Case Study

Natural Inhibitor; Antithrombin and Protein S Levels among Neonates Suffering from Sepsis

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Abstract

Neonatal sepsis is a fatal disease with significant neonatal morbidities and mortalities worldwide, despite preventive measures and efforts that target neonatal sepsis, morbidity and mortality statistics were not promising. Common clinical symptoms of sepsis make earlier diagnosis challenging.

***Objectives**: the study aimed to assess antithrombin (AT) and Protein S (PS) among Sudanese neonates with sepsis and compare them with healthy neonates to study the susceptible alteration in both natural inhibitors of hemostasis (AT and PS) considering different variables (gender, mode of delivery, gestational age, sepsis mode, outcome and causative bacterial agent)*

Methods: *a prospective case-control study achieved in the maternity hospital, Omdurman, Sudan in the period between Jun.2013 and Apr.2015 on a total of 100 samples divided into the case (neonates diagnosed with proven sepsis) and a control group of health neonates (50 for each) selected by non-probability sampling, Protein S was assessed by the clotting procedure using semi-automated coagulometer (Stago stat four), AT was assessed spectrophotometrically by the turbimetric method using semi-automated chemistry analyzer (Mindray BA-88A).*

Results: *the gender distribution was 23, 27 and 24, 26 males and females for case and control respectively, among case group; 17 neonates with early mode sepsis (0-7 days), and 33 with late onset (7-28 days). Considering the outcome in case group; 40 were recovered (80%) and 10 neonates dead (20%) of them; 4 (40%) with early onset sepsis, and 6 (60%) with late onset. Blood culture distributions were; Pseudomonas 23 (46%), Salmonella 9 (18), Klebsiella 7 (14%), Staph. epidermidis 3 (6%), Strep. fecailis 3 (6%), E. coli 2 (4%), Staph. aureus 2 (4%), and 1 Streptococci (Non group B) (2%).*

AT was significantly decreased in the case compared to the control group (mean; 183.9 and 221.5 Mg/ml) P. value 0.003. PS was insignificantly decreased (33.4 and 34.7%) P. value 0.76.

Among the case group; None of the gender, mode of delivery, Mode of sepsis, etiologic agent and sepsis outcome showed significant correlation with AT or PS.

Conclusion: it has been concluded that antithrombin was significantly decreased in septic neonates than healthy ones (P. value 0.003). It can be used as a diagnostic marker to offer quick reliable useful test feedback for septic neonates.

Keywords: *Neonatal sepsis, natural inhibitor, Antithrombin, Protein S, Sudan.*

Background:

Neonatal Sepsis is a life-threatening response to infection which causes tissue damage, organ failure, and death in the first twenty-eight days of life. It remains one of the significant morbidity and mortality causes among developing countries **(**1**)** accounts for the third common cause of neonatal deaths of annual deaths of 225 000 **(**2**)**. Worldwide highest rates of neonatal deaths in Sub-Saharan Africa (2, 3, 4, 5), although, it is declining of specific mortality rates annually **(**3**)**. Neonatal sepsis deaths account

1.6 times deaths with malaria and four times HIV deaths **(**6**)**. The pathophysiological mechanism of sepsis is not completely understood, but coagulation alterations are the general feature of the sepsis **(**7**)**. Both inflammation and hemostasis are strongly linked pathophysiologically and significantly affect each other. Coagulation system activation caused by the inflammation that in turn also considerably stimulate an inflammatory response, strong tight links between two systems significantly participate in disease pathogenesis and/or progression **(**8**)**. Activation of coagulation along with inhibition of fibrinolysis and increased possibility of organ dysfunction characterizes the sepsis and sepsis induced-DIC **(**9-11**)** a very serious condition results from coagulation activation followed by a continuation of coagulation which ends with consumption of coagulation elements later (consumption coagulopathy) **(**12, 13**)**, the later alterations occur in the delayed stages of sepsis-induced DIC **(**12, 14-16**)**. Antithrombin; a natural inhibitor plasma protein produce mainly in the liver, its main activity is to inactivate the thrombin which represents a sepsis important mediator **(**17, 18**)**, AT also serves as a local anti-inflammatory role in sepsis **(**19**)** Protein C pathways (PC and PS) also serves several biologic functions like; anti-inflammatory function **(**20, 21**)**. When activation of the hemostatic system changes from helpful to harmful represents an adequate moment for coagulation-targeted treatment or intervention where the immunological role of hemostasis is preserved and getting safe from the harmful effect of excessive activation of coagulation **(**22**)**. Analyzing blood coagulation found to be of more clinical significance than other classical tests **(**23**)**. Sepsis Diagnosis among critically ill septic patients is challenging, it can be complicated by inflammation **(**24**)**.

