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Cellular Immune Status and Inflammatory Markers in Herpes Simplex Virus Type-2 Sero-positive Pregnant Women

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Abstract

Objective: Herpes simplex virus type-2 (HSV-2) causes genital ulcer disease and has been hypothesized to cause increase in inflammatory markers that contribute to atherogenic process. This study aimed to determine the cellular immune status and levels of systemic inflammatory markers in HSV-2 sero-positive pregnant women attending the antenatal clinic of Central Hospital, Warri, Nigeria. **Method**: This study included three hundred and sixteen (316) pregnant women. The sero-prevalence of Herpes simplex virus type-2 was determined using an enzyme linked immunosorbent assay while levels of cellular immune status (CD_4^+) and inflammation markers (C-reactive protein, total leukocyte count, total lymphocyte count, plasma viscosity) were determined using standard procedures. **Result**: The prevalence of HSV-2 was 192 (60.8%). Mean C-reactive protein and lymphocyte concentration were significantly higher in HSV-2 sero-positive subjects than in sero-negative counterpart (15.63 vs. 11.02, $p< 0.001$) and (41.90 vs. 33.71, $p< 0.001$) respectively, but CD_4^+ count did not differ in both subject group ((1114.39 vs. 988.21, 95% C.I 55.493, 196.86, P=0.076). Also the levels of total white cell count and plasma viscosity were not significantly different in HSV-2 sero-positive and sero-negative subjects. **Conclusion**: Increased levels of C- reactive protein and total lymphocyte count were significantly associated with HSV-2 infection. Increased CRP levels are known to contribute to atherogenic process; its routine quantification in patients managed for herpes infection is however advised.

Keywords: HSV-2, Cellular immune status, CD_4^+, C-reactive protein, Genital ulcer.

1. Introduction

Herpes simplex virus type-2 is now recognized as a significant cause of genital ulcer disease worldwide [1]. Genital herpes can be caused by either herpes simplex virus type 1 (HSV-1) or type 2 (HSV-2) but, worldwide (over 90%) of cases are caused by HSV-2 [1].

The immunologic response against HSV-2 includes both innate and adaptive immune response. The innate immune response is more profound and constitutes a major factor in determining the prognosis of an infection with HSV-2. The profound production of interferons of α and β types has been shown to lead to protection against HSV-2 induced diseases in documented studies in mouse models and human studies [2]. Potent neutralizing antibody production via adaptive immune response has also been documented to negatively correlate with disease severity [2].

Also CD_4^+ T cells are very essential in controlling HSV-2 infection [3]. This is evident from the fact that CD_8^+ deficient human experimental subjects do not suffer from any viral infections caused by the *Herpesviridae*, whereas CD_4^+-deficient AIDS patients do [4]. CD_4^+ T-cell responses to HSV-2 appear to be directed against envelope glycoproteins, capsid proteins and regulatory elements within the tegument [3].

There is accumulating evidence that some agent of sexually transmitted diseases notably HSV-2 can cause induce inflammation in humans [5], with consequent induction of production of cytokines and acute phase response both locally and systemically with chemo tactic cytokine release into the peripheral circulation with the production of acute phase response (pyrexia, leukocytosis etc.) and mobilization of lymphocytes and neutrophil which may indirectly influence the course of atherosclerosis [6]. This could raise levels of inflammatory markers in systemic circulation with consequent deleterious activity on cardiovascular system and a possibility of coronary artery disease (CAD) [7]. Progressive depletion in polymorphonuclear cells has been hypothesized to be a significant risk factor for genital herpes acquisition [7]. Also, a low white blood cell count may lead to reactivation of genital herpes [7].

The study aimed to determine the cellular immune status (CD_4^+ T cell count) and levels of systemic inflammation markers (C-reactive protein (CRP), Total lymphocyte count, Plasma viscosity and Total white cell count) in Herpes simplex virus type-2 sero-positive pregnant women in Warri, Southern, Nigeria.

2. Materials and Methods

2.1 Study Area

This study was conducted at the antenatal clinic of the Department of Obstetrics and Gynaecology, Central Hospital, Warri, Delta state, Nigeria. This hospital is the major government owned tertiary heath facility in Delta central senatorial district, it also house the antenatal clinic annex of Delta state university teaching hospital, Oghara.

2.2 Study Design

This study was a cross-sectional descriptive study to evaluate cellular immune status and inflammatory markers in Herpes simplex virus type -2 positive pregnant women (15 to > 40 Yrs) attending antenatal clinic of Central Hospital, Warri, Delta state, Nigeria. The subjects used for this study was chosen by stratified random sampling technique at the antenatal clinic.

