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Acute Polycythemia Increases the Disappearance Rate of Clottable Fibrinogen in the Newborn Dog

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ABSTRACT. To explore the pathophysiology of the necrotizing enterocolitis caused by polycythemia in the newborn dog, the effect of acute polycythemia on fibrinogen disappearance rate was studied in 38 puppies (3–14 days). All pups received an exchange transfusion removing 65 ml/kg of blood and transfusing 85 ml/kg of either whole blood (control, resulting hematocrit = 37), or packed red blood cells (polycythemia, resulting hematocrit = 68). Necrotizing enterocolitis was found in 15 of 19 polycythemic and four of 19 control pups ($p < 0.01$). ^{125}I fibrinogen and Evan's blue (an albumin marker) were injected 2 h after transfusion and the concentration of clottable labeled fibrinogen and albumin tracer were measured at ½ and 2 h after injection. The fraction of the tracer that disappeared over the 1½-h period was calculated. In the polycythemic group 45 ± 18 SD% of the clottable fibrinogen disappeared *versus* only 28 ± 15% in the control group ($p < 0.01$). In the polycythemic group 36 ± 21% of the albumin tracer disappeared *versus* 31 ± 12% in the control group (NS). Thus polycythemia in the newborn dog is associated with an increased disappearance rate of clottable fibrinogen not associated with a general increase in protein disappearance rate. Thus an intravascular coagulopathy is evident in the polycythemic animals. Whether this coagulopathy is the cause of the necrotizing enterocolitis or is secondary to the necrotizing enterocolitis seen in this animal model cannot be determined from this experiment. (*Pediatr Res* 20: 151–154, 1986)

Abbreviation

pH_a, arterial pH

Polycythemia is a common condition in human newborns (1) that is thought to cause significant acute disease and possibly long-term neurologic sequelae (2–9). Hakanson and Oh (10) describe an association between polycythemic hyperviscosity and necrotizing enterocolitis in small for gestation age infants. We have demonstrated that polycythemia will cause necrotizing enterocolitis in the newborn dog (11). This article, as far as we are aware, was the only description of a disease seen in human infants with polycythemia, caused by polycythemia in an animal model. Since coagulation abnormalities have been associated with polycythemia *in vitro* (12–16) we sought to determine if coagulation abnormalities could be demonstrated in our model. Attempts to demonstrate coagulopathy in human infants with polycythemia have met with varying success (17, 18). However, since we would be using an animal model and thus would not be restricted to techniques that woud be safe for the subject, we would arguably have a better chance of detecting coagulopathy if it were present.

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METHOD

The pups were randomly assigned to a polycythemic or a control group. Thirty-eight, 3- to 14-day-old puppies were used. Carotid artery catheter was placed under local anesthesia and filled with 5% dextrose with 2 U of heparin/ml. After baseline measurements of hematocrit, arterial blood gases, whole blood lactate, and blood viscosity were performed all pups received an exchange transfusion through the arterial catheter in which 65 ml/kg of blood was withdrawn in 10 ml/kg increments and replaced with 85 ml/kg of either whole blood (19 pups, control group) or packed red blood cells (19 pups, polycythemic group). This procedure is shown in Figure 1. Blood was obtained from adult dog donors, preserved with CPDA, and cross-matched against the puppy's blood. The exchange transfusion was carried out over approximately 45 min alternating withdrawal and infusion of 10 ml/kg so the pup's blood volume was never markedly disturbed. At 2 h after the midpoint of the transfusion blood was drawn for hematocrit, and fibrin degradation products and 1 μCi of ^{125}I labeled fibrinogen and 2 mg of Evan's blue both dissolved in sterile water were injected. At 2½ h posttransfusion 0.6 ml of blood was drawn for determination of ^{125}I fibrinogen and Evan's blue concentration. At 4 h posttransfusion a second sample for ^{125}I fibrinogen and Evan's blue concentration was taken along with blood for hematocrit, arterial blood gases, blood lactate, viscosity, and fibrin degradation products.

