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Title: Anemia of prematurity : time for a change in transfusion management?

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**Erythropoietin levels in premature neonates
in relation to red blood cell transfusions**

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Submitted

Abstract

Objective: The role of erythropoietin (EPO) in physiological anemia of the premature newborn (AOP) and its relation with red blood cell (RBC) transfusions is still unclear and may depend on neonatal complications, frequent phlebotomies and transfusion policy.

Study design: Using waste material we frequently measured endogenous EPO levels in 46 premature neonates born < 36 gestational weeks, in their first month of life. These levels were correlated to hematological parameters when concomitantly determined, clinical parameters and administered transfusions.

Results: Thirty-six of 46 neonates received ≥ 1 transfusions starting at median day 4. EPO levels were not correlated with individual (pre-transfusion) hemoglobin levels, although overall higher EPO levels were present in the transfused cohort in the first 4 days after birth (8mU/mL; 5-95 percentile range <1.4–112.5 mU/mL) compared to the period thereafter (3mU/mL; 5-95 percentile range <1.4–23.9 mU/mL) ($p < 0.001$). In neonates with a lower Apgar score and respiratory failure higher EPO levels were observed. EPO levels declined after every administered RBC transfusion; albeit this was only statistically significant after the first transfusion. Incidental high EPO levels > 500mU/mL were associated with life-threatening conditions.

Conclusion: Newborns with AOP and requiring transfusions had higher initial EPO levels, which declined after starting transfusion treatment. We did not find a statistically significant suppressive effect of cumulative RBC transfusions in the first month after premature birth.

Introduction

Transfusion of red blood cells (RBC) is an important element in the treatment of premature neonates. These transfusions have been associated with potential negative clinical outcomes, although causality has not been proven.¹⁻² Introduction of transfusion guidelines has led to fewer administered transfusions. Nowadays, most transfusions are administered in the first month of life after premature birth.³ The transfusion needs in these first weeks result from a combination of physiological and iatrogenic factors. These include the short life span of fetal RBCs, an increasing blood volume due to relative rapid growth, an inadequate erythropoietin (EPO) response to the level of anemia and frequent phlebotomies for diagnostics.⁴⁻⁵ In premature neonates EPO is mainly produced by the liver, which is less prompt responsive to hypoxia. This results in a lower EPO level than would be expected for the degree of anemia.⁶ Per transfusion, premature neonates receive 10 to 20 mL packed RBCs (with an hematocrit (Ht) between 0.60 and 0.80 L/L) per kg bodyweight, depending on local transfusion practice and guideline.⁷ This results in a wide range of RBC volume administered. These differences in transfusion practice probably suppress EPO production in variable degrees. Few studies investigated the effect of RBC transfusion on EPO levels and they reported transfusion related suppression of EPO within 48 hours to 7 days after RBC transfusion.⁹⁻¹⁰ This suppression normalized 14 days after transfusion.¹⁰ However, most studies reported on premature neonates with a postnatal age of > 4 weeks who already had received multiple transfusions before the EPO levels were measured.⁹⁻¹⁰ A small recent study which used a RBC product with a Ht of 0.80-0.85 L/L, transfused approximately twofold of the hemoglobin (Hb) mass that was lost due to blood withdrawal and in this study the EPO production was found suppressed by every transfusion.⁸

The effects of a liberal transfusion policy, aiming to maintain a higher hematocrit, on the neuro-development of premature neonates have been studied. These studies however showed conflicting results. Maintaining a higher hematocrit was associated with a better cognitive development at the age of 18-21 months corrected age¹¹, but also associated with a worse neuro-cognitive profile and a reduced brain volume.¹²⁻¹³ A postulated explanation was the suppression of endogenous EPO production due to liberal transfusion management. Lower EPO levels could result in a decreased anti-inflammatory function and impaired cell recovery after brain injury.¹²⁻¹⁴ However, in a recent report on acute physiological effects of RBC transfusions, the mean EPO levels before RBC transfusion were similar between the patients in the group transfused at a higher hematocrit compared to the lower hematocrit group.¹⁵ Unfortunately, in this study the effects of multiple RBC transfusions on EPO levels were not reported, so a stronger reduction of EPO after a liberal transfusion strategy cannot be precluded.

