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Background: Nitric oxide (NO) and vascular endothelial growth factor (VEGF) are important mediators for hemodynamics and angiogenesis in the body. NO coming from endothelial cells and red blood cells is particularly effective in hypoxic vasodilation. VEGF has known effects on the induction of NO synthesis and is also known to be affected by blood product transfusions. The objectives of this study were to measure NO and VEGF levels before and after packed red blood cell (PRBC) transfusions.

Study design and methods: Blood was drawn from preterm newborns before and 30 min after PRBC transfusions and samples were used for NO and VEGF measurements. NO end products nitrite and nitrate were measured by modified Greiss method, VEGF levels measured by double sandwitch ELISA method. Vital signs including heart rate and blood pressure were also recorded.

Results: Thirty four newborns were included in the study and overall 54 transfusion episodes were assessed for mediator levels. No difference was observed between the mediator levels before and after PRBC transfusions. Vital signs were also unchanged.

Conclusion: As there was no change in NO end product levels with PRBC transfusions, it might suggest that hypoxia was not severe enough to cause nitrite increase; however, other NO sources might still be active. VEGF levels were found to be unchanged and may reflect a delayed effect of transfusion on VEGF induction.

Keywords: NO, VEGF, PRBC transfusion

Introduction

Nitric oxide (NO) and vascular endothelial growth factor (VEGF) are well known mediators of hemodynamics and vasculogenesis respectively. NO has various roles in the body having effects from the cardiovascular system to the neuronal tissues. It is the most important factor in the regulation of blood pressure and of the immune mediated response during infection and neuronal toxicity during hypoxia in the brain.[1–3] NO is synthesized from arginine and molecular oxygen by the enzyme nitric oxide synthase which has three isoforms.[4] Endothelial NOS is the constitutive which is activated by the calcium calmodulin complex, and it results in continuous NO production depending on the needs of the vascular system. Neuronal NOS which is also a

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constitutive form is primarily found in the brain and known to increase during hypoxia. Inducible NOS is primarily responsible for the abnormally increased NO levels during infection and inflammation. Bacterial lipopolysaccarides are well known inducers of inducible NOS.[4] The primary effect of NO on vascular system is vasodilation resulting in lower blood pressure.[5] It has previously been accepted that once produced NO would be inactivated by binding with hemoglobin (Hb) to form nitrosyl Hb which would later on be converted to nitrite and nitrate, well known end products of NO which are also used for quantitative NO measurements in the body.[6] However, current understanding about the fate of NO in the circulation is much less straightforward. Recent research suggests that red blood cells (RBCs) function both as scavengers for and as donors of NO. Once NO is in the RBC, it is bound with Hb to form either *S*-nitrosyl hemoglobin (SNO-Hb) or iron Hb and also stored in the form of nitrite.[7–9] During hypoxia NO is released from the RBC either from SNO-Hb by an unknown mechanism or from nitrite by the nitrite reductase effect of deoxyhemoglobin, resulting in the well-known survival mechanism in the circulation called hypoxic vasodilation to increase blood flow to the hypoxic tissue.[10,11]

Vascular endothelial growth factor is primarily known for its vasculogeneic effects in the body. There are five isoforms of VEGF with VEGF 165 being the most common one in human bodies.[12] VEGF synthesis is stimulated by hypoxia as a compensatory mechanism to induce angiogenesis thereby increase oxygen delivery to tissues. Pathological increases of VEGF in the body has been shown in immature retina of preterm infants causing retinopathy of prematurity and also in cancer patients resulting increased blood flow to the tumor tissue. The relationship between NO and VEGF is complex. VEGF is known to increase NO levels in the vascular system particularly during hypoxia, and NO once increased makes negative feed back on VEGF production resulting in decreased levels.[13–16]