The objectives of the study were to assess the natural inhibitor; antithrombin (AT) and protein S (PS) among Sudanese neonates with sepsis compared to healthy neonates, and to correlate gender, mode of delivery with that parameter among both Groups, and to correlate sepsis outcome, the onset of sepsis, and the etiologic agent of sepsis among case group.

Method:

A prospective cross-sectional hospital-based study was conducted in the maternity hospital, Omdurman. Sudan between June.2013 to April 2015. on a total of 100 Sudanese neonates divided equally into; cases (neonates with proven sepsis by blood culture), and controls (healthy neonates).
Blood culture for identification of microorganism was done, then positive culture included as a case sample. Venous neonatal blood collected and plasma prepared for AT and PS assessment. Blood culture, Gram stain, culture and sensitivity as well as biochemical tests for identification of microorganism was done for suspected neonates initially, then positive cultures were included as case samples. Platelet poor plasma collected was used for coagulation studies.

AT Procedure

1/10 diluted plasma was prepared (90 microliters of 0.15 M Saline + 10 microliter of plasma), Sufficient time was allowed for frozen plasma to thaw before use, Antithrombin reagent (working reagent) was prepared by transfer reagent two content (glycine buffer) into reagent one (contains micro latex particles coated with rabbit anti-human AT antibodies), the materials were allowed to stand at room temperature for 15 minutes. Then 50 microliter of 1/10 diluted plasma was obtained, and 250 microliters of antithrombin reagent were added, mixed and incubated for exactly 20 minutes, the absorbance then measured at 590nm using semi-automated pre-calibrated chemistry analyzer (Mindray BA-88A), calibrator as a test control (Uricalibrator, Stago) used, as well as all test reagents used were supplied by Stago. France). The result was interpreted in Mg/ml.

PS Procedure

1/10 diluted plasma was prepared (180 microliters of Owren-Koller buffer + 20 microliter of plasma) and mixed. Sufficient time was allowed for frozen plasma to thaw before use. Lyophilized reagents were left for about 15 minutes at room temperature before use. Then 50 microliter of 1/10 diluted pre-warmed plasma was added, a piece of the metal ball (Stago) was obtained, and reagent was incubated to reach 37+/- 0.2 C, then 50 microliters of protein S deficient plasma, factor V, then 50 microliters of PS activator were added, incubated for exactly 120 seconds, then 0.02 M calcium chloride was added, and the clotting time was counted in seconds using semi-automated pre-calibrated coagulometer (Stago. France), calibrator as a test control (Uricalibrator, Stago) used, as well as all test reagents used were supplied by Stago. France). The result was interpreted in percentage.

Ethical clearance:

Ethical clearance was obtained from the research ethical committee of Omdurman maternity hospital. The principal investigator obtained an informed consent form from the neonates' mothers who were included in the study before going on.

Data analysis

Data was entered and tabulated in an EXCEL sheet, then to SPSS (a statistical program for social and sciences). Data were analyzed by use of SPSS (IBM SPSS Statistics 20).

Results and discussion:

A total of 100 venous neonatal blood samples (50 for both groups) were included. Gender distributions were; 26 females (52%), and 24 males (48%), 27 females (54%), and 23 (46%) for case and control group respectively. Table (1)

Table (1): Distribution of study population according to their gender

Gender	Case group	Control group	Total
Female	26(52%)	27(54%)	53
Male	24(48%)	23(46%)	47
Total	50	50	100

In gestational age, case and control groups were classified into the term (37 weeks gestational age and above) and preterm (36 or less). 17 neonates were term (34%), and 33 were preterm (66%), 1 term neonate (2%) and 49 preterms (98%) for case and control. Table (2).

