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Elevated plasma tissue factor levels in neonates with umbilical arterial catheter-associated thrombosis

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Abstract

Catheterization of the umbilical artery has been a useful aid in the management of sick neonates for the past few decades. However, it is associated with various complications. Reported studies strongly suggest a significant role of intravascular catheterization in the development of aortic thrombi. Increase in thrombosis of large vessels is believed to be related to mechanical injury in the catheterized vessels, which provide direct exposure of blood to tissue factor (TF), the primary cellular initiator of the extrinsic coagulation pathway. This study was conducted to determine the levels of plasma TF, tissue factor pathway inhibitor (TFPI) and D-dimer (DD) in infants with umbilical arterial catheter (UAC)-associated thrombosis. Quantification of TF was carried out using an in-house sandwich ELISA, whereas TFPI and DD levels were measured with commercial immunoassay kits. Infants with UAC inserted were found to have significantly higher levels of plasma TF ($p < 0.001$) than baseline levels. However, there were no significantly elevated levels of TFPI or DD. Infants with UAC-associated thrombosis demonstrated a greater increase of TF level (median: 414.5 pg/mL; range: -76.0, 6667.0) than infants without UAC-associated thrombosis (105.0 pg/mL; -976.0, 9480.0; $p = 0.009$) following UAC insertion. Our findings indicate that quantification and monitoring of TF levels could predict thrombus formation in infants with indwelling UAC. Following umbilical arterial catheterisation, infants with an approximately 3-fold rise in plasma TF levels were most at risk of developing abdominal aorta thrombosis as confirmed by real-time abdominal ultrasonography.

Key words: tissue factor, tissue factor pathway inhibitor, D-dimer, umbilical arterial catheterisation, thrombosis and neonates.

INTRODUCTION

Catheterization of umbilical vessels to facilitate management of the sick or critically ill newborn infants has been a routine practice in most neonatal intensive care units (NICU). The use of an indwelling catheter in an umbilical vessel can lead to improved care of sick infants; however, this useful diagnostic and therapeutic tool may also cause serious complications. The potential complications include thrombosis; embolism; vasospasm; vascular perforation; infection; haemorrhage; gastrointestinal, renal, and limb tissue damage; hepatic necrosis; hydrothorax; cardiac arrhythmias; pericardial effusion and tamponade; and erosion of the atrium and ventricle.[1] In addition, the incidence of neonatal hypertension is significantly higher in infants inserted with umbilical arterial catheter (UAC).[2-4]

Thrombosis has been reported to be the most common complication, with an incidence ranging from as low as 4.7% to as high as 95%.[3,5-16] Since catheterization is undoubtedly of great usefulness, an understanding of the mechanisms and risk factors contributing to the development of thrombosis and infarction may help to reduce the incidence of such complications.

Tissue factor (CD142; tissue thromboplastin; coagulation factor III, TF), a ubiquitous membrane-anchored low molecular weight (45-47 kD) glycoprotein, is a high-affinity cellular receptor and cofactor for factor VII/VIIa.[17-20] This single chain glycoprotein is the primary cellular initiator of the coagulation protease cascade. Under normal circumstances, TF is present at sites anatomically separated from flowing blood.[21] It is constitutively expressed in a cell-specific manner

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in tissues surrounding vascular structures, and in cells delimiting organ boundaries. The physiological cellular distribution of TF appears to form a 'haemostatic envelope', ensuring prompt initiation of coagulation in the event of vascular rupture.[21,22] As a potent procoagulant, TF is believed to have a critical pathophysiological role in thromboembolic disorders and the lethal coagulopthy of septicemia in pathological conditions. Indeed, recent studies have suggested that TF also plays non-haemostatic roles in embryonic vascular development,[23] intracellular signaling,[24-26] neovascularization and tumour metastasis.[26-28]

The TF-VIIa complex initiates a cascade of proteolytic reactions resulting in thrombin production – the penultimate step in haemostasis.[29] TF function is regulated by feedback inhibition involving a Kunitz-type inhibitor, TF pathway inhibitor (TFPI).[30] Intricately interwoven with the clotting mechanism is the process of fibrinolysis, which is achieved by a potent plasma proteolytic enzyme, plasmin. The digestion of fibrin by plasmin produces fibrin degradation products, and one of them is fibrin D-dimer (DD), a cross-linked fragment that is not produced by the digestion of fibrinogen.[31] The fibrinolytic system is essential for removing fibrin deposits as part of vessel healing to reestablish blood flow and preserve vascular patency.