Recently it was proposed that administration of EPO after severe brain injury resulting from asphyxia or intra-ventricular hemorrhage could be neuro-protective, but the most optimal EPO dosage regimen is unclear. Besides a presumed deleterious effect of EPO treatment on retinopathy

of prematurity, also neuro-toxic effects of high dose EPO are possible.¹⁴ More subtle brain damage in newborns could however be negatively affected by early RBC transfusions by lowering the endogenous EPO level that may intercede with a potential physiological neuro-protective EPO effect.^{12-13, 16} Evaluation of intrinsic EPO levels after premature birth in presence or absence of RBC transfusions can attend studies investigating optimal EPO dosages, especially those focusing on early treatment. Few studies are available that report endogenous EPO levels and the effect of RBC transfusions through the first weeks of life after premature birth. Non-transfused premature neonates were reported to have a mean EPO level of 9.7 mU/mL (range ± 1 SE 7.4-10.3 mU/mL) during the first month of life.¹⁷ Anemic premature neonates were reported to have higher (anemic 20 ± 1.08 mU/mL vs non-anemic 14 ± 1.06 mU/mL) EPO levels.¹⁸ Yamashita studied both non-transfused and transfused very low birth weight premature neonates and reported EPO levels in the first week of life ranging from < 5 -307 mU/mL, while beyond this first week EPO levels were < 20 mU/mL (day 7-50 after birth).¹⁹

In this report we frequently measured EPO levels in the first weeks of life using waste material in relation to pre-transfusion Hb and administered RBC transfusions using a restrictive transfusion trigger in a group of 46 premature neonates born before 36 gestational weeks.

Material and Methods

Study population and material

In six consecutive months, 46 premature neonates born before 36 gestational weeks admitted at our neonatal intensive care unit were included in this study. Exclusion criteria were congenital abnormalities, hemolytic disease of the newborn and neonates in need of surgery. None of the neonates received recombinant human EPO. The follow-up period was up to 1 month after delivery or until discharge to another hospital or home. Clinical data of the study patients were collected retrospectively. Data on birth weight, gender, Apgar scores, number of RBC transfusions, type of respiratory support, sepsis (clinically suspect and/or positive blood culture), severe intra-ventricular hemorrhage ($\geq$ grade 3) and mortality were registered.

Transfusion policy and RBC product

All patients were transfused according to the operative Dutch guideline for blood transfusion. The triggers in short; within the first 24 hours after birth $< \text{Hb } 8 \text{ mmol/L}$ (13g/dL); stable neonates with cardio-respiratory problems and/or mechanical ventilation $< \text{Hb } 7 \text{ mmol/L}$ (11g/dL); postnatal age < 4 weeks: $< \text{Hb } 6 \text{ mmol/L}$ (10g/dL).²⁰ The standard transfusion product consisted of pre-storage filtered adult RBC stored in saline adenine glucose-mannitol with a hematocrit of $0.60 \pm 0.05 \text{ L/L}$, irradiated if transfused to an neonate weighing $< 1500 \text{ g}$ or gestational age < 32 weeks. The transfusion volume was 15 mL per kg body weight.

