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Diagnostic Value of Angiopoietin-1 and -2 as Markers for Disease Severity in Hemolytic Uremic Syndrome in Children

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ABSTRACT

Introduction: Hemolytic uremic syndrome (HUS) is defined as a triad of microangiopathic hemolytic anemia (MHA), thrombocytopenia, and acute renal failure. It is the most common cause of acute renal failure in children, and the incidence of this syndrome is increasing worldwide. Angiopoietin-1 and -2 each competitively bind to the endothelial Tie-2 receptor and play an important role in regulating endothelial cell function. The aim of this study was investigating the clinical significance of serum levels of angiopoietin (Ang) 1 and 2 in enterohemorrhagic Escherichia Coli (EHEC)-induced HUS and determining their correlation with disease severity.

Patients and Methods: Forty eight children aged between 1-16 years were included in the study and were divided into two groups, 24 patients with diagnosis of hemolytic uremic syndrome induced by EHEC infection and 24 healthy children as a control group. Serum samples were obtained from healthy control group and patients with EHEC-induced HUS at time of diagnosis. Serum samples from the patients were obtained for analysis for angiopoietin-1 and -2 by enzyme-linked immunosorbent assay.

Results: Our findings indicated that, serum Ang-1 levels might be useful for the prediction of the development of HUS. In HUS phase, in addition to more significant decrease of serum Ang-1 levels, serum Ang-2 level increased. These changes might be useful for the diagnosis of HUS and also be useful as a marker of disease activity of HUS.

Conclusion: Serum angiopoietin-1 and angiopoietin-2 levels and the Ang-2/Ang-1 ratio may be promising indicators of disease activity in HUS.

Keywords: Angiopoietin 1 and 2, EHEC infection, Hemolytic uremic syndrome.

INTRODUCTION

Hemolytic uremic syndrome (HUS) is a severe complication of Shigella toxin–producing enterohemorrhagic Escherichia coli (EHEC) infection and it is a clinical syndrome characterized with the triad of microangiopathy hemolytic anemia, thrombocytopenia and acute renal damage [(1)]. Hemolytic uremic syndrome is the most common cause of acute renal damage in children. The clinical finding in hemolytic uremic syndrome occurs as a result of thrombotic microangiopathy (TMA). The Pathological lesion is thickening of arterioles and capillary walls, endothelial swelling and detachment. Fibrin and platelet-rich thrombi leads to obstruction in the vascular lumen. Many tissues and organs including mainly the kidney are affected[(2)].

Microangiopathic hemolytic anemia of HUS has the following properties (Hemoglobin <10 g/dl, frequently <8 g/dl, negative Coombs test, increased reticulocyte count, increased serum lactic dehydrogenase (LDH) level, decreased serum hepatoglobulin level, fragmented erythrocytes on peripheral blood smear (helmet cells, schistocytes). In thrombocytopenia the platelet count is below 150,000/mm^3 (commonly<40,000/mm^3). Bleeding is rarely observed. In acute renal damage there are different degrees of renal involvement. Hematuria, proteinuria and increased serum creatinine are the most important indicators of renal damage. Oliguria or oligoanuria may occur. Dialysis is needed because of severe renal damage in more than half of cases [(3)].

Recent reports have highlighted the roles of cytokines and chemokines in the pathogenesis of EHEC- induced HUS. 174 serum cytokines were detected and five serum biomarkers have been identified to predict severity of EHEC-induced HUS. Among these biomarkers were angiopoietin (Ang)-2 [(4)]. Angiopoietin-1 and -2 each competitively bind to the endothelial Tie-2 receptor and play an important role in regulating endothelial cell function [(5)].

The aim of this study was investigating the clinical significance of serum levels of angiopoietin 1 and 2 in EHEC-induced HUS.

PATIENTS AND METHODS

This was a retrospective, analytical, case-control study. This study was carried out at Nephrology Unit at Children Hospital, Pediatrics and Clinical Pathology Departments, Faculty of Medicine, Zagazig University over a period of 7 months from January 2020 to July 2020.

Enrollment:

This study included 48 children aged between 1-16 years divided into two groups, 24 patients with diagnosis of hemolytic uremic syndrome induced by EHEC infection who fulfilled the inclusion criteria of

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the study and who were evaluated at the outpatient clinic in Pediatrics Department at Children Hospital and 24 healthy children as a control group.

Ethical considerations:

An informed consent was taken from parents or care-givers of children to be enrolled in the study. The study was approved by the Ethical Committee of Faculty of Medicine, Zagazig University. This work has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for studies involving humans.

