Normal thrombin generation in neonates in spite of prolonged conventional coagulation tests

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ABSTRACT

Conventional coagulation tests might be inadequate to explore mechanisms regulating thrombin generation in neonates, because they do not allow full activation of the reduced levels of protein C. Therefore, they do not reflect the action of pro- and anti-coagulants as does the endogenous thrombin potential assessed in the presence of thrombomodulin. Endogenous thrombin potential measured without thrombomodulin was greater than the lower-limit of the adult reference interval in 30% of 109 full-term and 49% of 55 pre-term neonates, a finding consistent with the reduced levels of procoagulants in this setting. When the test was modified adding thrombomodulin, endogenous thrombin potential reverted into the adult reference interval in 97% and 100% full-term and pre-term neonates. In conclusion, the coagulation balance in neonates is restored by the concomitant reduction of pro- and anti-coagulants. The restored balance can be shown *in vitro* by the endogenous thrombin potential test that includes thrombomodulin, but not by conventional coagulation tests.

Key words: newborns, coagulation balance, procoagulant factors, anticoagulant factors, activated partial thromboplastin.

Citation: Tripodi A, Ramenghi LA, Chantarangkul V, De Carli A, Clerici M, Groppo M, Mosca F, and Mannucci PM. Normal thrombin generation in neonates in spite of prolonged conventional coagulation tests. Haematologica 2008; 93:1256-1259. doi: 10.3324/haematol.12566

Introduction

The balance of coagulation is secured by the pro- and anti-coagulant drives operating in plasma that in normal conditions contrast with each other and prevent excessive thrombin generation (TG).[1] This balance may be perturbed as a consequence of the congenital deficiency of procoagulants leading to hemorrhage or of anticoagulants leading to thrombosis.[1] Although this concept is well recognized, few attempts have been made to design methods suitable to investigate the balance as it occurs *in vivo*. Coagulation is currently investigated by measuring separately the pro- or the anti-coagulants with such global tests as prothrombin and the activated partial thromboplastin times (PT, APTT), or through the assay of single pro- or anti-coagulants. Neither approach truly mimics what occurs *in vivo* as in both instances one measures the activity of one component which is barely or not at all contrasted by the other. For example, PT and APTT are prolonged in patients with congenital deficiencies of one or more procoagulants because these deficiencies result in reduced TG.[2] On the other hand, PT and APTT are normal in patients with congenital deficiencies of either naturally-occurring anticoagulant antithrombin and protein C (PC) pathway. This is not plausible as the above deficiencies support increased TG,[3] therefore, the PT and APTT should be shortened. How can this paradox be explained? It is reasonable to assume that PT and APTT, because of their design, are affected by the thrombin generated as a function of procoagulants, but much less by the inhibition of thrombin mediated by the anticoagulants. For example, neither the anticoagulant action of antithrombin nor that of PC can be fully expressed *in vitro* because these proteins are activated *in vivo* by glycosoaminoglycans[4] or thrombomodulin5 located on endothelial cells. Reagents/plasmas employed with PT and APTT do not contain sufficient amounts of such activators. Therefore, PT and APTT are able to indicate whether a patient is deficient in procoagulants, but not whether that deficiency is balanced by a deficiency in anticoagulants. PT and APTT maintain their value in the investigation of conditions characterized by congenital deficiencies of procoagulants (hemophilia, allied disorders), but much less in the investigation of those characterized by acquired deficiencies of both pro- and anti-coagulants, such as cirrhosis, neonatal period and others. The assay that would be suitable to account for both the action of pro- and anti-coagulants is the TG test.[6] Recently, we undertook studies aimed at investigating the balance in various acquired deficiencies of coagulation.[7,8] Here we report results on the TG assay in newborns.