2.3 Study Population and Selection Criteria

The study population included three hundred and sixteen (316) pregnant women between the ages of 15 and >40 years. Inclusion criteria included been pregnant, no drug history, no chronic disease, no pregnancy induced hypertension, non-smoking and no high risk pregnancy.

2.4 Ethical Approval

Informed consent (Written or verbal) was obtained from subjects before recruitment for this study. The protocol for this study was approved by the ethics committee of Delta State Hospitals Management Board, Warri Medical Zone

(Central hospital, Warri) in the letter CHW/ECC/VOL1/48.

2.5 Sample Collection and Preparation

About 10 ml of Blood sample was obtained by vein-puncture; equal volumes were dispensed into EDTA bottles and plain containers. Samples were securely and clearly labeled with codes corresponding to a subject name to prevent mismatch and misinterpretation of result. Grossly haemolysed and hyperlipaemic samples were discarded as they could generate false positive result. In general, phlebotomy errors that could undermine final results were minimized.

Total white cell, CD_4^+ and lymphocyte count were determined in the EDTA samples within 2 hrs of collection. Blood samples were allowed to settle down and Serum and plasma was separated using a bucket centrifuge calibrated to run at a speed of 2000 Revolutions per minute (RPM). . Remainder plasma was stored frozen for diagnosis of HSV-2 IgG and IgM. Serum samples were used to estimate levels of C-reactive protein and plasma viscosity.

2.6 Sample Analysis

Analysis of specimens was carried out at the Institute of human virology Nigeria (IHVN) laboratory located at Central Hospital, Warri and Immunology laboratory of Department of Chemical pathology, University of Benin Teaching Hospital, Benin City, Nigeria.

2.6.1 Herpes simplex virus type 2 IgG and IgM ELISA (Enzyme linked immunosorbent assay)

HSV-2 (IgG and IgM) antibodies was determined using type specific (HSV glycoprotein G) Herpes simplex virus type 2 ELISA IgG and IgM kits (Dia pro diagnostics, Milan Italy), and the results was interpreted following manufacturers specifications.

2.6.2 Principle of HSV-2 ELISA Test

The type specific HSV 2 IgG and IgM ELISA is based on a solid phase enzyme-linked immunosorbent assay (ELISA). The assay uses affinity purified HSV type 2 antibodies for solid phase (microtiter wells) immobilization and horseradish peroxidase (HRP) conjugated anti-HSV type 2 antibodies for detection. The test sample is diluted and incubated in the microtiter wells for 45 Min. The microtiter wells are subsequently washed and HRP conjugate is added and incubated for 30 minutes. This result in HSV type 2 molecules being sandwiched between the immobilization and detection antibodies. The wells are then washed to remove unbound HRP-labelled antibodies and TMB Reagent is added and incubated for 20 Min at room temperature. This results in the development of a blue colour. Colour development is stopped by the addition of Stop Solution, changing the colour to yellow, and optical density is measured spectrophotometrically at 405 nm. The concentration of HSV type 2 is proportional to the optical density of the test sample. A calibration curve, calibrated against an internal Gold standard, makes possible a quantitative determination of the antibodies in the patient.

2.6.3 CD_4^+ T Cell Count

Blood samples were analysed for CD_4^+ T cell estimation using flow cytometry. (Partec, GmbH, Germany). The partec Cyflow SL_3 is a single platform, three-parameter (SCC plus two colour fluorescence) desktop flow cytometer. It contains a solid laser for green excitation. It analyses concentrations of any particle or cell subpopulation of interest, using true volumentric absolute counting. The partec CD_4% reagent kit used contained direct immunofluorescence reagents for enumeration of mature CD_4^+ lymphocytes. Reference values for CD_4 T lymphocyte count in apparently healthy humans is > 450 CD_4^+ lymphocytes/µl.

2.6.4 Procedure for CD_4^+ T cell estimation

20 µl of CD_4^+ PE antibody was dispensed into a partec test tube and 20 µl of well mixed whole blood was added, mixed gently and incubated in the dark for 15 min at room temperature. The mixture was agitated during incubation every 5 Mins. 800 µl of CD_4 buffer was added to the mixture of antibody and sample and mixed gently. This was then plugged to the counter for counting.

2.6.5 Total leukocyte and Lymphocyte count

Total leukocyte and lymphocyte counts were determined in EDTA blood containers using Sysmex kx-21N hematology auto analyzer instrument [8]. Reference value for Total leukocyte count and total lymphocyte count in humans is 4000- 1000/l and 25-40 % respectively.

