



## **Neutrophils' Phagocytic Function of Hepatitis B Virus Infected Pregnant Women, Attending Specialist Hospital Sokoto, North Western, Nigeria**

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### **Authors' contributions**

*This research was carried out in collaboration among all authors. Authors COO and CCO designed the study. Authors COO, CCO, MHY and AAP managed the analyses of the study. Authors COO, CCO, MHY, AAP and MK managed the literature searches and wrote the protocols. Authors COO and ABS performed the statistical analysis and wrote the first draft manuscript. All authors read and approved the final manuscripts.*

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### **ABSTRACT**

Hepatitis B virus is the causative agent of hepatitis B infection. The virus is a major public health crisis in sub-Saharan Africa with high burden of morbidity and mortality. Vertical transmission is a significant contributor of new cases. This study was designed to access the innate immune

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competence of Hepatitis B viral infected pregnant women using neutrophil phagocytic function. In this cross sectional comparative observational study, simple random sampling technique was applied. A total of 100 Hepatitis B infected pregnant women and 100 controls were recruited. The data obtained were analysed using SPSS (version 23) software. A P probability value  $\leq 0.05$  was considered statistically significant. The results showed that proportion of phagocytic function was significantly lower ( $p < 0.05$ ) for HBV infected subjects compared with control. The findings also revealed that trimester does not influence the percentage neutrophil phagocytic function. Conclusively Hepatitis B infection affects innate immunity. Pregnant women should be screened for Hepatitis B surface Antigen (HBsAg) during routine Antenatal clinic and drugs should be recommended for Hepatitis B infected pregnant women.

**Keywords:** Neutrophil; NBT; hepatitis B; pregnant women; Sokoto.

## 1. INTRODUCTION

Hepatitis B virus (HBV) is a DNA virus belonging to the family *Hepadnaviridae* and the causative agent of Hepatitis B (HB) infection [1]. It is 50-100 times more infectious than Human Immunodeficiency Virus (HIV) and 10 times more communicable than hepatitis C virus (HCV). It is alluded to as a "quiet executioner" on the grounds that numerous individuals don't understand they are infected with the infection Samuel et al. [2]. Practices like sharing a tooth brush or extremely sharp objects can transmit infection in light of the fact that the base infectious dose is so low Pereira et al. [3]. HBV shares comparable courses of transmission with HIV Willey et al. [4]. Roughly 350 million individuals all through the world are infected with HBV with Nigeria arranged amongst the endemic nations Eke et al. [5]. Vertical transmission is a way of acquiring new HBV cases every year with 35% to 50% of transmission from mother to neonate in endemic nations ACOG, [6]. Vertical transmission happens to a great extent by means of a neonate's introduction to maternal blood and vaginal discharge during birth. The danger of progression to chronic infection is as high as 95% among neonates subject to vertical transmission as against 5% among grown-ups who contract the infection. The danger of viral transmission is around 10–20% when maternal blood is sure for HBsAg [6,7].

In Nigeria, the accompanying prevalence has been archived thus: Saidu et al. [8] announced 6.51% among pregnant ladies in Sokoto, Nigeria. Yakasai et al. [9] detailed a predominance of 7.9% among pregnant ladies in Kano, Nigeria. While in the South East, Ezeani et al. [10] revealed 7% among pregnant ladies and Oluboyo et al. [11] announced 6% in pregnant ladies in Nnewi, South East Nigeria.

The immune responses of HB viral infection are in two stages; the innate and the adaptive. The innate response act in the early intense stage where safe cells are enacted and the actuation of these cells assists with diminishing the viral burden through the discharge of cytokines, for example, IFN-gama [12].

It is realized that the assembly of the innate reaction has impressive significance in the accomplishment of resistant reaction against this viral disease. Along these lines, the current examination is intended to survey the neutrophils phagocytic capacity in HBV infected pregnant women utilizing nitroblue tetrazolium color decrease test.

When an individual is infected by the HBV, the intense period of the disease is joined by an underlying immunologic reaction that attempts to constrain the duplication of the viral particles inside the host. When this endeavor to clear the infection comes up short, the infected host immune framework will be undermined, attributable to exponential increase of the infection Ishikawa, [13]. This may prompt vertical transmission of HBV previously or at child birth [7,14].

The grimness and mortality because of HB viral infection are high in developing nations [15,16,17]. The infectivity spreads through the distinctive populace strata. Children, grown-ups and pregnant ladies are all infected by these viruses Ray and Bradley, [18]. Be that as it may, of significant concern is infectivity among pregnant ladies because of the condition of immune response during pregnancy and possible transmission to the infant Behrouz et al. [14]. There is scarcity of information with respect to neutrophils' phagocytic capacities in HBV infected pregnant women in the investigation

region which brought about the present study. The outcomes from this investigation may have potential advantage for the management of HBV viral infected pregnant ladies.

