saline; the cycle then begins again and repeats until the target yield is met. Donor pigs were used for 1 to 6 plateletpheresis procedures, with at least a week between each procedure. Pigs did not require calcium infusion during plateletpheresis.

There is an incompatibility between pig blood and the human blood-processing machine we used: because the instrument's sensor cannot detect the pig platelet–plasma interface, we manually selected (this screen is only accessible in the bootup process) a maximum volume of 25 to 35 mL, at which level the valve switched from plasma to platelets. This adjustment ensured that minimal RBC were collected in the platelet bag. Because normal pig hematocrits are lower than human hematocrits, a donor pig was unsuitable if its hematocrit was less than 27%. At such low hematocrits, the machine automatically aborts the collection of blood, given that more than 600 mL would be removed from the donor. This is an internal automatic setting for patient protection and is not adjustable.

The platelets were stored at 22° to 24 °C in a rotating (70 strokes per minute) platelet incubator (model PC100h, Horizon Series Countertop Platelet Incubator, and model PF15h, Horizon Series Platelet Agitator, Helmer, Noblesville, IN). Bags of collected platelets were weighed on day 0, and 20-mL samples were removed daily for blood gas analysis, CBC analysis, thromboelastography, and platelet aggregometry.

Recovery of fluorescently labeled platelets from recipient pig. Recipient pigs were anesthetized as described for donor pigs, and a Foley catheter was placed into the bladder and attached to a closed-system urometer (Professional Medical Products, Greenwood, SC). Two catheters (one for infusion of the labeled platelets and one for sample collection, to ensure no contamination) were inserted into the veins (either femoral or jugular) by using cutdown procedures. A sample from the bag of labeled platelets was tested by aggregometry (model 700, Chronolog Corporation, Havertown, PA) using collagen (final concentration, 8 µg/mL) as the agonist. A baseline blood sample (20 mL) was collected. The labeled platelets (120 mL) were infused at 40 mL/min (approximately 1 mL/kg/min) through one of the

Table 3. Coagulation parameters (mean ± 1 SD) of platelets on day 1 compared with those of the donor pig blood

	Pig plasma	Platelets day 1
PT (s)	11.2 ± 0.2	11.4 ± 0.4
PTT (s)	16.1 ± 0.6	18.3 ± 0.8
Fibrinogen (mg/dL)	260 ± 53	230 ± 21
D-Dimer (mg/L)	0.6 ± 0.5	0.6 ± 0.4
Antithrombin III (% dN)	95 ± 10	79 ± 14[a]
Protein C (% dN)	105 ± 27	72 ± 15
Factor II (% dN)	81 ± 20	73 ± 26
Factor V (% dN)	351 ± 38	408 ± 123
Factor VII (% dN)	66 ± 7	61 ± 10
Factor VIII (% dN)	451 ± 115	402 ± 43
Factor IX (% dN)	194 ± 42	201 ± 69
Factor X (% dN)	79 ± 20	71 ± 23
Factor XI (% dN)	80 ± 12	82 ± 14
Factor XII (% dN)	468 ± 52	423 ± 61
Factor XIII (% dN)	32 ± 5	65 ± 47
von Willibrand factor (% dN)	27 ± 2	41 ± 20

%dN, Dade normal (corresponds to normal human values as measured by our instrument).
[a]Value significantly ($P < 0.05$) different from that for pig plasma.

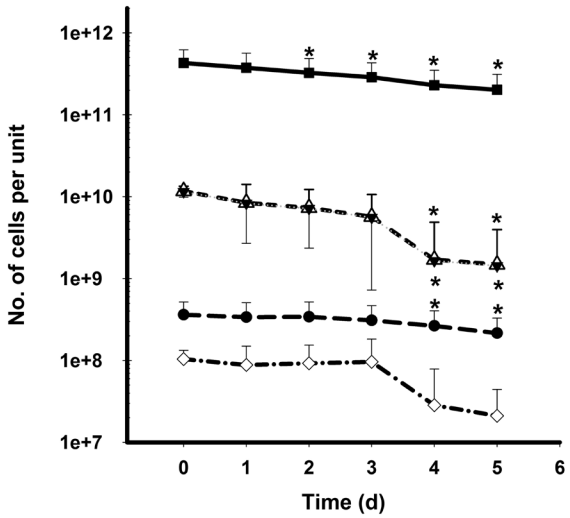

Figure 1. In vitro study. The numbers (mean ± 1 SD) of cells per unit in each bag ($n = 4$) over 5 d for platelets (solid squares), WBC (open triangles), lymphocytes (solid triangles), RBC (solid circles), and monocytes (open diamonds). Note that the lines for WBC and lymphocytes overlap. *, Value is significantly ($P < 0.05$) different from that on day 0.

catheters. At 3, 10, 30, and 60 min and then hourly until 5 h, venous samples (20 mL) were collected from the other catheter for CBC analysis, thromboelastrography, and percentage of labeled platelets according to flow cytometry (FACS Canto I, BD Biosciences, San Jose, CA). After each sample, 40 mL lactated Ringers solution was administered after each sample. The urine volume was recorded after each sample. At the end of 5 h, after collection of the final blood samples, recipient pigs were euthanized with sodium pentobarbital (90 mg/kg IV; 10 mL Fatal Plus, Vortech Pharmaceuticals, Dearborn, MI) while under surgical anesthesia.

