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**Research Article**

# Erythrocyte Methaemoglobin Concentration and Methaemoglobin Reductase Activity in the Presence of Nevirapine and Efavirenzin Wistar Rats

**Uwakwe, A. A., Wodu, E.*, Monanu, M. O.**
University of Port Harcourt, Choba, Rivers State, Nigeria

***Corresponding author**
Ebizimor Wodu
Email: woduinndu@gmail.com

**Abstract:** The effects of nevirapine and efavirenz on methaemoglobin concentration and methaemoglobin reductase activity on wistar rats were investigated. Ninety rats with average body weight 100 grams were allowed to acclimatize for two weeks and were distributed into two groups. Drugs at dosage 0.2, 0.4, 0.6 and 0.8mg/l00g body weightof nevirapine; and 0.6, 0.8, 1.0 and 1.2mg/l00g body weightof efavirenz were administered orally by intubation. Blood samples were collected from the experimental animals by cardiac puncture. Spectrophometric methods were used to ascertain the methhaemoglobin concentrations and the activity of NADH methaemoglobin reductase (NADH MR). The results revealed that both drugs increased methaemoglobin concentrations. The increase from 1.50±0.00 to 2.22±0.04 on the 21st day in the presence of nevirapine was significant ($p<0.05$), while efavirenz cause a non-significant increase throughout the experiment. Methaemoglobin reductase activity decreased significantly ($p<0.05$) from 5.46±0.80 to 4.74±1.09, 5.51±1.03 to 4.74±0.15 and 5.48±0.11 to 4.73±1.03 in the 7th , 14th and 21st days respectively week respectively in the presence of nevirapine, while the decrease due to efavirenz was not significant ($p<0.05$). Thus, to an extent both drugs adversely affected erythrocytes's ability to maintain haemoglobin in its oxygen carrying state.
**Keywords:** Antiretroviral drugs Nevirapine, efavirenz, methaemoglobin concentration, methaemoglobin reductase, Erythrocyte

## INTRODUCTION

Methemoglobin (MetHb) is hemoglobin that has been oxidized from the ferrous ($Fe^{2+}$) to the ferric ($Fe^{3+}$) state. It is unable to bind oxygen. The NADH-Methaemoglobin Reductase (NADH-MetHbR) enzyme reduces MetHb to hemoglobin in the body. Methemoglobinemia results due to either inadequate activity of this enzyme or due to much MetHb production. MetHb is continuously formed in the normal red blood cells by the process of autooxidation form and also MetHb is rapidly reduced to hemoglobin by intraerythrocytic MetHb reductase after sampling [1].

NADH-MetHbR (EC: 1.6.2.2) is a 300-amino acid, membrane-bound enzyme localized in the endoplasmic reticulum of all cells [2]. It transfers electrons from NADH to cytochrome *b*5 via its flavin adenine dinucleotide (FAD) prosthetic group [3]. Under normal conditions, this enzyme also referred to as ferricyanide reductase, NADH-diaphorase or cytochrome b5 reductase. It is the only system within the erythrocyte responsible for maintaining hemoglobin in its oxygen-carrying reduced state [4]. The second form of this enzyme is NADPH dependent, is less active in mammals in converting MHb back to hemoglobin than the NADH dependent form [5].

In general, celluar activity of NADH-MetHbR reflects an organism's capacity to reduce MHb. Thus, it is an important factor in evaluating MHb formation and etiology of methaemoglobinemia. It is the rate-limiting enzyme controlling the toxicokinetics of the reduction of MHbR. Borgese *et al*. [6] reported that the activity of NADH-MetHbR is generally reduced in red cells of patients with recessive hereditary methaemoglobinemia. Activity of this enzyme has been measured in a variety of non-human species [7, 8] and these studies have reported that species with lower NADH-MetHbR activities convert MHb back to haemoglobin at slower rates than the species with higher NADH-MetHbR activities [5].

Antiretroviral drugs are medication for treatment of infection by retroviruses, primarily human immunodeficiency virus (HIV) [9]. Nevirapine and efavirenz are dipryridodiazepinone and benzoxamine compounds respectively and they belong to the non-nucleoside reverse transcriptase inhibitor (NNRTI) class of antiretroviral drugs, active against human HIV-1. They bind noncompetitively to an active site of the

reverse transcriptase molecule. They binds directly to reverse transcriptase and blocks the RNA-dependent and DNA-dependent DNA polymerase activities by causing a disruption of the enzyme's catalytic site. This work looks at the effect of administration of nevirapine and efavirenz on the MHb concentration and NADH-MetHbR reductase in wistar rats.

## MATERIALS AND METHODS

### Experimental design

Ninety rats with average body weight 100 grams were allowed to acclimatize for two weeks and were distributed into two groups. Each group was divided into five subgroups. The first in each subgroup in each group served as control while the other four groups served as tests to which four different dosages (0.2, 0.4, 0.6 and 0.8mg/100g body weight of nevirapine; and 0.6, 0.8, 1.0 and 1.2mg/l00g body weight of efavirenz) of the drugs were administered to the rats by intubation. Blood samples were collected from the rats by cardiac punction each week for analysis.