The aim of the study was to assess the innate immune competence of HB viral infected pregnant women using the neutrophil nitroblue tetrazolium (NBT) dye reduction assay.

#### **The objectives are to:**

Assess the phagocytic functions of neutrophils in HB viral infected pregnant women.

Compare the phagocytic functions of neutrophils in HB viral infected pregnant women with non infected pregnant women.

## **2. MATERIALS AND METHODS**

### **2.1 Study Area**

The study was carried out in the Department of Obstetrics and Gynaecology, Specialist Hospital Sokoto in Sokoto State. The Hospital is a tertiary Hospital and a referral centre for all the General Hospitals in the state. It has a 120 bed capacity and the Department of Obstetrics and Gynaecology runs an Ante-Natal clinic with patient size of about 70-90 weekly. Sokoto state shares boundary with the Republic of Niger to the North, Kebbi State to the West and South, and Zamfara to the South and East. The total population and annual growth rate stood at 3.7 million and 3.0% respectively in the 2014 national population census NPC, [19].

### **2.2 Study Population**

The study recruited pregnant women who were screened for HBV using Onsite HBsAg Rapid Test Strip manufactured by CTK Biotech, Carlifonia USA. The pregnant women were sub-grouped into HBV infected group (Test) and HBV non infected group (control). The pregnant women were also grouped into five different age groups 16-20 years, 21- 25 years, 26-30 years, 31-35 years and >36 years old. The pregnant women were further grouped according to pregnancy trimester / gestational age. However, none in the first trimester was reported due to the requirement that patients need to report for antenatal in their second trimester. Samples obtained from each participant were evaluated for their neutrophils phagocytic function.

### **2.3 Study Design and Sampling Technique**

It was a cross sectional comparative observational study. Simple random sampling technique was applied for recruiting the pregnant women until the desired sample size was obtained.

### **2.4 Sample Size Determination**

The sample size for the study was calculated using the standard formula for minimum sample size Daniel, [20], using a previous prevalence of 6.51% Saidu et al. [8]. A minimum sample size of 98 was obtained. However, the sample size was rounded up to 100 for convenience.

### **2.5 Inclusion Criteria**

All consenting pregnant women between 16-45 years, visiting the Ante-natal centre of Specialist Hospital Sokoto were qualified to partake in the investigation.

### **2.6 Exclusion Criteria**

HBV positive pregnant women were likewise screened for Hepatitis A, Hepatitis C and HIV and positive ones were barred from the investigation.

### **2.7 Blood Sample Collection**

Using a sterile vacutainer, holder and needle, 4 ml of venous blood was aseptically collected from each subject (2 ml in a sterile plain and 2 ml in lithium heparinized vacutainers). The blood in the plain vacutainer was allowed to clot at room temperature, after which it was centrifuged at 3,000 rpm for 5 minutes to obtain a clear un-haemolyzed serum. The serum was transferred into a clean, dried serum vial and was rapidly used to determine HB status.

The 2 ml of blood sample in the lithium heparinized vacutainer was used for the neutrophil phagocytic function test.

#### **2.7.1 Procedure**

The test was completed at room temperature (25°C). The strip was drenched into the specimen, with the arrow pointing towards the specimen. It was taken out following 10 seconds and was laid on a level, perfect, dry, non-retained

surface. The outcome was perused following 15 minutes.

## 2.8 Neutrophil Phagocytic Function Test

Neutrophils' phagocytic function was determined using NBT dye reduction method. It was carried out using the method adopted by Onyenekwe et al. [21].

### 2.8.1 Procedure

Total white blood cell count and differential white cell count were enumerated for each sample. The absolute neutrophil count was also evaluated and the quantity of blood in ml that contained one million ( $1 \times 10^6$ ) neutrophil in each sample was calculated and dispensed into test tube. This was followed by addition of 15  $\mu$ l of pure isolate of *Escherichia coli* endotoxins, and 0.1 ml of 4 mmol of freshly prepared nitroblue tetrazolium reagent, the mixture was incubated at 37°C for 20 minutes. Five milliliter (5 ml) of dioxane was added, the content mixed gently and incubated in water bath at 70°C for 20 minutes with intermittent shaking. A blue brown colour developed. The insoluble crystal was removed by centrifugation at 4000g for 10 minutes at room temperature. The supernatant was filtered and the absorbance of the filtrate measured at 520 nm using spectrophotometer. The concentration of neutrophils that engulf the endotoxins were calculated by multiplying the absorbance of test with the conversion factor.