EPO measurements

Waste material from these neonates was collected from our clinical chemical laboratory. Before collection, all material was kept between 2-6°C up to 72 hours after blood withdrawal, thereafter all material was frozen and kept at -40°C. The EPO Enzyme Linked Immuno Sorbent Assay (ELISA) (IBL, Hamburg, Germany) was used for all measurements. The lower limit of detection of the EPO ELISA was 1.4 mU/mL. Per single measurement, at least 50 µL material was necessary. To validate the use of waste material refrigerated for up to 72 hours, we aliquoted plasma from premature neonates, cord blood and adult blood bank donors which was kept at varying time intervals at 2-6°C; <24 hours, between 24-48 hours and up to 72 hours until freezing at -40°C. Additional spiking experiments were performed with respectively 10 and 20 mU EPO alpha per sample. All tests were performed in duplicate. EPO levels remained stable at 2-6°C during the time series in all samples (R^2 : 0.998), indicating that the waste material could be used for testing.

Outcome parameters and statistical analysis

EPO levels were correlated to hematological parameters (Hb, hematocrit, number of nucleated RBC) if the waste material was drawn at the same time as the complete blood count. The first EPO value after birth was correlated to clinical parameters at birth (gestational age, birth weight, Apgar score ≤ 6 at 5 minutes after birth). We also examined EPO levels according to the method of respiratory support and to clinical and/or proven sepsis. Subsequent EPO levels were correlated to the number of administered RBC transfusions prior to EPO measurement. All EPO measurements that fell below the detection level of 1.4 mU/mL, were counted as 0 mU/mL. To test the correlation between continuous variables the coefficient of determination R^2 was calculated. We used a linear mixed model analysis for repeated measures to test changes in EPO levels before and after a RBC transfusion. The fixed effect was the number of administered RBC transfusions up to that moment. The number of measurements per patient differed as well as the number of administered transfusions per patient. Therefore both the patient variable and the number of administered transfusions at the time of EPO level measurement, were included as covariates to correct for inter-patient and intra-patient variability. The distribution of EPO levels was highly skewed, with the most data having low values and a some (extreme) high values. To normalize the data distribution, logarithmic transformation was applied to the values. To include the measurements of '0' mU/mL, a constant was added to all measurements. The log-transformed data were used in the analysis. The mean estimates were computed by back-transformation of the log-means. A p -value of <0.05 was considered statistically significant.

Ethics

In the Netherlands no ethical approval is required for this type of research with the use of waste material, as no new intervention or treatment is studied. All collected data were anonymous.

Results

Study population

The 46 neonates are described in Table 1. Thirty-six neonates (78%) received RBC transfusions, with a mean of 2.6 ± 1.5 transfusions per neonate in the first month after birth. The first RBC transfusion was administered at median day 4 after birth (95% range 1-9 days). The mean pre-transfusion hematocrit was 0.34 ± 0.04 L/L and the mean post-transfusion hematocrit was 0.40 ± 0.04 L/L. The mean Ht of non-transfused neonates was 0.46 ± 0.03 L/L in the first week after birth, and 0.36 ± 0.05 L/L in the weeks thereafter. The transfused neonates were more premature compared to the non-transfused neonates, had a lower birth weight and suffered from more complications. More material had been collected from transfused neonates compared to non-transfused premature neonates; reflecting that these children were sicker and exposed to more phlebotomies for diagnostic purposes and consequently more anemic. Transfused and non transfused neonates differed significantly (Table 1); both patient groups were analyzed separately as the non-transfused neonates could not serve as controls for the transfused neonates.