Manuscript arrived November 28, 2007. Revised version arrived on January 24, 2008. Manuscript accepted January 31, 2008.
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Design and Methods

Blood collection and plasma preparation

Cord-blood was collected after delivery from 109 full-term neonates (63 boys, gestational age range 37-41 weeks), and 55 pre-term neonates (28 boys, gestational age range 30-37 weeks and/or birth weight higher than 1.5 kg), who did not need ventilation assistance. Neonates received 1.0 mg vitamin K intramuscularly immediately after birth. None experienced hemorrhage/thrombosis, had received heparin/antithrombotic drugs or plasma products. Blood from each neonate was anticoagulated with citrate (0.109 M); proportion of 1:9 (anticoagulant:blood) and centrifuged at 2,880g for 20 minutes. Plasma was aliquoted in plastic tubes, snap-frozen and stored at –70° C. Plasmas prepared as above from venous-blood of 185 healthy adults (74 men) served to obtain adult reference intervals. When this was required for other purposes, venous-blood was collected from neonates 24-48 hours after birth and plasma was prepared and stored as above. Paired plasmas from cord- and venous-blood were available for 37 full- and 19 pre-term infants. Neonates were enrolled in the study after obtaining approval of our Institutional Review Board and informed consent of one of their parents.

Methods

TG was assessed according to Hemker *et al.*[6] as described by Chantarangkul *et al.*[9] with human recombinant (Recombiplastin, IL, Orangeburg, NY, USA) tissue factor (TF) (1 pM) in the presence of phospholipids (1.0 μM). TG was also assessed in the presence of thrombomodulin (ICN Biomedicals, Aurora, Ohio) (final concentration, 4 nM). Thrombin was measured as function of an internal calibrator (Thrombin Calibrator, Thrombinoscope BV, Maastricht, Netherlands). The area under the TG curve, called endogenous thrombin potential (ETP), was calculated with Thrombinoscope™ (Thrombinoscope BV) and reported as nM thrombin time minutes (nMmin). Measurements were taken within the same time-frame for neonates and adults. PT was measured with recombiplastin (IL) and APTT with automated APTT (bioMerieux, Durham, NC, USA); results for both were expressed as ratios of test-to-reference frozen normal-pooled (30 healthy adult donors) plasma. Factor (F) II was measured according to Bertina *et al.*;[10] FVIII, IX, VII and V were measured with one-stage coagulation assays; antithrombin was measured as heparin-cofactor by Electrachrome Antithrombin (IL); PC and α2-macroglobulin were measured as antigens by home-made ELISA and rocket-immunoelectrophoresis, respectively. Results for factor measurements were expressed as a percentage of the pooled-normal plasma arbitrarily set at 100%.

Statistical analyses

Results were expressed as medians and ranges. The Mann-Whitney U test was used to test for between-median differences. ETP values for neonates were considered to match the values found for the adult population if they were equal or greater than the lower-limit of the adult reference interval, defined as the 5th percentiles distribution of the adult population.

Results and Discussion

Levels of some pro- and anti-coagulants for a sub-sample of neonates for whom sufficient volumes of plasmas were available are reported in Table 1. Compared with adult reference intervals, neonates had prolonged PT and APTT and reduced levels of pro- and anti-coagulants, except FVIII, FV and α2-macroglobulin. Significant differences between full- and pre-term infants were found in all instances, except for PT-ratio and FVII. Pro- and anti-coagulants levels were higher in full- than in pre-term neonates; those differences were more pronounced for FVIII, FIX, antithrombin, PC and α2-macroglobulin ($p<0.001$). Distributions of ETP values for the whole population of neonates are reported in Figure 1. Median ETP values measured in the presence of thrombomodulin were in all instances slightly lower than those measured in the absence of thrombomodulin. Median (range) ETP values for pre-term were significantly higher than those for full-term infants both with [1,187 (756-1,524) *vs.* 1,089 (286-1,607), $p=0.002$] or without thrombomodulin [1,247 (818-1,567) *vs.* 1,171 (318-1,778), $p=0.01$]. Figure 1 also reports proportions of individual ETP results greater than the lower-limit of the adult reference interval; 30% of the values for full-term infants were greater than the lower-limit of the adult reference interval when the ETP was measured in the absence of thrombomodulin compared with 97% when the ETP was measured in the presence of thrombomodulin. The correspondent values for pre-term infants were 49% and 100% (Figure 1). Distributions of ETP values for 37 full- and 19 pre-term neonates for whom venous-blood was available are reported in Figure 2. The pattern of changes with respect to the TG in the absence or presence of thrombomodulin was substantially the same as that observed for cord-blood; the percentage of neonates for whom there was a recovery of the

Table 1. Median (range) values for coagulation parameters obtained for plasmas from full- or pre-term infants.