The pups were then anesthetized with 3 mg/kg of pentobarbital and 0.06 mg/kg of fentanyl and an endotracheal tube was placed and the pups were ventilated with a Harvard Instruments rodent ventilator. A 2-cm section of the right jugular vein was isolated and clamped with seraffine clamps, using the technique described by Wessler to determine physiologically the presence of activated clotting factors. The clamped section of vein was left *in situ* for 10 min, then it was removed, and opened into a Petri dish filled with 5% sodium citrate. The clots present in the vein were scooped into the Petri dish and graded 0 to 4 as described by Wessler *et al.* (20), with the grader blinded to the experimental group of the pup. A grade of 0 is assigned if no clotting is seen in the isolated vein. A grade of 4 is assigned if a complete cast of the vessel is seen. Ordinarily blood will not clot during this procedure. Clotting, a score of greater than 0, is thus abnormal and may indicate the presence of circulating activated coagulation factors.

As soon as the segment of jugular vein has been isolated from the rest of the circulation the pup was heparinized with 1000 U/kg of heparin and received an exchange transfusion of 500 ml/kg, removing blood from the carotid catheter and replacing it with heparinized saline (5 U/ml) through a catheter placed in

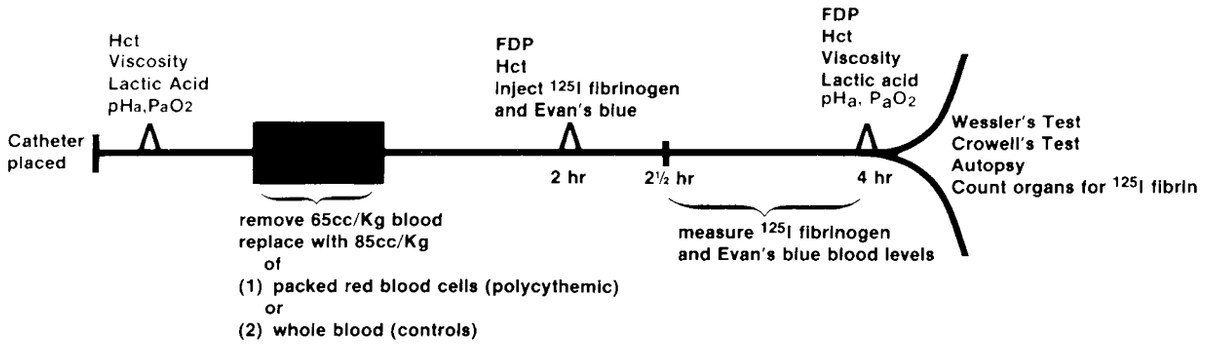

Fig. 1. Experimental protocol.

the left jugular vein. The infusion and withdrawal were syncronized to maintain vascular volume constant, although later in the exchange it became necessary for infusion to exceed withdrawal to maintain adequate output. By this method essentially all red cells were removed from the pup's body with the systemic hematocrit reduced to less than 2%. The pup was then sacrificed with 50 mg of pentobarbitol. Catheters were placed in the trachea, pulmonary artery, and the left atrium and tied so as to isolate the lungs from the rest of the circulation. The lungs were distended with 10 ml of air through the tracheal catheter, and then 20 ml of heparinized saline was infused into the left atrium and the effluent from the pulmonary artery was collected and filtered on Whatman no. 1 filter paper. The filter paper was examined under a dissecting microsope and the number of blood clots found were counted. This is the procedure described by Crowell and Reed (21) to search for microscopic clots in the vascular system. Clots that occurred in the small veins during the experiment were flushed into the lungs during the exchange transfusion with heparinized saline. Further clotting was prevented by injecting 1000 U/kg of heparin prior to the exchange. Clots that lodged in the small pulmonary arteries and arterioles were then flushed back out of the pulmonary artery on to the filter paper and counted.

Routine gross autopsy was then performed and all abnormal organs noted. The gut was examined and opened. After flushing all the blood from the animal the tissues appear very pale and it was easy to spot areas of tissue necrosis or extravascular blood because they stood out against the paleness. Areas with evident mucosal necrosis, hemorrhage into the tissues, and blood in the intestinal lumen were labeled as necrotizing enterocolitis. Gross examination for necrotizing enterocolitis was accurate in 19 of 20 cases in a previous experiment (11).