Table 1: Patient characteristics and measured erythropoietin levels

	Transfused (n=36)*	Non-transfused (n=10)*
Gestational weeks, mean (range)	27 4/7 (25 4/7-35 0/7)*	31 0/7 (29 1/7-32 2/7)*
Birth weight, g mean $\pm$ SD	1052 $\pm$ 256*	1359 $\pm$ 258*
Gender - Boys: Girls, n	26:10*	4:6*
Apgar score at 1 minute, median (range)	5 (1-9)	7 (2-10)
Apgar score at 5 minutes, median (range)	8 (6-9)	9 (5-10)
Respiratory support, % neonates (n)	100% (36)	70% (7)
Mechanical ventilation [‡] , days median (range)	9 (1-22)*	0 (0-3)*
Clinical Complications		
Sepsis [§] , % neonates (n)	64% (23)	40% (4)
Severe IVH ($\geq$ grade 3), % (n)	8% (3)	0% (0)
Mortality, % (n)	6% (2)	0% (0)
Mean Hb level, mmol/l, week 1	8.3 $\pm$ 1.0	10.1 $\pm$ 1.4
Mean Hb level, mmol/l, after week 1	8.3 $\pm$ 0.9	8.4 $\pm$ 1.5
Samples collected after premature birth, n	336	69
-Week 1, n samples	151	37
-After week 1, n samples	185	32
Median Erythropoietin levels mU/mL - first 4 days (5-95 percentile range)	8.0 (<1.4 – 112.5)*	<1.4 (<1.4 – 60.6)
Median Erythropoietin levels mU/mL - after day 4 (5-95 percentile range)	3.0 (<1.4 – 23.9)*	3.0 (<1.4 – 45.6)

Abbreviations: IVH: intra-ventricular hemorrhage

* *p-value* <0.05, † *p-value* <0.001

[‡] Synchronized Intermittent Mandatory Ventilation or High Frequency Oscillation, [§] defined by clinically suspect and/or positive blood culture

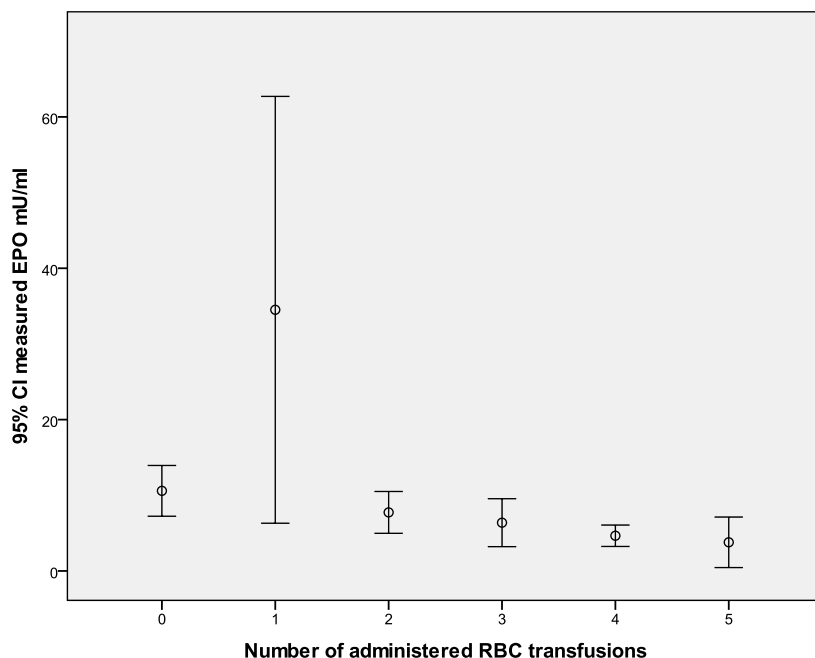


Figure 1: EPO levels measured in the group of transfused neonates (n=36).

The mean and 95% Confidence Interval of the EPO levels (mU/mL) were plotted against the number of administered RBC transfusions at the time of EPO measurement.

0 = before RBC transfusion

Endogenous EPO levels

Seventy percent (n=405; n=336 from transfused neonates / n=69 from non-transfused neonates) of the collected waste samples could be used for EPO measurements in duplicate. Most samples were collected in the first two weeks of life. In the group of neonates that required RBC transfusion (n=36), the first EPO levels measured within 24 hours after birth were not correlated with the first measured Hb concentration (R^2 : 0.102), birth weight (R^2 : 0.204), gestational age (R^2 : 0.102). EPO levels from neonates with an Apgar score of ≤ 6 at 5 minutes after birth were 25 ± 34.4 mU/mL (n=11) compared to the neonates with a better start after birth 8.6 ± 11.9 mU/mL (n=25) (p-value = 0.17).

