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Methaemoglobin Content and NADH-Methaemoglobin Reductase Activity of Three Human Erythrocyte Genotypes

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Abstract

Background: To study methaemoglobin content and NADH-methaemoglobin reductase activity of three human erythrocyte genotypes (HbAA, HbAS and HbSS).

Materials and Methods: Studies to ascertain methaemoglobin concentration and level of NADH-methaemoglobin reductase activity of three human erythrocyte genotypes (HbAA, HbAS and HbSS) were carried out in forty-three (43) healthy male participants of confirmed erythrocyte genotypes: HbAA (N=15), HbAS (N=15) and HbSS (N=13). Spectrophotometric method was used for determination of the two erythrocyte parameters.

Results: Whereas methaemoglobin concentrations in the three erythrocyte genotypes was in the order: HbAS<HbAA<HbSS, levels of NADH-methaemoglobin reductase activity was: HbSS<hbAS<HbAA. There was no statistically significant difference (p>0.05) in methaemoglobin concentration and NADH-methaemoglobin reductase activity between HbAA and HbAS erythrocytes.

Conclusion: The results showed a relationship between erythrocyte NADH-methaemoglobin reductase activity and methaemoglobin concentration.

Keywords: Methaemoglobin, Erythrocyte, Genotype, NADH-methaemoglobin Reductase.

Introduction

Methaemoglobin (MHb) is an altered state of haemoglobin in which the ferrous (Fe^{2+}) irons of heme are oxidized to the ferric (Fe^{3+}) state[1]. The ferric haem moities of MHb are unable to bind to oxygen molecules.

NADH-Methaemoglobin Reductase (NADH-MR) (EC: 1.6.2.2) is a 300-amino acid, membrane-bound enzyme localized in the endoplasmic reticulum of all cells[2]. This enzyme transfers electrons from NADH to cytochrome b_5 via its flavin adenine dinucleotide (FAD) prosthetic group[3]. Under normal conditions, this NADH-dependent enzyme also referred to as ferricyanide reductase, NADH-diaphorase or cytochrome b_5 reductase is the only system within the erythrocyte that maintains hemoglobin in its oxygen-carrying reduced state [4]. It is noteworthy that a second form of this enzyme, which is NADPH dependent, is less active in mammals in converting MHb back to hemoglobin than is the NADH-dependent form [5].

Generally, celluar activity of NADH-MR reflects an organism's capacity to reduce MHb and is therefore an important factor in evaluating MHb formation and etiology of methaemoglobinemia. From a toxicological standpoint, NADH-MR is the rate-limiting enzyme controlling the toxicokinetics of the reduction of MHb[4]. Prevoius report by Borgese *et al*. [6] stated that the activity of NADH-MR is generally reduced in red cells of patients with recessive hereditary methaemoglobinemia. Deficiencies in the reductase systems and resulting clinical manifestations have been described [7].

NADH-MR activity has been measured in a variety of non-human species[8,9,10] and these studies have revealed that species with lower NADH-MR activities convert MHb back to haemoglobin at slower rates than do species with higher activities [5].

The present study seeks to establish whether variability in NADH-MR activity exists amongst human erythrocyte genotypes, namely HbAA, HbAS and HbSS, in relation to their methaemoglobin contents. The findings of the present study will provide insights into the oxidant status of the three erythrocyte genotypes and deduce the degree of tolerance to toxic methaemoglobinemia by individuals who express these genotypes.

Materials and Methods

Selection of Volunteers/Collection of Blood Samples:

Forty-three healthy male (61-73kg) participants of confirmed HbAA (N=15), HbAS (N=15) and HbSS (N=13) genotypes between the ages of 20-28 years were enrolled for this study. Five milliliters (5.0ml) of human venous blood were obtained from participants by venipuncture and stored in EDTA anticoagulant tubes. Blood samples of HbSS genotype were from patients who attended clinics at the Federal Medical Center (FMC), Imo State University Teaching Hospital (IMSUTH), Orlu, St. John Clinic / Medical Diagnostic Laboratories, Avigram Medical Diagnostic Laboratories, and Qualitech Medical Diagnostic Laboratories. These centers are located in Owerri, Imo State, Nigeria.

