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Pattern of TNF-α and IL-1β Expression in Vaso-Occlusive Crises among 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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ABSTRACT

Introduction: Sickle cell disease (SCD) is characterised by chronic hemolysis, frequent infections, and recurrent occlusions of microcirculation, which cause a painful vaso-occlusive crisis and result in chronic organ damage and failure. Occlusions of the microcirculation and infections are important factors that stimulate the production of cytokines. Cytokines seem to be involved with several possible mechanisms in the pathogenesis of vaso-occlusive phenomena in sickle cell disease. Comparative analysis of pro-inflammatory cytokine production during sickle cell vaso-occlusive crisis gives an insight to the vaso-occlusive crisis markers for assessing disease severity.
Materials and Methods: TNF-α and IL-1β were measured by commercially available Enzyme-Linked Immunosorbent Assay (ELISA) kits in sickle cell disease patients (n = 49); in steady-state (n = 16) and in painful vaso-occlusive crisis (n = 33) and compared with age- and sex-matched normal healthy controls (n = 17).

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Results: Sickle cell disease patients in vaso-occlusive crisis showed elevated levels of TNF-α ($p < 0.05$) as compared to normal healthy control patients. However, levels of IL-1β in sickle cell disease patients in vaso-occlusive crisis compared to normal healthy controls remained similar ($p > 0.05$).
Conclusion: The study suggests that there is a relationship between the pro-inflammatory cytokine levels and vaso-occlusive crisis in sickle cell disease.

Keywords: Cytokines; TNF-α; IL-1β; sickle cell disease.

1. INTRODUCTION

Sickle cell disease (SCD) is a genetic disorder characterised by chronic hemolysis, recurrent occlusions of microcirculation with painful crises episodes and organ injury [1]. SCD results from point mutation of the gene encoding the β-globin subunit. The polymerisation of deoxygenated sickle hemoglobin leads to the decreased deformability of red blood cells [2]. Vaso-occlusion is a major cause for morbidity and mortality in SCD. It is a complex process governed by several factors which include adhesion molecules, receptors on erythrocytes, leucocytes, platelets and endothelial cells [3,4]. Vaso-occlusive episodes are the leading cause of admission to hospital, emergency room visits, missed school, and are associated with increased mortality rate [5]. Adhesion of sickle cells to the endothelium in steady-state (non-crises state) of SCD leads to chronic activation and damage of endothelium with subsequent release of pro-inflammatory cytokines such as IL-1, TNF- IL-8, IL-6 among others [6,7,8]. The production of these cytokines enhances the adhesiveness of sickle cells to the endothelium through induction of the vascular cell adhesion molecule (VCAM) expression, fibronectin and chemokine production [4,9].

In Nigeria, 78% of the nearly 200,000 annual emergency department visits for sickle cell disease are for a complaint of pain, yet there is no effective therapy that targets the underlying mechanisms of vaso-occlusive episodes [10,11]. The study assesses the pattern of pro-inflammatory cytokines in sickle cell disease patients during vaso-occlusive crisis and its associated pathogenesis with the aim of improving assessment of disease severity, treatment and prognosis in the local hospital practice.

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 over 500 bed capacity, several specialties and specialists. It serves as the main referral center for Rivers State and the neighbouring states.

2.2 Study Population and Sample

The study population is children attending the children emergency unit and out- patient clinic of the hospital. The study subjects were 66 children attending the out-patient clinic of UPTH between January to June 2016. The study subjects was divided into three groups as follows:

Group A: Healthy children attending UPTH for routine medical check.
Group B: Children with SCD attending the general out-patient clinic of UPTH.
Group C: Children with SCD presenting with vaso-occlusive crises at the children emergency unit of UPTH.

The sample size was calculated using the formulae below:

$$N = Z^2pq/d^2$$ as stated by Israel [12]

where N = desired sample size in a population greater than 1000.
Z = standard deviation usually set at 1.96, corresponding to the 95% confidence interval.
P = proportion of the target population estimated to have a particular characteristics.
q = 1 – p
d = degree of precision used (0.05).

In this study, p will be taken as 2% (0.02) representing the prevalence of sickle cell anaemia in Nigeria (Fleming et al. [13]).

Mathematically, the sample size is= 30.11816.

However, taking into consideration patients that may be lost to follow-up or lost samples, attrition rate of 10% will be used.

