

1 SUPPLEMENTAL METHODS

2

3 Detailed methodology for experimental procedures

4

5 *Exchange transfusion of 7- and 42- day old stored blood*

6 Commercially available canine universal donor (DEA1.1, ABRINT, Dixon, CA),
7 leukoreduced, red blood cells stored at 2-6 °C for either 7 or 42 days were used to perform
8 the four exchange-transfusions at 4, 7, 10 and 13 h as previously described ¹. Briefly, each
9 dog had 25% of total blood volume (20 ml/kg) removed via a femoral arterial catheter and
10 replaced within 30 minutes with an equivalent volume of stored red blood cells (10 ml/kg)
11 at room temperature followed by thawed fresh frozen canine universal donor plasma (10
12 ml/kg) at 37°C, both infused through the side arm of a catheter introducer placed in the
13 external jugular vein. All red blood cells and plasma units were cultured to detect
14 contamination within 5 minutes of opening prior to transfusion. This procedure was
15 repeated every 3 h for a total of 4 exchange transfusions over 12 h, resulting in a total blood
16 volume replaced of 80 ml/kg (reaching an equivalent of 68% total volume exchange when
17 accounting for mixing). This exchange transfusion was performed 4 to 16 h after bacterial
18 challenge.

19

20 *Blood cell washing*

21 RBC units were washed using the Food and Drug Administration-licensed Haemonetics
22 ACP215 automated cell processing system (Braintree, MA). The process of cell washing
23 introduces a unit of RBC (approximately 200-300 cc) into a centrifuge and adds isotonic
24 0.9% saline and 2% dextrose to the spinning RBCunit (1-2 liters depending on the volume

1 of the RBC unit). A disposable closed system circuit is used to ensure an aseptic procedure
2 (RBC De-Glycerol Set 325 mL BMB Ref.236, Haemonetics Corporation, Braintree, MA).
3 Briefly, the programmable instrument separates the RBC into a sterile container while
4 discarding supernatant plasma and wash solution. On completion, the washed RBCs are
5 transferred to the final container for storage or transfusion. Blood was stored in a CP2D
6 anticoagulant and AS-3 nutrient solution, an FDA-licensed anticoagulant preservative
7 solution for human red cells.

8

9 *Statistical methods*

10 Survival times were analyzed using stratified Cox proportional hazard model to
11 account for potential cycle effect. Linear mixed models (SAS PROC Mixed) were used for
12 the analysis of continuous variables to account for repeated measurements of each animal
13 and the actual pairing of animals within each cycle. To evaluate shock reversal, we
14 standardized MAP and NE using Z-scores and then calculated a “shock reversal” score
15 (higher score indicates improvement in shock reversal) based on the difference of the MAP
16 Z-score and NE Z-score, as done previously¹. To evaluate pulmonary function, we
17 constructed a “lung injury” score (lower score is better) based on the first principal
18 component of mPAP, arterial alveolar oxygen gradient, plateau pressures, breathing rates,
19 and oxygen saturation, as done previously¹. Standard residual diagnostics were used to
20 check model assumptions. Logarithm-transformation was used when necessary. SAS
21 version 9.3 (Cary, NC) was used for all analyses. All p-values are two-tailed and
22 considered significant if $p \leq 0.05$.

23

24 *Physiological and laboratory measurements*

1 Hemodynamic parameters [MAP, mPAP, PAOP, central venous pressure (CVP), heart rate
2 (HR)] were measured at 0, 2, 4, 7, 10, 13, 16 h and q2 h thereafter. Echocardiography was
3 performed at 0, 4, 24, 48, 72, 96 h. Cardiac output (CO) and blood sampling from the
4 animals [arterial and mixed venous blood gases, complete blood counts (CBC), and serum
5 chemistries] were measured in all animals at 0, 4, 7, 10, 13, 16, 24 h and q 24 thereafter.
6 Plasma cell-free hemoglobin (CFH), and haptoglobin were measured at 0 h, 10 h, 24 and 48
7 h. Plasma was collected to measure iron levels [non-transferrin bound iron (NTBI) and
8 plasma labile iron (PLI), and transferrin-bound iron (TBI) at 4 h, 13 h, 24 h and 48 h.
9 Sampling of the blood storage bags kept refrigerated for 7 or 42 days was performed at 4
10 and 10 h for potential bacterial contamination. Just prior to transfusion and q 24 h starting
11 at 0 h; blood cultures, sputum cultures and urine volumes were sampled.