Considering mechanical factors, there are reports concerning the ability of catheters to produce considerable trauma.[5,6] Additionally, increase in thrombosis of large vessels is believed to be related to direct mechanical injury and alteration of flow in catheterized vessels.[8] The traumatized vessels provide direct exposure of blood to collagen and TF, which will subsequently initiate the coagulation system. TF antigen in plasma may reflect endothelial injury and cell destruction in TF-containing tissues, and as yet there is no report demonstrating the correlation of TF, TFPI and DD levels with the occurrence of UAC-associated thrombosis. We therefore conduct this study to evaluate the clinical significance of TF, TFPI and DD levels, in newborn infants with UAC-associated thrombosis.

MATERIALS AND METHODS

Study subjects

A total of 94 consecutive newborn infants admitted to the NICU of Hospital Universiti Kebangsaan Malaysia from August 1998 to August 2000 with UAC inserted were eligible for the study. Infants who died before the removal of UAC, and who developed complication of septicemia were excluded from the study. Characteristics of the infants were summarised in Table 1.

TABLE 1. Characteristics of the infants with umbilical arterial catheter (UAC) inserted.

Characteristics	**Infants with UAC (*n* = 94)**
Gender	
Male (%)	60.6
Female (%)	39.4
Race	
Malay (%)	61.7
Chinese (%)	33.0
Indian and others (%)	5.3
Mode of Delivery	
SVD (%)	52.1
ID (%)	47.9
Birthweight (g)†	1973.5 ± 1019.7
Gestation (wk)†	33.2 ± 5.5
Apgar Score (1 min)†	5.2 ± 3.0
Apgar Score (5 min)†	6.7 ± 3.2

SVD, spontaneous vertex delivery; ID, instrumental delivery (lower segment caesarean section, breech, vacuum, forceps).
† Values are mean ± SD.

Blood collection and plasma preparation
Syringe technique was applied to draw 0.5-1 mL of blood from the UAC after obtaining consent from the infant's parents under study. Paired blood samples from the infants were taken at the time of insertion of UAC (initial-UAC), and just before the removal of UAC (end-UAC). Initial-UAC sample was taken before commencement of infusion of heparinised saline. For end-UAC sample, 1 mL of blood was withdrawn into a syringe before a sample of 0.5-1 mL of blood was collected into another sterile unused syringe for analysis. This is to prevent contamination of the specimen by the infusion fluid. Platelet-poor-plasma was subsequently obtained by drawing 9 vol of the blood into 1 vol of 3.8% (129 mM) trisodium citrate, and by centrifugation at 3000 rpm for 30 min at 4°C. The plasma was then aliquoted, snap-frozen and stored at -80°C for further testing. Blood separation was performed within 2 h of collection to minimize any effects of storage. Clotted and grossly haemolyzed samples were excluded from the study.

Umbilical arterial catheterization
The position of the tip of the UAC was confirmed by radiography. The UAC was inserted until its tip was located in the infant's aorta at a level between the sixth and ninth thoracic vertebrae. Whenever radiography showed that the tip of any UAC was at a level below the ninth thoracic vertebra, the UAC was withdrawn partially until its tip was at a level between the second and fourth lumbar vertebrae below the level of the infant's renal vessels. The tip of the UAC was positioned such to minimize the risk of causing thrombosis or arterial spasm to the origin of the renal arteries. Only polyvinyl Argyle catheters with end holes were used during the study period. All catheters were infused with heparinised intravenous fluid at a concentration of 1.0 U of heparin/mL. No infant received infusion of calcium solution via UAC.

Detection of thrombus formation
The first abdominal ultrasonography was performed shortly before or after the insertion of UAC. Within 24-48 h after removal of UAC, ultrasound scanning was repeated. This is the standard clinical pathway to monitor UAC-associated thrombosis in our NICU, based on the findings of our previous study.[3] Real-time ultrasound examination was performed in the NICU at the crib side by the attending neonatologists, using a 7.5 MHz linear probe (sometimes a 7.5 MHz sector probe was used for tiny infants) on an ultrasound machine (Apogee 800 Model, Advance Technology Laboratory, Bothell, Washington, USA). The abdominal vascular system was systematically examined utilising both 2-D and colour Doppler modes via the coronal views and transverse views from both flanks of the infant's abdomen, and by the transverse and longitudinal views at the infant's epigastric region. The following structures of each infant were scanned: abdominal aorta, origins of coeliac artery and superior mesenteric artery, proximal common iliac arteries, renal arteries, inferior vena cava, hepatic veins, portal vein, renal veins, the liver, adrenal glands, and kidneys. A thrombus was diagnosed ultrasonographically when a persistent intravascular echo was observed in two planes, attached either to the vessel wall or to the catheter.[32] Colour Doppler was used to confirm the presence of a mass effect of abnormal echoes at these sites.

