

Coagulation Activation, Fibrinolysis and Inhibitors in Neonates

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Abstract

Enhanced coagulation activation with reduced antithrombin III (ATIII) activity was seen in healthy neonates. Although systemic tissue plasminogen activator (t-PA) and urokinase-like plasminogen activator (u-PA) levels showed no significant differences from normal adults, enhanced fibrinolysis was indicated by elevated D-dimer and low plasminogen levels in the neonates in this study. Enhanced fibrinolysis observed was countered by elevated plasminogen activator inhibitor-1 (PAI-1) levels, a trend similar to that observed in the amniotic fluid during labour. The elevated PAI-1 level seen in neonates may have a beneficial effect in preventing haemorrhage in the neonatal period. The haemostatic and fibrinolytic mechanisms studied in normal pregnancy neonates were similar to neonates born to gestational diabetes mellitus (GDM) mothers. Further studies need to include neonates with poor outcome and low Apgar score to assess their haemostatic status.

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Introduction

The foetal coagulation system is an immature, developing system and thrombotic complications are not a feature of normal foetal life, possibly because the haemostatic system is in dynamic equilibrium.* Two forms of plasminogen activators have been described: tissue activator (t-PA) and urokinase-like (u-PA), both are serine proteases which converts the zymogen plasminogen into active enzyme plasmin. Plasmin, a proteolytic enzyme is responsible for the degradation of fibrin deposits in the blood vessels and form an inactive stoichiometric 1:1 complex with its efficient inhibitor α_2 -antiplasmin. t-PA is involved in fibrin clot lysis whilst u-PA is associated with a wide variety of biological functions which includes tissue remodelling, cell migration, tumour metastasis, ovulation and trophoblast implantation. Its principal inhibitor, plasminogen activator inhibitor-1 (PAI-1) is also synthesized by endothelial cells. Fibrinolysis which keeps the vascular compartment patent is regulated by the activities of both t-PA and PAI-1. D-dimer, a degradation product from cross-linked fibrin digested by plasmin is also an indicator of fibrinolytic activity *in vivo*. Fibrinolytic activity in the neonatal period is not fully understood. The levels of plasminogen and α_2 -antiplasmin were decreased compared to

adults,² whereas plasminogen activator activity was within normal to reduced levels.³⁻⁵ Fibrinolytic markers were significantly elevated⁶ indicating that newborns are able to remove fibrin efficiently. PAI-1 has been reported to be low⁵ or similar to adults³ whilst enhanced activation of coagulation, reflected by increased TAT-complex levels and D-dimer levels, suggests enhanced fibrinolysis.⁷ The activation of prothrombin to thrombin is a key event within the coagulation cascade. During this process, the amino-terminus of the prothrombin molecule is released as the inactive prothrombin fragment F_{1+2} (F_{1+2}) by factor Xa cleavage in parallel with the generation of thrombin. Thrombin produced are rapidly neutralised by antithrombin III, its physiological inhibitor by formation of an inactive thrombin-antithrombin III (TAT) complex. Both F_{1+2} and TAT-complex can be regarded as molecular markers of coagulation activation. There are wide variations in the haemostatic parameters reported and these could be due to the differences in the techniques used, but healthy neonates rarely show signs of abnormal haemorrhage or thrombosis unless obstetric or neonatal complications are present.

The aim of the study was to investigate coagulation activation, fibrinolytic activators and inhibitors in the cord blood of healthy newborns.

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Materials and Methods

Cord blood: Forty-four venous cord blood samples were collected from neonates delivered vaginally in the labour ward of the National University Hospital after obtaining full consent from the mothers of the neonates under study. Thirty cord blood from term neonates were used for analysis and 14 were not included due to pregnancy induced hypertension ($n = 4$), prematurity ($n = 3$), gestational diabetes with hypertension ($n = 1$), polyhydramnios ($n = 1$), oligohydramnios ($n = 2$), α -thalassaemia ($n = 1$), and sample clotted ($n = 2$). The cord blood from the infants were further divided as normal ($n = 20$) and high-risk pregnancy group of maternal gestational diabetes mellitus (GDM) ($n = 10$) and pregnancy-induced hypertension (PIH) ($n = 4$). GDM is diabetes mellitus occurring for the first time in pregnancy. The mean maternal age was 30.0 ± 4.8 years and the gestational ages ranged from 37 to 41 weeks (mean 39 ± 1.2 weeks). The Apgar score at 5 minutes was 9 in infants born to normal and high-risk pregnancy mothers under the study.