12

13 *Mechanical Ventilation, Fluid and Vasopressor Support*

14 Fractional inspired oxygen concentration (FiO₂), respiratory rate (RR), and positive end
15 expiratory pressure (PEEP) levels were titrated based on algorithms incorporating
16 scheduled SaO₂, and arterial blood gas measures as previously described^{1, 25}. All animals
17 received continuous maintenance fluids (Normasol-M + 27 mEq K⁺/L, 2 mL/kg/h, IV)
18 beginning at T₀. To simulate clinical hemodynamic support and equalize initial volume
19 status in all animals, up to three fluid boluses (0.9 % NaCl, 20mL/kg) were administered at
20 20 min intervals if PAOP measured at 4 h was < 10 mmHg. At 1 h the MAP was still < 80
21 mmHg after the three fluid boluses, a NE infusion was initiated at 0.2 µg/kg/min rate and
22 adjusted incrementally (0.2 to 0.4 to 1.0, to a maximum of 2.0 µg/kg/min) at 5-min
23 intervals to maintain MAP between 80 and 110 mmHg throughout the 96 h study. At

1 subsequent times (T6, T8, T10, T12, and every 4 hours thereafter), up to 3 IV fluid boluses
2 (20 mL/kg) were administered if PAOP < 10 mmHg.

3

4 *Sedation and analgesia management*

5 All animals were monitored by a clinician or trained technician at the bedside throughout
6 the study. Midazolam (0.2 mg/kg loading dose, 50 µg/kg/min infusion IV) sedation and
7 fentanyl (5 µg/kg loading dose, 0.7 µg/kg/min IV infusion) analgesia were titrated based on
8 an algorithm as previously described^{1,25}. Medetomidine infusion (2-5 mg/kg/min) was
9 used to supplement sedation as needed according to set criteria.

10

11 *Canine plasma Cell-free Hemoglobin (CFH), Haptoglobin, Transferring-bound Iron (TBI)* 12 *and Plasma Labile Iron (PLI) Assays*

13 Hb concentrations (µmol/L) in plasma or supernatant collected from stored RBC units were
14 determined by Drabkin's method as previously described¹. Canine haptoglobin levels were
15 determined using a commercial canine Hp ELISA kit (Abnova, Taipei City, Taiwan).
16 Canine NTBI and PLI were determined by commercial company using a proprietary assay
17 (aFerrix, Tel Aviv, Israel). TBI was measured using a spectrometer and measuring
18 transferrin electron paramagnetic resonance spectra as previously described¹.

19

20

1 **Supplemental Figures Legends**

2 ***Supplemental Figure 1. Serial mean \pm SE values of lung injury score (LIS) components.***

3 This figure uses the same format as Figure 2, except now respiratory rate (panels A and F),
4 alveolar-arterial oxygen gradient (panels B and G), plateau pressure (panels C and H),
5 SpO₂ (panels D and I) or oxygen saturation (panels E and J) are plotted on the y-axis.

6 ***Supplemental Figure 2. Mean (\pm SE) artery pressure and norepinephrine dose at serial***

7 ***timepoints.*** This figure uses the same format as Figure 2, except now mean artery pressure
8 (panels A and C) or norepinephrine rate (panels B and C) are plotted on the y-axis.

9 ***Supplemental Figure 3. Mean (\pm SE) levels of Alanine Aminotransferase (ALT) and***

10 ***Total Bilirubin at serial time points.*** The format is similar to Figure 2, except now levels
11 of ALT and Total Bilirubin are plotted on the y-axis of panels A and B. P-values are
12 denoted by asterisks and explained below the figures.

13

14

1 **Supplemental Tables Legends**

2 ***Supplemental Table 1. Laboratory parameters at 0h and 4h after S. aureus challenge***

3 ***(immediately before transfusion)***. Means and standard errors of blood chemistry, cellular

4 blood counts and proteins are shown for blood group at 0h (Table A) or 4h (Table B).

