

SERUM LEVELS OF PRO-INFLAMMATORY CYTOKINES CRISES IN CHILDREN WITH SICKLE CELL DISEASE IN RIVERS STATE, NIGERIA

ABSTRACT

Aim: To assess the serum levels of pro-inflammatory cytokines in children with sickle cell disease in steady state and vaso-occlusive crises in comparison to healthy children of the genotype HbAA.

Study Design: Case-Control study

Place and Duration of Study: University of Port Harcourt Teaching Hospital, January to June, 2016

Methodology: Enzyme-linked immunosorbent assay was used to assess serum levels of pro-inflammatory cytokines (IL-8, IL-6 and IFN- γ) in children with HbSS in steady state and vaso-occlusive crises (VOC) in comparison with healthy children with HbAA. Analysis of Variance was used to compare serum cytokine levels between the three groups and a p-value less than 0.05 was considered significant.

Results: The study showed that significantly higher levels ($p < 0.05$) of IFN- γ in HbSS subjects at steady state and vaso-occlusive crises ($112.3 \pm 23.75\text{pg/ml}$ and $596.5 \pm 225.2\text{pg/ml}$ respectively) in comparison with healthy children with HbAA ($97 \pm 27.1\text{pg/ml}$). IL-8 levels were also higher in steady state and children in VOC ($764.4 \pm 150.5\text{pg/ml}$ and $1076 \pm 216.2\text{pg/ml}$) compared to $502 \pm 179.40\text{pg/ml}$ in healthy HbAA subjects. The mean IL-6 concentrations were $30.82 \pm 10.06\text{pg/ml}$, $45.0 \pm 10.94\text{pg/ml}$ and $188.3 \pm 104.9\text{pg/ml}$ in HbAA subjects, HbSS steady-state and VOC patients respectively.

Conclusion: The finding of the study suggests that there is a relationship between the pro-inflammatory cytokine levels and vaso-occlusive crisis in sickle cell disease. Evaluation of the pro-inflammatory cytokines showed that IL-8 could be a useful marker for assessing disease severity and, consequently, therapeutic intervention.

Keywords: Sickle Cell Anemia, Cytokines, IL-8, IL-6.

INTRODUCTION

Sickle-cell disease (SCD) is an inherited blood disorder caused by a point mutation in the genes encoding the β -globin subunit leading to the polymerization of deoxygenated sickle hemoglobin 1S^{20} and decreased deformability of red blood cells (RBCs).^{1,2} It results in a myriad of clinical manifestations including haemolysis, anaemia and painful crisis causing significant morbidity and reduced quality of life in affected persons.³ In recent studies, the pro-inflammatory state has been significantly associated with vaso-occlusive complications that have been observed in patients with SCD.^{4,5,6} The release of pro-inflammatory mediators such as cytokines which initiate the transmission of painful stimuli have been associated with significant tissue damage. Several cytokines, such as interleukin-1 beta (IL-1 β) and tumor necrosis factor-alpha (TNF- α), are associated with the activation of leukocytes, particularly monocytes and neutrophils, in SCA.^{7,8} Several other cytokines are also involved in the chronic inflammatory state that is present in SCA. The activation of cells and the release of cytokines stimulate the NF- κ B transcription factor pathway, which regulates the production of interleukin-4 (IL-4) interleukin-6 (IL-6) and interleukin-8 (IL-8). IL-6 and IL-8 production are also enhanced by the STAT3 intra- cellular pathway and proinflammatory activities.^{9,10,11}

46 Recently, the involvement of several other cytokines, such as IL-18, IL-17, IL-23, IL-12 and
 47 IL10, in inflammatory responses in SCA patients has been described.^{5,7,9,10} Due to the
 48 extensive participation of cytokines in the inflammatory processes involved in SCA
 49 pathology, this study of the cytokine expression will contribute to an improved understanding
 50 of SCA pathology, especially the pathology of inflammatory vaso- occlusive events.

51 **METHODS**

52 **Study Center**

53 This study was conducted at the University of Port Harcourt Teaching Hospital, Port
 54 Harcourt (UPTH), a Federal Government owned tertiary institution, situated at Alakahia, Port
 55 Harcourt, Rivers State, Nigeria. It has over 500 bed capacity, several specialties and
 56 specialists. It serves as the main referral center for Rivers State and the neighboring states.