Blood was drawn from the umbilical vein with 18G needle and plastic syringe prior to placental separation after the cord was clamped and cut. A single puncture was made and care was taken to obtain the samples within seconds. Nine parts of blood were mixed with one part of 0.129 mol/L tri-sodium citrate (Merck) containing 0.21 mol/L HEPES (Sigma, USA) in a plastic tube. A total of 3 ml blood volume was immediately mixed by inverting the tube several times and left in crushed ice. The blood was spun in a refrigerated centrifuge at 2000 g for 15 min. The plasma was aliquoted and immediately stored at -70°C until assayed. For t-PA activity assay, the samples were acid-treated as previ-

ously described.⁸

Normal female adults: Twenty normal non-pregnant subjects were age-matched with the maternal age of the 20 normal infants under study.

Assays: The functional assays for t-PA activity,⁸ PAI-1⁹ and u-PA activity¹⁰ were determined as described. Enzyme-linked immunoassay (ELISA) kits for t-PA antigen (American Diagnostics Inc, CT, USA), PAI-1 antigen (Monozyme, Sweden), PAI-2 antigen (Biopools, Sweden), D-dimer (Stago, France), and F_{1+2} (Behringwerke, Marburg, Germany) were determined together with functional antithrombin III (ATIII) and plasminogen using chromogenic substrate assays.

Fibrin zymography: Plasminogen activators and their complexes from plasma euglobulin fractions were characterised by the previously described method.¹¹ Briefly, after sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), the fibrin-agar gel prepared earlier was layered onto SDS gel and incubated at 37°C for about 18 hours. The molecular masses were calculated from known markers; t-PA (67 kDa), and two chain u-PA (54 and 33 kDa) obtained from the National Institute of Biological Standards and Control (NIBSC), UK. The plasminogen activator/inhibitor complexes demonstrated in this study were referred to by earlier works.^{11,12} $20 \mu\text{l}$ volume samples were used for SDS-PAGE.

Statistical analysis: Independent t-test and Mann Whitney's U-Wilcoxon Rank sum W test were used for statistical analysis. A P value of less than 0.05 was considered statistically significant.

Results

The results of comparisons between normal and non-

TABLE I: COMPARISON OF CORD BLOOD OF NORMAL NEONATES ($n = 20$) FOR t-PA, u-PA AND PAI-1 WITH GDM NEONATES ($n = 10$) AND NORMAL ADULTS ($n = 20$) MATCHED FOR MATERNAL AGE

	t-PA		u-PA		PAI-1	
	Act (IU/ml)	Ag (ng/ml)	Act (IU/ml)	Ag (ng/ml)	Act (AU/ml)	Ag (ng/ml)
Normal Neonates						
Mean (SD)	1.73 (2.8)	5.24 (3.8)	0.47 (0.15)	0.98 (0.5)	17.2 (5.3)	14.9 (9.1)
Range	0.22 to 12.0	1.3 to 15.5	0.20 to 0.82	0.49 to 2.9	6.0 to 28.0	4.8 to 37.6
GDM Neonates						
Mean (SD)	0.55 (0.3)	4.7 (3.3)	0.50 (0.43)	0.89 (0.14)	18.7 (4.5)	17.2 (12.6)
Range	0.20 to 1.10	1.4 to 12.7	0.25 to 1.70	0.68 to 1.16	13.0 to 27.0	3.8 to 43.2
P	ns	ns	ns	ns	ns	ns
Normal Adults						
Mean (SD)	0.55 (0.25)	5.39 (3.1)	0.48 (0.20)	1.00 (0.22)	12.3 (4.0)	8.1 (4.4)
Range	0.24 to 1.02	0.4 to 11.5	0.27 to 0.86	0.70 to 1.50	7.5 to 20.0	1.2 to 16.6
P	ns	ns	ns	ns	<0.01	<0.01

Act: activity; Ag: antigen; SD: standard deviation; GDM: gestational diabetes mellitus; ns: not significant

TABLE II: COMPARISON OF CORD BLOOD FOR D-DIMER, PLASMINOGEN, AT-III AND F_{1+2} OF NORMAL NEONATES WITH GDM NEONATES AND NORMAL ADULTS AS IN TABLE I