Validation procedures. ***Plateletpheresed platelets.*** The AABB requires that each unit of platelets collected by apheresis must contain at least 3×10^{11} platelets, have a pH of ≥ 6.2 at the end of storage, and include less than 2 mL of RBC.[19]

Samples (20 mL each) were collected from each bag of platelets on the day of preparation (day 0) and daily for 5 d (days 1 through 5) from stored bags. Each sample underwent CBC analysis (Cell-Dyn 3700CS Hematology Analyzer, Abbott Laboratories, Abbott Park, IL), blood gas analysis (COBAS b221 Blood Analyzer System, Roche Diagnostics, Indianapolis, IN), assessment of platelet function by using thromboelastrography (Haemoscope model 5000, Haemonetics) at 37 °C with kaolin as the accelerator, and platelet aggregation analysis (Chronolog) using collagen as an agonist.

Fluorescently labeled platelets. The published techniques for fluorescently labeling platelets for baboon[24] and rabbit[31] were modified to label an entire bag of plateletpheresed pig platelets. We performed a dose–response test to determine the concentration of dye[16] that resulted in approximately 100% labeling of the platelets in a bag. We also performed serial dilution with labeled and unlabeled platelets to confirm that there was a linear relationship between the concentration of dye and the percentage of labeled platelets as determined by flow cytometry. In preliminary studies, washed platelets of known platelet count (Z2 Particle Counter, Beckman Coulter, Miami, FL) were diluted serially from 100% to 0% by using MTAB; and 100% labeled platelets of known platelet count were diluted by using nonlabeled washed platelets of known platelet count. The target platelet count of each dilution was verified (Z2 Particle Counter), and the percentage of labeled platelets at each dilution was measured by flow cytometry.

Washing of platelets. ***Reagents.*** Sodium chloride, potassium chloride, magnesium chloride, sodium phosphate monobasic, sodium bicarbonate, D(+)-glucose, HEPES sodium salt, prostaglandin I2 sodium salt, apyrase, BSA, trisodium citrate, and citric acid were purchased from Sigma–Aldrich Chemical (St Louis, MO). PBS was purchased from Fisher Diagnostics (Thermo Scientific, Middletown, VA).

Stock solutions. Stock solutions of modified Tyrode buffer (2.73 M NaCl, 53.6 mM KCl, 238 mM NaHCO3, 8.6 mM NaH2-PO4), magnesium chloride (0.1 M), HEPES buffer (0.5 M), and modified acidified citrate dextrose (30.8 mM trisodium citrate, 190 mM citric acid, 316 mM dextrose) were made weekly and kept refrigerated. BSA (20% w/v in distilled water), apyrase (20 U/mL in deionized water), and prostaglandin I2 (530 µM in 100% ethanol) stocks were frozen in 750-µL aliquots.

Working buffers. The modified Tyrode–HEPES–albumin buffer (MTAB; 137 mM NaCl, 2.7 mM KCl, 1.0 mM $MgCl_2$, 0.43 mM NaH_2PO_4, 12 mM $NaHCO_3$, 5.5 mM D-glucose, 5 mM HEPES, and 0.35% BSA; adjusted to pH 7.35) was made fresh daily from stock solutions and diluted with distilled water. CellTracker Green CMFDA Stock (Molecular Probes, Eugene, OR) was diluted to 10 mM in dimethyl sulfoxide and frozen in 70-µL aliquots.

Platelet washing procedure. A 'satellite' bag of platelets (that is, the XT-612 plastic bag from a Teruflex Triple Blood Bag System, catalog number 1BB*AGT456A2, Terumo Products, Somerset, NJ) was docked aseptically onto the bag of plateletpheresed pig platelets. Apyrase (final concentration, 0.02 U/mL) and prostaglandin I2 (final concentration, 1 µM) then were added to the plateletpheresed platelets. The 2 bags were centrifuged at room temperature at 750 × g for 10 min (model RC-3B, Sorvall, Thermo Scientific; no brake and level 1 acceleration speed). The plasma was transferred to the satellite bag and discarded, and remaining platelets were resuspended in 100 mL MTAB.

