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Evaluation of endothelial microparticles as a prognostic marker in hemolytic disease of the newborn in China

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Abstract

Objective: This study aimed to evaluate endothelial microparticles (EMPs) as a potential prognostic marker in hemolytic disease of the Chinese neonate.

Methods: We compared 29 newborns with ABO hemolytic disease of the newborn (ABO HDN), 22 newborns with Rh HDN, and 21 healthy newborns with matched mother and infant blood groups (controls). Markers of hemolysis and von Willebrand factor antigen (vWF Ag) were analyzed. EMP (CD144+) levels were measured before and after therapy.

Results: vWF Ag and pretherapy EMP levels were higher in the ABO HDN and Rh HDN groups than in the control group. Additionally, vWF Ag and pretherapy EMP levels were significantly higher in the ABO HDN group than in the Rh HDN group. Posttherapy EMP levels were decreased compared with pretherapy levels in the ABO HDN and Rh HDN groups. Moreover, hemoglobin and indirect bilirubin levels were independently correlated with pretherapy EMP levels in the ABO HDN group.

Conclusion: Our findings indicate that EMP measurement in neonates with HDN may provide a novel method of monitoring possible severe vascular dysfunction in patients in China. An external validation in larger datasets is necessary for further study.

Keywords

Endothelial microparticles (EMPs), hemolytic disease of the newborn (HDN), ABO HDN, von Willebrand factor antigen, hemoglobin, bilirubin

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Introduction

The immune reaction to incompatibility between blood types of the mother and

fetus leads to hemolytic disease of the newborn (HDN). Fetal red blood cells (RBCs) are destroyed by alloantibodies directed against RBC antigens acquired from the father.[1] ABO HDN is a common disease of the newborn in China. The Chinese population is the largest population worldwide. However, at present, there are no satisfactory methods to predict ABO HDN during the antepartum period.

Recently, a study reported that CD144+ can be used as a marker of endothelial injury in neonatal ABO blood group incompatibility.[2] Microparticles are vesicles that bud off from blood cells or the vascular endothelium and play important roles in various cellular functions, such as intercellular communication in inflammation and coagulation.[3,4] They are characterized by their size (0.2–2.0 μm) and expression on the surfaces of antigens.[5,6] Among these microparticles, EMPs have become interesting and important because they can be used as noninvasive biomarkers of vascular dysfunction.[7] Among multiple endothelial markers that have been used to detect EMPs (CD31, CD31, CD34, CD51, CD54, CD62E, CD105, CD106, CD144, and CD146), CD144 (VE-cadherin) is the most specific marker for EMP detection because it has not yet been found to be expressed in any other human blood cell.[3,5,8] However, the role of EMPs (CD144+) in endothelial injury in neonatal ABO blood group incompatibility in the Chinese population remains unclear.

Therefore, the present study aimed to determine whether EMPs can be used as a prognostic marker of ABO HDN in a Chinese population.

Materials and methods

Ethical approval

The study was approved by the Institutional Ethics Committees of Baoding First Central Hospital, and written informed consent was obtained from all participants.

In this study, 29 neonates with ABO HDN and 22 neonates with Rh HDN were enrolled. Both groups of neonates were admitted to the Department of Gynaecology and Obstetrics of Beijing China-Japan Friendship Hospital and Baoding First Central Hospital in China. The control group comprised 21 healthy neonates with matched infant and mother blood groups. The procedures were in accordance with the ethical standards of Beijing China-Japan Friendship Hospital and Baoding First Central Hospital.

Sample collection

Collection of samples was performed as previously described.[2] Briefly, ethylenediaminetetraacetic acid (1.2 mg/mL) was used for peripheral blood sample collection for hematological profiling and the direct Coombs test. For the flow cytometry assay, peripheral blood samples were collected into tubes with 0.2 mL of 3.8% trisodium citrate at a ratio of 9 volumes to 1 volume of citrate. For serum, the blood was allowed to clot for 1 hour and it was then separated by centrifugation at $80 \times g$ for 15 minutes. Serum was used for the indirect antiglobulin test, chemical analysis, and C-reactive protein (CRP) assays. Presurvey results showed $\delta = 2.6$, $\alpha = 0.05$ (bilateral), and $\beta = 0.2$. The appropriate sample size estimation was calculated by comparing the mean of two independent samples, and the result was $n = 70$. The sample size of each group was adjusted according to the actual situation.