5 ***Supplemental Table 2. Laboratory parameters at 24h and 48h after S. aureus challenge.***

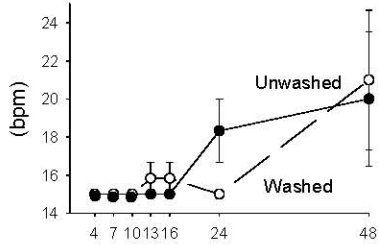
6 Means and standard errors of blood chemistry, cellular blood counts and proteins are shown

7 for blood group at 24h (Table A) or 48h (Table B).

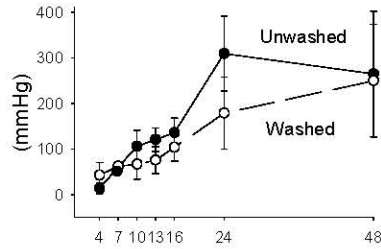
8

42 day old stored blood

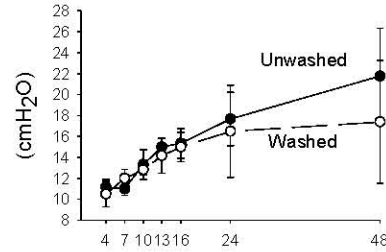
A. Respiratory Rate



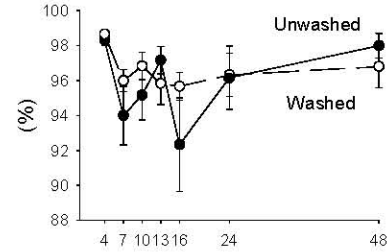
B. AaO2 gradient



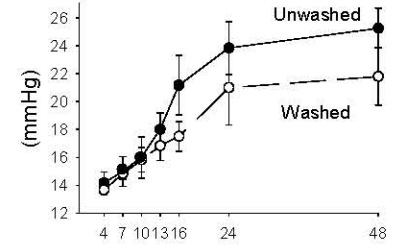
C. Plateau Pressure



D. SpO2

