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Serum Levels of Pro-inflammatory Cytokines in Children with Sickle Cell Disease in Rivers State, Nigeria

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Authors' contributions

This work was carried out in collaboration between both authors. Author OOS designed the study, wrote the protocol and the first draft of the manuscript and also managed the literature searches. Author OCLN managed the analyses of the study. Both authors read and approved the final manuscript.

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Original Research Article

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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- Y) 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.

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Results: The study showed significantly higher levels ($p < 0.05$) of IFN-γ in HbSS subjects at steady- state and vaso-occlusive crises (112.3 ± 23.75 pg/ml and 596.5 ± 225.2 pg/ml respectively) in comparison with healthy children with HbAA (97 ± 27.11 pg/ml). IL-8 levels were also higher in steady- state and children in VOC (764.4 ± 150.5 pg/ml and 1076 ± 216.2 pg/ml) compared to 502 ± 179.40 pg/ml in healthy HbAA subjects. The mean IL-6 concentrations were 30.82 ± 10.06 pg/ml, 45.0 ± 10.94 pg/ml and 188.3 ± 104.9 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 the 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.

1. 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 polymerisation of deoxygenated sickle haemoglobin with 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, reduced quality of life in affected persons and reduced lifespan [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 has been associated with significant tissue damage. Several cytokines, such as interleukin-1 beta (IL-1β) and tumour 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-κB 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 intracellular pathway and proinflammatory activities [9,10,11]. Recently, the involvement of several other cytokines, such as IL-18, IL-17, IL-23, IL-12 and IL10, in inflammatory responses in SCA patients has been described [5,7,9,10]. Due to the extensive participation of cytokines in the inflammatory processes involved in SCA pathology, this study of the cytokine expression will contribute to the appreciation of the importance of inflammation in the pathology of vaso-occlusive crises in sickle cell anaemia.

2. METHODS

2.1 Study Center

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

2.2 Study Population

The study population consisted of 90 children between 1 – 16 years with sickle cell disease (SCD). These patients visited the children emergency ward and the children out-patient clinic of UPTH between January to June 2016. The sample size was calculated with the formula; $n= (z^2 pq) /d^2$. Sample size was determination by proportion at a 95% confidence interval, based on a 2% proportion of sickle cell anaemia in children as stated by Rees et al. [5].

The study population was divided into three groups as follows:

Group A: 30 Healthy children attending UPTH for the routine medical check up.

Group B: 30 Children with SCD, who are otherwise healthy, with no presentations of occlusive crises attending the general out-patient clinic of UPTH

Group C: 30 Children with SCD presenting with vaso-occlusive crises at the children emergency unit of UPTH

2.3 Ethical Consideration

Informed written consent was obtained from all parents/caregivers of the patients and consent

was also obtained before recruitment of the study. Any one (parent/caregiver or patient) who refused consent were nor included in the study nor denied the appropriate medical care/attention. Ethical approval for the study was obtained from the Research Ethical Committee of the University of Port Harcourt Teaching Hospital (UPTH/ADM/90/S.IIVOL.X/713) and also from the University of Port Harcourt (UPH/R&D/REC/04) prior to commencement of the study.

2.4 Sample Collection and Analysis

Five millilitres of blood was collected from each subject in appropriate sterile containers and properly labelled. Serum was separated from each sample by centrifugation at 5000 rpm for 10 minutes and put in cryo-tubes and stored at -200C until the samples were pooled for cytokine analysis. Diagnosis of HbSS homozygous in the patients was confirmed based on electrophoresis results in the patient's medical records. Full blood counts of the blood samples were also assessed with the Beckman Coulter D × H 900 Hematology System (USA) at the haematology laboratory of the centre.

2.5 Cytokine Assay

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

2.6 Data Collection

Sociodemographic information and other relevant clinic information (HbSS diagnosis) were obtained from the medical records of the subjects included in the study.

2.7 Data Analysis

Serum cytokine levels and haematological variables were compared across the different groups using the Kruskal-Wallis ANOVA test. The Dunn's post test was used for comparisons between the 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.

3. RESULTS

Table 1 shows the demographic information of the study subjects. Of the sixty-six (66) subjects recruited in 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 2 shows the haematological parameters of the patients studied. The mean Hb levels, WBC, Neutrophils and lymphocytes counts were significantly different ($p < 0.0001$) across the groups, while mean eosinophil and basophil counts were not significantly different across the groups.

The mean IFN-γ concentrations were 97 pg/ml, 112.3 pg/ml and 596.5pg/ml in normal patients, steady-state and VOC patients. The mean IL-8 concentrations were 502 pg/ml, 764.4pg/ml and 1076 pg/ml in normal patients, steady-state and VOC patients. The mean IL-6 concentrations were 30.82 pg/ml, 45.0 pg/ml and 188.3 pg/ml in normal patients, steady-state and VOC patients. The mean IL-1β concentrations were 10.99 pg/ml, 13.41 pg/ml and 11.75 pg/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 4.0 shows the Pearson's Correlation value (r) between haematological parameters (Hb and WBC) and the different cytokines, (IFN-Ɣ, IL-8, and IL-6) in normal health patients, patients with VOC and steady- state patients. There were positive correlations between the haematological parameters and all cytokines except in IFN-Ɣ vs WBC, with IL-6 and Hb in VOC patients. In steady- state patients, IFN-Ɣ and IL-8 decrease as haematological parameters increase while WBC and Hb increase as IL-6 increases. 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-Ɣ and IL-6).

Table 1. Demographic information of patients

Groups	Age in years (Mean ±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. Hematologic parameters in studied subjects

Variable	Group A	Group B	Group C	ANOVA
Hb (g/dl)	13.8 ± 1.1	6.8 ± 0.7[a,b]	4.2 ± 0.9[a]	<0.0001*
WBC (10^9/liter)	7.3 ± 2.3	12.6 ± 2.8 [a,b]	27.6 ± 9.8 [a]	<0.0001*
Neutrophils (%)	53.4 ± 4.5	64.3 ± 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)*

4. DISCUSSION

Higher levels of IL-8, a chemotactic factor for neutrophils, during the 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 significant 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 are discordant with the study by Pathare et al., that reported increased concentrations of IL-6, IL-1β and IFN-γ in steady-state patients [15]. It also disagrees with a 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 the increase in IL-6 levels among patients in the steady state compared to normal control volunteers was not significant. This is consistent with the 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 patients in vaso-occlusive crisis and also steady-state and vaso-occlusive crisis patients. This is consistent with the study by Qari et al., which reported slight elevation of plasma levels of IFN-γ during a painful crisis when compared to the steady-state, the difference was however not statistically significant when healthy subjects were compared with patients in the

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- ϒ (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. Correlation of cytokines with white blood cell count and haemoglobin levels

Cytokines	VOC		Steady state		Control	
	WBC	HB	WBC	HB	WBC	HB
IFN- ϒ	-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

steady-state [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. This also suggests 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].

5. CONCLUSION

The finding of the study suggests that there is a relationship between the pro-inflammatory cytokine levels and the 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.

CONSENT AND ETHICAL APPROVAL

Informed written consent was obtained from all parents/caregivers of the patients and consent was also obtained before recruitment into the study. Any (parent/caregiver or patient) who refused consent were not included in the study nor denied the appropriate medical care/attention Ethical approval for the study was obtained from the Research Ethical Committee of the University of Port Harcourt Teaching Hospital (UPTH/ADM/90/S.IIVOL.X/713) and also from University of Port Harcourt (UPH/R&D/REC/04) prior to commencement of the study.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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