Diagnostic testing

We excluded patients with clinical evidence of infection or CRP levels >10 mg/L. Hematological laboratory data that were collected for the ABO group were generated

from measurements of parameters from neonates and mothers as previously described.[2] The Sysmex XT-1800i (Sysmex, Kobe, Japan) was used to perform a complete blood count. RBC morphology and the differential white blood cell count were determined by examination of Leishman-stained peripheral blood smears. Examination of a stained smear to determine the reticulocyte count was performed by peripheral blood staining with brilliant cresyl blue. For antibody detection in neonates and mothers, direct and indirect Coombs tests were used, respectively. Other laboratory investigations included liver and kidney function tests, markers of hemolysis (lactate dehydrogenase [LDH] and indirect bilirubin), and measurement of high-sensitivity CRP levels on a Cobas Integra 800 (Roche Diagnostics, Mannheim, Germany). A Sysmex CA-1500 Coagulation Analyzer (Siemens Healthcare Diagnostics, Marburg, Germany) was used to quantitate the level of von Willebrand factor antigen (vWF Ag).

Flow cytometric analysis

Anti-CD144 (anti-human VE-cadherin) was used as a specific marker in flow cytometric analysis of EMPs before and after therapy (exchange transfusion and/or phototherapy).[9–11] To adjust the final volume to 500 μL, citrated blood was diluted 1 : 50 in phosphate-buffered saline (Sigma-Aldrich, St Louis, MO, USA). Fifty microliters of dilution buffer was added to 5 μL of phycoerythrin-labeled anti-CD144 (R & D; Minneapolis, MN, USA). After 20 minutes of incubation in the dark at room temperature, the data were acquired by means of an EPICS-XL PROFILE II Coulter flow cytometer (Beckman Coulter, Miami, FL, USA). Isotype-matched control immunoglobulin G antibody was purchased from Beckman Coulter. Flow cytometric analysis of the EMPs is presented in Figure 1.

Statistics

SPSS version 15.0 (SPSS Inc., Chicago, IL, USA) for Windows was used for statistical analyses. The mean ± standard deviation was used to describe quantitative variables. ANOVA and the post hoc Tukey's test were used to compare differences among the three groups. The parametric Student's t-test was performed to quantitate variables between two groups. P (probability) <0.05 was accepted as a significant difference in the analyses. Correlations of variables

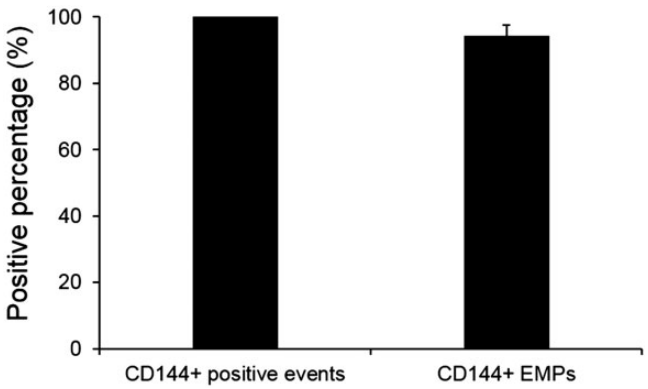

Figure 1. Flow cytometric analysis of endothelial microparticles. CD144+ endothelial microparticles are presented as a percentage of CD144+-positive events.

were analyzed by using the Spearman rank test.

Results

Birth weight and gestational age were not significantly different among the three groups (Table 1). We first determined the levels of hemoglobin and markers of hemolysis (LDH and indirect bilirubin). The levels of hemoglobin, LDH, and indirect bilirubin were remarkably different between the hemolytic groups and the control group ($P < 0.05$ overall) (Table 1). However, these variables were not significantly different between the ABO HDN and Rh HDN groups (Table 1). The reticulocyte count was significantly lower in the ABO HDN group than in the Rh HDN group ($P < 0.05$). The antiglobulin test in the Rh HDN group was positive. However, positive direct and indirect antiglobulin tests showed a low incidence in the ABO HDN group.

Furthermore, vWF Ag and pre-EMP levels were significantly higher in both hemolytic groups compared with the control group (all $P < 0.05$), and the ABO HDN group had the highest levels (Table 1). However, we did not find a remarkable difference in vWF Ag and pre-EMP levels between the ABO HDN and Rh HDN groups (Table 1).

We found that posttherapy EMP levels were strikingly decreased after exchange transfusion and/or phototherapy compared

Table 1. Clinical and laboratory characteristics of the ABO HDN, Rh HDN, and control groups.