2.6.6 C- reactive protein (CRP) estimation

The Turbitest AA Line (Wierner laboratories, S.AI.C, Argentina) C reactive protein test kit based on immunoturbidimetry was used for the quantitative determination of serum levels of C reactive protein in subjects studied. The detection limit of this kit i.e., the minimum analyte amount capable of being detected as a sample, different than zero, and corresponds to the concentration of 0.5 mg/l. Its linearity (Assay range) is from2 mg/l to 200 mg/l CRP approximately. Assay conditions: wavelength (340nm), reaction temperature (37^{O}c), reaction time (10mins), sample volume 80µl) and final reaction volume (1.28 ml). Provided reagents: Calibrator (Calibrador en serie, 1;3;5;6;7;and 8), Reagent A (Saline buffer solution, Ph 7.6) and Reagent B (monospecific anti-CRP antibodies). Chemwell 2910 (Automated EIA and chemistry analyser) (Manufactured by Awareness technology, Incorporated, Palm City, United States of America) was used for measurement of absorbance of test and calibrators.

The reference range for CRP in apparently healthy individuals is <10 mg/L.

2.6.7 Determination of Plasma Viscosity

A modification of the method of Reid and Ugwu., (1987) was used. A 1ml syringe with a hypodermic needle (21.6 × 0.8 × 4mm) was used. Briefly, plasma was drawn into the syringe, avoiding air bubbles, till the 1.0ml mark. The plunger was carefully, removed and the time taken for the entire plasma to drain was noted. This was done twice for each sample and the average taken for that sample. The entire process was repeated using distilled water. The plasma viscosity is the ratio of the flow rate of plasma to that of water.

2.7 Data analysis

Data was analyzed using SPSS v. 16. Sero-prevalence was reported in percentages and comparism of prevalence with cellular immune status and inflammatory markers was done using chi square test, ANOVA and Fischer's exact were necessary.

3. Result

The Mean age of the study subjects at enrolment was 29 years (SD± 4.8). Table 1 summarizes the obstetric characteristics of the study population. Among the enrolled subjects, 44.3% (n=140) was in their 2^{nd} trimester, 43.0 % (n=136) was in their third trimester while subjects in their 1^{st} trimester had the least numbers of participants in this study 12.7 % (n=40).

The prevalence of antibodies to HSV-2 is presented in Table 2. The total prevalence of HSV-2 was 192 (60.8%).

Table 3 summarizes the mean concentration of cellular immune parameters (CD_4^+cell count, Total Leukocyte count, Total lymphocyte count, C- reactive protein and Plasma viscosity) of study participants. The mean CD_4^+ and total white cell count were within reference range (400- 1800 /µl and 4000-10000/l respectively).

The association between Herpes simplex virus type-2 sero-positivity and inflammatory markers is presented in Table 4. Mean C-reactive protein and lymphocyte concentrations were significantly higher in Herpes simplex virus type-2 positive subjects than in sero-negative subjects (15.63 vs. 11.02, 95% CI= -5.997,-3.220, p<0.001) and (41.90 vs. 33.71, 95%, p<0.001) respectively, but there was no significant difference between total white cell count in HSV-2 positive and negative subjects (Mean: 5600.00 vs. 5618.75, p=0.895). CD_4^+ count were higher in Herpes simplex virus type-2 sero-negative subjects when compared to their sero-positive counterparts but this was not significantly different (1114.39 vs. 988.21, 95% CI=55.493, 196.864, P=0.076) Table 5.

Table 1: Obstetric characteristics of pregnant women attending the antenatal clinic of Central Hospital, Warri

Parameters	Frequency	Percentage (%)
Marital status		
Married	276	87.3
Single	32	10.1
Divorced	8	2.5
Trimester		
1st	40	12.7
2nd	140	44.3
3rd	136	43.0
Parity status		
Primigravidae	151	47.8
Multigravidae	165	52.2
Coitarchal age (Age at first sexual debut)		
≤16	103	32.5
>16	213	67.5
Symptomatic (Clinical) Genital Ulcer disease		
Yes	36	11.4
No	280	88.6
History of genital ulcer disease		
Yes	49	15.5
No	267	84.5
History of spontaneous Abortion		
Yes	28	8.9
No	288	91.1

Table 2: Prevalence of Latent, reactivated and new HSV-2 infection among pregnant women attending the antenatal clinic of Central Hospital, Warri

Herpes simplex virus type-2 sero-status	Number	Percentage
Positive	192	(60.8)
No infection	124	(39.2)

Table 3: Concentration of cellular immune parameters among pregnant women attending the antenatal clinic of Central Hospital, Warri