Parameter	*Full-Term*		*Pre-Term*		*p*
PT-ratio[a]	n=77	1.18 (1.02-3.64)	n=30	1.19 (0.97-1.61)	0.51
APTT-ratio[a]	n=77	1.60 (1.1-2.39)	n=30	1.78 (1.04-2.50)	0.02
Pro-coagulants[b]					
Factor II	n=77	47 (12-71)	n=30	42 (26-54)	<0.001
Factor VIII	n=77	85 (36-262)	n=30	64 (21-430)	<0.001
Factor IX	n=77	30 (11-76)	n=30	20 (12-63)	<0.001
Factor VII	n=77	45 (10-94)	n=30	45 (19-77)	0.72
Factor V	n=77	118 (24-171)	n=30	110 (63-144)	0.01
Anti-coagulants[b]					
Antithrombin	n=109	54 (30-78)	n=55	43 (17-80)	<0.001
Protein C	n=76	31 (17-55)	n=30	26 (13-38)	<0.001
α_2-macroglobulin	n=76	187 (66-342)	n=31	155 (85-283)	0.004

[a]Patient-to-reference coagulation times; [b]activity or antigen expressed as % of the normal pooled plasma set at 100%.

ETP into the adult reference interval when the test was performed in the presence of thrombomodulin was 100%, both for full- and pre-term infants (Figure 2). Compared with adult populations, neonates are characterized by an impaired synthesis of coagulation factors which is responsible for the prolongation of PT and APTT.[11-13] However, despite these abnormalities, neonates have normal hemostasis even after surgery or trauma and may even develop thrombosis.[14] The reasons for this discrepancy are still unclear, but the concomitant reduction of the naturally-occurring anticoagulants observed in neonates might play a role. Recently, investigation of plasmas from neonates with TG assays that are also capable of exploring the anticoagulant systems have contributed to develop the concept that in the neonates the coagulation balance may be restored by the concomitant deficiencies of pro- and anti-coagulants.[15-18] These studies had some limits. First, TG was measured by methods that employed the sub-sampling technique where plasma was defibrinated before assay or fibrin formation was inhibited by addition of exogenous agents;[15-17] these methods are cumbersome and may be subjected to artifacts. Second, these studies examined plasmas pooled from many neonates instead of single donations;[15-18] in this material the variable deficiency of one or more pro- or anti-coagulants in individual plasmas might be compensated.This means that experimental conditions may not be exactly comparable to a population of individual neonates. Third, pooled plasmas were manipulated by increasing or decreasing the anticoagulant factors. Fourth, these studies used cord- instead of venous-blood. All the above conditions might have influenced the results to such an extent that the conclusions may not necessarily be applicable to the situation operating *in vivo*.

To overcome these limitations, we investigated individual plasmas from a cohort of neonates; plasma was derived from cord-blood, but for a subsample of neonates we also used plasma derived from venous-blood. Apart from the difference due to the effect of the administration of vitamin K that affects venous-, but not cord-blood, the differences between the two types of blood samples are not well established and one might suspect that coagulation factors in cord-blood may become more easily activated than in venous-blood. Therefore, the conclusions derived from results obtained for venous-blood are likely to be more applicable to the real situation. In this respect, it is of interest to note that the conclusions from the analysis of cord-blood in our study are also applicable to venous-blood. We used an assay that mimics what occurs *in vivo* more closely than the PT or APTT. The method is based on the continuous registration of TG in plasma that is minimally manipulated, avoiding defibrination or addition of exogenous pro- or anti-coagulants, except for thrombomodulin. Furthermore, TG curves are calculated by a specialized software[19] able to correct for the amidolytic activity that the α2macroglobulin-thrombin complex still retains on the synthetic substrate. However, it should be acknowledged that the conditions used in this assay may differ from those operating *in vivo*. First, the appropriate concentration of TF mimicking *in vivo* conditions is unknown; the concentration used in this study (1 pM) is much smaller than that used in the PT, making ETP more suitable than PT to reflect *in vivo* conditions. Second, the effect that soluble instead of cell-bound thrombomodulin may have on PC activation, as well as the appropriate concentration of thrombomodulin needed to mimic *in vivo* PC activation, are unknown. The final concentration of thrombomodulin used in this study (4 nM) was derived from our previous experience in which this concentration gave the best TG discrimination between healthy subjects and patients with congenital PC deficiency.

