Alterations of Hemostatic and Inflammatory Markers in Neonatal Infections in Egypt

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Abstract: Infection often causes systemic activation of both inflammation and coagulation that may have major consequences for the pathogenesis of microvascular failure and subsequent organ failure. Studies on coagulation focus mainly on adults, seldom have been done on sick newborns. This research was designed to investigate the effect of infection on the balance between tissue factor (TF) and its inhibitor, tissue factor pathway inhibitor (TFPI) (measures of coagulation) and to elucidate the association between TF, TFPI and C-reactive protein (CRP, measure for inflammation) in jaundiced newborns. Anthropometric data including birth weight, length, and head circumference showed non significant changes between infected jaundiced newborns (cases) and jaundiced neonates (controls). Meanwhile plasma TF, TFPI, CRP, bilirubin, and blood hemoglobin (Hb) levels were extremely elevated in infected jaundiced newborns as compared to jaundiced neonates. TFPI/TF ratio was extremely low in cases as compared to controls. Plasma calcium concentration exhibited no changes in both groups. TFPI in cases displayed significant positive correlations with TF and CRP and a marked negative correlation with Hb. No correlations were found between TFPI/TF and CRP and other determined parameters in cases. These observations suggest that each of TF, TFPI and CRP is of diagnostic value for infections. In addition, anti-inflammatory and anticoagulant therapies besides antibiotics may be adjuvant management strategies for infections.

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1. Introduction:

Infection is a common and life threatening condition with a high mortality rate. Infection involves uncontrolled host defense responses that lead to inflammation, endothelial damage, enhanced coagulation, diminished fibrinolysis, and fibroproliferation (Maclaren and Stringer, 2007). The immune response to infection includes activation of the blood clotting system, leading to extravascular fibrin deposition to limit the spread of invasive microorganisms (Yun *et al*., 2009). The early activation of coagulation is triggered by tissue factor expression and secondary impaired fibrinolysis by the upregulation of fibrinolysis inhibitors. This imbalance is a major cause of subsequent organ dysfunction (Fourrier, 2006). Increasing evidence points to an extensive cross-talk between those two systems, whereby inflammation not only leads to activation of coagulation, but coagulation also considerably affects inflammatory activity. The intricate relationship between inflammation and coagulation may have major consequences for the pathogenesis of microvascular failure and subsequent multiple organ failure, as a result of severe infection and the associated systemic inflammatory response (Levi, 2010). Consistent evidence has demonstrated an inverse association between postnatal and childhood infections and adulthood liver enzymes levels. There is also evidence that intrauterine and early life exposures affecting birth size and growth may also affect liver development because it remains plastic in first years of life (Fraser *et al*., 2008a). Besides, this immediate postnatal infection may be particularly important in determining adult cardiovascular disease risk (Fraser *et al*., 2008b).

Inflammatory responses to infection include expression of tissue factor on activated monocytes as well as increased acute phase proteins such as C-

reactive protein which contribute to hypercoagulable states (Levi *et al* 2004). Tissue factor is a potent initiator of the coagulation cascade which can trigger the hemostatic system to generate thrombin. Also in severe conditions, including infection and lung injury. TF may induce fibrin deposition in organs which are believed to have determining impact on patients outcome. In addition, TF acts as a signaling receptor and is involved in the systemic inflammatory response (Laterre *et al*., 2006). Tissue factor pathway inhibitor is a negative regulator of coagulation mediated by TF. The changes in plasma TFPI concentrations during inflammation are not well defined. Patients with chronic inflammation have increased plasma concentrations of TFPI (Novo *et al*., 2005). However, low TFPI concentrations have been reported in some cases of acute inflammatory process (Sandset and Andersson, 1989).

Studies on TF and TFPI focus mainly on adult subjects, seldom have been done on newborns. Therefore, the aim of this study was designed to evaluate the crucial role of TF and TFPI plasma levels in diagnosis of neonatal infection and to evaluate to what extent an inflammatory marker C-reactive protein, hemoglobin, and calcium may be altered in the infected newborns. Besides, our aim included studying how the previously mentioned biochemical parameters may correlate with each others. These parameters seem to be quite helpful for proper treatment, correction of any metabolic disturbances and preventing any dangerous complications.