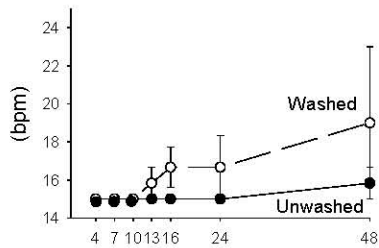


E. Mean Pulmonary Artery Pressure

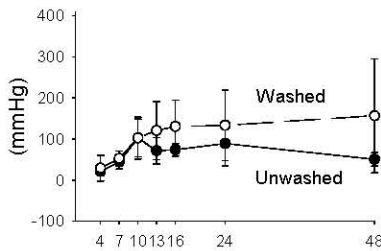


7 day old stored blood

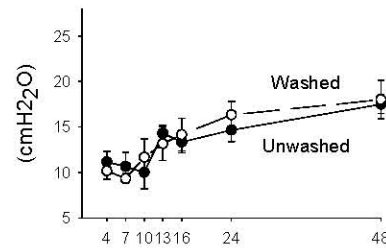
F. Respiratory Rate



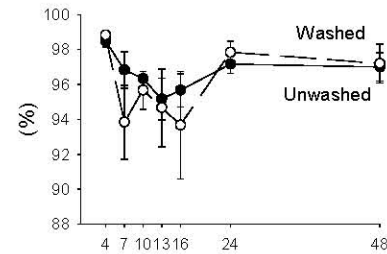
G. AaO2 gradient



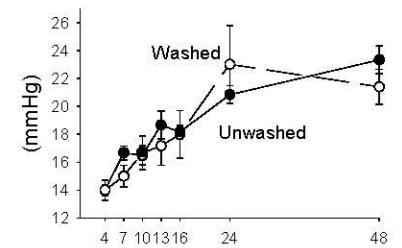
H. Plateau Pressure



I. SpO2

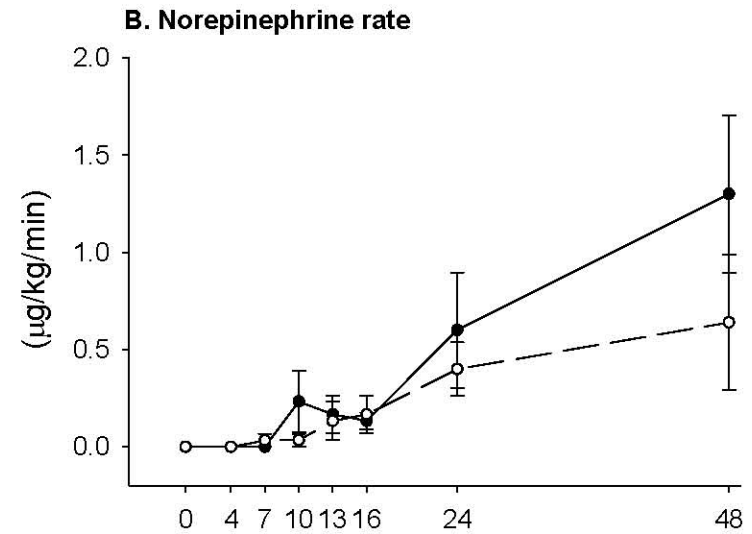
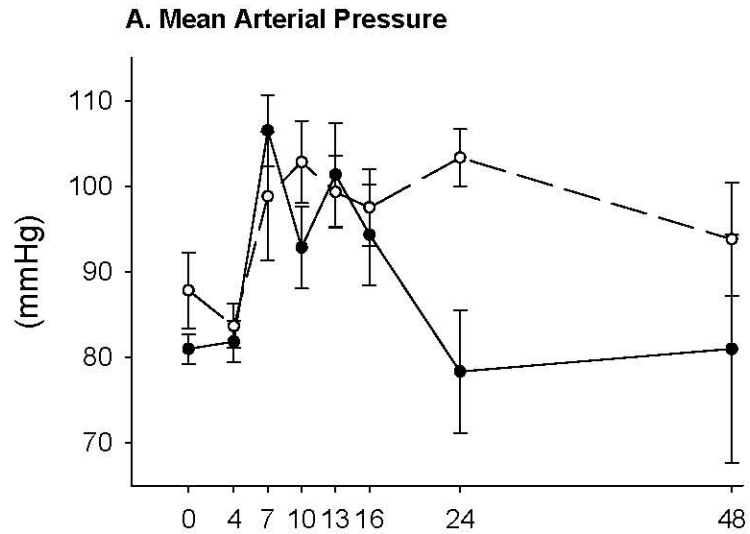


J. Mean Pulmonary Artery Pressure

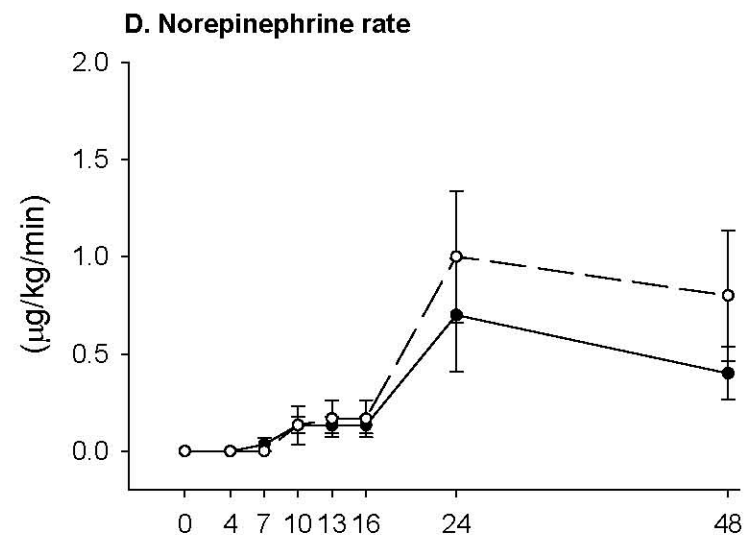
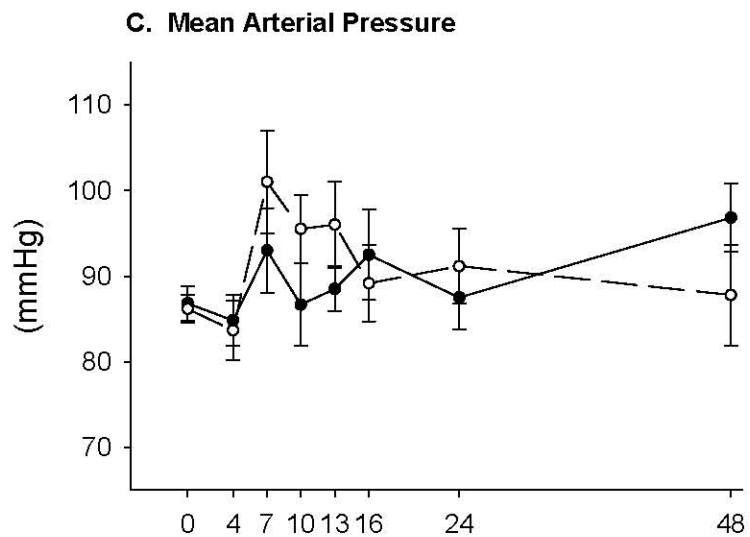