Inclusion criteria:

This study included healthy control group and patients with EHEC-induced HUS with the following characters: (1) Age (1-16) years. (2) Sex: both male and female, (3) with or without encephalopathy.

EHEC infection is diagnosed by one or both of the following: Bloody diarrhea, vomiting, and bowel cramps with microbiological identification of EHEC infection.

HUS is diagnosed by presence of: Thrombocytopenia (platelet count <150,000/mm^3), acute renal dysfunction (renal injury evidenced by hematuria, proteinuria, elevated creatinine levels), and peripheral blood examination suggestive of hemolytic anemia (hemoglobin< 10 g/dl).

Exclusion criteria:

Included infants and children with any condition or disease state, which may cause impaired renal function and anemia without being eligible to the diagnostic criteria of hemolytic uremic syndrome including: Disseminated intravascular coagulopathy (DIC). Septicemia. Bilateral renal vein thrombosis. Chronic renal failure**.** Chronic hemolytic anemia. Tumor lysis syndrome. Children taking nephrotoxic drugs, and also, patients who had any disease that affects level of angiopoietin-1 and -2 as cardiovascular disease was excluded from the study.

All patients included in this study were subjected to:

1- Full history taking.

2- Overall clinical examination:

- ***General examination particularly:*** Blood pressure. Body weight daily, and pallor.
- ***Systematic examination:*** Abdominal examination. Neurological examination (cases with CNS involvement were presenting with disturbed conscious level, seizures, and were examined for focal motor or sensory deficits). Chest examination, and cardiac examination.

3- Radiological investigations:

- ***Plain chest radiography:*** Plain X-ray chest posteroanterior view was done for all studied patients to reveal pulmonary consolidation (pneumonic patches) suggestive of pneumococcal HUS. Signs of volume overload as a result of renal failure (pulmonary edema and cardiomegaly). Site of hemodialysis catheter immediately after insertion and to exclude catheter related complications.
- ***Pelvi-abdominal ultrasonography:*** was done for all patients in order to: Evaluate the size and echogenicity of both kidneys. Measure parenchymal thickness. Assess corticomedullary differentiation. Exclude renal stones, masses, backpressure changes, obstructive uropathy. Evaluate size and echogenicity of other abdominal organs. Exclude abdominal masses and lymphadenopathy.

Methodology:

Serum samples were obtained from healthy control group and patients with EHEC-induced HUS at time of diagnosis. Serum samples from the patients were separated, divided into aliquots, frozen, and stored at -80° C until analysis for angiopoietin-1 and -2 by enzyme-linked immunosorbent assay (ELISA) **(4).**

The kit of angiopoietin-1 uses a double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) to assay the level of human angiopoietin-1(Ang-1) in samples. Add angiopoietin-1 to monoclonal antibody enzyme well which is pre-coated with human angiopoietin-1 monoclonal antibody, incubation; then, add angiopoietin-1 antibodies labeled with biotin, and combined with streptavidin-HRP to form immune complex; then carry out incubation and washing again to remove the uncombined enzyme. Then add chromogen solution A, B, the color of the liquid changes into the blue, and at the effect of acid, the color finally becomes yellow. The chroma of the color and the concentration of the human substance angiopoietin-1 of sample were positively correlated **(6).**

The kit of angiopoietin-2 uses the same methods as that used to assess angiopoietin-1, where angiopoietin-2 replaces angiopoietin-1 in everything **(7).**

Stool samples were collected form the patients under complete aseptic conditions in sterile containers for cultures to detect toxin producing E-coli. Stool samples first cultured on selenite broth over night to exclude the non-pathogenic strains.

After that, subculture on sorbitol-MacConkey agar (Oxoid)^{TM} culture (SMAC) was done for 24 hours. Isolation of STEC colorless, sorbitol-negative colonies were done. By slide latex agglutination test (Thermo Fisher)^{TM} the sorbitol negative colonies were examined for toxin production. CBC (complete blood count) and reticulocytic count for patients were done. Peripheral blood smear for features of hemolysis (i.e. fragmented RBCs) were tested. Coombs' test (direct and indirect) was performed. Kidney and liver function tests were done. PT (prothrombin time) and PTT (partial

thromboplastin time) were measured. C-reactive protein was assessed. Complement (C3) concentration was done [4].