Platelet staining procedure. The platelets were resuspended in 100 mL MTAB, CellTracker Green CMFDA was added (final

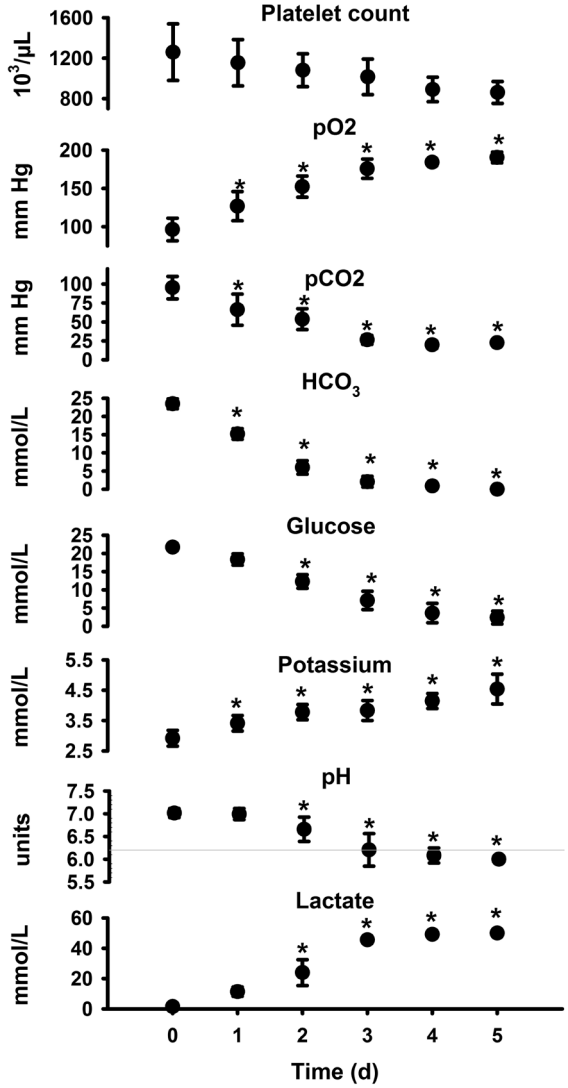

Figure 2. In vitro study. Platelet counts and the pO_2, pCO_2, HCO_3, glucose, potassium, pH, and lactate concentrations (mean ± 1 SD) of samples from plateletpheresis bags (n = 4) on the day of collection (day 0) and days 1 through 5 of storage at 22 °C in a platelet incubator. *, Value is significantly ($P < 0.05$) different from that on day 0.

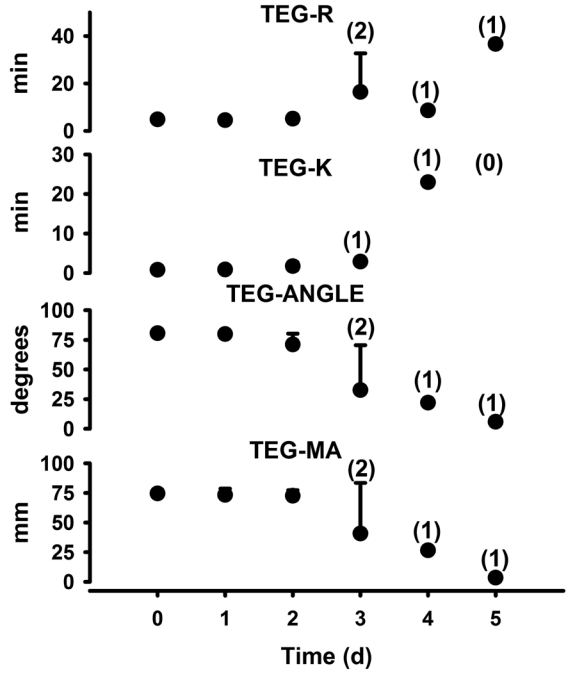

Figure 3. In vitro study. Thromboelastographic (TEG) variables (mean ± 1 SD) of R time (onset of coagulation), K time (kinetics of coagulation), angle (rate of clot formation), and maximal amplitude (MA; strength of the clot) of samples from plateletpheresis bags (n = 4) on the day of collection (day 0) and on days 1 through 5 of storage at 22 °C in a platelet incubator. Only one bag gave any TEG results after day 3; numbers in parentheses indicate the number of samples represented in each result. *, Value is significantly ($P < 0.05$) different from that on day 0.

concentration, 5 mM), and the platelets and dye were mixed gently. The platelets were incubated at 37 °C for 30 min in the dark. Steps 2 through 4 of the platelet washing procedure (that is, addition of apyrase and prostaglandin I2, centrifugation, transfer of plasma, and resuspension in MTAB) were repeated twice with the resuspension volume being 100 mL of MTAB after the first wash; after the second wash, the platelets were resuspended in 125 mL MTAB, and apyrase (0.005 U/mL) was added. The stained platelets were incubated at 22 °C (Horizon Series Countertop Platelet Incubator, Model PC100h, Helmer) overnight in the dark.