30 x 10% = 3

This gave a minimum of 33 subjects for the healthy Group (control) and 33 subjects for patients with sickle cell disease which were subdivided into patients in steady-state who have not had crisis nor blood transfusion since the last hospital visit of at least 2 months (Group B) and patients in vaso-occlusive crises (Group C).

2.3 Ethical Consideration

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

2.4 Sample Collection and Analysis

Five milliliters of blood were collected from each subject in EDTA for full blood count and HbSS determination, sterile plain bottles for cytokine studies and properly labeled. Diagnosis of HbSS homozygous in the patients was confirmed by genetic analysis in the molecular laboratory of the study center. Full blood counts of the blood samples were also assessed at with the Operon Bio Tech Auto-analyzer KT-6400 at hematology laboratory of the center.

2.5 Cytokine Assay

Serum levels of TNF-α and IL-1β were quantified using capture Enzyme Linked Immunosorbent Assay (ELISA) kits, according to the manufacturer's instruction (Avivabio 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/mg.

2.6 Data Collection

Socio-demographic information and other relevant clinic information were obtained from the medical records of the subjects included in the study.

2.7 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 0.05level 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 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 ± 3.5years. The mean age of the entire study sample was 6.5 ± 3.2 years.

Table 2 shows the hematological parameter of the patients studied. The mean Hb levels in subjects presenting with vaso-occlusive crises 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 X 10^9/L. Patients in steady state had a mean WBC of 12.6 ± 2.8 X 10^9/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 ± 9.2. Steady state patients had a mean lymphocyte of 28.9 ± 10.3 and normal patients had a mean lymphocytes of 40.9 ± 4.0. The mean eosinophil differential in VOC patients was 3.1 ± 2.2, in 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.

The median TNF-α levels between normal healthy control and steady-state patients were not statistically significant ($p > 0.05$), but there was a statistically significant difference ($p < 0.05$) in the median TNF-α concentrations between normal healthy control and VOC patients and

also between steady-state and VOC patients as shown in Fig. 1.

Fig. 2 shows that the median IL-1β concentrations were 10.91pg/ml, 11.88pg/ml and 12.03pg/ml in normal healthy control, steady-state and VOC patients respectively. The median IL-1β concentrations among normal healthy control and steady-state patients, normal healthy control and VOC patients and also steady-state and VOC patients were not statistically significant ($p > 0.05$).

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)*

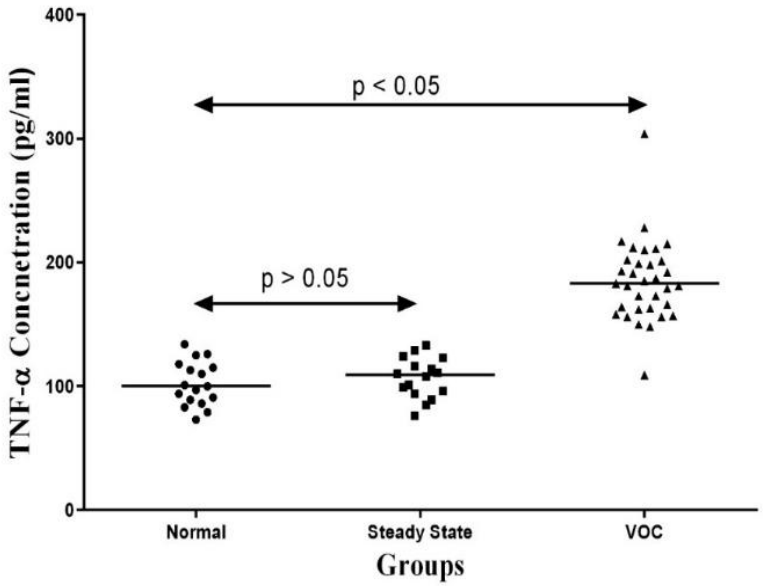

Fig. 1. Column Scatter Plot of TNF- α Concentration in normal healthy control, steady-state and vaso-occlusive crisis
Solid lines indicate median values for each group

