Effects of Antioxidants on Drug-Induced Glutathione Instability and Lipid Peroxidation in Erythrocytes of Sickle Cell Patients

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Abstract

Glutathione (GSH) instability leading to its depletion, and enhanced lipid peroxidation, are associated with the pathogenesis, progression and therapy of many diseases / disease conditions – including sickle cell disease. In this study, the effects of two antioxidants, ascorbic acid and α - tocopherol, on GSH instability and lipid peroxidation were assessed before inducing oxidative stress with acetylphenylhydrazine (APHZ) (Pre-APHZ), post APHZ, and post – APHZ followed with ascorbic acid and α - tocopherol respectively. The mean pre-APHZ GSH concentration of 26 $\pm$ 2.32 mg/100ml was significantly ($p < 0.05$) reduced to 13 $\pm$ 1.98mg/100ml post – APHZ. This post – APHZ value was insignificantly ($p > 0.05$) elevated to 16 $\pm$ 1.82 mg/100ml when treated with ascorbic acid, while α - tocopherol significantly ($p < 0.05$) elevated it to 22 $\pm$ 2.24 mg/100ml. APHZ significantly ($p < 0.05$) elevated the level of lipid peroxidation (MDA) from 210.91 $\pm$ 23.43 nmol/hr (pre-APHZ) to 284 $\pm$ 31.00 nmol/hr post-APHZ. Both ascorbic acid and α - tocopherol did not reduce this post – APHZ lipid peroxidation level significantly ($p > 0.05$) as the post APHZ + ascorbic acid and post – APHZ + α - tocopherol levels of MDA remained at life – threatening levels of 266.70 $\pm$ 31.00 nmol/hr and 238.50 $\pm$ 26.37 nmol/hr respectively. In this study, α - tocopherol was a better antioxidant than ascorbic acid but it appears that elevation of GSH level per se is not enough to arrest drug – induced lipid peroxidation in sickle cell patients and 238.50 $\pm$ 26.37 nmol/hr respectively. In this study, α - tocopherol was a better antioxidant than ascorbic acid but it appears that elevation of GSH level per se is not enough to arrest drug – induced lipid peroxidation in sickle cell patients.

Keywords:

Introduction

Glutathione, a tripeptide of glutamic acid, cysteine and glycine (Panela *et al.*, 2005), is synthesized in the cytosol as part of the ϒ- glutamylcysteine cycle of GSH metabolism (Voet and Voet, 1994). Glutathione (GSH), a tripeptide of glutamic acid, cysteine and glycine (Pamela, *et al,* 2005), is synthesized in the cytosol as part of the μ – glutamy/ cycle of GSH metabolism (Voet and Voet, 1994). Many physiological processes, especially in the highly oxidizing environment of the erythrocyte, oxidize GSH to GSSG, but the cell is able to maintain a GSH: GSH: GSSG ratio of over 100, which permits GSH to function as an intracellular reducing agent, through the action of glutathione reductase (Pamela et al., 2005; Voet and Voet, 1994). By cycling between the reduced thiol forms (GSH) and the oxidized form (GSSG), glutathione, which is usually present at high levels (~ 5nM) in animal cells, serves as a sulphydryl (SH) buffer (Stryer, 2000). Erythrocyte membranes, thiols (e.g. cysteine, cysteinylglycine, μ – glutamylcysteine), thiol – containing enzymes, and other proteins (e.g. haemoglobin), are maintained in the reduced state by GSH because they are not subject to direct reduction by NADPH (Griffith, 1991).It has been established that the α – glutamyl /cysteine cycle serves as a vehicle for the energy –driven transport (group transfer) of amino acids into cells through the synthesis and breakdown of GSH (Voet and Voet, 1994) GSH is also conjugated to drugs in the second phase of xenobiotic transformations, but unlike other conjugating agents, it has no identified nucleotide intermediate (De Leve and Kaplowitz, 1991). In the synthesis of peptidoleukotrienes, GSH is the source of the SH group added to leukotriene A4, an epoxide, to form the first of the leukotrienes, leukotriene C4 (Voet and Voet 1994).

Peroxidative modification of unsaturated phospholipids, free fatty acids, glycolipids, lipoproteins (especially low-density lipoprotein), and cholesterol, is triggered by free radicals (e.g. O_2, OH, $OONO^-$) non-free radical species (e.g. O_2, O_3, O^1), and oxidant drugs (Kappus and Sies 1981). Lipid peroxidation occurs in a variety of cells and tissues, including microsomal membranes, peroxisomes, mitochondria, lysosomes, and erythrocyte membranes (Monaharan *et al.,* 2002). The intermediates or products of lipid peroxidation, mainly hydroperoxides, ketones, aldehydes and diadehydes, cause covalent intra – and inter – molecular bonds/cross-linking with proteins and other biomolecules (Siroev and Makarova, 1989), and this calls for cytoprotection to avert apoptotic death (Lee, *et al,* 2002). Accordingly, oxidative stress engendered by an imbalance between the rate of production of free radicals and anti-peroxidative defences has been implicated in the pathogenesis of many diseases, ageing, and disease complications – including sickle cells syndrome (H_o, et al, 2007). The primary line of defence for counteracting free radical damages is a system of enzymes – glutathione peroxidase (GPx), superoxide dismutase (SOD), and catalase; GPx,

for example, catalyzes the reaction of hydroperoxides swith GSH to form GSSG and either an alcohol or water (H_o, *et al,* 2006). The secondary line of defence against free radical damages is provided by the antioxidants/free radical scavengers (Bilgin-Karabulut, *et al,* 2001), About 400 antioxidants have been identified, but the best known /studied are GSH,α - tocopherol (Vit. E), ascorbic acid (Vit. C) retinol (Vit. A), carotenoids (pro-vit. A), carotenoids (pr0-vit.A), ubiquinol and uric acid (Van – Acker, *et al,* 2000). In spite of the roles played by free radicals in pathology, they are however necessary for the maturation of cellular structures, and released by macrophages and neutrophils to destroy invading pathogenic microbes (Bagchi and Puri, 1998).

