Erythropoietin responses to progressive blood loss over 10 days in the ovine fetus

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Sohl, Bryan D., Cecilia Y. Cheung, John A. Widness, and Robert A. Brace. Erythropoietin responses to progressive blood loss over 10 days in the ovine fetus. Am J Physiol Regulatory Integrative Comp Physiol 281: R1051–R1058, 2001.—Long-term loss of fetal blood can occur with fetomaternal hemorrhage, vasoprevia, or placental previa. Our objective was to determine the effects of progressive fetal blood loss over 10 days on fetal plasma erythropoietin (EPO) concentration and its relationship to arterial PO2, hematocrit, and the volume of blood loss. Late-gestation fetal sheep (n = 8) were hemorrhaged daily at a rate of 1 ml/min over 10 days. The extent of hemorrhage differed in each fetus and ranged from 30 to 80 ml/day, with the cumulative volume removed ranging from 78 to 236 ml/kg estimated fetal weight. Four fetuses served as time controls. EPO concentration measurements were by radioimmunoassay. Statistical analyses included regression, correlation, and analysis of variance. We found that EPO and arterial PO2 were unchanged until the cumulative hemorrhage volume exceeded 20–40 ml/kg. Once this threshold was exceeded, plasma EPO concentration increased progressively throughout the study and averaged 14.3 ± 3.2 times basal values on day 10. EPO concentration, arterial PO2, and hematocrit changes were related curvilinearly to cumulative hemorrhage volume (P < 0.01), whereas the relationship between plasma EPO and arterial PO2 was log linear (P < 0.001). We conclude that 1) fetal plasma EPO concentration and arterial PO2 are insensitive to a slow, mild-to-moderate blood loss over several days; 2) unlike the rapid return of EPO to normal within 48 h after acute hemorrhage, fetal EPO concentration undergoes a progressive increase with moderate-to-severe blood loss over several days; 3) the long-term hemorrhage-induced changes in EPO are best correlated with arterial PO2; and 4) the fetal EPO response to hemorrhage does not appear to be limited by the fetus’s ability to produce EPO.

hemorrhage; fetal hypoxia; oxygen tension; sheep

THE REGULATION of plasma erythropoietin (EPO) concentration in the fetus is not well understood. Several studies have shown that fetal EPO levels are elevated after acute hemorrhage (5, 13, 14, 19). In contrast, it is not known whether fetal EPO concentrations would be elevated after a slow loss of fetal blood over many days as could occur under a number of conditions, including fetomaternal hemorrhage, vasoprevia, or placental previa. This lack of knowledge occurs in part because fetal EPO data from human and animal studies appear to be conflicting. In humans, fetal anemia or nonanemic hypoxia is associated with elevated EPO levels (15, 21, 23–25), and these elevated EPO levels have been suggested as an index of chronic fetal hypoxia. In fetal sheep, after an initial rise, plasma EPO returns to normal or near normal at ≥48 h posthemorrhage even though the fetuses remain moderately to severely anemic for many days (19, 20). Thus chronic anemic hypoxia does not appear to be associated with elevated EPO levels in the ovine fetus. Further, in fetal sheep subjected to long-term hypoxia induced by lowering maternal inspired oxygen content, plasma EPO concentration initially increases and subsequently returns to near-normal levels within a few days after initiating the hypoxemia even though fetal oxygen levels remain reduced (11). These observations suggest that chronic nonanemic hypoxia does not appear to be associated with elevated EPO levels in fetal sheep. Because the ovine fetus has not demonstrated a maintained, long-term increase in plasma EPO levels, whereas elevated EPO levels in the human fetus have been suggested to be an index of long-term hypoxia, the EPO system in human and ovine fetuses may respond differently to long-term anemic and nonanemic hypoxia. This could occur if the ovine fetus was unable to sustain high levels of EPO production for prolonged periods.

The present study was designed to explore this possibility. More specifically, we tested the hypothesis that, when subjected to progressive, moderate-to-severe blood loss over 10 days, the ovine fetus would undergo sustained or progressive increases in plasma EPO concentration rather than a return to near-normal levels after the initial increase. Another objective was to determine whether the relationship between blood loss and fetal plasma EPO concentration differs when the fetus is subjected to a slow loss of blood over many days vs. a rapid loss of blood over a few hours. To do this, we compared the relationships of fetal plasma EPO concentration with arterial PO2, hematocrit, and the volume of blood lost in the present study and in a recently reported acute hemorrhage study (5).