Since no changes occur in plasma volume in unfed pups between 2 and 4 h after exchange transfusion induced polycythemia (19), a change in the concentration of a plasma tracers during this period implies a change in the total amount of the tracer in the blood stream. Fibrinogen was used as a marker since it participates in blood coagulation, both as fibrin monomer in platelet aggregation and as fibrin in the final clot. Evan's blue was chosen since it binds to albumin in the blood. Albumin was used as a representative, noncoagulable plasma protein. ^{125}I fibrinogen was measured on an automated γ counter after allowing the blood to clot at room temperature for 2 h. Clot and plasma were counted separately. Evan's blue concentration was measured spectroscopically as previously described (19). The fractional disappearance rate of clottable fibrinogen is the concentration of ^{125}I fibrinogen in the bood clot at 2½ h minus that at 4 h, the quantity divided by the concentration of ^{125}I fibrinogen in the clot at 2½ h. The fractional disappearance rate of total fibrinogen is calculated using a similar formula on the concentration of fibrinogen in the combined serum and clot. Evan's blue fractional disappearance rate was also calculated using this procedure. If a change in the fractional disappearance of fibrinogen occurs in parallel with a change in the fractional disappearance of albumin, some general change in protein loss or catabolism can be implied, most probably increased leakage of protein into the interstitial fluid and lymphatics. If, on the other hand, a change in fibrinogen disappearance rate occurs without a change in albumin disappearance then it is presumably due to the special use of fibrinogen in blood coagulation.

Hematocrit was spun at 12,000 $\times$ *g* for 10 min and corrected for trapped plasma (19). Blood viscosity was measured on a Wells Brookfield cone-plate microviscometer with a 1.5° cone angle at 115, 46, 23, 11.5 s^{-1}. The viscometer was calibrated using standard oils. Whole blood lactate was measured by standard enzymatic assay as previously described (22). Fibrin degradation products were measured using the tanned red cells hemagglutination inhibition immunoassay (23) using a kit from Burroughs Wellcome.

Organs were then removed and counted on the γ counter to assess what portion of the radiolabeled fibrinogen had been deposited in each organ (essentially all blood had been washed from the organs). Results were expressed as counts/g tissue divided by injected counts/g body weight. The ear was used as the skin sample. The right flexor carpi ulnaris muscle was used for the muscle sample.

Statistics for two group comparisons were made by two tailed *t* test if conditions of normality (Kolmogorov-Smirnov, $p > 0.05$) and homoscedasticity (F test $p > 0.05$) prevailed, and otherwise by two tailed Mann Whitney U test. Comparison of discrete variables were done by the χ^2 test. Results are expressed as mean $\pm$ SD.

RESULTS

The control and polycythemic animals did not differ significantly in age, weight, sex, or initial hematocrit or viscosity, blood lactate, or pH_a, base deficit and PaO_2 (Table 1). The posttransfusion hematocrit was 39 $\pm$ 7 SD in the control group and 72 $\pm$ 5 in the polycythemic group ($p < 0.01$), and this difference persisted to the 4-h specimen (see Table 2). Posttransfusion viscosity in the polycythemic group was approximately double that in the control group ($p < 0.01$) at all shear rates. The posttransfusion pH_a was lower in the polycythemic group (7.12 $\pm$ 0.2 *versus* 7.39 $\pm$ 0.06 in the controls, $p < 0.01$) and the blood lactate levels was higher in the polycythemic group (47 $\pm$ 34 mg *versus* 23 $\pm$ 10 mg/100 ml in the controls, $p < 0.05$). Posttransfusion base deficit was higher in the polycythemic group (19.7 $\pm$ 11.4 mEq/liter in the polycythemic group *versus* 4.01 $\pm$ 2.60 mEq/liter in the control group, $p < 0.01$). Final PaO_2 was similar in the two groups. Results of the physiologic coagulation test of Wessler (*in situ* coagulation in the clamped jugular vein) and Crowell (number of blood clots flushed from the lungs) were not statistically different in the two groups. The concentration of fibrin degradation products was not statistically different in the two groups either at 2 h (22 $\pm$ 23 mg/liter in polycythemic, 21