57 **Study Population**

58 The study population consisted of children between 1 – 16 years with sickle cell disease
 59 (SCD) patients presenting at the children emergency ward and the children out-patient clinic
 60 of UPTH between January to June 2016. The study population was divided into three groups
 61 as follows:

62 **Group A:** Healthy children attending UPTH for routine medical check.

63 **Group B:** Children with SCD attending the general out-patient clinic of UPTH

64 **Group C:** Children with SCD presenting with vaso-occlusive crises at the children
 65 emergency unit of UPTH

66 **Ethical Consideration**

67 Informed written consent was obtained from the patients or parents/caregivers of the patients
 68 before recruitment into the study. Ethical approval for the study was obtained from the
 69 Research Ethical Committee of the University of Port Harcourt Teaching Hospital and also
 70 from University of Port Harcourt prior to commencement of the study.

71 **Sample Collection and Analysis**

72 Five milliliters of blood were collected from each subject in appropriate sterile containers and
 73 properly labelled. Diagnosis of HbSS homozygous in patients was confirmed by genetic
 74 analysis in the molecular laboratory of the study center. Full blood counts of the blood
 75 samples were also assessed at the hematology laboratory of the center.

76 **Cytokine Assay**

77 Serum levels of the cytokines were quantified using capture Enzyme Linked Immunosorbent
 78 Assay (ELISA) kits, according to the manufacturer's instruction (Avivabio systems, San
 79 Diego, CA, USA). Concentrations for each sample were extrapolated from the standard curve
 80 and expressed as mg/ml and were ultimately normalized to total protein in the sample and
 81 expressed as pg/mg.

82 **Data collection**

83 Sociodemographic information and other relevant clinic information were obtained from the
 84 medical records of the subjects included in the study.

85 **Data Analysis**

Serum cytokine levels and hematological variables were compared across the different groups using the Kruskal-Wallis ANOVA test. The Dunn's Post's test was used for comparisons between two groups. All statistical tests were performed at a 95% confidence interval at a 0.05 level of significance with the Graphpad Software Version 6.0.

RESULTS

Table 1 shows the demographic information of the study subjects. Of the sixty-six (66) subjects recruited in the period of the study, Group A (Normal healthy control patients of HbAA genotype), consisted of 17 (25.8%) children with the mean age of 6.0 ± 3.1 years. Group B (SCD patients in steady-state) consisted of 16 (24.2%) while Group C (patients with SCD and vaso-occlusive crises) consisted of 33 (50.0%) subjects with a mean age of 7.0 ± 3.5 years. The mean age of the entire study sample was 6.5 ± 3.2 years.

Table 1: Demographic Information of Patients

GROUPS	Age in years (Mean $\pm$ SD)	Frequency (%)
Children without SCD	6.0 ± 3.1	17 (25.8%)
SCD with Steady State	7.0 ± 3.3	16 (24.2%)
SCD with vaso-occlusive crises	7.0 ± 3.5	33 (50.0%)
Total	6.5 ± 3.2	66 (100.0)

SCD: Sickle cell disease

Table 2 shows the hematological parameter of the patients studied. The mean Hb levels in VOC patients was 4.2 ± 0.9 g/dl. Patients in steady state had an average Hb of 6.8 ± 0.7 g/dl and Normal patients had a mean Hb of 13.8 ± 1.1 g/dl. Mean WBC in VOC patients was 27.6 ± 9.8 10⁹/L. Patients in steady state had a mean WBC of 12.6 ± 2.8 10⁹/L and Normal patients had a mean WBC of 7.3 ± 2.3 . Mean neutrophils in patients with VOC was 73.8 ± 9.5 , patient's in steady state had a mean neutrophil percentage of 64.3 ± 7.9 and normal patients had a mean neutrophil of 53.4 ± 4.5 . Mean lymphocytes in patients with VOC was 21.8 ± 9.1 , steady state patients had a mean lymphocyte of 28.9 ± 10.3 and normal patients had a mean lymphocytes count of 40.9 ± 4.0 . The mean eosinophil differential in VOC patients was 3.1 ± 2.2 , steady state patients, mean eosinophil differential was 4.1 ± 2.3 and in normal patients it was 4.1 ± 1.8 . basophil differential was highest in normal patients (1.6 ± 0.8), followed by steady state patients (1.5 ± 0.9) and VOC patients (1.2 ± 1.0). There were statistically significant differences ($p < 0.05$) in all the haematological parameters except the eosinophil and basophil differentials which had no significant differences ($p > 0.05$) between the different groups of patients.