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MATERIAL AND METHODS

These studies were conducted in 12 chronically catheterized, late-gestation fetal sheep that were 124.7 ± 0.6 (SE) days gestation on the first day of the study (term = 150 days). Each ewe carried a single fetus. The protocol was approved by the University of California San Diego Animal Subjects Committee before the studies were initiated, and we followed the National Research Council’s Guide for the Care and Use of Laboratory Animals throughout the study.

The surgical preparation of the animals has been described in detail elsewhere (1, 2, 21). In brief, under inhalation anesthesia using aseptic techniques, indwelling plastic catheters were placed through fetal and maternal femoral arteries into the descending aorta. Prophylactic antibiotics (22) were given at the time of surgery and on the 5 subsequent days. Amniotic fluid volume responses in these animals have been reported elsewhere (22).

Experiments began 5 or 6 days after catheter placement. On experimental days 1–10, 2 ml of fetal arterial blood were removed for determination of microhematocrit in triplicate, blood gases, and pH (Nova, Stat Profile Ultra Analyzer, corrected to fetal body temperature of 39.5°C), plasma EPO concentration by radioimmunoassay (10), and plasma iron concentration by electrochemical detection (10). After daily sampling, eight fetuses were subjected to a fixed daily hemorrhage at a rate of 1 ml/min for the first 9 days and were subjected only to sampling on day 10. The extent of the daily hemorrhage differed among individual fetuses to explore the effects of different degrees of anemia and included 30 (n = 1), 40 (n = 1), 60 (n = 4), 70 (n = 1), and 80 (n = 1) ml/day so that the total volume of blood removed ranged from 290 to 740 ml over 10 days. For comparison, ovine fetuses at this gestational age have an average blood volume of ~375 ml (1). In two of the eight hemorrhaged fetuses, the daily volume of blood removed was reduced from 60 to 25–35 ml/day for days 4–9 because low arterial Po2 values raised concerns about impending fetal death. The remaining four fetuses served as time controls with no additional blood removed. The cumulative volume removed by hemorrhage represents the total volume removed at the time each sample was taken, including the sample itself. The volume of blood removed by hemorrhage after the daily measurements is included in the following day’s cumulative volume.

After the final blood sample and measurements on experimental day 10, the animals were euthanized (130 ml/kg pentobarbital sodium given intravenously). Fetal weight was determined after towel drying.

Data presentation and statistical analyses. The data were divided into control and hemorrhage treatment groups and are presented as means ± SE. Changes over time in a single treatment group were analyzed with a two-factor repeated-measures analysis of variance (ANOVA), with time and subject being the factors tested. To compare the effects of treatment over time, a three-factor repeated-measures ANOVA was used, with time, treatment, and subject being the factors. Differences between treatment groups were determined by a significant interaction between time and treatment. Significance was accepted at P = 0.05. When the null hypothesis was rejected with the ANOVA, post hoc testing used Fisher’s least significant difference for multiple comparisons.

To explore relationships between and among variables, bivariate, polynomial, multivariate, and nonlinear correlation and least squares regression were used to analyze both absolute values and changes from the initial values. A nonlinear regression model that allowed for a flat relation with no change over a portion of the curve followed by a straight line sloping either upward or downward was used because some fetal variables were insensitive to mild or moderate hemorrhage but changed with more severe hemorrhage. This model provided a better fit than the other regression models and has the added advantage that it helps define the threshold required for a response to occur. For the correlation and regression analyses, data from the control and hemorrhaged fetuses were combined by using 120 values (12 animals × 10 days) for each variable.

Multivariate regression was also performed on data from our recent acute hemorrhage study (5) to explore potential differences in responses to acute, moderately severe hemorrhage over 2 h vs. repeated hemorrhage over 10 days. The multivariate analysis was performed by starting with the independent variable that had the strongest correlation (i.e., the greatest r value), adding variables one at a time, and removing those that did not achieve statistical significance. This process was repeated until all of the included independent variables were significantly related to the dependent variable and none of the excluded were.