2. Subjects and Methods:

2.1 Subjects:

The target population in this study was newborn babies. Thirty neonates (13 boys and 17 girls) with jaundice and infection were considered as cases (jaundiced newborns with infection). Their gestational age was 38.8± 1.27 (mean ± SD) weeks. They were chosen from the Neonatology Unit Al-Azhar University Hospital during the period 2008-2009. Twelve of them were infected due to premature rupture to maternal membrane, ten due to maternal urinary tract infection with fever, and eight of them due to maternal vaginal infection with fever. Other twenty neonates (9 boys and 11 girls) were selected from the same unit and in the same period with gestation age matched to the patient's group 39.2 ± 0.92 weeks and were considered as control group. Full ethical permission for the study was granted by National Research Centre Ethical Committee for research on human subjects and another local ethical approval was received from Al-Azhar University Hospital. The mothers were informed about the purpose of the study and their verbal informal consents were obtained. All individuals included were subjected to anthropometric measurements that included length, weight, and head circumference using standardized equipments and following the formal recommendations of Fomon and Nelson (1993).

Infected neonates were defined as belonging to one of the following groups (Mathers and Pohlandt, 1987) to select neonates most fitted to the criteria of current study, 1- culture proven septicaemia, babies in whom a generalized bacterial infection resulted in clinical signs of infection documented by a positive blood culture. 2- Clinical septicaemia; babies in whom a generalized bacterial infection resulted in clinical signs of infection and haematological changes supporting the diagnosis. 3- Congenital pneumonia; babies in whom a congenital focal infection of the lungs resulted in respiratory distress and signs of infection documented by a positive tracheal aspirate culture obtained within 4h of delivery (Sherman *et al* 1980) and by haematological changes supportive of infection together with x-ray changes consistent with the diagnosis.

2.2 Blood Sampling:

On the third day after delivery venous blood samples were withdrawn from cases and control newborns into heparinized tubes. Part of heparinized blood was used for leukocytes count and hemoglobin determination. The other part of blood was centrifuged at 3000 rpm for 15min and plasma was separated and stored at -70°C for analysis.

2.3 Analytical determinations:

The plasma level of tissue factor was evaluated by means of Quantikine® Human Tissue Factor ELISA Kit (R & D, system, Europe, Catalog number DCF, 300), Briefly, a marine antihuman tissue factor monoclonal antibody had been precoated onto a microplate wells. Standards and samples were pipetted into wells and any TF present was bound by

immobilized antibody. After washing away any unbound substances, the conjugate (polyclonal antibody against TF conjugated to horse-radish peroxidase) was added to the wells. Following a wash to remove any unbound antibody enzyme reagent, a substrate solution, H_2O_2 and tetramethylbenzdine is added to the well. The sensitivity was increased by addition of 0.5M sulfuric acid stop solution, turning colour to yellow. Quantitative data were obtained by measuring the solution absorbance at 450nm, and relating it to standard curve. Also, plasma concentration of TFPI was measured using Quantikine® Human Total Tissue Factor Pathway Inhibitor Immunoassay [ELISA] kit (R & D, system, Europe, Catalog number DTFP10) employed a mouse monoclonal antibody against TFPI as the capture antibody. Samples and standards were incubated in microtest wells precoated with the capture antibody. TFPI was detected using conjugate polyclonal antibody against TFPI conjugated with horse-radish peroxidase. The subsequent steps were identical with those described above. Plasma C-reactive protein concentration was quantitatively determined by an enzyme immunoassay method using ELISA Diamed Eurogen (Belgium) Kit. Calcium level in plasma was evaluated colorimetrically by reacting with methylthymol blue according to Gindler and king (1972) method. Total bilirubin plasma concentration was evaluated using the procedure of (Walter and Gerarade, 1970). Finally, blood hemoglobin was estimated using the method of Betke and Savelsberg (1956).

2.4 Statistical analysis:

The data in the present work were statistically analyzed using SPSS computer program. Values were expressed as means $\pm$ S.D. Differences between cases and control were compared with a Student's two tailed t-test. Correlations between TF and TFPI and other biochemical variables in cases were done according to Pearson correlation coefficient. A probability value of $P \leq 0.05$ was considered to be statistically significant.