Ethics:

The institutional review board of the Department of Biochemistry, University of Port Harcourt, Port Harcourt, Nigeria, granted approval for this study and all participants signed an informed consent form. This study was in accordance with the ethical principles of the Helsinki Declaration. Individuals were enrolled from Imo State University, Owerri, Nigeria and environs. The research protocols were in collaboration with registered and specialized clinics and medical laboratories.

Preparation of Erythrocyte Haemolysate:

The erythrocytes were washed by methods as described by Tsakiris *et al.*[11] and Chikezie[12]. Within 2hours of collection of blood samples, portions of 1.0 ml of the samples were introduced into centrifuge test tubes containing 3.0ml of buffer solution pH=7.4: 250mM tris-HCl (Tris-HCl)/140mMNaCl/1.0mM$MgCl_2$/10mMglucose. The erythrocytes were separated from plasma by centrifugation at 1200g for 10minutes, washed three times by three similar centrifugations with the buffer solution. The erythrocytes re-suspended in 1.0ml of this buffer were stored at 4^0C. The washed erythrocytes were lysed by freezing/thawing as described by Galbraith and Watts [13] and Kamber *et al.* [14]. The erythrocyte haemolysate was used for the determination of NADH-methaemoglobin reductase activity and methaemoglobin concentration.

Determination of Methaemoglobin Concentration of Erythrocyte Lysate:

Determination of methaemoglobin content of red cell lysate was by modification of the method of Evelyn and Malloy[15] as described by Akomopong *et al.*[16] and Chikezie[12]. A total of 400µl of 0.5M Phosphate buffer (pH 6.5) was added to 600µl of the cell lysate and the mixture was centrifuged at 16,000g for 5minutes to sediment debris. A total of 700µl of the supernatant fraction was used to measure the absorbance at λmax=630nm (the absorbance maximum for methaemoglobin) and the reading was recorded as S1. A total of 50 µl of 10gpercentageKCN was added and after 5minutes at room temperature (24^0C), a second reading (S2) was recorded. KCN converts methaemoglobin to cyanomethaemoglobin, which does not absorb at 630nm; hence, 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 λmax=630nm was recorded and then KCN was added to form cyanomethaemoglobin. Specifically, 70 µl of the supernatant fraction was diluted 10-fold into 600 µl of 0.1M phosphate buffer (pH=6.5). Next, 30µl of freshly prepared 20g% $K_3Fe(CN)_6$ was added and incubated for 5minutes at room temperature (24^0C) and an initial reading (T1) was recorded. A total of 50 µl of 10% KCN was subsequently added, and a second reading (T2) was recorded. The percent methaemoglobin in the sample was calculated as [100(S1-S2)] / [10(T1-T2)].

Determination of Erythrocytes Haemolysate Haemoglobin Concentration:

A modified method of Baure[17] described by Chikezie *et a*l.[18] based on cyanomethaemoglobin

reaction was used for the determination of haemolysate haemoglobin concentration. The expressed values were in grams per deciliter (g/dl). A 0.05ml portion of human red blood cell haemolysate was added to 4.95ml of Drabkin reagent (100mgNaCN and 300mg $K_4Fe(CN)_6$ per liter). The mixture was left to stand for 10minutes at room temperature and absorbance read at λmax=540 nm against a blank. The absorbance was used to evaluate for haemolysate haemoglobin concentration by comparing the values with the standards.

Determination of NADH-Methaemoglobin Reductase Activity of Erythrocyte Lysate:

The enzyme activity represents the rate of oxidation of NADH and is described as follows:

$$2K_3Fe(CN)_6 + NADH + H^+ \xrightarrow{\text{NADH-MR}} 2K_2HFe(CN)_6 + NAD^+$$
$$(Fe^{3+}) \qquad\qquad (Fe^{2+})$$

NADH-MR activity of erythrocyte lysate was assayed according to the method of Board [19]. A mixture of 0.2ml tris-HCl/EDTA buffer pH=8.0, 0.2ml NADH and 4.35ml of distilled water was introduced into a test tube and incubated for 10minutes at 30^0C. The whole content was transferred into a cuvette and the reaction above started by adding 0.2ml of $K_3Fe(CN)_6$/0.05ml erythrocyte haemolysate. The increase in absorbance of the medium was followed spectrophotometrically at λmax=340nm per minute for 10 minutes at 30^0C against a blank solution.