To summarize, the results of this large study of plasmas from individual neonates and with a relatively newer and simpler method for TG reinforce the concept that the coagulation balance in neonates, usually regarded as perturbed because of the deficiency of procoagulants, might be restored by the concomitant deficiency of the naturally-occurring anticoagulants. This finding questions the usefulness of procoagulant agents in controlling bleeding. This restored balance can only be demonstrated using an assay that reflects the action of the pro- counteracted by

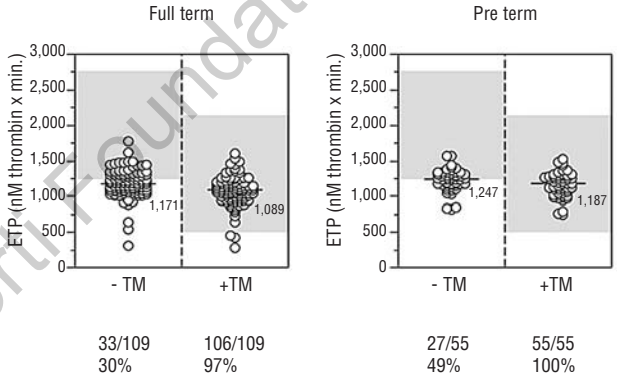

Figure 1. **Endogenous thrombin potential (ETP) for full- (n=109) or pre-term (n=55) neonates when the test was performed for cord blood-derived plasma in the absence (–TM) or presence (+ TM) of soluble thrombomodulin. Horizontal bars represent median values; shaded areas represent the adult reference interval. Numbers on the bottom represent the proportions of neonates identified as having ETP levels greater than the lower-limit of the adult reference interval.**

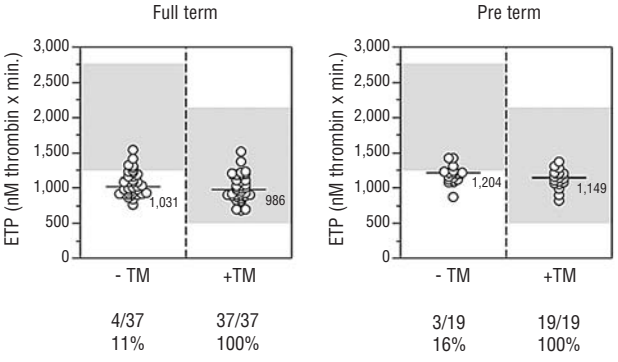

Figure 2. **Endogenous thrombin potential (ETP) for full- (n=37) or pre-term (n=19) neonates when the test was performed for venous blood-derived plasma. See also legend to Figure 1.**

the action of the anti-coagulants. Consequently, such traditional tests as PT and APTT might not be completely adequate to investigate coagulation in neonates. Perhaps the measurement of TG in the presence of thrombomodulin might be more suitable to assess hypo- or hyper-coagulability in this setting. Although plausible, this hypothesis needs to be substantiated in clinical studies. Recently, we came to the same conclusion by investigating the balance of coagulation in cirrhosis,[7,8] a clinical condition that shares similarities with the neonatal period concerning the perturbation of pro- and anti-coagulants.[20]

Another important and new finding of the study is that pre-term infants generate slightly but significantly more thrombin than their full-term counterpart. The practical implications of this finding are unknown, but may form the basis for clinical studies in this category of neonates whose outcome is not devoid of complications.

In conclusion, the coagulation balance in neonates is restored by the concomitant reduction of pro- and anti-coagulants. The restored balance can be shown *in vitro* by TG tests that include thrombomodulin, but not by such conventional coagulation tests as PT or APTT.

Authorship and Disclosures

AT: conception of the study, analyzing and interpretation of the data, writing the manuscript. PMM, FM: contribution to the conception of the study, data interpretation and manuscript revision. LAR, ADC, MG: management of patient recruitment and data collection. VC: data management, statistical analysis and contribution to data interpretation. MC: management of laboratory investigations. The authors declare no potential conflict of interest.