Flow cytometry. Whole-blood samples collected in citrate tubes were centrifuged at 200 × g for 10 min at room temperature, and the PRP was transferred to a polystryrene tube (Falcon, BD). Then 5 µL of PRP was added to 500 µL of PBS (Thermo Scientific, Waltham MA) in a 5-mL polystyrene round-bottom tube (BD Falcon, BD Biosciences) and vortexed for 10 s. The samples were analyzed by flow cytometry (FACSCanto I, BD Biosciences) using FACSDiva software (version 6.1.3, BD Biosciences). The light scatter and fluorescence channels were set at logarithmic gain, and a minimum of 200,000 platelets events at a rate of less than 3000 events per second were acquired. The platelets were gated on their characteristic forward- and side-scatter properties and analyzed for FITC fluorescence (emission at 530 nm). Because there were WBCs in the plateletpheresis product and because CellTracker stains label both of these cell types, platelets and WBC were differentiated according to size and internal granularity.

Percentage recovery of infused CMFDA-labeled platelets. Percentage recovery of infused labeled platelets at each time point was determined by

$$\left(\left[\%\ \text{CMFDA-positive platelets}\right]\times\left[\text{platelet count}/\mu\text{L}\right]\times\left[\text{blood volume }(\mu\text{L})\right]\right)/\left(\text{number of CMFDA-positive platelets infused}\right).$$

Baseline blood volume was estimated by assuming 70 mL/kg as the estimated total blood volume (EBV) of the pig. The blood volume for each subsequent time point was adjusted for the 20-mL sample volume removed, urine volume lost, and the flush volume administered, assuming that one-third of the flush volume remained in the plasma compartment. The following equations were used.

$$\text{Baseline EBV}_0\,(\text{mL})\quad 70\ \text{mL/kg}\times\text{body weight (kg)},$$

where the subscript 0 refers to baseline.

$$\text{Estimated plasma volume }(\text{EPV}_i,\ \text{mL})$$
$$\left\{\left[100\quad\text{hematocrit }(\%)\right]/100\right\}\times\text{EBV}_i,$$

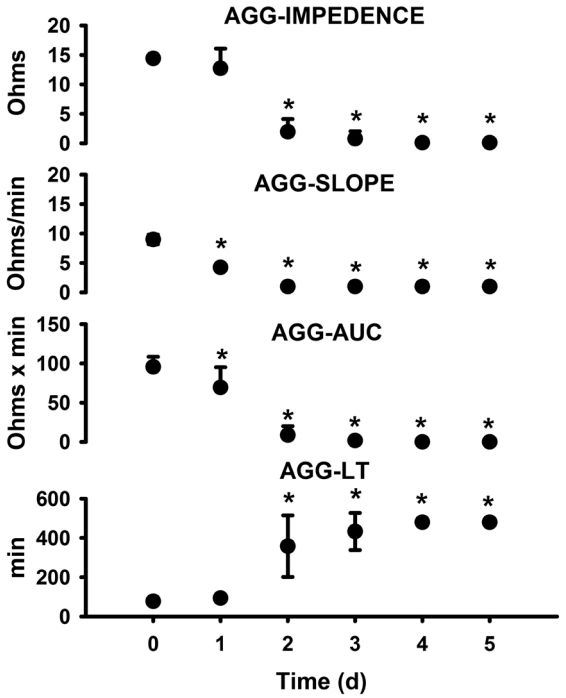

Figure 4. In vitro study. Platelet aggregation (AGG) responses (mean ± 1 SD) according to impedance (peak height), slope, AUC, and lag-time (LT) of samples from plateletpheresis bags (n = 4) on the day of collection (day 0) and on days 1 through 5 of storage at 22 °C in a platelet incubator. *, Value is significantly ($P < 0.05$) different from that on day 0.

where subscript *i* refers to the sample.

$$\text{Estimated RBC volume } (\text{ERBCV}_i) \quad [\text{hematocrit } (\%)/100] \times \text{EBV}_i.$$

The sample volume (whole blood) is subtracted from each subsequent EBV_i. For each subsequent EBV_i, EPV_i and $ERBCV_i$ are calculated. The urine volume is subtracted from and 1/3 of the lactated Ringers flush volume is added to the EPV_i to calculate the adjusted EPV_i. The adjusted EPV_i and the $ERBCV_i$ are summed to determine the EBV_i for each sample. The adjusted EBVi is used in place of the blood volume (µL) in the percentage recovery formula given earlier. The maximal recovery percentage was calculated by setting the 3-min results to 100%. Loss of fluorescence in urine was not determined.

Data analysis. Data are expressed at mean ± 1 SD. Paired *t* tests were used to compare values before and after apheresis or other treatment. One-way ANOVA for repeated measures was used for assessing changes over time (SAS Institute, Cary, NC). The Mauchly test for sphericity could not be performed because it requires that the number of subjects in the study exceeds the number of time points. Therefore, the conservative Greenhouse–Geisser correction was performed in all cases. If significance was found with ANOVA, then post hoc comparisons with either Dunnett (to baseline) or Tukey (all pairwise) tests (SigmaPlot, version 12, Systat Software, San Jose, CA) were performed. A P value of less than 0.05 was considered statistically significant.