Variables	ABO HDN (I) n=29	Rh HDN (II) n=22	Controls (III) n=21	P value			
				Overall	I vs III	II vs III	I vs II
Gestational age (weeks)	38.1±1.2	39±1.1	40±1.5	0.921	0.654	0.518	0.721
Sex, n							
Male	14	13	8	0.203	–	–	–
Female	15	9	13				
Weight (kg)	3.3±0.9	3.0±0.3	3.3±0.2	0.241	0.153	0.085	0.078
Therapy, n							
None	10	5	21				
Phototherapy	9	8	0	<0.02	<0.01	<0.05	0.062
Exchange transfusion	10	9	0				
Positive direct Coombs test n (%)	5 (13.7)	22 (100)	0 (0)	<0.05	<0.05	<0.05	<0.05
Positive indirect Coombs test, n (%)	6 (20.6)	22 (100)	0 (0)	<0.05	<0.05	<0.05	0.058
WBC count ($\times 10^9$/L)	16.0±5.8	15.5±3.2	16.1±7.1	0.092	–	–	–
Hemoglobin (g/dL)	16.1±4.2	14.9±1.12	16.8±1.02	<0.05	<0.05	<0.05	0.812
Platelets ($\times 10^9$/L)	225±90.2	256±62.5	265±65.6	0.075	–	–	–
Reticulocyte count (%)	6.1±1.9	7.1±2.9	4.1±1.1	<0.05	<0.05	<0.05	<0.05
LDH (IU/L)	1178±612	1311±491	701±195	<0.05	<0.05	<0.05	0.092
Indirect bilirubin (mg/dL)	8.2±3.1	11±8.1	3.2±2.1	<0.05	<0.05	<0.05	0.212
vWF Ag (%)	128±15	102±15	80±11	<0.05	<0.05	<0.05	0.051
EMP pretherapy (%)	6.9±2.1	5.8±2.9	3±1.1	<0.05	<0.05	<0.05	0.059
EMP posttherapy (%)	5.1±2.6	4.9±1.8	–	0.098	–	–	–

Values are mean ± standard deviation, n, or n (%). HDN: hemolytic disease of the newborn; WBC: white blood cell; LDH: lactate dehydrogenase; vWF Ag: von Willebrand factor antigen; EMP: endothelial microparticle.

with pretherapy levels (both $P < 0.05$) in the ABO HDN and Rh HDN groups (Table 1).

To determine whether there were significant relationships between pretherapy EMP levels and these clinical factors, we performed correlation analyses. We found significant positive relationships between pretherapy EMP levels and LDH levels ($r = 0.392$, $P < 0.05$), and between indirect bilirubin and vWF Ag levels in the ABO HDN and Rh HDN groups. However, pretherapy EMP levels were negatively correlated with hemoglobin levels in the ABO HDN group ($r = -0.322$, all $P < 0.05$) (Figure 2).

Additional analysis showed that hemoglobin, LDH, and indirect bilirubin levels were independently correlated with pretherapy EMP levels in the ABO HDN group ($r^2 = 0.821$, all $P < 0.05$) (Table 2). However, only hemoglobin and LDH levels were strongly correlated with pretherapy EMP levels in the Rh HDN group ($r^2 = 0.869$, both $P < 0.05$) (Table 2).

Discussion

The ABO blood group system is the most important system for human blood transfusion in China. A and B blood group antigens are histo-blood group antigens and are widely disturbed in human tissues and organs. These antigens are strongly expressed on the surfaces of endothelial cells.[12,13] In this study, we evaluated EMP levels as an indicator of endothelial dysfunction.[2] We also analyzed the relationship between ABO HDN and the integrity of vascular endothelium and the association between possible endothelial injury and hemolytic disease severity in China.

In the present study, the ABO HDN and Rh HDN groups showed significantly lower hemoglobin levels compared with the control group. The reticulocyte count in the Rh HDN group was higher than that in the Rh HDN group. Additionally, the antiglobulin test in the Rh HDN group was positive. However, both positive direct and indirect antiglobulin tests showed a low incidence in the ABO HDN group. Our results suggested that the ABO HDN and Rh HDN groups had red cell hemolysis, which is consistent with previous findings.[2,14,15] Recently, a previous study reported that the Coombs test showed either negativity or only weak positivity in ABO HDN neonates.[16] This result indicates that the Coombs test cannot be used as the only diagnostic test to determine ABO HDN in neonates.

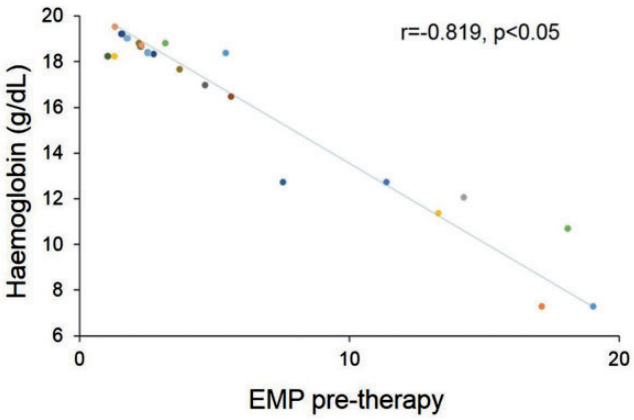

Figure 2. Significant correlation between the pretherapy level of EMP and hemoglobin (g/dL).