3.Results:

Table (1) shows different anthropometric measures in the cases (infected jaundiced neonates) and control group (jaundiced neonates). Gestational age, birth weight, length, and head circumference were approximately the same in both groups. There were no significant differences between cases and control.

Hematologic and biochemical variables are presented in table (2). Blood hemoglobin concentration was not significantly changed in infected neonates (17.2 $\pm$ 3.08) as compared to control neonates (14.9 $\pm$ 1.10). Meanwhile, leukocytes count was significantly elevated in infected newborns ($P < 0.001$) ($11.2 \times 10^3 \pm 5.50$) as regards to control value ($7 \times 10^3 \pm 4.50$) indicating inflammation and infection. Besides, a strong inflammatory marker, plasma C-reactive protein value in infected newborns was extremely increased (2 $\pm$ 0.643) ($P < 0.001$) when compared to control neonates (0.21 $\pm$ 0.10). Also, plasma TF and TFPI levels were extremely elevated ($P < 0.001$) (176 $\pm$ 67 and 20.9 $\pm$ 3.23, respectively) in newborns with infection as compared to control newborns (36.9 $\pm$ 12.9 and 7.5 $\pm$ 1.54, respectively). By contrast, TFPI/TF ratio in patients was significantly reduced ($P < 0.001$) (139 $\pm$ 57.3) when compared to control value (212 $\pm$ 108). Hyperbilirubinemia was noticed in patients ($P < 0.001$) (16.4 $\pm$ 3.23) as compared ~~to to~~ control (4.03 $\pm$ 0.93) ~~was not~~ significantly differed in both groups.

Table 3 presents the correlations between tissue factor, tissue factor pathway inhibitor, and their ratio with other determined parameters in infected neonates. Where, it shows that plasma tissue factor had a significant positive correlation with plasma tissue factor pathway inhibitor (r 0.487, $P \leq 0.01$). Also, a marked positive correlation (r 0.611, $P \leq 0.001$) was found between plasma TFPI and plasma C-reactive protein levels. Controversially, a negative marked correlation exhibited between plasma TFPI and blood hemoglobin (r -0.452, $P \leq 0.01$).

4.Discussion:

Aside from its role in minimizing blood loss, the clotting system is also an effector arm of the immune system that modulates release of inflammatory mediators and prevents the spread of invasive microorganisms through extravascular fibrin deposition. Evidence is also robust that infection is a clinical syndrome characterized by a systemic inflammatory response and activation of coagulation

(Levi, 2010). However, up to our best knowledge, this aspect has not been extensively studied in newborns, therefore we designed this work to evaluate the changes in coagulation fibrinolysis factors, TF and TFPI, respectively, and in inflammatory markers, C-reactive proteins and to find the correlation between those various parameters in infected jaundiced newborns.

The emerged data of the present study showed that plasma tissue factor concentration in cases was significantly higher than those of controls. Our results are consistent with those of Yue *et al.* (2003) and Levi (2010). TF is the primary initiator of the extrinsic coagulation pathway. It is a glycoprotein expressed in the subendothelial structure throughout the vasculature. When the lining of the blood vessel wall is damaged TF comes in contact with blood stream and binds to coagulation factor VII. Once coagulation factor VII is complexed with TF, it is converted to the active form which proteolytically activates downstream coagulation factors, ultimately leading to the conversion of prothrombin to thrombin and fibrin clot formation (Ware *et al*., 2006). Also, during the initial phase of injury due to infection the host defense response produces inflammatory cytokines from lymphocytes, activated macrophages and endothelium. These cytokines include interleukin-1, interleukin-6, tumor necrosis factors (TNF-α). These inflammatory cytokines further contribute to endothelial damage that results in exposure of the cell surface and the production of TF (Bastarache *et al*., 2006). Evidence in support of this view includes the following concerns. The administration of TNF-α to healthy volunteers induces thrombin generation and the activation of coagulation (Vanderpoll *et al*., 1990). In addition, proinflammatory cytokines, such as IL-1B and TNF-α, increase mRNA and protein expression of TF by monocytes (Gando *et al*., 2003) and macrophages (Levi *et al*., 2006). Furthermore, the blocking of IL-6 by specific antibodies attenuates the activation of coagulation in chimpanzee model of endotoxemia (Vander Poll *et al*., 1994). Finally, the administration of low dose endotoxin to normals is associated with a 125-fold increase in TF mRNA monocyte concentration (Franco *et al*., 2000). Indeed, the activation of the coagulation cascade by acute systemic inflammation during infection (i.e. endotoxemia) is mediated through the TF pathway (Esmon *et al*., 1999).