References

1. Dahlback B. Blood coagulation and its regulation by anticoagulant pathways: genetic pathogenesis of bleeding and thrombotic diseases. J Intern Med 2005;257:209-23.
2. Al Dieri R, Peyvandi F, Santagostino E, Giansily M, Mannucci PM, Schved JF, et al. The thrombogram in rare inherited coagulation disorders: its relation to clinical bleeding.Thromb Haemost 2002;88:576-82.
3. Tripodi A, Martinelli I, Chantarangkul V, Battaglioli T, Clerici M, Mannucci PM. The endogenous thrombin potential and the risk of venous thromboembolism. Thromb Res 2007;121:353-9.
4. Huntington JA. Mechanisms of glycosaminoglycan activation of the serpins in hemostasis. J Thromb Haemost 2003;1:1535-49.
5. Dahlback B. Progress in the understanding of the protein C anticoagulant pathway. Int J Hematol 2004;79: 109-16.
6. Hemker HC, Giesen P, Al Dieri R, Regnault V, de Smedt E, Wagenvoord R, et al. Calibrated automated thrombin generation measurement in clotting plasma. Pathophysiol Haemost Thromb 2003;33:4-15.
7. Tripodi A, Salerno F, Chantarangkul V, Clerici M, Cazzaniga M, Primignani M, Mannucci PM. Evidence of normal thrombin generation in cirrhosis despite abnormal conventional coagulation tests. Hepatology 2005; 41:553-8.
8. Tripodi A, Primignani M, Chantarangkul V, Clerici M, Dell'Era A, Fabris F, et al. Thrombin generation in patients with cirrhosis: the role of platelets. Hepatology 2006;44:440-5.
9. Chantarangkul V, Clerici M, Bressi A, Giesen PL, Tripodi A. Thrombin generation assessed as endogenous thrombin potential (ETP) in patients with hypo- or hyper-coagulability. Effects of phospholipids, tissue factor and residual platelets on the measurement performed in platelet-poor and platelet-rich plasma. Haematologica 2003;88:547-54.
10. Bertina RM, van der Marel-van Nieuwkoop W, Loeliger EA. Spectrophotometric assays of prothrombin in plasma of patients using oral anticoagulants. Thromb Haemost 1979; 42:1296-305.
11. Andrew M, Paes B, Milner R, Johnston M, Mitchell L, Tollefsen DM, et al. Development of the human coagulation system in the full-term infant. Blood 1987;70:165-72.
12. Nardi M, Karpatkin M. Prothrombin and protein C in early childhood: normal adult levels are not achieved until the fourth year of life. J Pediatr 1986;109:843-5.
13. Petaja J, Manco-Johnson MJ. Protein C pathway in infants and children. Semin Thromb Hemost 2003;29: 349-62.
14. Nowak-Gottl U, Kosch A, Schlegel N. Neonatal thromboembolism. Semin Thromb Hemost 2003;29:227-34.
15. Cvirn G, Gallistl S, Muntean W. Effects of antithrombin and protein C on thrombin generation in newborn and adult plasma. Thromb Res 1999; 93:183-90.
16. Cvirn G, Gallistl S, Leschnik B, Muntean W. Low tissue factor pathway inhibitor (TFPI) together with low antithrombin allows sufficient thrombin generation in neonates. J Thromb Haemost 2003;1:263-8.
17. Cvirn G, Gallistl S, Rehak T, Jurgens G, Muntean W. Elevated thrombin-forming capacity of tissue factor-activated cord compared with adult plasma. J Thromb Haemost 2003;1:1785-90.
18. Fritsch P, Cvirn G, Cimenti C, Baier K, Gallistl S, Koestenberger M, et al. Thrombin generation in factor VIII-depleted neonatal plasma: nearly normal because of physiologically low antithrombin and tissue factor pathway inhibitor. J Thromb Haemost 2006;4:1071-7.
19. Hemker HC, Willems GM, Béguin S. A computer assisted method to obtain the prothrombin activation velocity in whole plasma independent of thrombin decay processes. Thromb Haemost 1986;56:9-17.
20. Tripodi A, Caldwell SH, Hoffman M, Trotter JF, Sanyal AJ. The prothrombin Time Test as a measure of bleeding risk and prognosis in liver disease. Aliment Pharmacol Ther 2007;26: 141-8.