Base 10 logarithmic transformation was used to normalize distributions before statistical analysis if needed. Cumulative hemorrhage volumes were normalized by dividing by the estimated fetal weight on each day of the study. Estimated daily fetal weights were calculated from the weight at autopsy assuming that the control fetuses grew 3%/day, that the fetuses that hemorrhaged <60 ml/day grew at 2%/day, and that the fetuses that hemorrhaged >60 ml/day grew at 1%/day. Although the accuracy of these estimates is unknown, they were based on the observations that our ovine fetuses normally grow at ~3%/day (6) and that ovine fetuses made severely anemic by hemorrhage over 7 days reduced their growth rate to ~1%/day (8). Hematocrit units are percent, and a 10% decrease in hematocrit corresponds to a decrease from 30% to 20%.

RESULTS

The cumulative volume of fetal blood removed over the 10-day study was 6.3 ± 0.3 ml/kg estimated fetal weight (20 ml/fetus) for the control animals compared with 143 ± 19 ml/kg in the hemorrhaged fetuses (range 290–740 ml/fetus).

Fetal hematocrit in the latter group decreased progressively from its prehemorrhage value of 33.9 ± 1.1% to 17.5 ± 1.3% on day 10 for a net decrease of 16.4 ± 1.2% (Fig. 1A), while hematocrit in the control fetuses underwent a small but significant increase (2.3% ± 0.8%, P = 0.012). Compared with control fetuses, hematocrit became significantly lower from day 2 onward when hemorrhaged volume was 19.6 ± 1.9 ml/kg. Over the 10-day study, the relationship between change in hematocrit (ΔHct) and cumulative hemorrhage volume (Fig. 1B) was nonlinear (r = 0.967, P < .0001), with hematocrit decreasing as hemorrhage volume exceeded ~10 ml/kg. On a linear scale (not shown), the decrease in hematocrit was approximately linearly related to the volume of blood removed until the hemorrhage volume exceeded 100 ml/kg, after which the change in hematocrit per unit volume removed decreased. As hemorrhage volumes exceeded 150 ml/kg, there was little further change in hematocrit.

Fetal arterial Po2 was 23.0 ± 0.5 mmHg on day 1 and decreased significantly in the hemorrhaged but not the control fetuses (Fig. 2A). In hemorrhaged fetuses, ar-
arterial PO₂ became significantly lower than in control fetuses from day 5 onward when hemorrhaged volume was 71.6 ± 7.6 ml/kg. This insensitivity of arterial PO₂ to blood loss is reflected by the relatively flat regression relation with cumulative blood loss for hemorrhage volumes up to ~40 ml/kg as shown in Fig. 2B. Afterward, arterial PO₂ decreased sharply as increasingly larger cumulative blood volumes were removed. Arterial pH was unchanged with time in both groups as was arterial PCO₂, averaging 7.354 ± 0.014 and 55.7 ± 2.1 mmHg, respectively, over the 10 days.

Fetal plasma EPO concentration did not change significantly with time in the control fetuses, averaging 25 ± 2 mU/ml. In contrast, in the hemorrhaged fetuses, EPO increased progressively over the 10-day study, averaging 354 ± 82 mU/ml on day 10 (Fig. 3A). When we compared changes with time in the hemorrhaged and control fetuses, EPO became significantly higher from day 3 onward when hemorrhaged volume was 38.8 ± 3.8 ml/kg. From regression analysis, plasma EPO concentration was unchanged until the hemorrhage volume exceeded 20 ml/kg (Fig. 3B), after which it increased exponentially with the greater volumes removed.

Changes in fetal plasma iron concentrations were reciprocal to those of plasma EPO concentration in hemorrhaged fetuses, with values greatly decreasing to average 26 ± 2 μg/dl on days 6–10 (Fig. 4A). In the
control animals, plasma iron remained above 100 μg/dl and did not change with time. There was a significant correlation between plasma iron concentration and the cumulative hemorrhage volume (Fig. 4B).

At autopsy, the four control and eight hemorrhaged fetuses were 132.0 ± 0.4 and 134.5 ± 1.8 days gestation and weighed 3.22 ± 0.17 and 3.26 ± 0.08 kg, respectively. The weights did not differ significantly although the hemorrhaged fetuses were slightly older (P = 0.025).