Table 2. Multiple regression analysis of the relationships between pretherapy endothelial microparticle levels and laboratory variables in the ABO and Rh HDN groups.

Group	Independent variables	Unstandardized coefficients		Unstandardized coefficients		
		B	Standard error	Beta	P value	r^2
ABO HDN	Constant	9.123	3.102		<0.05	0.821
	Hemoglobin (g/dL)	−0.689	0.079	−0.537	<0.05	
	LDH (IU/L)	0.081	0.032	0.158	<0.05	
	Indirect bilirubin (mg/dL)	0.195	0.029	0.502	<0.05	
Rh HDN	Constant	6.102	1.098		<0.05	0.869
	Hemoglobin (g/dL)	−0.199	0.042	−0.398	<0.05	
	LDH (IU/L)	0.001	0.000	0.512	<0.05	

HDN: hemolytic disease of the newborn; LDH: lactate dehydrogenase.

LDH and indirect bilirubin levels were lower in the ABO HDN group than in the Rh HDN group, but this difference did not reach the level of significance. This finding is in line with the results of previous studies.[2,15]

Recently, CD144 has attracted interest because anti-CD144 is used as a marker of endothelial injury in various diseases, such as diabetes mellitus, coronary artery disease, ischemic stroke, and end-stage renal disease.[10,11,17] vWF Ag is another marker of endothelial dysfunction.[18] In our study, we found that vWF Ag and EMP levels were notably higher in the ABO HDN and Rh HDN groups than in the control group, and that these levels were highest in the ABO HDN group. However, differences in vWF Ag and EMP levels were not found between the ABO HDN and Rh HDN groups, which are in line with a previous report.[2] These increased levels of vWF Ag and EMP represented endothelial injury caused by anti-A and anti-B antibodies with their corresponding antigens located over the endothelium.

A previous study reported that ABO antigens were strongly expressed on vascular endothelium, while Rh antigens were not.[17] This means that the antigen–antibody reaction in Rh HDN only occurs on the surfaces of red blood cells and leads to hemolysis and mild endothelial injury in an indirect manner.[2] However, a much more severe and direct endothelial injury results from the antigen–antibody reaction that occurs on endothelial cells together with RBCs.[2] Although previous studies have shown that ABO HDN might have a serious effect on the endothelium,[19] few investigations on neonates in the Chinese population have been conducted.[20]

A previous study suggested that levels of EMPs were positively correlated with markers of hemolysis and inversely correlated with hemoglobin levels in sickle-cell disease.[21,22] Additionally, pretherapy EMP, vWF Ag, and D-dimer levels were found to be significantly increased in patients with paroxysmal nocturnal hemoglobinuria.[23] In this study, pretherapy EMP levels were positively correlated with the reticulocyte count and indirect bilirubin, LDH, and vWF Ag levels in both groups. However, pretherapy EMP levels were negatively correlated with hemoglobin levels in the ABO HDN group. This is consistent with the findings of a previous study.[2]

Pretherapy EMP levels were remarkably higher than posttherapy levels in the ABO HDN and Rh HDN groups in our study. However, the greatest decrease in EMP

levels was found after exchange transfusion in the ABO HDN group compared with baseline or postphototherapy levels. This finding might be because the antigen–antibody reaction on endothelium and EMPs had been washed away by the exchange transfusion. Additionally, phototherapy only decreased bilirubin levels, but did not eliminate the causative antigen–antibody reaction.

The present study has several limitations. We analyzed a relatively small cohort of patients in few locations (Beijing and Wuhan Cities). Additionally, we did not use other soluble endothelial cell markers, such as vascular cell adhesion molecule-1, intercellular adhesion molecule-1, and P-selection. Therefore, further analysis of other markers to provide additional information and a larger sample size are required to represent more areas of China.

In conclusion, the present study suggests that binding of anti-A and anti-B antibodies with their relative antigens on the endothelium leads to endothelial dysfunction. Increased EMP levels in ABO HDN may be representative of immunoglobulin G-mediated erythrocyte destruction. Therefore, EMP measurement in neonates with HDN may be a novel method that provides an early initial stage to monitor possible severe vascular dysfunction in patients in China. However, external validation of our results in larger datasets is necessary in future studies.

Declaration of conflicting interest

The authors declare that there is no conflict of interest.

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