During the first month in general, EPO levels were not significantly correlated to Hb concentration (R^2 : 0.003) and nucleated RBC count (R^2 : 0.153) when examined concurrently.

EPO levels during the first week after premature birth were highly variable and after the first week of life we observed overall lower EPO concentrations. As the first RBC transfusion was administered at median day 4, we examined the EPO levels in the first 4 days and the period thereafter. Median EPO level in the first 4 days was 8 mU/mL (5-95 percentile range <1.4 – 112.5 mU/mL). In the period thereafter the median EPO level was significantly lower, 3 mU/mL (5-95

percentile range $<1.4 - 23.9$ mU/mL (p -value <0.001). EPO levels measured in neonates requiring mechanical ventilation (22.4 ± 97.2 mU/mL) was higher compared to neonates requiring CPAP (8.7 ± 52.8 mU/mL) or requiring no respiratory support (7.2 ± 13 mU/mL). This however was not statistically significant (p -value 0.147, one way ANOVA for differences between groups). Neonates in the transfused group who suffered from sepsis had similar EPO levels compared to the other neonates in the same group. (data not shown)

The non-transfused neonates ($n=10$) were analyzed in a similar manner. EPO levels after birth were not correlated with the first Hb level ($R^2:0.044$), birth weight ($R^2: 0.511$), gestational age ($R^2:0.011$). Only 1 out of 10 neonates had an Apgar score of ≤ 6 at 5 minutes after birth. Median EPO level in the first 4 days after birth was <1.4 mU/mL (5-95 percentile range $<1.4 - 60.6$ mU/mL) and in the period thereafter 3 mU/mL (5-95 percentile range $<1.4 - 45.6$ mU/mL).

A significant proportion of the samples had EPO levels below the detection limit of 1.4 mU/mL. This was the case in respectively 35% of the samples from the transfused neonates and 48% of the non-transfused neonates.

EPO levels were correlated to the number of RBC transfusions administered prior to EPO measurement with the use of a linear mixed model to correct for repeated measurements within the patients. Both the patient variable and the number of administered transfusions were included to correct for random effects. Per transfusion administered, the decline in EPO level was between -1.3 and -1.9 mU/mL. The decline in EPO after every administered RBC transfusion is reported in Table 2. This decline in EPO was only statistically significant after the first RBC transfusion, a drop of -1.3 mU/mL (p -value 0.018). The decline in EPO values after subsequent RBC transfusions were not significantly correlated.

In the first week after birth, incidental high EPO levels were observed, exceeding adult reference values, ranging from over 100 mU/mL to 1200 mU/mL. Two out of three extremely high EPO levels (>500 mU/mL) were observed in neonates who died early after birth. One neonate born at a gestational age of 26 weeks suffered from severe intra-ventricular hemorrhage ($\geq$ grade 3); the other neonate was born after 32 gestational weeks but had severe intra-uterine growth retardation and died because of cardio-respiratory failure. The third surviving neonate was born after 30 gestational weeks and suffered from severe intra-ventricular hemorrhage ($\geq$ grade 3) and sepsis.

Table 2: EPO in relation to administered RBC transfusions.

	Mean estimate	95% confidence interval	<i>p</i> -value
After 1 RBC transfusion	-1.3 mU/ml	-1.9 ; -0.45	0.018
After 2 RBC transfusions	-1.5 mU/ml	-2.0 ; -0.76	0.066
After 3 RBC transfusions	-1.7 mU/ml	-2.1 ; -0.95	0.166
After 4 RBC transfusions	-1.6 mU/ml	-2.1 ; -0.95	0.128
After 5 RBC transfusions	-1.9 mU/ml	-2.3 ; -1.34	0.606

Mean estimates (back transformation of the log means) were calculated after every increase in number of administered RBC transfusions

Discussion

In this observational study we measured endogenous EPO levels in premature neonates born before 36 gestational weeks during their first month of life. To avoid extra bloodletting we validated EPO measurement in waste material from blood withdrawn for other clinically indicated diagnostic tests. This enabled us to determine EPO levels approximately 4 times during the first week of life and 1-3 times per week in the period thereafter.