### Sample collection and preparation

Blood sample were obtained by cardiac puncture was stored in EDTA anticoagulant tubes. The erythrocytes were washed by methods as described by Tsakiris*et al*. [10]. 1.0 ml portion of the sample was introduced into centrifuge test tubes containing 3.0 ml of buffer solution pH=7.4: 250mM tris (hydroxyl methyl) amino ethane–HCl (Tris-HCl) /140mMNaCl/I.0mMMg$Cl_2$/10mMglucose). The separation of the erythrocytes from plasma was done by centrifugation at 1200g for 10 minutes, washed three times by three similar centrifugations with the buffer solution. The erythrocytes were resuspended in 1.0 ml of this buffer and were stored at $4^{O}$C. The washed erythrocytes were lysed by freezing and thawing as described by Galbraith and Watts [12]. The erythrocyte haemolysate was used for the determination of the erythrocyte enzyme activities.

### Determination of Methaemoglobin Concentration

Method used in the determination of methaemoglobin content of red cell haemolysate was a modified form of the method of Evelyn and Malloy [12] as described by Chikezie PC *et al*. [13]. 400µl of 0.5M Phosphate buffer (pH 6.5) was added to 600µl of the cell haemolysate and was centrifuged at 16,000g for 5 minutes to sediment debris. 700µl of the supernatant was used to measure the absorbance at 630nm and the reading was recorded as S1. 50 µl of 10g% KCN was added and after 5minutes at room temperature the second reading S2 was recorded. KCN converts methaemoglobin to cyanomethaemoglobin, which does not absorb at 630nm. The difference between absorbance readings of S1 and S2 represents the absorbance due to methaemoglobin.

To measure total hemoglobin levels, all of the hemoglobin was converted to methaemoglobin. The absorbance of the sample at 630nm was recorded and then KCN was added to form cyanomethaemoglobin. 70 µl of the supernatant fraction was diluted 10-fold into 600 µl of 0.1M phosphate buffer (pH=6.5). Then, 30µl of freshly prepared 20g% $K_3Fe(CN)_6$ was added, then incubated for 5minutes at room temperature and an initial reading (T1) was taken. After that 50 µl of 10% KCN was subsequently added, and the second reading (T2) was recorded. The percent methaemoglobin in the sample was calculated by the formula [100(S1-S2)] / [10(T1-T2)].

### Determination of Haemolysate NADH-Methaemoglobin Reductase Activity

NADH-MR activity of erythrocyte haemolysate was assayed according to the method of Board *et al*. [14]. The reaction mixture contained 0.2ml tris-HCl/EDTA buffer pH=8.0, 0.2ml NADH and 0.35ml of distilled water, 0.2ml of $K_3Fe(CN)_6$ and 0.05ml of erythrocyte haemolysate.

The 0.2ml tris-HCl/EDTA buffer pH=8.0, 0.2ml NADH and 0.35ml of distilled water were introduced into a test tube and incubated for 10minutes at $30^{O}$C. The content was transferred into a cuvette and the reaction was started by adding 0.2ml of $K_3Fe(CN)_6$ followed by 0.05ml of erythrocyte haemolysate. The increase in absorbance of the medium at $30^{O}$C was followed spectrophotometrically at 340nm for 10 minutes at 60 seconds intervals against a blank solution.

### Calculation of NADH-Methaemoglobin Reductase Activity

The equation below was used to evaluate erythrocyte NADH-MR activity in international unit per gram haemoglobin (IU/gHb).

$$E_A = \frac{100}{[Hb]} \times \frac{O.D/min}{\Sigma} \times \frac{V_C}{V_H}$$

Where,

EA is the Enzyme activity in IU/gHb

[Hb] is the Haemolysate haemoglobin concentration (g/dl)

0. D/min is the Change per minute in absorbance at 340nm.

Σ is the Millimolar extinction coefficient = 6.22, in reaction in which 1mole of $NADH^{+}H^{+}$ is oxidized.

$V_C$ is the Cuvette volume (total assay volume) = 1.0ml.

$V_H$ is the Volume of haemolysate in the reaction system (0.05ml).

## RESULTS AND DISCUSSION

The mean ± S.D of methaemoglobin concentration, expressed as percentage of total haemoglobin concentration and the activity of NADH

methaemoglobin reductase in the presence of nevirapine and efavirenz in albino rats are presented in Table 1, 2, 3 and 4. Erythrocyte methaemoglobin concentration in the presence of nevirapine (Table 1) increased in a dose dependent fashion. In the 7th and 14th days of the experiment, there was a non-significant ($p>0.05$) increase in methaemoglobin concentration when compared to control values, whiles the increase on the 21st day was significant ($p<0.05$). Efavirenz caused a non-significant ($p>0.05$) dose dependent increase all through the experimental weeks when compared to the control values.