Table 2: Hematologic parameters in Studied Subjects

Variable	Group A	Group B	Group C	ANOVA
Hb (g/dl)	13.8 ± 1.1	$6.8 \pm 0.7^{a,b}$	4.2 ± 0.9^a	$<0.0001^*$
WBC (10 ⁹ /liter)	7.3 ± 2.3	$12.6 \pm 2.8^{a,b}$	27.6 ± 9.8^a	$<0.0001^*$
Neutrophils	53.4 ± 4.5	$64.3 \pm 7.9^{a,b}$	73.8 ± 9.5^a	$<0.0001^*$

Lymphocytes	40.9 ± 4.0	28.9 ± 10.3 ^{a,b}	21.8 ± 9.2 ^a	<0.0001*
Eosinophils	4.1 ± 1.8	4.1 ± 2.3 ^{c,d}	3.1 ± 2.2 ^c	0.1347**
Basophils	1.6 ± 0.8	1.5 ± 0.9 ^{c,d}	1.2 ± 1.0 ^a	0.2680**

All values are present in mean ±SD

*Difference across the groups is statistically significant

**Difference across the groups is not statistically significant

^a Difference compared to Group A is statistically significant ($p < 0.05$)

^b Difference compared to Group C is statistically significant ($p < 0.05$)

^c Difference compared to Group A is not statistically significant ($p > 0.05$)

^d Difference compared to Group C is not statistically significant ($p > 0.05$)

The mean IFN- γ concentrations were 97pg/ml, 112.3pg/ml and 596.5pg/ml in normal patients, steady-state and VOC patients. The mean IL-8 concentrations were 502pg/ml, 764.4pg/ml and 1076pg/ml in normal patients, steady-state and VOC patients. The mean IL-6 concentrations were 30.82pg/ml, 45.0pg/ml and 188.3pg/ml in normal patients, steady-state and VOC patients. The mean IL-1 β concentrations were 10.99pg/ml, 13.41pg/ml and 11.75pg/ml in normal patients, steady-state and VOC patients. There was no significant differences ($p > 0.05$) in all cytokine concentration between normal healthy control patients and steady-state patients except in the IL-8 concentration ($p < 0.05$) as shown in Table 3.

Table 3: Pattern of Serum Cytokine Levels in Subjects

CYTOKINES	NORMAL (Group A, n =17)	STEADY STATE (Group B, n =16)	VOC (Group C, n =33)	Multiple Comparisons		
				A v B	A v C	B v C
IFN- γ (pg/ml)	97.94 ± 27.11	112.3 ± 23.75	596.5 ± 225.2	0.230**	0.003*	0.0002*
IL-8 (pg/ml)	502 ± 179.40	764.4 ± 150.5	1076 ± 216.2	0.003*	0.002*	0.0001*
IL-6 (pg/ml)	30.82 ± 10.06	45.00 ± 10.94	188.3 ± 104.9	0.145**	0.002*	0.0014*

* $p < 0.05$. The difference between the cytokines concentration of the different groups is statistically significant

** $p > 0.05$. The difference between the cytokines concentration of the different groups is not statistically significant

Table 4.0 shows the Pearson's Correlation value (r) between haematological parameters (Hb and WBC) and The different cytokines, (IFN- γ , IL-8, and IL-6) in Normal health patients, Patients with VOC and steady state patients. There were positive correlations between the blood parameters and all cytokines except in IFN- γ vs WBC, with IL-6 and Hb in VOC patients. However, there were negative correlations between, IFN- γ and WBC with all blood parameters, with IL-6 having positive correlations ($r \leq 0.31$) with WBC and Hb levels in Steady State patients. In the Normal individuals, there were negative correlations ($r \leq -0.24$) between WBC and all cytokines. There were negative correlations ($r \leq -0.59$) between Hb levels and IL-8, and positive correlations ($r \leq 0.36$) between Hb levels and other cytokines (IFN- γ and IL-6).