	D-dimer (ng/ml)	Plasminogen %	AT-III %	F_{1+2} nmol/L
Normal Neonates				
Mean (SD)	264 (95)	62 (9.8)	71 (11.0)	1.6 (1.1)
Range	143 to 465	47 to 77	54 to 90	0.82 to 5.80
GDM Neonates				
Mean (SD)	228 (115)	59.6 (8.9)	75.7 (7.5)	1.42 (0.43)
Range	114 to 396	46 to 71	64 to 83	0.84 to 2.00
<i>P</i>	ns	ns	ns	ns
Normal Adults				
Mean (SD)	162 (90)	113 (11.4)	109 (8.0)	0.89 (0.5)
Range	70 to 375	87 to 132	96 to 120	0.42 to 2.40
<i>P</i>	<0.01	<0.001	<0.001	<0.05

SD: standard deviation; GDM: gestational diabetes mellitus;
ns: not significant

pregnant adults and neonates are shown in Tables I and II. Distribution of t-PA and PAI-1 levels is shown in Figure 1, D-dimer, ATIII, plasminogen and F_{1+2} levels are shown in Figure 2, and fibrin zymography is shown in Figure 3. The D-dimer level (3253 ng/ml) of one normal neonate was not included in the final analysis as this would have caused a wide variation in standard deviation, the other parameters of the same neonate was included in the final analysis.

Plasminogen Activators and Plasminogen Activator Inhibitors

There were no statistical significant differences for t-PA and u-PA activities and antigen levels between

neonates born to normal pregnant mothers and adults. There was a rising mean trend in t-PA activities in normal neonates of mean 1.73 IU/ml as compared to mean 0.55 IU/ml in adults but it did not reach statistical significance possibly due to the wide variations in t-PA activities observed in neonates (Table I).

PAI-1 activity and antigen levels were significantly elevated ($P < 0.01$) in neonates compared to normal adults (Table I). PAI-2 antigen levels were not detectable in the cord blood of neonates under study.

There were no significant differences for t-PA, u-PA, PAI-1 and PAI-2 levels between normal and healthy GDM neonates under study. The distribution of t-PA and PAI-1 levels is shown in Figure 1.

D-dimer, Plasminogen, ATIII and F_{1+2}

Significantly elevated ($P < 0.01$) D-dimer levels in neonates with decreased plasminogen ($P < 0.001$), and AT111 ($P < 0.001$) and enhanced coagulation activation evidenced by F_{1+2} levels were observed when compared to normal adults (Table II). One normal neonate with the only D-dimer level of 3253 ng/ml of plasma was not included in the final analysis.

There were no significant differences in D-dimer, plasminogen, AT111 and F_{1+2} levels between normal and GDM neonates. Their distributions are shown in Figure 2.

Zymography

Cord blood of normal, GDM and PIH neonates showed similar lytic bands as in non-pregnant and pregnant adults. Free t-PA (67 kDa) and single chain u-PA (54 kDa) were observed together with t-PA/PAI-1 (110 kDa) and plasmin/ α_2 -antiplasmin complexes

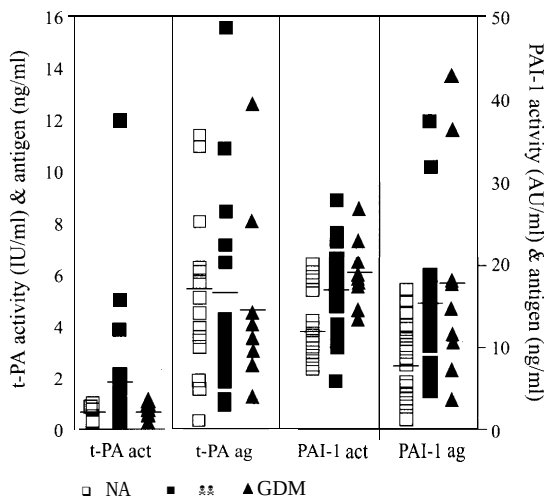


Fig. 1. Distribution of t-PA and PAI-1 levels in cord blood of healthy term neonates born to mothers with normal pregnancy (■), and gestational diabetes mellitus (▲) compared to normal adults (□). The mean levels are shown.

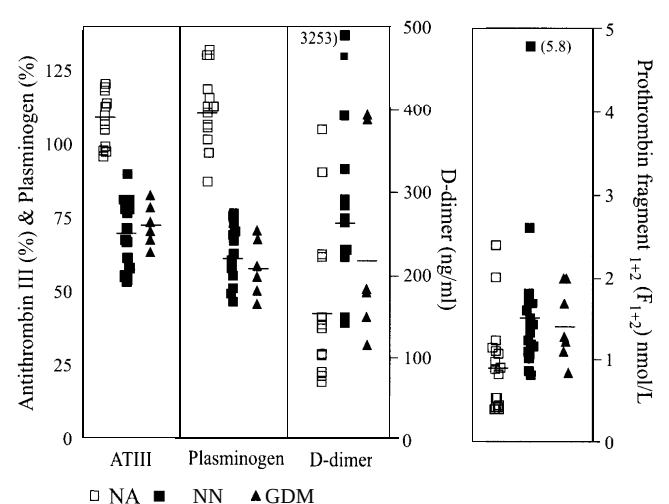


Fig. 2. Distribution of ATIII, plasminogen, D-dimer and F_{1+2} levels in cord blood of the same subjects shown in Figure 1. The mean levels are also shown.