To explore potential factors associated with the increase in plasma EPO concentration, bivariate and multivariate regression were used. With bivariate regression, we found the strongest relationships between the logarithm of EPO concentration and either ΔHct or the absolute arterial Po2 (Fig. 5). Multivariate regression yielded the following equation: log [EPO] = 2.523 − 0.0495 × PaO2 (P < 0.0001) − 0.0297 × ΔHct (P < 0.0001) + 0.015 × ΔPaCO2 (P = 0.003), where PaO2 and PaCO2 are arterial Po2 and arterial PCO2, respectively, and with r = 0.853, P < 0.0001 overall (n = 120 values). When analyzed simultaneously, none of the other variables, including gestational age, cumulative hemorrhage volume, the duration of hemorrhage, absolute arterial pH, ΔpH, ΔPaO2, absolute arterial PCO2, or absolute Hct were statistically related to EPO concentration.

Because the above statistical relationship between EPO and ΔPaCO2 was unexpected, multivariate regression was also performed on the same variables from
our recently reported 10-day study in which similarly aged ovine fetuses were either control, hemorrhaged 40% of their blood volume over 2 h on day 3, or hemorrhaged 40% plus given 60 mg of iron intra-amniotically on day 3 (5). For combined data from the latter three groups of animals, the regression equation was \[ \log [EPO] = 1.955 - 0.018 \times Hct + 0.0403 \times \Delta Hct + 0.0202 \times \Delta PaCO_2 \] with \( r = 0.623, P < 0.0001 \) overall (\( n = 198 \) values).

**DISCUSSION**

In the present study of ovine fetuses subjected to daily blood loss, plasma EPO concentration not only increased in response to hemorrhage but also increased progressively by 1 order of magnitude as the cumulative blood loss increased over the 10-day study. This response is different from the transient increase in EPO concentration with a return to normal within a few days in fetal sheep subjected to a fixed level of maternal hypoxemia (11). The response is also different from the initial increase and subsequently returned to near-normal levels at \( \geq 48 \) h posthemorrhage in fetal sheep subjected to a fixed level of maternal hypoxemia (11). The response is also different from the initial increase and subsequently returned to near-normal levels at \( \geq 48 \) h posthemorrhage in fetal sheep subjected to a fixed level of maternal hypoxemia (11). The response is also different from the initial increase and subsequently returned to near-normal levels at \( \geq 48 \) h posthemorrhage in fetal sheep subjected to a fixed level of maternal hypoxemia (11). The response is also different from the initial increase and subsequently returned to near-normal levels at \( \geq 48 \) h posthemorrhage in fetal sheep subjected to a fixed level of maternal hypoxemia (11). The response is also different from the initial increase and subsequently returned to near-normal levels at \( \geq 48 \) h posthemorrhage in fetal sheep subjected to a fixed level of maternal hypoxemia (11). The response is also different from the initial increase and subsequently returned to near-normal levels at \( \geq 48 \) h posthemorrhage in fetal sheep subjected to a fixed level of maternal hypoxemia (11). The response is also different from the initial increase and subsequently returned to near-normal levels at \( \geq 48 \) h posthemorrhage in fetal sheep subjected to a fixed level of maternal hypoxemia (11). The response is also different from the initial increase and subsequently returned to near-normal levels at \( \geq 48 \) h posthemorrhage in fetal sheep subjected to a fixed level of maternal hypoxemia (11). The response is also different from the initial increase and subsequently returned to near-normal levels at \( \geq 48 \) h posthemorrhage in fetal sheep subjected to a fixed level of maternal hypoxemia (11). The response is also different from the initial increase and subsequently returned to near-normal levels at \( \geq 48 \) h posthemorrhage in fetal sheep subjected to a fixed level of maternal hypoxemia (11). The response is also different from the initial increase and subsequently returned to near-normal levels at \( \geq 48 \) h posthemorrhage in fetal sheep subjected to a fixed level of maternal hypoxemia (11). The response is also different from the initial increase and subsequently returned to near-normal levels at \( \geq 48 \) h posthemorrhage in fetal sheep subjected to a fixed level of maternal hypoxemia (11). The response is also different from the initial increase and subsequently returned to near-normal levels at \( \geq 48 \) h posthemorrhage in fetal sheep subjected to a fixed level of maternal hypoxemia (11). The response is also different from the initial increase and subsequently returned to near-normal levels at \( \geq 48 \) h posthemorrhage in fetal sheep subjected to a fixed level of maternal hypoxemia (11). The response is also different from the initial increase and subsequently returned to near-normal levels at \( \geq 48 \) h posthemorrhage in fetal sheep subjected to a fixed level of maternal hypoxemia (11). The response is also different from the initial increase and subsequently returned to near-normal levels at \( \geq 48 \) h posthemorrhage in fetal sheep subjected to a fixed level of maternal hypoxemia (11). The response is also different from the initial increase and subsequentl...
in hematocrit may have allowed fetal cardiac output and regional blood flow to increase (8) so that tissue oxygen delivery was unchanged, whereas fetal cardiac output may not have increased further with the more severe hemorrhages. Alternatively, increased fetal erythropoiesis over the 10-day study may have contributed to the insensitivity to mild or moderate hemorrhage. Although increased erythropoiesis undoubtedly occurred as evidenced by the very large fall in plasma iron concentration, hematocrit decreased in the present study with mild-to-moderate hemorrhage, while arterial Po2 was unchanged so that an increased cardiac output with increased regional blood flow appears the more likely explanation. Another possible contributor is that EPO clearance may have increased so the increases in EPO production with mild-to-moderate hemorrhage may not have been reflected in an increased plasma concentration. These possibilities have yet to be explored. However, it should be noted that, in fetal sheep in which hemoglobin is converted to methemoglobin, EPO increased only after blood oxygen content was reduced by 33% (16). This observation is consistent with the threshold observed in the present study and suggests that there may be a general insensitivity of the fetal EPO system to mild-to-moderate changes in blood oxygen content until the threshold is exceeded. Alternatively, there may have been a small transient increase in plasma EPO concentration after the mild hemorrhage, with a return to normal before sampling at 24 h posthemorrhage. However, this possibility is not supported by a study of acute hemorrhage in which EPO was not significantly altered at 2, 4, 6, or 24 h posthemorrhage (13).