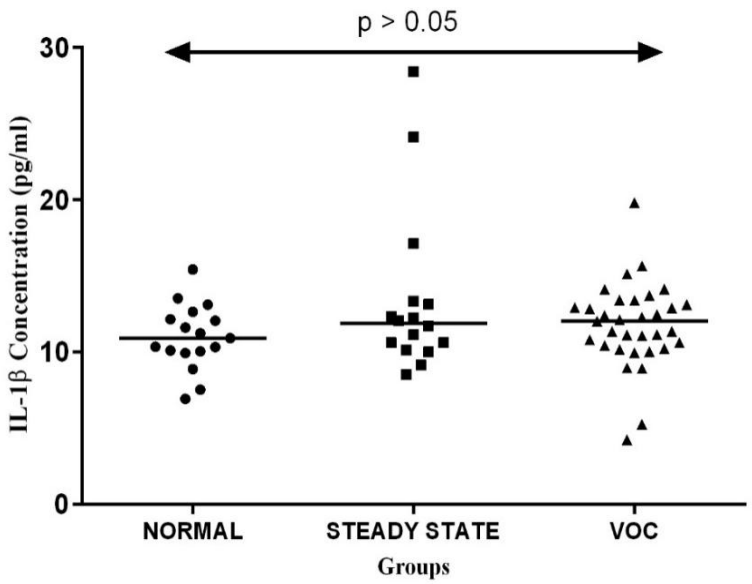

Fig. 2. Column Scatter Plot of IL-1β Concentration in normal healthy control, steady-state and vaso-occlusive crisis patients

Solid lines indicate median values for each group

4. DISCUSSION

Sickle cell disease is a chronic inflammatory state with significant inflammatory components including elevated leukocyte counts, abnormal activation of granulocytes, monocytes and endothelial cells, and increased levels of multiple inflammatory mediators [5,9,14]. Acute painful sickle cell crisis, with its accompanying regional ischemia, represents the most common presentation of sickle cell anemia patients to the emergency department [15,16]. It has been reported that vaso-occlusive crisis is a multi-factorial process involving haematological, inflammatory and thrombotic disturbances [17,18].

It is believed that there are significant subclinical microvascular occlusions in steady-state with ongoing local tissue ischaemia and necrosis. These subclinical micro-infarctions are induced by the enhanced adhesiveness of sickle reticulocytes and irreversibly sickled erythrocytes to the vascular endothelium, along with the associated chronic endothelial activation and the production of pro-inflammatory cytokines (IL-1β, IL-6, IL-8, TNF-α) by activated endothelial cells [15,19,20]. The degree of stimulation and production of cytokines is not high enough to trigger clinically evident vaso-occlusions in the steady-state. However, this balance can be very easily tilted and additional small insults may be enough to precipitate a crisis [21].

Evaluation of serum cytokines in all sickle cell disease patients in this present study, either in a steady-state condition or in vaso-occlusive crisis revealed an increased level of concentrations which were significantly higher in patients with vaso-occlusive crisis when compared to TNF-α levels in steady-state and normal healthy control patients. There was no significant difference in TNF-α concentrations between steady-state and normal healthy control patient which is in agreement with findings of Keikhaei et al. [**Error! Bookmark not defined.**]. However, it is not consistent with the findings of previous study by Tavakkoli et al., which reported higher levels of TNF-α in sickle cell patients with vaso-occlusive crisis than in steady-state group but this difference was not statistically significant [22].

IL-1β remained relatively the same, in the three groups studies. Likewise, significant differences were found between vaso-occlusive crisis patients and normal healthy controls and also between steady-state and vaso-occclusive crisis patients for all pro-inflammatory cytokines except IL-1β. This observation is consistent with the findings of previous studies by Taylor et al. [23]. Keikhaei et al. [**Error! Bookmark not defined.**], which reported no significant detectable levels of IL-1β in the serum of patients in steady-state condition or normal healthy controls. Qari et al. observed higher plasma concentrations of IL-1β

in sickle cell anaemia patients during both steady-state and vaso-occlusive crisis than in normal healthy control subjects [22].

5. CONCLUSION

The results of this study support the view that the chronic inflammatory response is an ongoing process, not only during vaso-occlusive crisis, but also during steady-state conditions in sickle cell disease patients. These data also suggest that there is a relationship between the pro-inflammatory cytokine levels and vaso-occlusive crisis in sickle cell disease. However, the finding presented here should be interpreted cautiously as there are a wide variety of major contributors to the pathogenesis of sickle cell disease such as adhesion molecules, other plasma proteins, leukocyte activation status, chronic inflammation, oxidative stress, procoagulant state and multiple organ damage endothelial injury, that needed to be investigated further for a better understanding of the pathophysiology of SCD.

CONSENT AND ETHICAL CONSIDERATION

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

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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