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ANTIOXIDANT-MEDIATED GLUTATHIONE LEVELS OF SICKLE ERYTHROCYTES UNDER DRUG-INDUCED OXIDATIVE STRESS.

[1]Aloh, G. S, [2*]Obeagu, Emmanuel Ifeanyi, [3]Okoroiwu, I.L., [1]Odo, Christian Emeka, [2]Odo Matthew Chibunna, [4]Kanu,S.N, [5]Elemchukwu, Q., [5]Okpara,K.E., [6]Ugwu, Getrude Uzoma,

[1]Department of Biochemistry, Michael Okpara University of Agriculture, Umudike, Abia State, Nigeria.

[2]Diagnostic Laboratory Unit,University Health Services, Michael Okpara University of Agriculture, Umudike, Abia State, Nigeria.

[3]Department of Medical laboratory Science,Imo State University,Owerri,Nigeria.

[4]Abia State University Teaching Hospital,Aba,Abia State,Nigeria.

[5]Rivers State College of Health Technology,Port Harcourt.

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***Correspondence for Author**
Obeagu,E.I.
Diagnostic Laboratory Unit,University Health Services, Michael Okpara University of Agriculture, Umudike, Abia State, Nigeria.+2348037369912
emmanulobeagu@yahoo.com
obeagu.emmanuel@mouau.edu.ng

ABSTRACT

The effects of two antioxidants, ascorbic acid and α-tocopherol, on the glutathione (GSH) levels of sickle erythrocytes were studied before and after inducing oxidative stress with 5mg of acetylphenylhydrazine (APHZ) per ml of blood. Pre –APHZ GSH levels of the control and sickle erythrocytes were 39.82 ± 1.48 mg/100ml and 26.00 ± 2.32 mg/ml respectively. Their respective post – APHZ levels were 37.00± 2.35 mg/ml and 13.00 ± 1.98 mg /ml. Post – APHZ +ascorbic acid , their mean GSH levels were respectively 37.75 ± 2.50 mg/ml and 16.00 ± 1.83 mg/ml , while post- APHZ + α- tocopherol significantly ($p<0.05$)elevated their GSH levels to 39.76 ± 2.80 mg/ml and 22.00 ± 2.24 mg/ml respectively. These results indicate that whether defective or not , the initial effect of an oxidant drug is to lower the GSH levels of red cells. Because the sickle erythrocytes had a subsisting redox stress ,the effect of APHZ could not be fully reversed by both ascorbic acid and α - tocopherol as occurred in the control. α – Tocopherol was more effective in counteracting the effect of APHZ , probably because it is a fat – soluble vitamin.

KEYWORDS: Antioxidants; ascorbic acid; alpha-tocopherol; glutathione; oxidative stress; Sickle cell.

INTRODUCTION

Although the peoples of Africa have known sickle cell disease/syndrome(SCD/S) for centuries and given it names (USDHHS, 1989), western literature ascribes its discovery to James B. Herrick who, in 1910, reported that one of his patients from the West Indies had an anaemia characterized by unusual red cells that were sickle-shaped (Kark,2000). The relationship of red cell sickling to low oxygen tension was reported in 1927 by Hahn and Gillepsie while Sherman, in 1940, associated alteration in haemoglobin structure with deoxygenation (Kennedy *et al.*,1986). Today, SCD/S is a generic reference to a group of haemoglobin disorders in which the β-globin gene at chromosome 11 of haemoglobin (Hb) is inherited and Hb "Senegal" or sickle Hb (HbS) is predominant(USDHHS,1989). The clinical manifestations of SCD include anaemia, vaso-occlusive crises, bacterial infections (septicaemia), acute chest syndrome, stroke, acute splenic sequestration and aplastic crisis, retinopathy, ischemic tissue injury, leg ulcers, priapism etc, (Satyen *et al.,* 2007) These clinical presentations are precipitated by intracellular polymerization of HbS that occurs when erythrocytes are partially deoxygenated under hypoxia (Taha & Kazzi, 2007). Repeated cycles of oxygenation/deoxygenation result in irreversibly sickled cells (Ho *et al.,* 2007), intracellular water and potassium loss, progressively dehydrated dense cells that are less deformable and which cause micro-vascular occlusion and haemolytic anaemia (Powars & Hiti, 1993). These effects are due to the increased production of reactive oxygen species (Kark, 2000).

A battery of enzymes – glutathione reductase (GPx), superoxide dismutase (SOD), and catalase – provide the first line of defence against these reactive oxygen species (Dhalla *et al.,* 2000). The second line of defence is provided by the antioxidants or free radical scavengers, including ascorbic acid, α- tocopherol, glutathione (GSH),etc, (Uday *et al., 1999).* The oxidative stress caused by the imbalance between the rates of production and scavenging of free radicals has been implicated in the pathogenesis and progression of many diseases and disorders, including sickle cell disease/disorders (Bilgin-Karabulut *et al.,* 2001).

In this study, the effects of two antioxidants, ascorbic acid and *α*-tocopherol, on the glutathione levels of sickle erythrocytes under drug-induced oxidative stress were

investigated. Oxidative stress was induced in the red cells with 5mg of acetylphenylhydrazine (APHZ), a classical inducer, (Beutler,1955), per ml of whole blood.

MATERIALS AND METHODS

Volunteers and Blood Samples: The consents of the 30 non-sickle volunteers and the authorities of the hospitals used for the study were obtained. The 30 sickle cell patients used for this study were confirmed ones on routine visits to the hospitals used.