In 37% of all samples, EPO levels were below the detection limit of our assay of 1.4 mU/mL (35% in the transfused group and 48% of the samples in the non-transfused group). Other studies reported higher¹⁷⁻¹⁸ or similar¹⁹ ranges of endogenous EPO levels during the first month of life. These differences could be secondary to the use of different techniques as in most studies a radio-immuno assay (RIA) was used. Comparison of EPO measurement kits showed that the performance of RIA methods was best across the range of 40-150 mU/mL, but more poorly at the lower end of the detection range. The ELISA method we used showed good precision across lower EPO levels.²¹

Thirty-six neonates received RBC transfusions during their first month of life. Ten neonates did not receive transfusions. Both groups differed significantly in basic demographics and were analyzed separately. Transfused neonates suffered from more respiratory and infectious complications and more blood samples had been withdrawn for diagnostic tests, which probably contributed to the transfusion needs. During the first 4 days after premature birth, EPO levels were higher in the anemic neonates who needed transfusions, albeit with a wide range. We observed no relationship between individual EPO concentrations and the Hb levels. Although the differences were not statistically significant; the EPO levels of both neonates with an Apgar score of ≤ 6 at 5 minutes after birth and neonates requiring mechanical ventilation, were clearly higher. This rather suggests that the higher EPO concentrations in the first week of life may be explained by a higher clinical stress level and/or more other sources of hypoxic stimuli. Higher EPO levels in cord blood have previously been found associated with neonatal stress and severe intra-ventricular hemorrhage.²²⁻²³ Also in septicemia higher EPO levels have been recorded.²⁴ In our cohort EPO levels were however not higher in neonates suffering from sepsis. Extremely high EPO levels in three of our patients were probably indicative for life-threatening events and two of these three neonates died. Because in extremely stressful situations EPO levels can be extraordinary high, it may be advised to measure EPO prior to administration of recombinant human EPO for neuro-protective indications to avoid extreme high EPO levels in severely ill premature neonates.

In the transfused neonates the EPO concentrations declined significantly after the first week of life. To unravel cause or coincidence, we performed a linear mixed model analysis to examine the relation between measured EPO levels and the administered transfusions. Per administered RBC transfusion the EPO levels declined. After the first RBC transfusion this decrease was statistically significant. The continuing decline in EPO after following transfusion was however

not significantly related to the number of administered RBC transfusions. Although statistically not significant the cumulated decrease in EPO could still be clinically important; i.e. after the mean number of administered transfusions (approximately 3), the cumulative decline in EPO level could be more than 4 mU/ml. It is possible that the decline in EPO in our cohort is less distinct after transfusion due to the relative low RBC volume administered per transfusion compared to a RBC product with a higher hematocrit or transfused at a higher volume per kg bodyweight, which also could worsen the suppression of nucleated red blood cells.^{8, 25}

In the non-transfused premature neonates EPO levels slightly increased and after the first week of life the EPO levels in both transfused and non-transfused neonates were similar, albeit that after day 18 no samples were available of non-transfused patients.

In conclusion, in a large group of prematurely born infants, we showed by frequent determination of EPO levels in waste material, an initial higher EPO value in the first week of life in the premature newborns that needed RBC transfusions, without a relationship with the individual Hb levels. EPO levels tended to be higher with other causes of hypoxic stress, although we did not find a significant relationship with a low Apgar score at 5 minutes after birth, need for respiratory support or sepsis. In agreement with other studies, EPO levels declined after every RBC transfusion.⁸⁻¹⁰ This decline could only be significantly correlated to the first RBC transfusion. It is however possible that differences in transfusion policy (amount of RBC transfused per kg body weight) result in variable degrees of EPO suppression.

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