Results

Plateletpheresed platelets. The baseline blood values from donor pigs (n = 9) and machine-derived parameters are shown in Table 1. Overall, the swine platletpheresis procedure took 90 to 180 min, depending on the number of cycles required to meet the target collection. Approximately 5 cycles were needed to collect the target yield of platelets in a volume of 200 to 300 mL, resulting in a platelet count per bag on day 0 (mean ± 1 SD) of $870 \pm 523 \times 10^3$/ µL. The AABB requirement for humans is 3×10^{11} apheresed platelets per unit.[19] Assuming that the typical human weighs 70 kg, the target dose for pigs as adjusted for body weight yields a dose of 4×10^9 platelets per kilogram. Although the actual yield of the swine platelets was slightly less than the AABB target (Table 1), when adjusted for the 40-kg pigs used in our studies, the calculated platelet dose (6×10^9 platelets per kilogram) exceeded the weight-adjusted target required by the AABB.

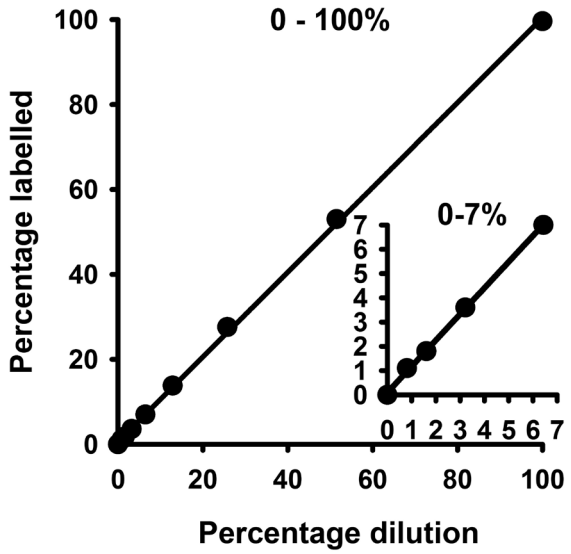

Figure 5. The percentage of fluorescently labeled platelets that were detectable by flow cytometry was linear for dilutions from 0% to 100% (r = 0.9995; slope = 1.0756; $P < 0.0001$).

We found no relationship between pigs' blood platelet count and the concentration of the platelets in the product bag, even though the final volume in the platelet bag (approximately 200 mL) and number of cycles run (4) were similar among donor pigs. For example, for 3 pigs that donated multiple times, their baseline platelet counts were 392, 300, and 357×10^3/ µL, yet the concentration in each platelet bag was 519, 1369, and 341 $\times 10^3$/ µL, respectively, with consistent platelet counts from individual pigs.

The mean platelet volume in the platelet bag on day 0 was the same that of platelets in pig plasma (Table 2), indicating that the plateletpheresis procedure did not provoke a shape change. However, our CBC machine did not report any results on regarding mean platelet volume (although it did report platelet counts) on subsequent days from the platelet bags, so a change in morphology cannot be ruled out on these subsequent days.

Of note is that the leukoreduction filter that is part of the kit used in this study worked only to remove neutrophils, eosinophils, and basophils (Table 2). The filter allowed 21% of donor pigs' prepheresis blood content of WBC through to the platelets bag: 98% of these were lymphocytes and 2% were monocytes. The AABB requires that each leukoreduced unit of platelets should contain fewer than 5×10^6 leukocytes per unit. Therefore units of swine platelets cannot be considered to be leukoreduced when prepared as we described.

Although the hematocrit of the platelet bag was less than 2% (Table 2), the volume of RBC in each unit of swine platelets on day 0 was 5 ± 2 mL (n = 4), which is higher than the 2-mL limit recommended by the AABB. Therefore, the blood type of the