### 2.9 Determination of Conversion Factor

A quantity of 150  $\mu$ mol ascorbic acid was dispensed into a tube containing 0.2 ml of 4 mMol NBT solutions and mixed thoroughly. Two (2) milliliter of 0.1 M sodium hydroxide in 24 mMol sodium bicarbonate solution was dispensed into the reaction mixture and incubated at room temperature for 10 minutes. Four (4) milliliter of dioxane was added and the mixture was thoroughly shaken. Subsequently,

1:10 dilution of this suspension was made in dioxane and incubated at 70°C for 20 minutes. This was cooled at room temperature and the absorbance was measured at 520 nm using spectrophotometer. Dioxane was used as blank. The conversion factor was determined to be 42.

### 2.10 Calculation

Functional activities of neutrophils were calculated as follows:

Functional activity of neutrophils = Absorbance of test  $\times$  Conversion factor

### 2.11 Data Analysis

Data was analyzed using SPSS (version 23) software. Descriptive analysis of percentages, mean, standard deviation and standard error of mean were carried out. The results of percentage neutrophil NBT dye reduction obtained from HBV positive pregnant subjects were compared with values of controls using independent sample T-test, while one way analysis of variance (ANOVA) was used for comparisons of three or more groups. In each case where there were significance differences, a post-hoc analysis was carried out using LSD multiple comparisons test, p-value less than or equal to 0.05 ( $P \leq 0.05$ ) was regarded as statistically significant.

## 3. RESULTS

The result in Table 1 shows comparison of Mean ( $\pm$  SD) Nitroblue Tetrazolium Reduction among HBV infected pregnant women and HBV non-infected pregnant women.

The mean ( $\pm$  SD) of formazan generated by neutrophils between HBV infected pregnant women ( $8.50 \pm 2.41$  Fmol/Phag), was significantly lower ( $P < 0.05$ ) compared with the corresponding value of control participants ( $10.59 \pm 7.43$  Fmol/Phag).

**Table 1. Mean nitroblue tetrazolium reduction among hepatitis B infected pregnant women and non-infected pregnant women**

Groups	NBT Formazan (Fmol/Phag)
Test (n=100)	8.50 $\pm$ 2.41
Contrl(n=100)	10.59 $\pm$ 7.43
P-Value	0.008

Values are Mean  $\pm$  SD, n= Number of subjects, NBT= Nitroblue Tetrazolium, Test = HBV infected pregnant women, Contrl = HBV non-infected pregnant women

**Table 2. Mean nitroblue tetrazolium reduction among hepatitis B Infected pregnant women at different age ranges**

Age (years)	NBT Formazan (Fmol/Phag)
16-20 (n=18)	7.64 ± 1.16
21-25 (n=34)	8.52 ± 2.90
26-30 (n=17)	9.12 ± 2.71
31-35 (n=22)	8.49 ± 2.33
36-above (n=9)	9.00 ± 1.66
F-Value	0.951
P-Value	0.438

Values are Mean ± SD, n=Number of subjects, NBT =Nitroblue Tetrazolium, % = percentage

**Table 3. Mean nitroblue tetrazolium reduction among hepatitis B infected pregnant women and non-infected pregnant women at different trimesters**

Trimester	NBT Formazan, (Fmol/Phag)
2nd TT (n=45)	8.21 ± 1.60
3rd TT (n=55)	8.74 ± 2.91
2nd TC (n=50)	10.71 ± 7.02
3rd TC (n=50)	10.47 ± 7.90
F-Value	2.459
P-Value	0.064
Post hoc Test Using LSD	
2nd TT vs 3rd TT	0.636
2nd TC vs 3rdTC	0.834
2nd TT vs 2nd TC	0.030
3rd TT vs 3rd TC	0.111

Values are Mean ±SD, n = Number of subjects, NBT = Nitroblue Tetrazolium, 2nd TT = second trimester test, 3rd TT = third trimester test, 2nd TC = second trimester control, 3<sup>rd</sup> TC = third trimester control

The result in Table 2 shows Mean (± SD) Percentage Nitroblue Tetrazolium Reduction in HBV infected pregnant women of different age ranges.

The mean (± SD) percentage rate of formazan production by neutrophils between the different age groups showed no significant difference (P > 0.05).

The effect of Trimester on Mean (± SD) Percentage Nitroblue Tetrazolium Reduction between HBV infected pregnant women (Test), HBV non-infected pregnant women (Control) is presented in Table 3.