Time (hours) after *S. aureus* (1.5×10^9 CFU/kg) intrabronchial challenge

42 day old stored blood

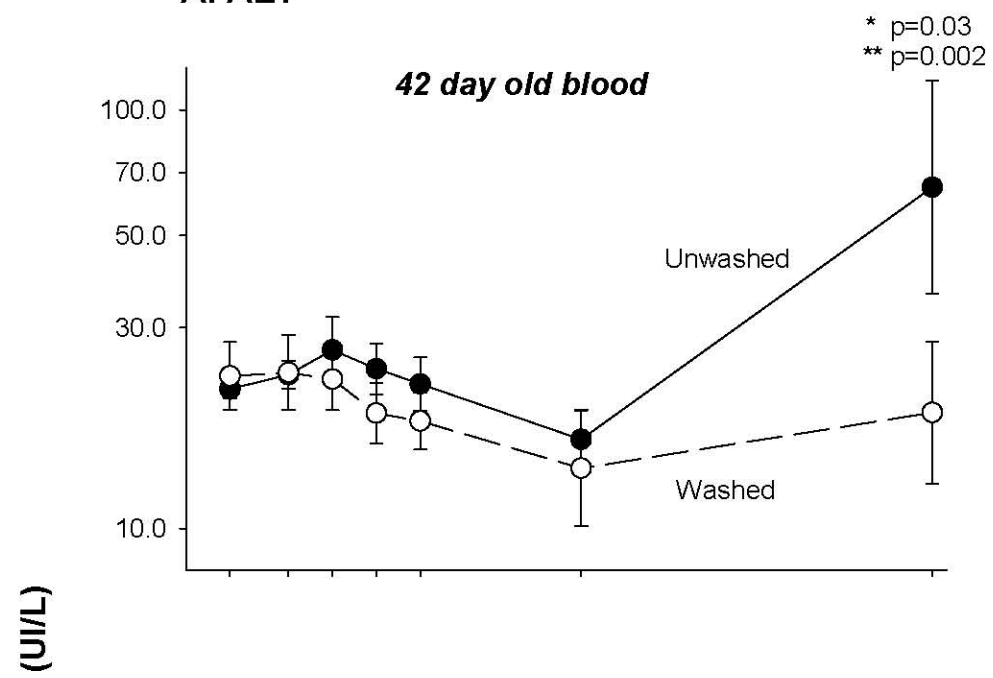