With blood losses more extensive than 20–40 ml/kg, fetal EPO concentration began to increase as arterial Po2 began to decrease, suggesting that the fall in arterial Po2 and oxygen delivery was driving the rise in EPO. This is consistent with our observation that EPO was negatively correlated with fetal arterial Po2 and is similar to reports of EPO being inversely correlated with cord blood Po2 in human fetuses (17). EPO was also correlated with ΔHct and ΔPaCO2 in the present study. The correlation of EPO with ΔHct is expected because a change in hematocrit is an important determinant of blood oxygen content and hence oxygen delivery to the EPO-producing tissues. The correlation of EPO with ΔHct, but not absolute hematocrit, is interesting in that it suggests that each fetus adapted to its normal hematocrit, whatever that might be. This may occur through changes in blood flow due to viscosity changes as fetal anemia has been shown to produce major changes in fetal cardiac output and regional blood flow distribution (8).

The positive statistical correlation of fetal arterial plasma EPO concentration with ΔPaCO2 but not with absolute arterial PCO2 is difficult to interpret. In diabetic pregnancies without labor at Cesarean delivery, human umbilical EPO concentration correlated positively with arterial PCO2, and in offspring of hypertensive patients, cord EPO correlated negatively with arterial pH and base excess (23). It remains unclear whether there might be mechanistic reasons for fetal EPO to correlate with either ΔPaCO2 or absolute arterial PCO2. The multivariate regression finding that log [EPO] was positively correlated with ΔPaCO2 in both this study and in a reanalysis of data from our recent 2-h fetal hemorrhage study (5) suggests this may not be due to random chance. However, the EPO gene has not been demonstrated to be sensitive to changes in arterial PCO2 (9). The relationship between EPO and ΔPaCO2 may be due to the unique sources of EPO in the fetus. In the adult, the kidneys are the primary source of EPO. In the fetus, the placenta (which is fetal tissue), kidneys, and liver may all be major sources of circulating EPO because these tissues express EPO mRNA (7, 12, 24). Maternal plasma is not a source because EPO does not cross the placenta in humans and sheep (18, 27). Furthermore, plasma EPO concentration and hematocrit are normal in anephric human and ovine fetuses (12, 15, 26, 29). Studies are needed to identify the source(s) of EPO in the fetus and clarify why plasma EPO concentration may be related to arterial PCO2 changes.