Table (2): Distribution of study population according to their gestational age

Gestational age	Case group	Control group	Total
Term	33(66%)	49(98%)	82
Preterm	17 (34%)	1 (2%)	18
Total	50	50	100

Groups were categorized according to delivery mode; cesarean section and normal vaginal delivery neonates. 17 were delivered normally (34%), and 33 were delivered by cesarean sections 66%), 7 were delivered normally (14%), and 43 were delivered by cesarean sections (86%) for case and control. (Table 3)

Table (3): Distribution of study population regarding their mode of delivery

Mode of delivery	Case group	Control group	Total
Normal vaginal delivery	33 66%)	43(86%)	76
Caesarean section	17(34%)	7 (14%)	24
Total	50	50	100

In the case group, it is categorized additionally to the outcome, the case group was classified into dead and recovered neonates. The case group was classified considering their outcome; dead and recovered neonates. Dead were constituted 10 (20%), 40 were recovered (80%). The case group was classified according to sepsis onset into; early-onset (0-7 days) represented 17 (34%), late-onset (7-28 days) represented 33 (66%). The etiologic agent of sepsis among the case group was classified into; Gram-negative 41 (82%), and Gram-positive 9 (18%). Table (4).

Mortality among sepsis onset distributed in case group into; early onset 4 (40%), late onset 6 (60%). The causative bacterial agents were distributed as; *Pseudomonas* 23 (46%), *Salmonella* 9 (18%), *Klebsiella* 7 (14%), 3 (6%) *Staph.epidermidis* 3 (6%) *Strep. fecailis* 2 (4%), *E.coli* 2 (4%), *Staph.aureus* 2 (4%), and 1 (2%) *Streptococci (Non group B).* Mortality caused by *Pseudomonas* 3 (30%), *Salmonella* 3 (30%), *S.Epidermidis* 2 (20%), *Klebsiella* 1 (10*%),* and *E.Coli* 1 (10%). Table (4).

Table (4): Frequency of bacterial isolates (n=50) considering its etiologic agent

Gram negative	**No (%)**	**Gram positive**	**No (%)**
Pseudomonas	23 (46%)	Staph epidermidis	3 (6%)
Salmonella	9 (18%)	Strepococcus fecailis	3(6%)
Klebsiella	7 (14%)	Staph. aureus	2 (4%)
E.coli	2 (4%)	Streptococci (Non group B)	1 (2%)
Total	**41**		**9**

AT mean showed a significant decrease in the case group compared to the control (183.9 and 221.5 Mg/ml) (P value 0.00). Table (5).

PS mean showed an insignificant decrease in the case compared to the control (33.4% and 34.7%) (P value 0.76). Table (5).

Table (5): Antithrombin and protein S mean and P. value among both groups

Parameter	Mean	P. value
AT (case) AT (control)	183.9 Mg/ml 221.5 Mg/ml	0.00
PS (case) PS (control)	33.4% 34.7%	0.76

The gender didn't show a significant decrease with AT or PS (AT mean was; 186.5 and 181.7 Mg/ml for males and females, and PS; 33.8 and 33.1%). (P-value; 0.81, 0.89 for AT and PS).

Mode of delivery didn't show a significant decrease with AT or PS (AT mean; 176.8 and 189.1 Mg/ml and for normal vaginal delivery and caesarian section and PS; 32.5 and 34.1%). (P.value; 0.54, and 0.79). Among case sepsis, the outcome showed a significant decrease in PC between dead and recovered neonates (mean; 25.4 and 36.2% for dead and recovered). (P.value 0.04).

Fibrinogen, PS, or AT didn't show a significant correlation with PC among the same group (mean; 517.3, 473.4 mg/dl in fibrinogen for dead and recovered septic neonates, 30.4 and 34.2% PS, 176.9 and 185.7 Mg/ml in AT). (P.value; 0.49, 0.61 and 0.72 for fibrinogen, PS and AT respectively).