Chemicals: The chemicals used for this study were of analytical grades and products of reputable companies.

GSH Assay (Beutler, 1966): One ml of whole venous blood was drawn into 1ml of acid-citrate-D-glucose (ACD) solution constituted with 16.0g of sodium citrate, 4.8 g of citric acid, and 29.5g of D-glucose in one litre of distilled water. Five milligrammes (5mg) of acetylphenylhydrazine (APHZ) per ml of blood were added to induce oxidative stress, mixed, shaken to ensure thorough oxygenation, and incubated for 2 hours at 37^{o}C in a water bath. To test the effect of ascorbic acid on APHZ-induced oxidative stress and on GSH level after inducing stress, 0.8mg of ascorbic acid per ml of blood was added to the reaction mixture after the addition of APHZ, and incubated for 2 hours. For the effect of α-tocopherol, 0.1mg of vitamin E per ml of blood was added at that point into a separate reaction mixture and also incubated for 2 hours. At the end of the incubation, 2ml of distilled water was added. Five minutes later, 5ml of 3% glacial metaphosphoric acid was added with agitation, followed with 3g of NaCl. The reaction mixture was shaken, filtered, and 2ml of the filtrate added to 6ml of saturated NaCl solution in a 25-mm cuvette at 20-25^{o}C. One ml of 2% sodium nitroprusside and 1ml of 0.67M NaCN-5M $NaCO_3$ (1:1,v/v) solution were added to develop colour. Absorbance was read at 525nm within one minute.

RESULTS

The data obtained were subjected to a t-Test at $p<0.05$ to compare the differences between their means.

Table 1: Mean glutathione levels of erythrocytes before APHZ treatment, after APHZ treatment, and after APHZ treatment followed with ascorbic acid and α-tocopherol treatments.

Erythrocyte Type	Pre- APHZ (mg/100ml)	Post –APHZ (mg/ml)	APHZ+ Ascorbic acid (mg/ml)	APHZ + α- tocopherol (mg/ml)
Control	39.82± 1.48	37.00± 3.25	37.75± 2.50	39.76± 2.40
Sickle Cell	26.00± 2.32	13.00± 1.98	16.00± 1.82	22.00± 2.24

DISCUSSION

As depicted on Table 1,sickle erythrocytes had a lower mean GSH level than the control red cells prior to the induction of oxidative stress. This would suggest that these cells had a high subsisting oxidative challenge when compared with the control red cells. Earlier studies (Aslan *et al.*, 2000), suggest that sickle haemoglobin (HbS) auto-oxidizes 1.7 times the rate of normal/ non - sickle haemoglobin (HbA), and therefore has a higher propensity to produce damaging oxidants, such as O_2^- and H_2O_2. This also agrees with similar studies by Dhalla *et al.*,(2000), which demonstrated that increased production of reactive oxygen species by these cells under hypoxia is responsible for the pathogenesis and progression of sickle cell complications.

However, when both cell types (Table 1) were challenged with APHZ (the oxidant stressor), their mean GSH levels decreased but to varying extents. The decrease in the mean GSH level of the control red cells was insignificant ($p>0.05$). This was indicative of an effective system of enzymes (the first line of defence) and the second line - antioxidant defences against offending redox species. On the contrary, APHZ treatment of the sickle erythrocytes caused their mean GSH levels to be drastically depleted by 50% (26.00$\pm$ 2.32 to 13.00$\pm$1.98 mg/100ml). This must be due to an overwhelming level of oxidants which, according to Schacter *et al.*, (1988) accumulate when the activities of red blood cell (RBC) antioxidant enzymes are low. Irrespective of red cell types and mechanism of depleting their GSH levels, it is noteworthy that the first effect of the oxidant stressor (APHZ) was to lower their GSH levels. Since RBC glutathione is maintained in the functional reduced state (GSH) by the NADPH generated in the pentose phosphate pathway (PPP), it is most probable that APHZ caused a defect in this pathway, especially at the step catalyzed by glucose-6- phosphate dehydrogenase.

Introducing ascorbic acid into the APHZ – treated reaction mixture shored up the mean GSH levels in both cell types but that of sickle red cells still remained far below the pre-APHZ level.

α- Tocopherol had a more profound effect in elevating the mean GSH levels of both the control and sickle erythrocytes. The mean GSH level in the control red cells was almost restored to its pre – APHZ level. There was also a significant elevation ($p<0.05$) of its mean level in sickle red cells but could not be restored to its pre-APHZ level. The oxidant species generated were obviously overwhelming to both the enzymatic and antioxidant defences of

the red cells. Although ascorbic acid ameliorated the effect of APHZ, α-tocopherol was more effective, probably because of its fat- solubility which must have enabled it to penetrate the erythrocyte membrane lipids to either scavenge the offending redox species or reduce GSSG to GSH.

In conclusion, APHZ exerted a greater oxidative (GSH – depleting) effect on sickle red cells than on the control (non-sickle) ones. The initial effect of an oxidant drug (eg,APHZ), irrespective of red cell type, is to lower its GSH level. Both ascorbic acid and α- tocopherol reduced the effect of APHZ but the latter was more effective, probably on account of its fat – solubility. Based on the results of this study, it is advisable to co – administer, or follow, an oxidant drug with an erythro-protective antioxidant, especially α- tocopherol which, in this study, proved to be very effective in counteracting APHZ – induced oxidative stress / GSH depletion.

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