7 day old stored blood

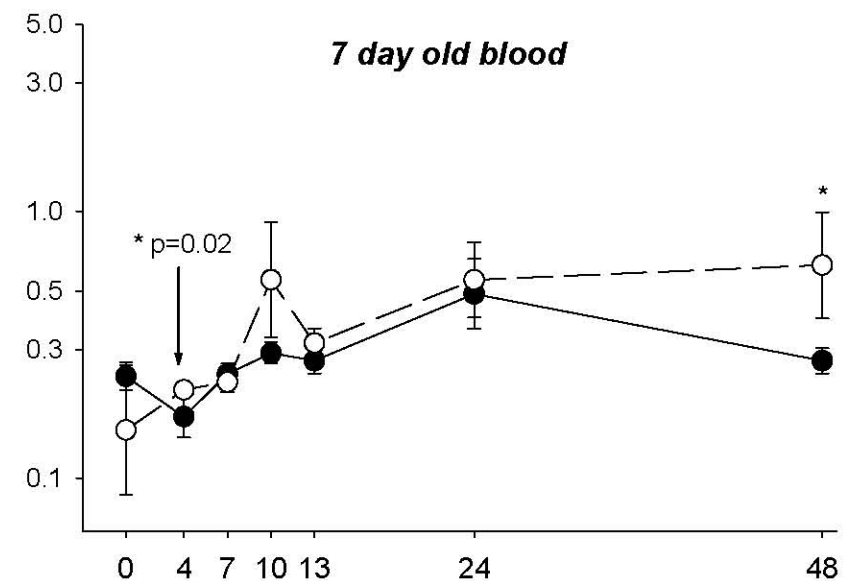
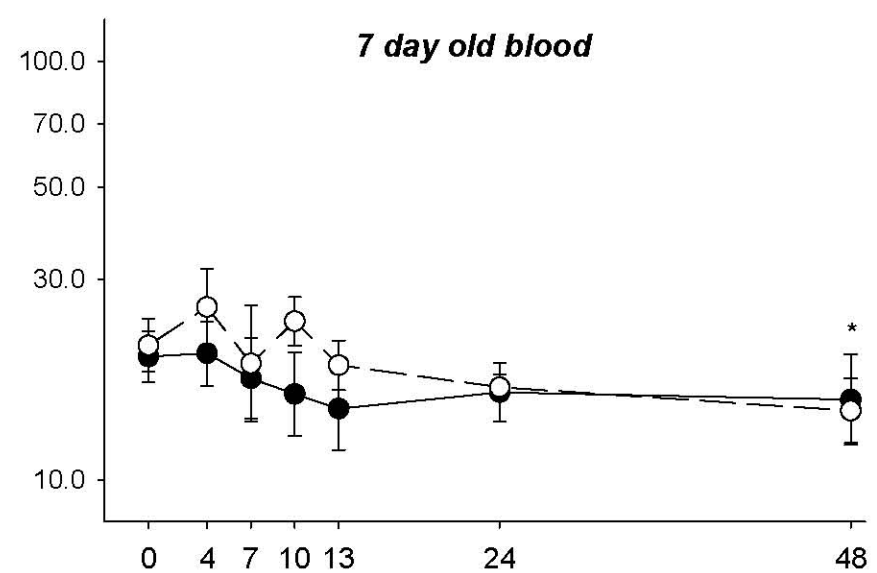
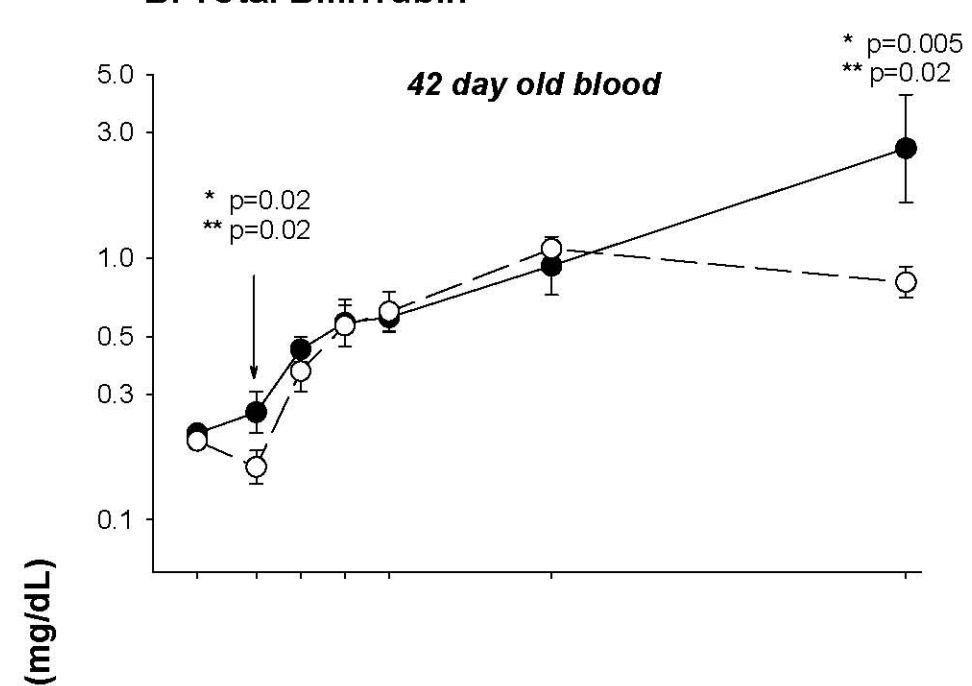