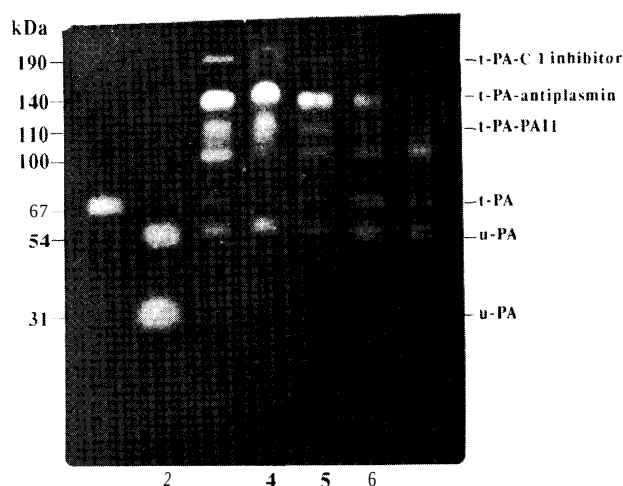


Fig. 3. Zymography. Lane 1, t-PA (1 IU/ml). Lane 2, two chain u-PA (0.25 IU/ml) and plasma euglobulin fractions from Lane 3, normal non-pregnant subject. Lane 4, during labour. Lane 5, normal pregnancy neonate. Lane 6, neonate of gestational diabetes mellitus mother. Lane 7, neonate of pregnancy-induced hypertension mother.

(140 kDa) (Fig. 3). Their lytic zones observed were evaluated with reference to earlier works.^{11,12} The t-PA/inhibitor complex at 100 kDa may possibly arise from complexation of sct-PA with the binding protein in plasma which exists in excess over t-PA in normal plasma.¹³

Discussion

Previous studies have shown enhanced activation of coagulation in healthy neonates,^{7,14} and reduced vitamin-K dependent coagulation factors^{14,15} have been suggested to be mainly due to low levels of mRNA in the immature foetal rather than in adult liver.¹⁶ No evidence of differences in enhanced coagulation activity was observed in neonates born to pre-eclamptic mothers compared to the control group.¹⁷ The fibrinolytic mechanism of newborns was reported to have slow plasmin generation" and has been suggested to favour fibrin formation rather than fibrinolysis.¹⁸ In our study of healthy normal and GDM neonates with Apgar score of 9 at 5 minutes after delivery showed enhanced activation of coagulation as evidenced by elevated F_{1+2} levels with reduced AT111 levels compared to normal adults. The fibrinolytic activities in the neonates under study did not show any statistical significant differences in systemic t-PA and u-PA levels compared to adults. However, t-PA activity although showed higher mean trends in the normal neonates did not reach statistical significance, possibly due to wide variation in activity level seen. Enhanced fibrinolysis was indicated by the significant increase in D-dimer and reduced plasminogen levels of neonates compared to adults. In a normal neonate, the D-dimer (not included in the final analysis) reached 3253 ng/ml of plasma and t-PA activity 4.9 IU/ml suggesting en-

hanced fibrinolysis. The enhanced fibrinolysis status observed in neonates is countered by the elevated PAI-1 levels which was similar to the trend observed in amniotic fluid of termed normal pregnancy.¹² PAI-2, although present in amniotic fluid during labour was not detected in cord blood. The elevated PAI-1 levels seen in neonates could have a beneficial effect in preventing haemorrhage in the neonatal period. Zymography demonstrated similar lytic zones of free t-PA, SCU-PA, t-PA/PAI-1 complexes, observed in adults and neonates. Moreover, the reduced vitamin-K dependent coagulation factor VII and fibrinogen observed in our earlier study¹⁶ together with low plasminogen level suggest limited optimal thrombin generation and fibrinolysis and that the coagulation and fibrinolytic mechanism is in dynamic equilibrium.

The haemostatic and fibrinolytic mechanisms studied in normal pregnancy neonates were similar to neonates born to GDM mothers. However, further studies need to include neonates with poor outcome and low Apgar score to assess their haemostatic status

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