Tissue factor pathway inhibitor is a three kunitz domain glycoprotein which inhibits thrombin generation through the inhibition of the F VII/TF complex and activated factor (F)x. Under normal conditions, approximately 85% of TFPI is bound to endothelial cell surfaces, 5% is stored in platelets, and 10% circulates bounds to lipoproteins (Lindahl *et al*., 1992). The primary function of this protein is the inhibition of tissue factor (Ware *et al*., 2006). The overall balance between the concentration of the coagulation factors and the anticoagulation proteins is one of the determining factors of thrombin generation. In normal state, the immunoreactive concentrations of TFPI in the plasma are 500-1000 times higher than that of TF (Shimura *et al*., 1997). In the current study our data demonstrated that infected newborns had higher plasma TFPI levels than controls which is in agreement with those of Yue *et al*., (2003). During infection, increased levels of cytokines (Levi, 2010) prompt the release of TFPI from the surfaces of endothelial cells. This process ultimately depletes tissue factor pathway inhibitor from the endothelium. Therefore, the rational for administering this inhibitor to patients with severe sepsis is to restore TFPI and to protect the endothelium from TF-mediated injury (Abraham *et al*., 2001). The current results showed that plasma TFPI/TF ratio was lower in infected jaundiced neonates than that of controls mainly due to the increase in both TFPI and TF plasma concentrations. The same result was previously reported in neonates by Yue *et al* (2003) and in similar inflammatory conditions by Erez *et al*., (2008). The lower TFPI/TF ratio in cases occurred despite the increase in the plasma TFPI concentration observed in these patients. This suggests that the balance between TF and its natural inhibitor may better reflect the overall activity of TF pathway of coagulation, than the individual concentrations. Also, this lower ratio may contribute to the increased thrombin generation.

C-reactive protein is one of the acute phase proteins that increased during systemic inflammation (Libby and Ridker, 2004). It is synthesized by the liver in response to factors released by fat cells and it is a member of the pentraxin family of proteins. A number of claims have been published for the role of CRP in the diagnosis and management of neonatal

infection as its determination is of value in the early diagnosis of neonatal infection (Speer *et al*., 1983), sequential CRP determinations may be used to monitor the course of neonatal infection (Sann *et al*., 1984) and neonatal infection with a low CRP implying a poor prognosis (Philip, 1979). Our data demonstrated increased levels of CRP in cases as compared to controls suggesting good prognosis due to CRP role in protection against inflammation caused by infection. Previous reports confirm ours (Kebapcilar *et al*., 2010 and Kim *et al*., 2010). However, our data are not in consistent with those of Mathers and Pohlandt (1987) who reported low levels of CRP in neonates with infection. The most plausible explanation for this discrepancy is that their samples were collected in the very early stages of the infective process, meanwhile ours were collected on the third day after infection as CRP rises above normal limits within 6 hours and peaks at 48 hours and its half life is constant (Pepys and Hirsch field, 2003).

Our data demonstrated a strong positive correlation between TFPI level and CRP concentration in cases which confirms the association between inflammatory and hemostatic markers (Levi, 2010). The elevation in TFPI and CRP has a positive improved outcome against the pronounced inflammatory and coagulation responses of increased TF level in our cases (Abraham *et al*., 2001 and Philip, 1979).