Time (hours) after *S. aureus* (1.5×10^9 CFU/kg) intrabronchial challenge

A. ALT



B. Total Bilirubin



Time (hours) after *S. aureus* (1.5×10^9 CFU/kg) intrabronchial challenge

* Qualitative Interaction; significantly different and opposite effect of washing older vs. fresher stored blood
 ** Washed stored blood has a significantly different effect than unwashed stored blood

A. 0 hours (before *S. aureus* intrabronchial challenge)

Parameter	Blood transfused			
	[mean (+ SE)]	Old Blood	New Blood	Washed Old
Blood Chemistry				
Na (mmol/L)	145.6 (1.2)	147.5 (0.6)	148 (1.3)	146 (1.6)
K (mmol/L)	3.87 (0.13)	3.99 (0.17)	4.07 (0.07)	4.13 (0.17)
Cl (mmol/L)	120.2 (1.6)	120.2 (0.4)	121.3 (1.4)	121.1 (1.8)
Glu (mg/dL)	108.8 (10.8)	107.2 (6.7)	113.8 (10.0)	110.2 (4.9)
Lac (mmol/L)	0.94 (0.14)	1.58 (0.31)	1.30 (0.30)	1.26 (0.23)
Cellular Blood Counts				
RBC ($10^6/\mu\text{L}$)	5.47 (0.32)	5.90 (0.45)	6.53 (0.47)	5.4 (0.21)
Hb(g/dL)	11.63 (0.5)	12.65 (0.9)	12.6 (1.0)	11.7 (0.48)
Hematocrit (%)	39.35 (2.7)	42.8 (2.7)	46.9 (3.8)	40.1 (1.9)
WBC	5.58 (0.57)	6.43 (0.99)	5.02 (0.87)	5.52 (0.95)
PLT ($10^3/\mu\text{L}$)	293.7 (32.8)	356.5 (31.9)	379.5 (104.7)	324.2 (24.2)
Proteins				
Total Proteins (g/dL)	4.4 (0.16)	4.9(0.19)	4.7 (0.17)	4.8 (0.16)
Albumin (g/dL)	2.5 (0.08)	2.6 (0.09)	2.6 (0.14)	2.6 (0.14)
CK (UI/L)	623.8 (64.9)	613.5 (58.6)	870.8 (116.5)	744.7 (152.6)

B. 4 hours after *S. aureus* intrabronchial challenge

Parameter	Blood transfused			
	[mean (+ SE)]	Old Blood	New Blood	Washed Old
Blood Chemistry				
Na (mmol/L)	144.9 (0.7)	147 (1.7)	146.7 (1.0)	147.7 (0.7)
K (mmol/L)	3.92 (0.15)	3.75 (0.14)	3.95 (0.07)	3.86 (0.07)
Cl (mmol/L)	121.4 (0.9)	121.3 (0.7)	121.6 (0.9)	122.1 (1.1)
Glu (mg/dL)	129 (14.4)	116.7 (8.4)	120.5 (7.0)	122.2 (5.8)
Lac (mmol/L)	1.30 (0.29)	1.30 (0.22)	1.13 (0.19)	1.08 (0.24)
Cellular Blood Counts				
RBC ($10^6/\mu\text{L}$)	6.02 (0.47)	6.39 (0.58)	5.77 (0.36)	6.18 (0.17)
Hb(g/dL)	13.0 (1.1)	13.9 (1.1)	12.0 (0.7)	13.2 (0.4)
Hematocrit (5)	43.7 (3.6)	46.9 (3.9)	41.5 (2.5)	46.6 (1.5)
WBC	4.72 (0.73)	4.9 (0.69)	4.39 (0.27)	5.25 (0.86)
PLT ($10^3/\mu\text{L}$)	306.8 (26.1)	293.5 (21.1)	278 (18.6)	335.2 (22.5)
Proteins				
Total Proteins (g/dL)	4.2 (0.15)	4.6 (0.15)	4.4 (0.16)	4.6 (0.15)
Albumin (g/dL)	2.3 (0.07)	2.5 (0.05)	2.4 (0.08)	2.5 (0.07)
CK (UI/L)	480 (75.9)	438.7 (36.9)	655.7 (63.3)	596.5 (98.1)