Statistical Analysis:

Data collected throughout history, basic clinical examination, laboratory investigations and outcome measures were coded, entered and analyzed using Microsoft Excel software. Data were then imported into Statistical Package for the Social Sciences (SPSS version 20.0) software for analysis. According to the type of data, qualitative were represented as number and percentage and quantitative were represented by mean ± SD, median, and range. Difference and association of qualitative variables were tested by Chi square test (X^2). Differences between quantitative independent groups were compared by t-test or Mann-Whitney test. P value was set at <0.05 for significant results and <0.001 for high significant result.

RESULTS

There was no statistically significant difference between the cases and the control group as regard both age and sex (**Table 1)**.

Table (1): Comparison of age and sex between studied groups

			Case (N=24)	Control (N=24)	P
Age		**Mean± SD**	6.0±3.35	7.1±2.85	0.412
		Median (Range)	5.0 (0.6-13)	6.0 (1-14)	
Sex	**Male**	**N**	10	13	
		%	41.6%	54.2%	
	Female	**N**	14	11	0.386
		%	58.4%	45.8%	
Total		**N**	24	24	
		%	100.0%	100.0%	

This table shows that the most prevalent symptom were pallor 100%, then diarrhea 66.7% then oliguria with 58.3% and anuria with 54.2% (**Table 2)**.

Table (2): Clinical characters distribution among studied cases (N=24)

		N	%
Diarrhea	**-VE**	8	33.3
	+VE	16	66.7
Vomiting	**-VE**	17	70.8
	+VE	7	29.2
Abdominal pain	**-VE**	20	83.3
	+VE	4	16.7
Fever	**-VE**	18	75.0
	+VE	6	25.0
Pallor	**-VE**	0	0.0
	+VE	24	100.0
Hematuria	**-VE**	17	70.8
	+VE	7	29.2
Anuria	**-VE**	11	45.8
	+VE	13	54.2
Oliguria	**-VE**	10	41.7
	+VE	14	58.3
HTN	**-VE**	13	54.2
	+VE	11	45.8
Seizures	**-VE**	19	79.2
	+VE	5	20.8

This table shows levels of laboratory parameters among studied cases (**Table 3)**.

Table (3): Laboratory distribution among studied cases (N=24)

Urea (mg/dl)	Mean± SD	204.0±8.5
Creatinine (mg/dl)	Mean± SD	4.57±1.83
HB (gm/dl)	Mean± SD	6.38±1.22
WBCS (thousands/cmm)	Mean± SD	19.36±4.21
PLT (thousands/cmm)	Mean± SD	75.16±6.84
Reticulocytic Count (%)	Mean± SD	8.17±1.74
PT (sec)	Mean± SD	12.02±1.23
PTT (sec)	Mean± SD	33.91±3.33
C3 (g/L)	Mean± SD	107.25±23.11
Na (mmol/L)	Mean± SD	131.7±9.15
K (mmol/L)	Mean± SD	4.02±1.15

79.2% had improved and 20.8% didn't improved (CKD 8.3% and ESRD 12.5%) (**Table 4).**

Table (4): Outcome distribution among studied cases (N=24)

		N	**%**
Outcome	**CKD**	2	8.3
	ESRD	3	12.5
	Improved	19	79.2
Overall outcome	**Not**	5	20.8
	Improved	19	79.2
	Total	24	100.0

Cases were significantly lower than control as regard angiopoietin-1 but significantly higher as regard angiopoietin-2 and ratio (**Table 5)**.

Table (5): Comparison of angiopoietin 1, angiopoietin 2 and ratio between studied groups

	Case (N=24)	**Control (N=24)**	**T**	**P**
Ang-1	346.58±11.25	1817.97±68.6	9.790	<0.01**
Ang-2	6976.75±146.73	1403.72±46.98	11.171	<0.01**
Ratio Ang-2/-1	26.11±5.65	0.72±0.087	6.779	<0.01**

**: Significant

There was a perfect area under curve with significant cutoff <786.5 with sensitivity 100% and specificity 100.0% (**Table 6).**

Table (6): AUC, cutoff and validity of angiopoietin 1

AUC	**Cutoff**	**P**	**95% Confidence Interval**		**Sensitivity**	**Specificity**
			Lower Bound	**Upper Bound**		
1.000	<786.5	<0.01**	1.000	1.000	100.0%	100.0%

: Significant- There was significant area under curve with significant cutoff >2835.0 with sensitivity 92.5% and specificity 98.8% as regard angiopoietin 2, and as regard ratio there was perfect area under curve with significant cutoff <786.5 with sensitivity 100% and specificity 100.0% (Table 7).**