Among case group, sepsis onset didn't show significant decrease in fibrinogen, PC, PS or AT (mean; 491.2 and 477.5 mg/dl in fibrinogen for early and late onset sepsis respectively, 32.2 and 34.9% in PC, 28.7 and 35.9% PS, 173.7 and 189.2 Mg/ml in AT). (P value; 0.80, 0.56, 0.25 and 0.46).

The etiologic agents of sepsis in the case group didn't show significant difference with fibrinogen, PC, PS or AT (mean was; 475.6 and 512.0 mg/dl in fibrinogen for Gram-negative and Gram-positive respectively, 35.5 and 27.4% in PC, 34.3 and 29.3% PS, 187.9 and 165.7 Mg/ml in AT). (P-value; 0.59, 0.16, 0.52 and 0.39).

Discussion:

Fibrinogen showed a significant increase in the case compared to the control (P.value 0.00), the result was in line with Krishna I et al **(**25**)**, Charan P A et al **(**26**)**, and Mondal et al **(**27**)** who concluded increased fibrinogen level in sepsis, fibrinogen is an acute-phase protein it increases in inflammatory conditions like sepsis.

AT showed a significant decrease in the case group compare to the control (P.value 0.00), this result was in line with Warkentin et al **(**28**)**, Nishida et al **(**29**)**, Elbeshlawy et al **(**30**)**, and Hagag et al **(**31**)** who concluded reduced AT level in sepsis cases. Significant AT reduction in septic patients was suggested, it can be justified due to the consumption and impaired synthesis of AT in sepsis **(**28**)**. AT can be useful as a biomarker in the diagnosis of neonates suffering from sepsis.

PC showed a significant decrease in dead septic neonates compared to the recovered septic (P.value 0.04), this result was matched with Griffin et al **(**32**)**, M De La Torre-Prados et al **(**33**)**, William L **(**34**)**,

Sundaram, et al (35) who correlated PC (low PC) with poor outcome and mortality in septic patients. Consumption of PC may be clarified the decreased PC level which accompanied with mortality, PC has an anti-inflammatory function, cytoprotective, antithrombotic (20, 21), anti-apoptotic function (21) among other functions i.e. regulating gene expression, these function gives PC to play a crucial role in sepsis progression. PC can be a useful marker in neonatal sepsis mortality.

The insignificant decrease in PC and PS among both groups (P.value; 0.41 and 0.76), the study decreased level of both parameter, the insignificance may due to the physiological decrease of healthy neonates (control) which confirmed in the study (normal PC in an adult population usually between 70% and 130% (33) (according to the used kit's manufacturer), whereas; most of the PC studies conducted on adult, relatively smaller sample size could also be contributed in PC insignificance, along with that a lot of studies reported PC and its cofactor PS reduced significantly in sepsis (28, 30, 34), and several studies confirmed it among septic neonates (30, 35)

On my view, it is noted that PC correlation is a much stronger correlation with poor outcome and mortality more than the disease itself (sepsis), another meaning that I noted that several studies focused targeted PC in treatment and management of sepsis mortality than using PC as a marker in the diagnosis of sepsis, moreover; recombinant studies and approved as a novel therapy for sepsis (37), then later it withdrawn from the market (38) subsequently recombinant protein C introduced (39). Insufficient data reported to use of recombinant PC for the management of severe sepsis in neonates, and it's reported that neonates shouldn't be treated with recombinant PC (40). This discussion can reinforce the PC correlation with sepsis outcome (discussed above).

Conclusion:

Fibrinogen showed a significant increase in septic neonates compared to healthy. AT showed a significant decrease in septic neonates compared to the healthy. PC showed a significant decrease in dead septic neonates compared to the recovered septic. Both fibrinogen and AT can be a useful biomarker in sepsis diagnosis, PC can be a useful biomarker in neonatal sepsis mortality.

Conflict of interest:

Nothing to disclose.

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