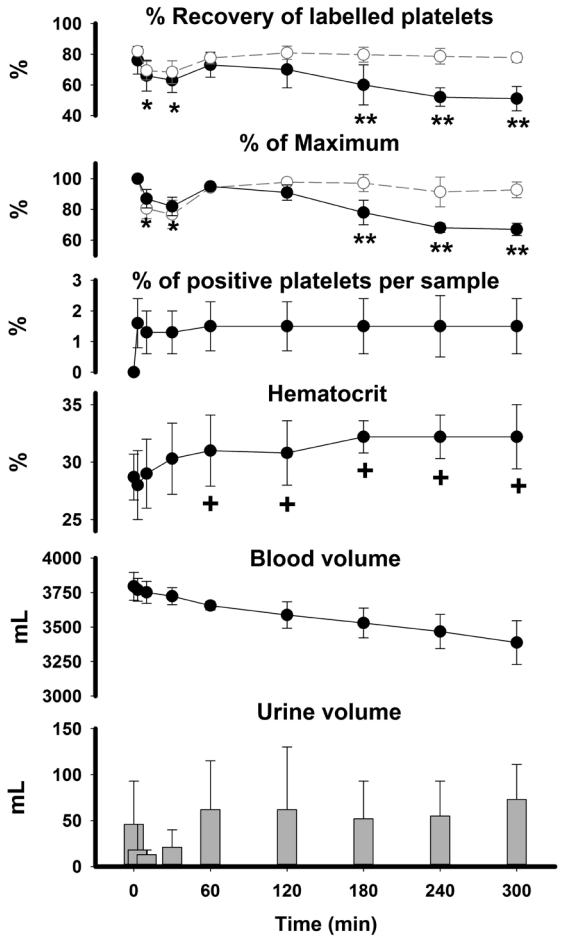

Figure 6. In vivo study. Parameters measured over 5 h in pigs (n = 3) that received a complete plateletpheresis bag of fluorescently labeled, nonautologous platelets. The percentage recovery or percentage of maximum (maximum, 3 min) of labeled platelets was calculated either from the total injected sample adjusted for the changes in estimated blood volume at each time point (closed circles) or from the total injected sample at time 0 without correction for changes in blood volume during the 5-h study (open circles). *, Value significantly ($P < 0.05$) different from that at 3 min; **, value significantly ($P < 0.05$) different from those at 3 and 60 min; +, value significantly ($P < 0.05$) different from that at 0 min. Data are given as mean ± 1 SD.

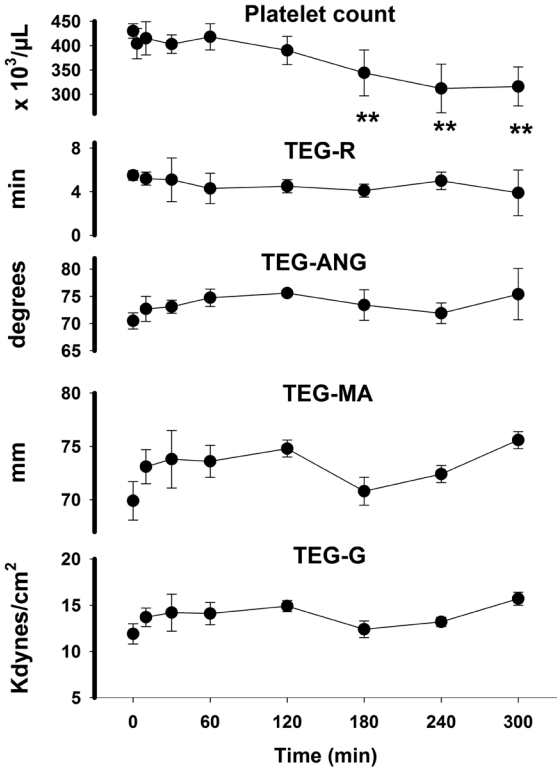

Figure 7. In vivo study. Platelet count and thromboelastographic (TEG) values (mean ± 1 SD; R time, angle [ANG], maximal amplitude [MA], and elasticity [G]) of blood samples from the recipient pig after infusion of labeled platelets. **, Value significantly ($P < 0.05$) different from those at baseline and 60 min.

donor and recipient pigs should be determined so that only type-matched swine platelets are administered.

The platelet content of the bag, calculated by multiplying the volume of the bag by the platelet concentration, was significantly ($P < 0.05$) lower on days 2 and later compared with day 0 (Figure 1). The percentage of platelets on days 1 through 5 (that is, after storage) compared with that on day 0 was 87% ± 10%, 76% ± 10%, 67% ± 9%, 54% ± 10%, and 47% ± 9%, respectively. The number of each other cell type, present at about 10% of the number of platelets, also decreased over the 5 d. The RBC count showed a gradual decline and became significantly ($P < 0.05$) reduced compared with that on day 0 by day 4 (72% ± 14%). The WBC, lymphocytes, and monocytes showed a sudden, large decline on day 4 (Figure 1) to 16% ± 30%, 16% ± 30%, and 21% ± 34% of day 0 numbers, respectively.

As shown in Figure 2, the pH was significantly lower than that on the collection day by day 2 and reached the AABB limit of pH = 6.2 on day 3. The pCO_2, pO_2, HCO_3, and potassium levels changed significantly ($P < 0.05$) by day 1, and lactate and glucose levels changed significantly ($P < 0.05$) by day 2. There were no significant changes in platelet function as measured by thromboelastrography (Figure 3) for the first 3 d. After day 3, only one of the bags yielded a measurable thromboelastrogram, whereas the other 3 bags did not clot at all during the analysis. In the one bag that was evaluable, the thromboelastrographic parameters of reaction time, angle and K value (that is, speed of clot formation), shear elastic modulus, and maximal amplitude trended toward hypocoagulation. The ability of the platelets to aggregate to collagen showed marked changes by day 2 (Figure 4). Based on these functional and biochemical results, only day 1 platelets met the AABB standards and demonstrated normal function. In addition, the coagulation parameters of day 1 platelets remained comparable to normal pig values (Table 3), except for a decrease in antithrombin III in day 1 platelets.