In-house TF ELISA
TF antigen was measured by a previously published in-house direct sandwich ELISA,[33] which employed two antibodies against human TF. Briefly, the microtiter plate (Immulon® 2HB Removawell®, Dynex Technologies, USA) was coated at 4ºC overnight with 1250 ng/mL mouse anti-human TF MAb (Enzyme Research Laboratories Inc., Indiana, USA). After blocking with buffer containing 5% (wt/vol) skimmed milk and 0.5% (vol/vol) Tween 20, samples diluted with PBS containing 1.5% skimmed milk and 0.5% Tween 20 were incubated for 1 h at 27ºC. After five washing steps, 2 µg/mL of horseradish peroxidase-labelled sheep IgG anti-human TF (Affinity Biologicals Inc., Ontario, Canada) was added and incubated for 1 h at 27ºC. TMB (3,3',5,5'-tetramethylbenzidine; Sigma Co., USA) was utilized as substrate. Absorbance was measured spectrophotometrically in Multiskan MS microplate reader (Labsystem, Finland). Samples were assayed in triplicate and the respective TF levels were interpolated from the linear portion (10-4000 pg/mL) of the standard curve constructed from recombinant TF (American Diagnostica Inc., Greenwich, CT, USA). The assay demonstrated low intra- (3-9 CV%; coefficient of variation) and inter-assays (6-14 CV%) variability, as well as satisfactory analytical recovery (92-104 %).

TFPI and DD
Total TFPI and DD were measured with Asserachrom® Total TFPI (Diagnostica Stago, Asnières-Sur-Seine, France) and TintElize® D-dimer (Biopool® International, CA, USA) commercial ELISA kits respectively.

Statistical analysis
Statistical analysis was carried out using SPSS Version 9.0. Tests of normality were performed to verify the distribution of continuous variables. Paired Student's *t*-test (or Wilcoxon signed-rank test for not-normally distributed variables) was applied to assess the differences between initial-UAC and end-UAC samples. Unpaired Student's *t*-test (or Mann-Whitney U test for not-normally distributed variables) was used to compare variables between two groups (with thrombus *vs*. without thrombus; alive *vs*. dead). All statistical tests were two-tailed, and p value < 0.05 was considered to be statistically significant.

RESULTS

Initial-UAC vs. end-UAC samples
Table 2 compares the levels of plasma TF, TFPI and DD in initial-UAC and end-UAC samples. The levels of TF were significantly increased after UAC insertion ($p < 0.001$). However, TFPI and DD levels showed no significant increase.

Development of UAC-associated aortic thrombus
No thrombus was detected by ultrasonography in any infant at the time of insertion of UAC. Abdominal aortic thrombi were detected in 22 infants upon removal of UAC. The duration of UAC *in-situ* was longer in infants who developed thrombi (median: 5.5 days, range: 1, 24) than those without thrombi (median: 4 days, range: 1, 25), although the difference was not statistically significant ($p = 0.137$).

Infants with UAC-associated thrombus vs. infants without UAC-associated thrombus
Infants who developed UAC-associated thrombosis demonstrated greater increase in TF levels after catheterization (median: 414.5 pg/mL; range: -76.0, 6667.0) than infants without UAC-associated thrombosis (median: 105.0 pg/mL; range: -976.0, 9480.0; $p = 0.009$) (Table 3). TF levels in the initial-UAC samples were not significantly different between infants who developed thrombus (median: 232.0 pg/mL; range: 66.0, 1579.0), and those without thrombus (median: 253.0 pg/mL; range: 36.0, 2125.0; $p = 0.9$). Meanwhile, there were no significant differences in the increase of TFPI and DD levels after catheterization, between these two groups of infants.

Alive vs. dead infants
During the study, 89 infants receiving UAC insertion survived until discharge while 5 died subsequently after the removal of UAC. Infants who survived showed non-significant difference of initial-UAC TF levels (median: 251.0 pg/mL; range: 36.0, 2125.0) when compared with those who died (median: 589.0 pg/mL; range: 153.0, 1442.0; $p = 0.2$). There were no significant differences in the increase in levels of TF, TFPI and DD levels after catheterization, between infants who survived and those who died (Table 4).