The results indicate the Mean (± SD) Percentage Nitroblue Tetrazolium Reduction between HBV infected pregnant women (Test), HBV non-infected pregnant women (Control) based on trimester shows no significant difference (P=0.064). However, since it was close to 0.05 a posthoc analysis was carried out.

The results indicate that the mean (± SD) percentage rate of formazan production by

neutrophils between 2<sup>nd</sup> trimester test group compared with 3<sup>rd</sup> trimester test group and between 2<sup>nd</sup> trimester control group compared with 3<sup>rd</sup> trimester control group shows no significant difference (P > 0.05).

On the other hand, the mean (± SD) percentage rate of formazan production by neutrophils between 2<sup>nd</sup> trimester test group and 2nd trimester control group shows a significantly lower difference (P < 0.05).

Also the mean (± SD) percentage rate of formazan production by neutrophils between 3<sup>rd</sup> trimester test group and 3<sup>rd</sup> trimester control group shows no significant difference (P > 0.05).

#### 4. DISCUSSION

Results from this study showed a significantly lower (P < 0.05) neutrophil ingestion rate of NBT in HBV infected pregnant women compared to the control subjects. This is consistent with the previous study of Pawlowska et al. [22] who

determined the functional state of peripheral blood neutrophils in patients with chronic active viral hepatitis. The metabolic activity of neutrophils was examined in NBT reduction tests stimulated with lipopolysaccharide of *Escherichia coli* and majority of the patients had reduced values, which suggested a lack of response to lipopolysaccharide of *Escherichia coli* by neutrophils. However the findings were in variance with Tefanova et al. [23] who evaluated the functional metabolic activity of peripheral blood neutrophils in acute virus HBV and/or HCV in 48 patients and recorded pronounced functional dysbalance of neutrophil leukocytes. The difference in these findings may reflect the different state of inflammation in the body. Neutrophils, as a major component in the mammalian innate immune system, have essential roles in the battle with invading bacteria, fungi as well as viruses. More attentions has been drawn to dissecting the roles of neutrophils in bacterial pathogenesis; however, very little is still known about neutrophils during the course of viral infections. The reduced phagocytic function of neutrophils could also be looked as in terms of HBV infecting the neutrophils and declining their activity. WNV, H5N1 and EBV have been found to productively infect neutrophils, thus these viruses may utilize the infected neutrophils as transport vehicles for dissemination in the body Brandon and Fengwei, [24].

The observations in this study showed no significant difference ( $P > 0.05$ ) for neutrophil NBT dye reduction in second trimester HB infected pregnant women compared with third trimester HB infected pregnant women. This was in agreement with Okamura et al. [25] who demonstrated no significant difference in neutrophil ingestion rate activity between pregnancy periods.

This study also disclosed that neutrophil NBT dye reduction was significantly lower ( $P < 0.05$ ) in second trimester HB infected pregnant women compared with second trimester HB non-infected pregnant women that served as control subjects. This also consolidated the fact that the lowered neutrophil NBT dye reduction between the test and the control was brought about by the viral infection thereby in agreement with Pawlowska et al. [22] and Tefanova et al. [23] who reported lowered functional metabolic activity of peripheral blood neutrophils in both chronic and acute virus HB infection respectively.

## 5. CONCLUSION

The following conclusions were deduced from the study:

1. The percentage neutrophils' phagocytic function was significantly reduced in HB infected pregnant women compared to the non-infected pregnant women.
2. This indicated that the innate immune response was negatively affected in HB infection.

## 6. RECOMMENDATION

The following recommendations were made from the findings of this study:

1. Pregnant women ought to be screened for HBsAg during routine ANC.
2. Drugs ought to be prescribed for HB infected pregnant women.
3. Further investigations may concentrate on stimulating both T and B cells, in order to have additional understanding on commitment of T cells and B cells immunity to Hepatitis infection.
4. In addition, studies should be carried out using floctometry and RT-PCR in the measurement of neutrophils phagocytic surface molecules.

## CONSENT AND ETHICAL APPROVAL

The ethical approval for this examination was acquired from the Ethics and Research Committee of the Sokoto State Ministry of Health (SKHREC/024)016) dated 1/08/16 and from the Specialist Hospital Sokoto (SHS/SUB/133/VOL.1) dated 9/6/16.

Informed consent for incorporation into the investigation was received, for every patient, utilizing a standard educated assent structure. At the enrolment, an organized questioner controlled poll was utilized to gather information on subjects socio-economic and demographic qualities including age, conjugal status, occupation, instructive level, the status of Hepatitis A and B, and clinical phase of infection whether acute or chronic, regardless of whether on treatment or not, were also recorded.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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