Table 1. *Initial values (mean ± SD)*

	Control	Polycythemic
Age (day)	8 ± 3	7 ± 3
Wt (g)	571 ± 117	505 ± 110
Sex (M/F)	11/8	12/7
Hematocrit	34 ± 8	38 ± 6
Viscosity (cp)		
115 s^{-1}	3.4 ± 0.6	3.7 ± 0.9
46 s^{-1}	3.9 ± 0.9	4.5 ± 1.2
23 s^{-1}	4.6 ± 1.3	5.3 ± 1.7
11 s^{-1}	5.7 ± 2.2	7.0 ± 2.3
P_aO_2 (torr)	101 ± 15	105 ± 17
pH_a	7.38 ± 0.10	7.39 ± 0.12
Base deficit	4.16 ± 3.37	4.29 ± 3.12
Lactate (mg/dl)	27 ± 15	26 ± 12

Table 2. *Final values (mean ± SD)*

	Control	Polycythemic
Hematocrit		
2 h	38 ± 7	72 ± 5*
4 h	37 ± 6	68 ± 7*
Viscosity (cp)		
115 s^{-1}	4.0 ± 0.5	8.7 ± 1.4*
46 s^{-1}	4.9 ± 1.0	10.7 ± 1.7*
23 s^{-1}	6.1 ± 1.7	13.3 ± 2.5*
11 s^{-1}	7.8 ± 3.5	17.1 ± 3.7*
PaO_2	98 ± 23	97 ± 11
pH_a	7.39 ± 0.06	7.12 ± 0.10*
Base deficit	4.01 ± 2.60	19.7 ± 11.4*
Lactate (mg/dl)	23 ± 10	47 ± 34†
Clotting score	1.1 ± 0.5	1.6 ± 0.6
Clots in lungs	0.6 ± 0.6	0.8 ± 0.6
Fibrin degradation products (mg/liter)		
2 h	21 ± 22	22 ± 23
4 h	19 ± 15	31 ± 22

* $p < 0.01$.
† $p < 0.05$.

± 22 mg/liter in controls, $p > 0.1$) or at 4 h (31 ± 22 mg/liter in polycythemics, 19 ± 15 mg/liter controls, $0.05 < p < 0.1$), although the 4-h value could be considered borderline statistically significant. The fractional disappearance rate of clottable fibrinogen was significantly higher in the polycythemic group (45 ± 18% *versus* 28 ± 15% in the controls, $p < 0.01$) (Table 3). A smaller although still significant, $p < 0.05$, increase in the disappearance rate of total fibrinogen (clottable + nonclottable) was also seen. There was no significant change in the disappearance rate of the albumin marker. Increased fibrin deposition, roughly twice that of the control group, was seen in the jejunum, ileum, liver, heart, kidney, cerebral hemisphere, and cerebellum in the polycythemic group ($p < 0.01$ all values). No significant difference in fibrin deposition was noted for the skin, muscle, and brainstem.

Necrotizing enterocolitis was diagnosed at autopsy in 79% of the polycythemic animals but in only 21% of the controls (Table 4, $p < 0.01$).

DISCUSSION

A coagulopathy was present in the polycythemic group as evidenced by an increase in the disappearance rate of clottable fibrinogen with no change in the disappearance rate of albumin. As discussed earlier this implies that the increased disappearance of fibrinogen is due to it's utilization in blood coagulation. That is, the increase in the disappearance rate of fibrinogen cannot be explained by a general increase in protein disappearance rate. This was accompanied by an increased deposition of the marked

Table 3. *Final values (mean ± SD)*

	Control	Polycythemic
Fractional disappearance (%)		
Clottable fibrinogen	28 ± 15	45 ± 18*
Total fibrinogen	22 ± 5	31 ± 14†
Albumin marker	31 ± 22	36 ± 21
Fibrin deposition		
Jejunum	0.36 ± 0.23	0.92 ± 0.71*
Ileum	0.47 ± 0.37	0.94 ± 0.57*
Liver	0.31 ± 0.14	0.73 ± 0.66*
Heart	0.24 ± 0.11	0.53 ± 0.41*
Kidneys	0.86 ± 0.30	1.45 ± 0.73*
Cerebrum	0.03 ± 0.01	0.05 ± 0.03*
Cerebellum	0.02 ± 0.01	0.04 ± 0.04*
Brain stem	0.005 ± 0.02	0.004 ± 0.003
Skin	0.38 ± 0.09	0.38 ± 0.18
Muscle	0.29 ± 0.10	0.28 ± 0.09

* $p < 0.01$.
† $p < 0.05$.