Packed red blood cell (PRBC) transfusions are very frequently administered in intensive care units with various consequences. In preterm newborns PRBC transfusions are known to cause iron overload and also release free radicals which might impact on the developing brain and retina. The risk of transmission of infection is also another problem in all age groups. The reason for PRBC transfusion in intensive care units maybe variable within a wide spectrum including symptomatic anemia, hemodynamic instability

and respiratory problems. However, very little is known about what happens to the vasoactive mediators, such as NO and VEGF during anemia and after correction of anemia. Hypothetically since anemia is a reason for tissue hypoxia NO might increase during anemia both from the red cell source and also from the endothelium, to improve blood flow to end organs. After transfusions since blood flow to tissues has been restored to appropriate levels, the endothelial NO synthesis might reduce and NO released from RBCs might return to the storage site on the Hb resulting in overall decreased NO end product levels in the serum. The objectives of this study were to measure serum NO and VEGF levels in newborns in a neonatal intensive care unit (NICU) before and 30 min after PRBC transfusions and compare the results to assess the effect of erythrocyte transfusions on these mediators.

Materials and method

Newborns with birth weight >750 g, admitted to our NICU for various reasons were considered eligible for the study after informed parental consent. The study was approved by hospital ethics committee. After inclusion in the study, 2 ml blood was obtained from each infant before PRBC transfusions and 30 min after the end of transfusions. The decision to transfuse the baby was based on the following criteria as well as the attending neonatologist's decision:

- transfuse if on the ventilator or severe heart disease with Hb<15 g;
- transfuse if on nasal CPAP with increased oxygen requirement with Hb<10 g;
- transfuse if on room air with Hb<7 g with symptoms which could be attributed to anemia.

All babies received 15 ml/kg PRBCs over 3 h. PRBCs used for transfusion were <7 days old. If an infant required more than one PRBC transfusion during hospital stay, the same amount of blood was drawn again before and after transfusions up to two transfusion episodes. Serum was separated by 3500 rpm centrifugation and stored at −80° till the time of measurement. The hemodynamic parameters of the infants including heart rate, blood pressure before and after transfusions were also recorded and analyzed.

NO measurements

Modified Greiss method developed by Miranda *et al.* was used for NO end product measurements nitrite and nitrate.[17] After protein denaturation with ethanol and centrifugation at 13400 rpm for 5 min,

samples were placed on ELISA plate and 100 µl vanadium chloride was added, followed by 50 µl naftilethilendiamine dihydrochloride and 50 µl sulfanilamide. The plates were read at ELISA reader at 540 nm wavelength after 30 min incubation at 37°C.

VEGF measurements

VEGF 165 levels were measured by sandwich ELISA method using the appropriate commercial kit (Human CYT ELISA, Biosource International Inc., Camarillo, CA, USA) according to the manufacturer's guidelines. The ELISA plates were read at 450 nm wavelength. The VEGF values were calculated by the readings using Microsta Statistical package.

Statistics

SPSS for windows 11·0 version was used for statistical analysis. Paired *t*-test was used for before and after transfusion group analyses and $P<0·05$ was considered significant. The values are reported as mean $\pm$ SD unless indicated otherwise.

Results

Thirty four newborns were included in the study (24 male, 10 female). The birth weight was 1296 ± 659 g and gestational age was $29·6\pm2·9$ weeks. Blood sampling was done on all of the 34 newborns included in the study for the first transfusion; however, during the second transfusion sampling was performed on 20 babies secondary to clinical problems. Altogether 54 transfusion episodes were included in the study. Hb values before the first and the second transfusions were $9·3\pm1·6$ g and $10·7\pm1·9$ g respectively. In all of the measurements no difference was observed between pre-transfusion and after transfusion levels of both of the mediators (Table 1). The hemodynamic parameters including heart rate and blood pressure were also unchanged (Table 1).