Table 7: AUC, cutoff and validity of angiopoietin 2 and ratio

	Area	**Cutoff**	**P**	**95% Confidence Interval**		**Sensitivity**	**Specificity**
				Lower Bound	**Upper Bound**		
Angio2	0.989	>2835.0	<0.01**	.969	1.000	92.5%%	98.8%
Ratio	1.000	>3.94	<0.01**	1.000	1.000	100.0%	100.0%

**: Significant

DISCUSSION

In this study, the demographic characteristics of participants revealed that age was 6.0±3.35 and 7.1±2.85 for cases and control respectively without significant difference between groups, also, as regard sex distribution there was no significant difference between cases and control, this indicates matching between groups. In comparison to our findings, the study of **Pundzienėa *et al.*** [8] reported that total of 62 pediatric patients were enrolled into their study, including 24 males (38.7%) and 38 females (61.3%). The mean time of follow-up was 12.4 ± 4.5 years (range, 6.2–18.9 years). The mean age at diagnosis was 2.9 ± 3.9 years (range, 0.3–14.3 years). Less than half (45.2%, 28/62) of the children were aged less than 1 year at the onset of the disease, and 82.3% (51/62) were younger than 5 years.

In the current study, we found that the most prevalent symptom were pallor 100%, then diarrhea

66.7% then oliguria with 58.3% and anuria with 54.2%, 25% of studied group had fever and 83.3% had abdominal pain also 70.8% had dialysis and 20.8% had encephalopathy. In contrast to the study of **Vaterodt *et al.*** [9] which reported that most patients with STEC-HUS presented with a prodromal phase of 5 days with diarrhea (90.5%), often bloody, vomiting and fever. These symptoms were seen less frequently in patients with non-STEC-HUS (diarrhea 33.3%). Acute renal failure was similar in both groups [STEC-HUS 44/62 (71.0%), Non-STEC-HUS 10/13 (76.9%)], but the need of antihypertensive medication was higher in non-STEC-HUS-group (35.1% vs. 56.3%). Extrarenal manifestations were encountered more frequently in Non-STEC-HUS-group. Decrease in complement factor C3 was seen in 19% of STEC-HUS- and 50% of non-STEC-HUS-group.

In the present study, as regard outcome; 79.2% had improved and 20.8% didn't improved (CKD 8.3% and ESRD 12.5%). Comes in agreement with our findings, the study of **Ahmed Ali *et al.*** [10] which reported that 51.3% had complete renal recovery, 15.4% CKD 3-4, 12.8% CKD 5 requiring renal replacement therapy (RRT), and 20.5% died.

In the present study, cases were significantly lower than control as regard Ang-1 but significantly higher regard Ang-2 and ratio. Our results are supported by the study of **Shimizu *et al.*** [11], which reported that serum Ang-1 levels were significantly decreased, whereas serum Ang-2 levels were significantly elevated in HUS. Consequently, the Ang2/Ang-1 ratio, which reflects a relative excess of Ang-2, was also increased. Also our results are consistent with the results in a previously study of **Page *et al.*** [12], their findings are consistent with the pathophysiological roles of Ang-1 and -2 in endothelial cell function and pathogenesis of HUS and indicated that endothelial cell dysfunction by Ang-2/Ang-1 dysregulation is already present during the pre-HUS phase. Furthermore, the Ang-2/Ang-1 ratio profoundly and rapidly increased as HUS developed. In another study done by **Kümpers *et al.*** [13] reported that median serum Ang-2 concentrations were increasingly higher across the following groups: healthy controls, patients without sepsis, patients with sepsis and patients with septic shock. In contrast, Ang-1 concentration was significantly lower in all patient groups compared with healthy controls.

Moreover, in the present study, and as regard validity of Ang-1, we found that there was a perfect area under curve with significant cutoff <786.5 with sensitivity 100% and specificity 100.0%, meanwhile, there was a significant area under curve with significant cutoff >2835.0 with sensitivity 92.5% and specificity 98.8% as regard Ang-2, and as regard ratio there was perfect area under curve with significant cutoff <786.5 with sensitivity 100% and specificity 100.0%.

CONCLUSION

Disruption of homeostasis of vascular endothelial function by angiopoietin-1 and angiopoietin-2 may be closely associated with the development of hemolytic uremic syndrome. Serum angiopoietin-1 and angiopoietin-2 levels and the Ang-2/Ang-1 ratio may be promising indicators of disease activity in HUS.

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