C. 24 hours after *S. aureus* intrabronchial challenge

Parameter	Blood transfused			
	[mean (+ SE)]	Old Blood	New Blood	Washed Old
Blood Chemistry				
Na (mmol/L)	145.9 (1.5)	153.7 (4.5)	147.1 (2.3)	150.8 (1.8)
K (mmol/L)	3.46 (0.18)	3.53 (0.24)	3.62 (0.25)	3.32 (0.18)
Cl (mmol/L)	127.1 (1.7)	134.6 (4.5)	128.9 (2.2)	131.4 (2.4)
Glu (mg/dL)	143.2 (8.0)	156.5 (7.5)	147.5 (7.4)	155.5 (5.4)
Lac (mmol/L)	0.56 (0.05)	0.64 (0.07)	0.73 (0.06)	0.70 (0.14)
Cellular Blood Counts				
RBC ($10^6/\mu\text{L}$)	5.32 (0.28)	6.2 (0.54)	5.23 (0.44)	5.58 (0.35)
Hb(g/dL)	11.0 (0.6)	12.3 (0.7)	10.7 (0.8)	11.4 (0.6)
Hematocrit (5)	37.7 (2.0)	43.4 (4.0)	37.9 (3.4)	39.3 (1.9)
WBC	2.01 (0.65)	5.41 (1.63)	2.6 (0.52)	4.01 (0.99)
PLT ($10^3/\mu\text{L}$)	132.8 (11.7)	148.3 (11.3)	145.7 (11.3)	127.2 (9.3)
Proteins				
Total Proteins (g/dL)	3.6 (0.23)	3.6 (0.22)	3.7 (0.14)	3.7 (0.13)
Albumin (g/dL)	1.6 (0.11)	1.8 (0.1)	1.6 (0.04)	1.8 (0.08)
CK (UI/L)	582 (160.7)	533.3 (144.8)	717.5 (144.7)	356.3 (78)

D. 48 hours after *S. aureus* intrabronchial challenge

Parameter	Blood transfused			
	[mean (+ SE)]	Old Blood	New Blood	Washed Old
Blood Chemistry				
Na (mmol/L)	145.8 (2.6)	150.3 (3.4)	146.1 (2.3)	150.7 (4.4)
K (mmol/L)	3.37 (0.74)	3.45 (0.43)	3.33 (0.31)	3.21 (0.18)
Cl (mmol/L)	130.0 (2.8)	133.7 (4.0)	128.6 (2.1)	133.4 (4.0)
Glu (mg/dL)	139.2 (19.8)	153.3 (10.1)	154.2 (10.9)	155.5 (5.4)
Lac (mmol/L)	0.67 (0.15)	0.48 (0.06)	0.47 (0.03)	0.67 (0.17)
Cellular Blood Counts				
RBC ($10^6/\mu\text{L}$)	4.39 (0.32)	4.86 (0.39)	3.55 (0.25)	4.79 (0.41)
Hb(g/dL)	8.7 (0.5)	9.8 (0.88)	7.14 (0.48)	9.82 (1.02)
Hematocrit (5)	30.8 (1.6)	34.22 (3.69)	25.0 (2.0)	33.5 (2.5)
WBC	4.96 (1.57)	4.82 (1.06)	3.5 (0.46)	4.32 (0.77)
PLT ($10^3/\mu\text{L}$)	97.7 (7.9)	94.17 (6.27)	66.2 (7.4)	108.2 (15.7)
Proteins				
Total Proteins (g/dL)	3.4 (0.25)	3.3 (0.14)	3.2 (0.17)	3.5 (0.29)
Albumin (g/dL)	1.5 (0.08)	1.6 (0.6)	1.4 (0.06)	1.5 (0.13)
CK (UI/L)	610 (184.9)	343.8 (151.7)	604.6 (53.7)	105.2 (9.8)