Discussion

Blood product transfusions are given very frequently in adult, pediatric and NICUs for various indications. PRBCs are mostly used to correct anemia and increase tissue oxygen delivery. The body has very efficient compensatory mechanisms to maintain adequate tissue oxygenation during diseases. The RBC has a key role in maintaining tissue perfusion and oxygenation together with the endothelial cell. During hypoxia NO increases to cause vasodilation and thus increasing tissue oxygenation. The increased NO comes from the endothelium and more recently has been shown to come from RBCs which are now considered important NO reservoirs. NO from the RBC comes from either SNO-Hb or nitrite, the relative importance of each is not known.[9,11] Therefore, it is reasonable to hypothesize that NO should increase during anemia as it is a cause of tissue hypoxia. The same assumption can also be made for VEGF which is known to increase during hypoxia as another compensatory mechanism in the body. Thus in this study we investigated NO and VEGF levels in newborns during anemia and following correction of anemia. However, our findings were not consistent with our hypothesis. We did not observe any significant difference between mediator levels before and after PRBC transfusions. Hemodynamic parameters such as heart rate and blood pressure were also similar before and after transfusions. Previously, Nielsen *et al.* have reported increased VEGF levels in patients who received blood product transfusions; however, PRBCs were not included in their study.[18] On the other hand, Patel *et al.* have shown increased VEGF release and increased angiogenesis 24 h after PRBC transfusions.[19] The reason that VEGF increase was not observed in our study could be due to the timing of sampling which was 30 min after completion of the transfusion. That amount of time might have been inadequate for VEGF synthesis induction. Our findings on NO product levels including nitrite and nitrate did not support our hypothesis suggesting NO levels should increase during anemia and decrease once anemia and consequent tissue hypoxia has been corrected. This could be due to the possibility that the main effector in mild hypoxia during anemia is SNO-Hb which was not measured in our study. The

Table 1 Pre- and post-transfusion values of NO, VEGF and vital signs

$n=54$	NO (µg/l)	VEGF (pg/ml)	HR (beats/min)	SBP (mmHg)	DBP (mmHg)
Pre Tx	18·65 ± 10·25	857·98 ± 607·97	153·56 ± 14·88	60·9 ± 8·5	38·06 ± 8·7
Post Tx	18·1 ± 14·3	946·09 ± 652·98	152·16 ± 15·62	60·9 ± 8·6	39·87 ± 6·9
P	>0·05	>0·05	>0·05	>0·05	>0·05

NO: nitric oxide; VEGF: vascular endothelial growth factor; HR: heart rate; SBP: systolic blood pressure; DBP: diastolic blood pressure; Pre Tx: pre-transfusion; Post Tx: post-transfusion.

mechanism by which hypoxic vasodilation is achieved via NO has been studied by different groups within the last decade. Basically there are two widely accepted mechanisms:

(i) Nitrite induced vasodilation where nitrite is reduced to NO by deoxyhemoglobin or xanthine oxidoreductase during hypoxia.[7,9,10] Huang *et al.* have shown that at levels slightly above physiological concentrations nitrite causes hypoxic vasodilation where the PaO_2 is as low as 20–40 mmHg.[20] Recently, Chen *et al.* have developed a kinetic model for nitrite reduction by Hb and diffusion of NO from RBC.[21]

(ii) SNO-Hb induced vasodilation hypothesis, which is caused by NO stored in the RBC in the form of SNO-Hb and released during hypoxia by an, as yet, unknown mechanism to cause hypoxic vasodilation. SNO-Hb is formed when Hb is S-nitrosated at the β-93 cysteine and in case of hypoxia NO is transferred to circulation, making Hb a transporter rather than a destroyer of NO.[7-9] Nitrite is also known to react with thiols during acidosis and produce SNO-Hb.[11,22] Since SNO-Hb measurements are difficult, research investigating this mechanism has been inconsistent. However, the results suggest that the absolute amount of NO bioactivity released from SNO-Hb is small but sufficient to interfere with hypoxic conditions. As our results show an unchanged NO end product levels before and after PRBC transfusions, in the group studied, we might speculate that during mild anemia the hypoxic vasodilation caused by anemia is obtained by the SNO-Hb mechanism rather than nitrite itself; however, this requires further investigation.

In conclusion our results show that during mild anemia neither VEGF nor NO product levels change with PRBC transfusions. The fact that no change in heart rate and blood pressure was observed with transfusions could demonstrate that tissue hypoxia was indeed mild. The effect of PRBC transfusions should also be investigated in patients with lower Hb levels resulting in more profound tissue hypoxia.

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