The donor pigs (70 ± 4 kg) tolerated the plateletpheresis collection procedure well, as shown by the changes before and after collection of selected parameters in Table 2 for 4 animals for which postplateletpheresis samples were collected during model development. There were no adverse events during the procedure, and the pigs maintained their hematocrit and platelets counts at acceptable levels. Only the platelet and calcium levels were significantly ($P < 0.05$) reduced acutely but remained at clinically normal levels. Blood platelet counts immediately after collections were reduced to 61% ± 12% (n = 4) of pretreatment values. However, in 3 pigs that donated multiple (2 to 6) times, the pigs' platelet counts remained at 94% of the first-donation value (range, 79% to 108%) over the 2 to 6 mo that these pigs were donors.

Platelet survival in circulation. Platelets were collected from 3 donor pigs. Before fluorescent labeling of platelets, the average volume of the plateletpheresed platelets was 219 ± 39 mL, the platelet count was 404 ± 305 × 10^3 platelets per microliter,

and the average total number of platelets per bag was $0.9 \pm 0.6 \times 10^{11}$. After washing and labeling of the platelets, the total volume was 120 mL, the average platelet count was $269 \pm 159 \times 10^3$ per microliter, and the total number of platelets per bag was $0.4 \pm 0.2 \times 10^{11}$. The average percentage recovery after washing and labeling was 40% ± 9%. The average percentage of labeled platelets in the bags was 99.0% ± 0.2%. Samples collected from each bag on the experimental day demonstrated sufficient aggregometry function (peak height, 40 ± 4 Ω). The percentage of labeled platelets detectable by flow cytometry was linear to 0, as determined in validation studies (Figure 5).

Each of 3 recipient pigs received the entire volume of fluorescently labeled platelets over 3 min. With the timer started immediately after infusion, there was an initial peak recovery of labeled platelets equal to 76% at 3 min, a slight decrease to 63% at 30 min but a return to 73% at 1 h and a slow decline to 51% at 5 h (Figure 6). Taking the 3-min value as the maximum, we found that there was likewise a reduction at 30 min, a transient rise over the next 2 h, and a slow decrease to 67% recovery of labeled platelets at 5 h after transfusion. In the recipient pigs, the percentage of labeled platelets remained constant for the entire 3 h, but there was a slow decline in platelet counts to approximately 73% of baseline over the 5-h period. There was a trend ($P = 0.0758$) toward a decrease in calculated total blood volume to 89% of baseline at 5 h, likely due to the dehydration that occurred during the experimental period as indicated by a 3% increase in hematocrit. The urine volume voided remained consistent. Had we not used the approximately 11% reduction in blood volume over the 5 h, but only the baseline estimated blood volume (as is done in most studies[24,31]), our percentage recovery and maximal recovery would have been stable over the 5 h, at 79% and 95%, respectively, after the transient decreases at 10 and 30 min (gray dotted lines, Figure 6).

There was a trend ($P = 0.0769$) for an increase in the strength of the clot (that is, maximal amplitude of the thermoelastogram) after infusion of the dose of plateletpheresed platelets (Figure 7). The maximal amplitude of the thermoelastogram is highly dependent on the number of platelets in the circulation[35]. There was no increase in the blood platelet count after the infusion of labeled platelets, and a significant ($P < 0.05$) decrease occurred after 180 min.

Discussion

These results demonstrate that swine platelet production methods using commercially available equipment for humans, the modifications described in this study, and storage of apheresed platelets for a maximum of 24 h meet the AABB standards for nonleukoreduced apheresed human platelets. Preliminary studies on swine platelets derived from single units and tested on days 0, 3, and 5 indicated that swine platelets exhibited extensive storage-associated lesions such that by day 3, the ATP levels were greatly reduced, in contrast to human platelets in which ATP levels remain stable. By adding additional time points between days 0 and 3, we found that there was little difference between day 1 (24 h of storage) compared with day 0 (freshly prepared) swine platelets and that the day 1 platelets met AABB standards. Therefore our procedure accommodates a lag of at least 1 d between collection and use for experimental purposes and makes use of standard, commercially available human equipment and supplies.

Several factors may explain the failure of human reagents to support the energy requirements of swine platelets to yield a prolonged storage time. Swine RBC lose their intramembrane glucose transporter shortly after birth and therefore switch to liver-derived inosine as their preferred energy substrate.[32,42,44] Whether swine platelets membranes similarly lack the glucose transporter is unknown. In fact, several studies on the energy metabolism of swine platelets indicate that they do appear to use glucose.[27,28] In our current study, the glucose concentration in the bag declined in a linear fashion as the lactate concentration increased, indicating that the blood cells in the bag indeed were utilizing glucose for energy production.