Currently, it is not known whether the regulation of EPO production in the fetus adapts over time to accommodate changes in oxygen availability or other environmental factors. The present study does not suggest such adaptation because the multivariate regression relationships between fetal plasma EPO concentration and the other fetal variables during the present 10-day progressive hemorrhage study was surprisingly similar to the multivariate regression relationship for fetuses subjected to a 40% hemorrhage over 2 h. Both experiments yielded similar dependencies of EPO on ΔHct and ΔPaCO2. The only difference in the multivariate regression equations was that one showed dependence on arterial Po2, whereas the other depended on Hct. This may not be a true difference as both arterial Po2 and Hct are major determinants of blood oxygen content.

In the hemorrhaged fetuses, plasma iron concentration decreased dramatically, averaging only 25% of its day 1 value on days 6–10. This decrease of 75% in plasma iron concentration is much greater than the transient 20–30% decrease seen in ovine fetuses 2–3 days after an acute 40% hemorrhage (5) and is greater than the 50–60% decrease after a 105% hemorrhage over 3 days (19). Although blood volume was not measured in the present study, the cumulative blood loss of 143 ± 19 ml/kg corresponds to a loss of 127% of the fetus's estimated day 1 blood volume as ovine fetal blood volume averages 113 ml/kg fetal body wt (1). Thus it appears that the fall in fetal plasma iron concentration is proportional to the extent of blood loss over a wide range in the sheep fetus. Furthermore, erythropoiesis may be severely compromised at such low iron concentrations because fetal red blood cell mass expansion is linearly related to plasma iron concentration for iron concentrations below 200 μg/dl (5).

In the present study, the changes in hematocrit were correlated with the cumulative hemorrhage volumes, raising the possibility that the extent of the blood loss
can be estimated from the changes in hematocrit. From regression analyses, the standard error of the estimate (SEE) was 13.5 ml/kg for volumes up to 150 and 21.2 ml/kg fetus for hemorrhage volumes up to 235 ml/kg. From these values, the errors in estimating the extent of blood loss would range from ±27 to ±42.4 ml/kg (i.e., ±2 × SEE). We conclude that the volume of blood lost over 10 days cannot be accurately estimated from changes in hematocrit. On the basis of the changes observed in plasma iron, this is probably due to ongoing and augmented fetal erythropoiesis combined with an expanding blood volume as the fetus grows.

The major finding of this study is that, in contrast to the transient increase in fetuses subjected to either sustained hypoxia or to sustained anemia produced by hemorrhages over 2 h or 3 days, fetal plasma EPO concentration undergoes a sustained and progressive 10- to 15-fold increase when repeated fetal blood loss occurs over many days. This most likely is due to a sustained reduction in blood oxygen content and tissue oxygen delivery as suggested by the significant correlations observed for plasma EPO concentration with hematocrit change and with arterial Po2.

Perspectives

In the human fetus, umbilical cord blood sampling often reveals that fetal EPO levels are elevated, sometimes grossly so. In the sheep fetus, hemorrhage that removes 40% of the fetus’s initial blood volume over 2 h or removes 105% of the initial blood volume over 3 days is followed by a sharp rise in fetal plasma EPO concentration at 24 h posthemorrhage, with a return to near-normal EPO levels at 48 h posthemorrhage and thereafter, even though fetal anemia persists. The present study clearly shows that this posthemorrhage return to near-normal fetal plasma EPO concentrations is not due to a limited ability of the fetus to produce EPO. Instead, the stimulus to the EPO gene must be returning to near-normal levels. A large adaptive reduction in fetal oxygen consumption in the posthemorrhage period may be the primary mechanism that mediates the return of plasma EPO concentration to near-normal levels. Furthermore, if the EPO-regulatory mechanisms are similar, the present study suggests that a grossly elevated plasma EPO concentration in the human fetus reflects either an acute stress or a severe chronic stress, such as continuing blood loss. This implies that such fetuses need to be carefully managed.

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