TABLE 2. Comparison of tissue factor (TF), tissue factor pathway inhibitor (TFPI) and D-dimer (DD) levels in initial-umbilical arterial catheterization (UAC) and end-UAC samples.

	Initial-UAC (n = 94)	**End-UAC (n = 94)**	p values
TF (pg/mL)†	253.0 (36.0, 2125.0)	441.5 (39.0, 9645.0)	$p < 0.001$*
TFPI (ng/mL)‡	55.9 ± 31.0	59.0 ± 32.6	0.4
DD (ng/mL)†	406.8 (47.1, 3815.4)	432.4 (61.0, 3507.7)	0.8

Data are expressed as † median (range) or ‡ mean ± SD.
* Denotes statistical significance.

TABLE 3. Comparison of the increase of plasma tissue factor (TF), tissue factor pathway inhibitor (TFPI) and D-dimer (DD) levels between infants with and without umbilical arterial catheter (UAC)-associated thrombosis.

Difference between end-UAC and initial-UAC samples	**Infants with UAC-associated thrombus (*n* = 22)**	**Infants without UAC-associated thrombus (*n* = 72)**	*p* values
TF (pg/mL)†	414.5 (-76.0, 6667.0)	105.0 (-976.0, 9480.0)	0.009*
TFPI (ng/mL)‡	4.1 ± 47.7	2.7 ± 34.2	0.9
DD (ng/mL)†	23.9 (-3117.2, 1714.4)	32.5 (-3065.0, 3168.3)	0.9

Data are expressed as † median (range) or ‡ mean ± SD.
* Denotes statistical significance.

TF/TFPI ratio

Infants with UAC-associated thrombosis showed greater increase of TF/TFPI ratio than infants without UAC-associated thrombosis; however, this difference did not reach the statistical significant level ($p = 0.06$; Table 5). This ratio was also not statistically different between infants who were alive and those who died.

DISCUSSION

To our knowledge this is the first study to demonstrate a significant association between umbilical arterial catheterisation and an increase in plasma TF levels ($p < 0.001$) in newborn infants. In an experimental study reported by Chidi *et al*.,[34] catheterization of the abdominal aorta with small umbilical artery catheters consistently produced significant intimal injury and endothelium disruption. The increased end-UAC TF levels in the present study suggested that direct mechanical injury of vascular endothelium, which was the extravascular TF reservoir, eliciting acute response to vascular injury. The finding was unlikely due to the heterogeneous study population (Table 1). There was no significant correlation noticed between the TF levels and birthweight or TF levels and gestational week, although the infant's birthweight and gestational week ranged widely from 265-4890 g (birthweight ≥ 2500 g: n = 31; birthweight < 2500 g: n = 63) and 24-43 wk (term gestation: n = 35; preterm gestation: n = 59) respectively. Similarly, no significant correlation was observed between the TF levels and Apgar scores despite of the wide ranges of Apgar scores at 1 min (0-9) and 5 min (0-10).

It is interesting to note that there were no significant differences between the end-UAC TFPI and DD levels and those in the initial-UAC samples. We speculated that the measured TFPI concentration was the total TFPI presented in plasma, which comprised the TF-FVIIa

TABLE 4. Comparison of the increase of plasma tissue factor (TF), tissue factor pathway inhibitor (TFPI) and D-dimer (DD) levels between umbilical arterial catheterized-infants who survived and those who died.

Difference between end-UAC and initial-UAC samples	**Alive (*n* = 89)**	**Dead (*n* = 5)**	*p* values
TF (pg/mL)†	133.0 (-976.0, 9480.0)	10.0 (-114.0, 5553.0)	0.3
TFPI (ng/mL)‡	3.1 ± 37.0	1.6 ± 51.1	0.9
DD (ng/mL)†	37.2 (-3117.2, 3168.3)	-51.2 (-2957.2, 540.0)	0.4

Data are expressed as † median (range) or ‡ mean ± SD.

complexes, bound and free forms of TFPI. The active form of TFPI could not be determined in the present study. Meanwhile, the DD had most probably reached its maximum levels where the production of this marker had achieved the saturation state, as its levels measured in the neonates in the present study, whether in initial or end-samples were very high when compared with levels in normal adults [mean 40 ng/mL[35]].