Parameter	Mean ± Standard Deviation
CD_4^+ count	1037.72 ± 317.394
Total White cell count	5611.39 ± 1224.925
Total Lymphocyte count	38.68 ± 11.880
Plasma Viscosity	1.66 ± 0.134
C-reactive protein	13.82 ± 6.517

Table 4: Association between HSV- 2 status and systemic inflammatory markers among pregnant women attending the antenatal clinic of Central Hospital, Warri

Parameter HSV-2 status	Mean± S.E	P value
White cell count		
Negative 124	5600.00± 93.199	0.294
Positive 192	5618.75 ± 96.307	
Lymphocyte count		
Negative 124	33.71± 0.684	0.020*
Positive 192	41.90± 0.938	
C- reactive protein		
Negative 124	11.02± 0.310	0.024*
Positive 192	15.63± 0.530	
Plasma Viscosity		
Negative 124	1.62± 0.010	0.267
Positive 192	1.69± 0.010	

***Statistically significant**

Table 5: Association between HSV- 2 status and cellular immune status among pregnant women attending the antenatal clinic of Central Hospital, Warri

Parameter	Mean± S.E	95%C.I	P value
CD_4^+			
Negative 124	1114.39± 21.172	55.493, 196.864	P=0.076
Positive 192	988.21± 25.420		

4. Discussion

This study determined the association between HSV-2 sero-positivity and cellular immune parameters with focus on CD_4^+ T lymphocyte count and inflammatory markers among pregnant women. Increased lymphocyte count in HSV-2 sero-positive subjects in this study agrees with the report of an earlier study [9]. Lymphocytes are made by the immune system to fight disease. An increase in circulating lymphocytes is a sign of viral infection and is most commonly seen in lymphoproliferative disorders or in recurrent or new Herpes simplex virus infection [9].

In this study C- reactive protein was significantly higher in HSV-2 sero-positive subjects than those without infection. C-reactive protein (CRP) is a biomarker of acute phase response which is known to typically elevate in blood as a result of inflammation. It has been observed that pathogen induced inflammation may contribute to atherogenic process that may lead to coronary heart disease (CAD) [6]. An association between increasing burden of antibodies to persistent viral infections and increased levels of CRP and CAD was found in a study that tested the association of a five panel of pathogens (Cytomegalovirus, HSV-1, HSV-2, *Chlamydia* and Hepatitis A virus) to CAD risk [10].

Other studies [11,12], have also shown that increasing burden of persistent viral infection such as HSV-2 leads to increased endothelial dysfunction which is one of the earliest signs of atherosclerosis. Epstein [6], summarized that apart from the ability of persistent viral infections to contribute to atherosclerosis, they can also trigger plaque rupture and acute thrombotic occlusion which are major factors in acute myocardial infarction and for sudden deaths in patients with CAD.

The CD_4^+ cell count in HSV- 2 sero-positive pregnant women was lower (988.21± 25.42) than their sero-negative counterparts (1114.39± 21.172) but were not statistically different. Our study shows that HSV-2 does not lead to CD_4^+ cell decline unlike the Human immunodeficiency virus (HIV) with tropism for CD_4^+ cells. In a recent study, there was no association between CD_4^+ T cell counts and HSV-1 or HSV-2 sero-status in a proof of concept study among pregnant women in Limpopo, South Africa [13]. Similarly a study among postpartum women in Kenya did not find any significant association between HSV-2 infection and CD_4^+ T cell decline [14]. Our result does not agree with the study of Barnabas *et al* [15], which reported a an association between CD4 count decrease and HSV-2 infection [15]. The non- association between HSV-2 infection and CD4+ cell decline has also been proven in a United States epidemiology study Hoots *et al*., [16].

Total white cell count and plasma viscosity were not associated with HSV-2 sero-positivity, this indicates that they may not be useful indicators for non-specific evaluation for HSV-2. Plasma viscosities have been hypothesized to be a useful non-specific marker for inflammation in HIV [17], that assertion was unable to be proven in this study for its utility in HSV-2. The low concentration of plasma viscosity in subjects in our study is thought to be influenced by packed cell volume which has been found to be generally low among pregnant women in sub-Saharan Africa [18].

5. Conclusion

There were no statistically significant association between HSV-2 sero-positivity and CD_4^+ T cell counts levels, plasma viscosity and total white cell count. It can be hypothesized that HSV-2 does not cause CD_4^+ cell decline and its routine measurement in management of HSV-2 mono-infection in a resource limited setting such as ours might be of no diagnostic value except when there is HIV co-infection. C-reactive protein and total lymphocyte count were significantly associated with HSV-2 sero-positivity indicating a causal role of HSV-2 in inflammation. Increased CRP levels are known to contribute to atherogenic process; its routine quantification in patients managed for herpes infection is however advised.

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