Table 4.0: Correlation of cytokines with White Blood Cell Count and Haemoglobin levels

Cytokines	VOC		Steady State		Control	
	WBC	HB	WBC	HB	WBC	HB
IFN- γ	-0.34	0.35	-0.24	0.36	-0.06	-0.04
IL-8	0.25	0.03	-0.22	-0.59	0.45	-0.26
IL-6	0.47	-0.21	-0.24	0.12	-0.43	0.31

DISCUSSION

Higher levels of IL-8, a chemotactic factor for neutrophils, during vaso-occlusive painful crisis in sickle cell disease have been reported independently of the crisis-inducing factors.¹² This study observed higher levels of IL-8 in vaso-occlusive crisis patients compared to steady-state and normal healthy control patients. There was a significant difference found between normal healthy control and steady-state patients for all pro-inflammatory cytokines studied. This finding is supported by Goncalves et al., who showed a significantly elevated concentrations of IL-8 in vaso-occlusive crisis versus steady-state patients.¹³ However, another study by Michaels et al., demonstrated no differences between vaso-occlusive crisis and steady-state patients.¹⁴ The steady-state patients in this study showed increased levels of IL-8 versus normal healthy control patients, which is discordant with the study by Pathare et al., that reported increased concentrations of IL-1 β and IFN- γ in steady-state patients.¹⁵ It also disagrees with study by Keikhaei et al., which reported increased levels of TGF- β and IL-17 in steady-state versus normal healthy control patients.¹⁶ There were progressively increased levels of IL-8 from normal healthy control patients to steady-state patients and then in vaso-occlusive crisis patients. The elevated concentrations of IL-8 are associated with increased haemolysis, vascular occlusion and inflammation in sickle cell disease.¹⁷ IL-6 concentrations were also significantly higher in vaso-occlusive crisis patients compared to IL-6 levels in steady-state and normal healthy control patients but there was an increase in IL-6 levels between steady-state and normal healthy control patients which was not statistically significant. This is consistent with findings of Keikhaei et al.,¹⁶ Pathare et al.,¹⁵ and Hibbert et al.,¹⁸ which showed higher mean serum concentrations of IL-6 in sickle cell anaemia patients than in normal healthy controls, and there was also a significant increase in IL-6 levels in vaso-occlusive crisis patients when compared to steady-state patients.

There was no significant difference in the mean concentrations of IFN- γ between steady-state and normal healthy control patients but there was a significant difference in the mean concentrations of IFN- γ between normal healthy controls and vaso-occlusive crisis patients and also steady-state and vaso-occlusive crisis patients. This is consistent with study by Qari et al., which reported slight elevation of plasma levels of IFN- γ during painful crisis when compared to the steady-state, this difference was not statistically significant when compared to the healthy, age-matched control group.¹⁹ Increased levels of pro-inflammatory cytokines such as, IL-6, IL-8 and IFN- γ in vaso-occlusive crisis patients compared to the normal healthy controls and steady-state patients could be indicative of a chronic inflammatory response and further production of these cytokines from activated endothelial cells, platelets and accumulated monocytes/macrophages in the vaso-occlusion area.^{11,12,15} These inflammatory mediators enhance red blood cell adhesiveness to endothelium and form a vicious cycle leading to more dense aggregations of sickle erythrocytes, platelets and neutrophils, and eventually to clinical vaso-occlusion.^{15,18}

194 **CONCLUSION**

195 The finding of the study suggests that there is a relationship between the pro-inflammatory
 196 cytokine levels and vaso-occlusive crisis in sickle cell disease. Evaluation of the pro-
 197 inflammatory cytokines showed that IL-8 could be a useful marker for assessing disease
 198 severity and, consequently, therapeutic intervention.

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