Table 4. *Incidence necrotizing enterocolitis (NEC)*

	No NEC	NEC*	%NEC
Controls	15	4	21
Polycythemic	4	15	79

* $p < 0.01$.

fibrinogen in all of the tissues of the body except skin, muscle, and brainstem. Since the increased disappearance of fibrinogen was due to coagulation, this deposition of labeled fibrinogen is presumably intravascular coagulation. However, since most of the polycythemic animals already had necrotizing enterocolitis at the time the coagulopathy was diagnosed three possibilities remain: 1) polycythemia causes coagulopathy which then causes necrotizing enterocolitis, 2) polycythemia causes necrotizing enterocolitis which then causes a coagulopathy, and 3) polycythemia, as induced in this experiment, directly causes both coagulopathy and necrotizing enterocolitis by independent mechanisms. Since necrotizing enterocolitis is a condition in which a large amount of dead tissue exists within the body and in which bacterial sepsis is common, the second possibility is at least a plausible as the first.

There was a lower pH_a and a higher blood lactate in the polycythemic group. Very low pHs, below 7.0, are capable of causing intravascular coagulopathy (24). In theory the low pH_a in the polycythemic group could be due to an effect of polycythemia, an effect of necrotizing enterocolitis, or to a difference in administered base or acid at the time of transfusion. The principle base in CPDA blood is sodium citrate, which is present in 13 mmol/liter in whole stored blood. Citrate concentrations at least in short-term blood storage are greater in the plasma than in the packed cell fraction (References 25 and 26; 1–12 h storage *versus* 1–7 days storage in this experiment). Much of the citrate in the blood may be converted to bicarbonate in the kidney (26, 27). Red cells have more buffering capacity and thus the plasma from stored blood would have less titratable acidity than the red cells. Thus pups administered packed cells could have received more acid and less base than those receiving whole blood. In a previous experiment using a similar protocol where base excess and lactate were measured at 1 h posttransfusion, before necrotizing enterocolitis was evident although perhaps not before the pathologic process had begun, a rise in base deficit of 6 mEq/liter and a fall in pH_a of 0.07 was seen in the polycythemic group, but not in the control group [95% confidence interval (4, 8) mEq/liter base deficit, (0.00, 0.14) pH_a, study described in Reference 22, LeBlanc MH, *et al.* unpublished data]. No differences in blood lactate were seen [+1 mg/dl, 95% confidence

interval (−4, 6) mg/dL relative to controls]. Similar findings were seen in the few pups in the polycythemic group in the current experiment that did not get necrotizing enterocolitis (fall in pH_a 0.1, 95% confidence interval (−0.05, 0.25), rise in base deficit 5.1 mEq/liter, 95% confidence interval (0, 10) mEq/liter, rise in lactate −2 mg/dl, 95% confidence interval (11, −15) mg/dl, n = 4). Thus, the difference in transfused acid and base could conceivable account for as much as 50% of the acidosis seen in the polycythemic group in this study, if one uses the upper limit of the confidence interval for change in base deficit from my earlier experiment (22) attributes it all to difference in transfused acid and base and assumes no metabolic compensation occurs during the 4-h experiment. This would account for a pH_a difference of 0.14 of the 0.27 fall in pH_a seen. The remaining acidity must be attributed directly to polycythemia or to necrotizing enterocolitis caused by polycythemia. The role of these pH_a differences in the coagulopathy seen in this experiment is speculative at this time. Since only severe acidosis has been associated with intravascular coagulopathy, it is unikely that the small pH_a differences that could be attributable to the transfusion were, by themselves, a cause of the coagulopathy.

This experiment was designed to study acute changes caused by polycythemia and thus will not reflect chronic changes such as vascular remodeling (28) or hypervolemia caused by renal sodium retention (19) or other events taking more than 4 h. An increase in blood volume to approximately 110% of pretransfusion levels or 130% of control levels would be expected at 2 to 4 h posttransfusion in this animal model (19). In our study of vascular volume after induction of acute polycythemia in the newborn dog, the degree of hypervolemia present at 2 to 4 h posttransfusion was the same whether the polycythemia was induced by transfusion or by exchange transfusion.

In summary although polycythemia was associated with coagulopathy in this animal model, this experiment cannot answer the question, "does the coagulopathy cause the necrotizing enterocolitis or does the necrotizing enterocolitis cause the coagulopathy." Further experiments will be required to test these hypotheses.

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