In a previous study, Boo *et al*.[3] reported that, after controlling for various potential confounders, the only significant risk factor associated with the development of abdominal aortic thrombosis following insertion of UAC was the duration of UAC *in situ*. They found that for every additional day of UAC remaining *in situ*, the adjusted odds ratio of developing aortic thrombosis was 1.2 (95% C.I.: 1.1, 1.3; $p = 0.002$). In the present study, infants who developed aortic thrombi demonstrated greater increase of TF levels than those who did not develop such thrombus ($p = 0.009$), after umbilical arterial catheterization (Table 3). Although not statistically significant, infants with UAC-associated thrombosis in the present study also had their UAC left *in-situ* for longer duration than those without thrombosis. The most likely explanation for this lack of statistical significance was the much smaller sample size of infants with thrombosis in the present study than our previous one.

The findings of the present study suggest that serial measurement of TF may help to identify infants at risk of developing aortic thrombosis when there is a need of a UAC to remain *in situ* for longer period of time. An approximately 3-fold increase of TF levels demonstrated moderate diagnostic indices of thrombus formation (sensitivity = 55%, specificity = 83%, positive predictive value = 50%, negative predictive value = 86%). These infants therefore may need to be monitored more closely by abdominal ultrasonography as well. The finding of this study was in agreement with the accumulating evidence that increased TF production could be an important causative factor of thromboembolic state in pathological conditions.[20,36,37] Conversely, neither the increase in TFPI nor DD levels was significantly different between infants with or without thrombosis (Table 3).

On the other hand, no significant differences were shown in the increase of these three haemostatic markers between infants who survived and those who died (Table 4). In a study on patients with disseminated intravascular coagulation (DIC), there was no significant difference in plasma TF levels between the DIC patients with good outcome and those with poor outcome.[38] Thus, based on our data, we might surmise that these markers were not useful in predicting the survival of infants receiving UAC insertion. However, the number of infants who died in this study was too small ($n = 5$) to draw any definite conclusion.

The activity of TF is tightly regulated *in vivo* by TFPI, which inhibits the activity of TF-VIIa complex by forming a stable, inactive quaternary complex composing of TFPI, TF, Factor VIIa and Xa, thereby blocking the generation of thrombin.[30] Thus, the increase TF/TFPI ratio might contribute to a markedly hypercoagulable state that could progress to thrombus. In infants with UAC-associated thrombosis, the thrombotic tendency may be induced by uncontrolled elevation of TF levels that are not sufficiently balanced by TFPI. In

TABLE 5. Relationship between tissue factor (TF) / tissue factor pathway inhibitor (TFPI) ratio and outcome of infants receiving umbilical arterial catheter (UAC) insertion.

Outcome	TF/TFPI ratio†	*p* values
UAC-associated thrombosis		
Yes ($n = 22$)	8.9 (-4.5, 151.3)	0.06
No ($n = 72$)	1.6 (-40.1, 150.5)	
Died		
Yes (n = 5)	0.4 (-6.5, 150.5)	0.3
No (n = 89)	2.6 (-40.1, 151.3)	

Data are expressed as median (range).
† Difference between TF/TFPI (end-UAC) and TF/TFPI (initial-UAC) ratio

the present study, a greater increase of TF/TFPI ratio was noted in infants with UAC-associated thrombus than those without UAC-associated thrombus. The borderline lack of statistical significance ($p = 0.06$; Table 5) could be due to inadequate sample size.

In summary, infants inserted with UAC showed elevated levels of TF after catheterization. In particular, infants who developed aortic thrombus demonstrated a significantly greater increase in TF levels than infants who did not develop thrombus ($p = 0.009$). Our findings indicated that serial measurement of TF levels following insertion of UAC could predict possible impending thrombus formation, particularly when there is a 3-fold rise in TF levels. Based on this observation, the institution of prophylactic measures such as anti-TF or TFPI therapy, may help to deter the development of thrombus and minimize the morbidity of infants receiving UAC insertion. Although raised levels of plasma TF in infants with UAC-associated thrombosis may reflect the aetiopathogenesis of thrombosis, measurement of TF levels has moderate sensitivity to predict the development of thrombosis. The reason for this moderate sensitivity could be due to the fact that we are measuring the total amount of TF, but not the functional component of TF. In this regard, functional activity measurement of TF,[39,40] may complement excellently to the immunochemical analysis of TF antigen levels, and thus will clarify some of the ambiguities that remain.

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