Calculation of Enzyme Activity:

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

$$E_A = \frac{100}{[Hb]} \times \frac{0.D/min}{\sum} \times \frac{V_C}{V_H}$$

Where,

E_A = Enzyme activity in iu/gHb

[Hb] = Haemolysate haemoglobin concentration (g/dl)

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

∑ =Millimolar extinction coefficient = 6.22, in reaction in which 1mole of NADH + H^+ is oxidized.

V_C = Cuvette volume (total assay volume) = 1.0ml.

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

Statistical Analysis:

The experiments were designed in a completely randomized method and data collected were analyzed by the analysis of variance procedure while treatment means were separated by the Least Significance Difference (LSD) incorporated in the Statistical Analysis System (SAS) package ver. 9.1.

Results

The mean (±S.D) of methaemoglobin concentration, expressed as percentage (MHb%) of total haemoglobin concentration of three erythrocyte genotypes (HbAA, HbAS and HbSS) was in the order: HbAS<HbAA<HbSS. Wheras there was no significant difference (p>0.05) in MHb% between HbAA and HbAS erythrocytes, level of methaemoglobin in sickle cell genotype exhibited significantly higher (p<0.05) concentration compared with the HbAA and HbAS genotypes. (Table 1).

The three human erythrocyte genotypes showed varied levels of NADH-MR activity that was in the order: HbSS<HbAS<HbAA. Similar to methaemoglobin concentrations, there was no significant difference (p>0.05) in NADH-MR activity between HbAA and HbAS erythrocytes (Table 1). Therefore, there was an inverse relationship between MHb% and NADH-MR activity amongst the three human erythrocytes genotypes.

Table 1: Methaemoglobin Concentration (MHb %) and NADH-Methaemoglobin Reductase Activity of Male Erythrocyte Haemolysate.

Genotype	MHb%	NADH-MR (IU/gHb)
HbAA (N=15)	1.48±0.14[a]	14.77±1.47[a]
HbAS (N=15)	1.45±0.13[a]	14.47±0.98[a]
HbSS (N=13)	2.50±0.43[b]	8.86±2.49[b]

Means±S.D in the column with the same letter are not significantly different at p< 0.05 according to LSD. n= number of blood samples.

Discussion

Previous reports have revealed an association between dysfunctional erythrocytes (HbSS genotype) and raised levels of oxidized haemoglobin[12, 20]. They noted that the primary

reason for the relatively raised concentration of oxidized haemoglobin (methaemoglobin) in HbSS erythrocytes was higher production of superoxide ion by these erythrocytes compared to those of HbAA and HbAS erythrocyte genotypes. Specifically, sickle erythrocytes spontaneously generate approximately twice as much superoxide, peroxide and hydroxyl radicals when compared with normal erythrocytes. In addition, HbSS erythrocytes contain increased amounts of malondialdehyde, a by-product of lipid peroxidation and evidence of abnormal amino-group cross-linking by malondialdehyde has been demonstrated in lipid extract of HbSS erythrocytes membranes[21]. This phenonmenone confirms the susceptibilty of sickle erythrocytes to endogenous free radical mediated oxidative damage that correlates with the proportion of irreversible sickled erythrocytes [22].

The relative lower activity of NADH-MR of sickle erythrocytes compared with the HbAA and HbAS erythrocytes suggests an inverse relationship between the methaemoglobin concentration of human erythrocytes and NADH-MR activity. In concordance with this finding, previous reports by Agar and Harley [8] Lo and Agar[9] and Whittington *et al*.[10] 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. In addition, Geetha *et al*. [23] averred that high level of methaemoglobin concentration in cirrhotic erythrocytes might be partly due to low level of methaemoglobin reduction engendered by low level of NADH-MR activity. Therefore, this study suggests that persons who express the sickle erythrocytes will exhibit more susceptibilty and slower rate of recovery when challenged with agents that causes toxic methaemoglobinemia. This suggestion agrees with the findings of Daly *et al.* [24] who reported that several members of a family, who showed decreased activities of erythrocyte NADH-cytochrome b5 reductase, were predisposed to the development of clinically significant methaemoglobinaemia when they were administered oxidant drugs.

Conclusion

The present study showed that in the absence of exogenous oxidants, there was an inverse relationship between erythrocyte methaemoglobin concentration and NADH-MR activity.

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