Another potential reason for the failure of the human storage media of acid citrate dextrose to support swine platelets is that swine platelets demonstrate a marked Crabtree effect, where in the presence of high external concentrations of glucose, glycolysis via the Emden–Meyerhof pathway is accelerated and oxidative phosphorylation via the Krebs cycle–tricarboxylic acid pathway is inhibited. This outcome was corroborated by the fact that the platelets did not appear to use O_2 and CO_2 in light of their increase and decrease, respectively, by day 1. The pO_2 concentration began at a relatively high level, probably because the pigs were on 100% O_2 and because the venous blood pO_2 was greater than 80 mm Hg. If the platelets were metabolically active, the pO_2 would be expected to stay constant as long as the platelet bags themselves were sufficiently permeable to oxygen.[43] The bags that we used are rated to be able to store a maximum of 5×10^{11} platelets at a maximal concentration of 2600×10^3 platelets per microliter for 5 d. Instead, the pO_2 and pCO_2 gradually increased to equilibrate to those of atmospheric air, such that the pO_2 in all 4 bags exceeded that of atmospheric oxygen (assumed to be 160 mm Hg) by day 3. We do not have an explanation for this implausible response, but perhaps the blood gas machine that we used wasn't calibrated to measure samples that weren't whole blood. We interpret our results as indicative of a trend to equilibration with atmospheric levels, indicating that essentially no oxidative metabolism was occurring in the stored platelets.

A plateletpheresis technique using an Amicus system was applied previously to swine.[40] The cited authors used the instrument to make the pigs thrombocytopenic and to produce platelet product; the platelets were used 3 d after apheresis, and the quality of their platelets was not reported. However, the platelets appeared to be functional when they were transfused into the pigs because the platelet count and maximal clot strength (as measured by rotational elastometry) were both increased.[40] In addition, the rate of blood loss in swine after liver injury after platelet transfusion was reduced significantly compared with that after saline treatment. To our knowledge, the cited study[40] and our current one are the only studies in which functional platelets have been isolated from swine by using commercially available equipment designed for human use. The plateletpheresis technique using a commercially available human instrument (COBE Spectra, Gambro BCT) was well tolerated in dogs as long as calcium was administered.[8]

Our study has some limitations. We did not use flow cytometry to test for the expression of P-selectin and other receptors—as is done in human cases—because we have been unable to find any antibodies that crossreact with swine platelets. This observation of poor immunologic reaction of swine platelets to human antibodies has been documented by others.[25] In contrast, standard functional coagulation assays designed for human platelets work well for swine platelets.

Because platelets degrade significantly after the first 24 h of storage, it was important to evaluate whether 24-h platelets would stay in circulation for the 5-h duration that we needed to study their hemostatic activity. In addition, because we planned to use nonautologous platelets to mimic what

happens in the normal trauma bay, it was important to ascertain that nonautologous swine plateletpheresed platelets were viable and efficacious in a normal animal. The experiment using fluorescently tagged platelets demonstrated that the swine platelets remained in the circulation at levels (greater than 66%) that are deemed acceptable for human products. There is a great heterogeneity in response among patients that receive platelets transfusions, usually because the recipients have underlying disease processes; in addition, the standard human protocol uses autologous platelets to evaluate the viability and efficacy of platelets products in normal humans.[5,26,34] Because we lack the capacity to use radiolabeled platelets at our facility, we used a fluorescent-labeled platelet method to calculate the percentage of circulating platelets, as has previously been used in other animal studies of autologous platelets.[16,24] Our results using nonautologous platelets indicated that the apheresed platelets remained in the circulation of normal pigs for the duration of the study. These pigs were not splenectomized; the pig has a highly contractile spleen, which may have led to platelets sequestration by the reticuloendothelial system.[14]

One aspect of the plateletpheresis method that differs between swine and humans is that the leukoreduction filter removed only neutrophils from swine and not lymphocytes or monocytes also. In addition, the WBC count dropped rapidly on day 3 of storage. Human WBC are known to disappear rapidly, probably because of apoptosis, when stored for more than 72 h.[37,38] Recent proteomic studies demonstrated fewer proteins in the supernatants of leukoreduced RBC than nonleukoreduced RBC,[11] indicating that the degradation products may be associated with cell death.

In conclusion, we have described procedures for producing units of apheresed platelets from swine blood by using standard equipment for human blood and have characterized the activity of the platelets so obtained. We found that these platelets, when stored for as long as 24 h, produce viable and active platelets that meet the AABB standards for platelets function and are suitable for swine studies investigating platelets transfusions.

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The authors report no conflicts of interest.

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