The present research reveal significant decrease ($p<0.05$) in activity of NADH MR in the presence of nevirapine when compared to the control (Table 3), while the decrease due to the presence of efavirenz (Table 4) was not significant ($p>0.05$). The decrease indicates that both drugs have inhibitory effects on the enzyme.

**Table 1: Rat erythrocyte methaemoglobin concentration in the presence of nevirapine**

| Dosage (mg/100g body weight) | % Methaemoglobin | | |
|---|---|---|---|
| | Week 1 | Week 2 | Week 3 |
| 0.00 | 1.51±0.11[a] | 1.53±0.06[a] | 1.50±0.00[a] |
| 0.20 | 1.60±0.10[a] | 1.61±0.02[a] | 1.61±0.01[a] |
| 0.40 | 1.86±0.12[a] | 1.85±0.10[a] | 1.97±0.25[b] |
| 0.60 | 2.04±0.15[b] | 1.87±0.20[a] | 2.10±0.05[b] |
| 0.80 | 2.12±0.20[b] | 2.10±0.11[b] | 2.22±0.04[b] |

Values are recorded as MEAN±SD. Means with same superscript letters are not statistically different at 95% confidence limit ($p<0.05$)

**Table 2: Rat erythrocyte methaemoglobin concentration in the presence of efavirenz**

| Dosage (mg/100g body weight) | % Methaemoglobin | | |
|---|---|---|---|
| | Week1 | Week 2 | Week 3 |
| 0.00 | 1.50±0.03[a] | 1.48±0.01[a] | 1.49±0.10[a] |
| 0.60. | 1.53±0.23[a] | 1.54±0.12[a] | 1.56±0.01[a] |
| 0.80 | 1.57±0.12[a] | 1.60±0.10[a] | 1.61±0.21[a] |
| 1.00 | 1.58±0.12[a] | 1.61±0.20[a] | 1.65±0.20[a] |
| 1.20 | 1.60±0.05[a] | 1.64±0.18[a] | 1.82±0.13[b] |

Values are recorded as MEAN±SD. Means with same superscript letters are not statistically different at 95% confidence limit ($p<0.05$)

**Table 3: Rat erythrocyte NADH methaemoglobin reductase activity in the presence of nevirapine**

| Dosage (mg/100g body weight) | NADH Methaemoglobin Reductase Activity (Iu/gHB) | | |
|---|---|---|---|
| | Week1 | Week 2 | Week 3 |
| 0.00 | 5.46±0.80[a] | 5.51±1.03[a] | 5.48±0.11[a] |
| 0.20. | 5.37±1.04[a] | 5.30±0.16[a] | 5.21±1.01[a] |
| 0.40 | 5.10±0.10[b] | 5.02±0.10[b] | 4.90±0.25[b] |
| 0.60 | 5.00±0.14[b] | 5.22±0.25[a] | 4.90±0.20[b] |
| 0.80 | 4.74±1.09[c] | 4.73±0.15[c] | 4.73±1.03[b] |

Values are recorded as MEAN±SD. Means with same superscript letters are not statistically different at 95% confidence limit ($p<0.05$)

**Table 4: Rat erythrocyte NADH methaemoglobin reductase activity in the presence of efavirenz**

| DOSAGE (mg/100g body weight) | NADH Methaemoglobin Reductase Activity (Iu/gHB) | | |
|---|---|---|---|
| | Week 1 | Week 2 | Week 3 |
| 0.00 | 5.48±0.02[a] | 5.48±0.15[a] | 5.46±0.12[a] |
| 0.60 | 5.39±0.33[a] | 5,35±0.06[a] | 5.21±1.11[a] |
| 0.80 | 5.26±1.10[a] | 5.07±0.11[a] | 5.01±0.09[a] |
| 1.00 | 5.02±0.12[a] | 4.92±0.35[a] | 4.88±0.22[a] |
| 1.20 | 4.91±0.05[b] | 4.87±0.15[a] | 4.87±1.03[b] |

Values are recorded as MEAN±SD. Means with same superscript letters are not statistically different at 95% confidence limit ($p<0.05$)

Early studies have reported that certain xenobiotics are capable to elicit the formation and elevation of erythrocyte methemoglobin concentration and thus distort the normal plasma hemoglobin/methemoglobin ratio. Callister [15] reported that nitrates and anilines as the most common causes of methemoglobin toxicity in human.

The increase in methaemoglobin concentration in the rats studied was expected in the presence of both

drugs since they decreased the activity of NADH methaemoglobin reductase, an enzyme also responsible for the conversion of methaemoglobin back to haemoglobin. Increased methaemoglobin concentration in the erythrocytes suggests an inverse relationship between the methaemoglobin concentration and NADH-MR activity. In concordance with this finding, previous reports by Lo and Agar [16] and Whittington *et al*. [7] showed that the capacity of non-human species to revert oxidized haemoglobin-$Fe^{3+}$ (MHb) to the reduced form haemoglobin-$Fe^{2+}$ is inextricably connected with the NADH-MR activity of the corresponding erythrocytes.

## CONCLUSION

To an extent both drugs adversely affected erythrocytes's ability to maintain haemoglobin in its oxygen carrying state.

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