Despite the extensive existing knowledge of the structure of Hb and its function within the erythrocytes, relatively little is known about pathophysiologic interactions involving extraerythrocytic Hb and host tissue. Clinical evidence of reticuloendothelial system damage has suggested that extraerythrocytic Hb can be toxic (Feola *et al*., 1988). The emerged results showed an increase in Hb concentration in cases. Blck (1988) reported that Hb can be released from erythrocytes during infection as a result of coagulation-mediated intravascular hemolysis or bacterial hemolysin activity. Evidence was exist that bacterial endotoxin (lipopolysaccharides [LPS]) and Hb may coexist in blood stream during pathologic conditions. Hb has been shown in vitro to enhance LPS initiated activation of coagulation and to stimulate production of mononuclear cell TF (Roth *et al*., 1993). In vivo, synergism of the toxicities of Hb and LPS has been proven and may contribute to the observed thrombosis and organ failure (White *et al*., 1986). Our data showed an inverse association between TFPI and Hb which was conceivable and was previously reported (Leurs *et al*., 1995 and Pawlak *et al*., 2007).

Concerning plasma bilirubin, our cases showed significantly higher levels of bilirubin than non infected jaundiced neonates. Our finding was in agreement with those of Yue *et al*., (2003). Yue documented that severe hyperbilirubinemia cases had higher TF levels than that in mild hyperbilirubinemia cases indicating that hyperbilirubinemia can aggravate the imbalance between TF and TFPI induced by infection through increasing plasma TF level.

In conclusion, our study provided consistent evidence that points to an extensive interactions between inflammation and coagulation during infection. Outcomes of this research showed significant increase in TF, TFPI and CRP levels in jaundiced infected newborns. In addition, more pronounced hypebilirubinemia and hemoglobin were in cases than jaundiced neonates. However, calcium levels and anthropometric measures exhibited no changes in both, groups. With this in mind, it may be worthwhile to use TF, TFPI and CRP measurements for diagnosis of infections. Moreover, anti-inflammatory and anticoagulant therapies besides antibiotics may be adjunctive management strategies for infectious diseases.

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Table (1): Different anthropometric measures of all enrolled neonates (mean ± S.D.)

Parameters	Neonates with infection N = 30	Control neonates N = 20
Gestational age (weeks)	38.8 ± 1.27	39.2 ± 0.92
Birth weight (kg)	3.38 ± 0.82	3.28 ± 0.35
Length (cm)	48.9 ± 2.99	49.3 ± 1.46
Head circumference (cm)	34.4 ± 2.04	35.1 ± 1.12

Values were compared relative to control neonates.

Table (2): Hematologic and biochemical parameters for infected and non infected neonates.

Parameters	Infected neonates N = 30	Non infected neonate N = 20
Hemoglobin (g/dl)	17.2 ± 3.08	14.9 ± 1.10
WBCs (cmm)	$11.2x10^3$ ± 5.50***	$7x10^3$ ± 4.50
C-reactive protein (mg/dl)	2.00 ± 0.643***	0.21 ± 0.101
Tissue factor (ng/l)	176 ± 67***	36.9 ± 12.9
Tissue factor pathway inhibitor (ng/ml)	20.9 ± 3.23***	7.50 1.54
Tissue factor pathway inhibitor/tissue factor	139 ± 57.3***	212 ± 108
Calcium (mg/dl)	8.44 ± 0.913	8.9 ± 0.79
Bilirubin (mg/dl)	16.4 ± 3.23***	4.03 ± 0.93

Values are expressed as mean ± S.D. and compared to control values.
*** P < 0.001

Table (3): Pearson correlation coefficients (r values) relating tissue factor, tissue factor pathway inhibitor and their ratio with the determined parameters in infected neonates.

Variables	Tissue factor ng/L	Tissue factor pathway inhibitor ng/ml	Tissue factor pathway inhibitor/tissue factor
Tissue factor pathway inhibitor (ng/ml)	0.487**	0	0.211
Bilirubin (mg/dl)	-0.261	0.228	0.217
WBCs cmm	0.141	-0.399	-0.159
C-reactive protein (mg/dl)	-0.019	0.611***	0.159
Hemoglobin (g/dl)	-0.268	-0.452**	0.133
Calcium (mg/dl)	-0.056	0.068	0.131

Correlation coefficients were significantly different from zero with ** P < 0.01 or *** P < 0.001.

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