Materials and Methods

A total of thirty (30) sickle cell patients between the ages of 10 and 25 years was used for this study. All the patients were attending regular checkups at the Federal Medical Centre, Abakaliki, and were not having crises at the time of collecting their blood samples. They all consented to the requests to use their blood for research purposes only. Five milliliters (5ml) of venous blood were drawn from each patient – 3ml for GSH and 2ml for lipid peroxidation (TBARs) assays respectively. Oxidative stress was induced for each assay with 5mg of acetyphenythydrazine (APHZ) per ml of whole blood. Each parameter (GSH and TBARs) was measured pre-APHz treatment, post – APHZ, and post – APHZ follow by a ascorbic acid and α - tocopherol in different assay test tubes.

Glutathione determination was done according to the acid – citrate – D – glucose (ACD) method of Beutler (196). Five milliliters 95ml) of venous blood were drawn into 1ml of an aqueous ACD solution containing 16. 0g of sodium citrate, 4.8g of citric acid, and 29.5g of glucose per litre. 5mg of APHZ were added to the test tube, mixed, shaken to ensure thorough oxygenation, and incubated for 2hours at 37^0C in a water bath. At the end of the incubation period, 2ml of distilled water were added. Five minutes later, 5ml of 3% glacial metaphosphoric acid (equivalent to 35% H_2 PO_3) were added with agitation, followed by 3g of NaCI. The reaction mixture was shaken. The mixture was filtered, and 2ml of the filtrate added to 6ml of saturated NaCI solution in a 25 – mm cuvette, while the solution was equilibrated at 20 - 22^0C.1ml each of 2% sodium nitroprusside and 0.67 M NaCN – 5M Na_2C0_3 were added to develop colour. A spectrophotometer reading was made at 525nm within one minute and the values read off from a standard curve.

Erythrocyte lipid peroxidation, estimated as the rate of malondialdehyde (MDA) formation per unit time (nmol/hour), was determined according to the method described by Siroev and Makarova (1989). 2ml of oxalated (1.34%) blood were placed in a centrifuge tube and centrifuged for 10 minutes at 3000 x g. The supernatant was carefully drawn off with a Pasteur pipette. The erythrocyte residue was washed three consecutive times with three volumes of 0.9% NaCI, and centrifuged again for 10 minutes at 3000x g to further precipitate the crythrocytes. Next, 0.50ml of the erythrocyte precipitate and an equal volume of distilled water were placed in a clean centrifuge tube and allowed to stand for 30 minutes to allow complete haemolysis to occur. This mixture was centrifuged for 30 minutes at 3000 x g and, with a Pasteur pipette, the supernatant, together with grey-coloured intermediate layer of the haemolyzed erythrocyte membrances were transferred into a clean test tube. Two test tubes were labeled samples 1 and 2 respectively. In tube 1 were placed 0.3ml of 4 x 10^{-5}M Mohr salt solution, 0.3ml of 0.04M tris-HCI buffer, 0.3ml of 2.6mM ascorbic acid solution, and 0.1ml of erythrocyte membrane suspension. To tube 2, the control, was added 0.3ml of Mohr solution, 0.3ml of tris-HCI buffer, 0.3ml of ascorbic acid solution, 0.1ml of erythrocyte membrane suspension, and 1ml of 40% trichloroacetic acid solution, which was added immediately. The two test tubes were placed in a water bath at 37^0C for 20 minutes. Thereafter, the reactions in both tubes were stopped with 1ml of trichloroacetic acid solution. The samples were further centrifuged for 15 minutes at 3000 x g, the supernatant liquids were decanted into separate clean test tubes, and 1ml of 0.8% thiobarbituric acid added to each tube. The tubes were placed in a boiling water bath for 10 minutes and then cooled in ice – cold water. The absorbance in tube 1 was measured against the control (tube 2) at 532nm and read off from a standard curve.

Results and Discussion

Most of the complications of sickle cell syndrome are traceable to repeated cycles of oxygenation/deoxygenation which result in irreversible membrane damage (USDHHS, 1989). Its exacerbation is in turn linked to low GSH levels of erythrocytes and the concomitant low level of pentose phosphate pathway – derived NADPH. The consequence, membrane lipid peroxidation among others, leads to both intravascular and macrophage – mediated splenic haemolyses which ultimately precipitate haemolytic crisis (Satyen *et al.,* 2006).

The low mean GSH level of 26 $\pm$ 2.32 mg /100ml pre – APHZ treatment (Table 1) when compared with 40.00 mg/100ml in non – sickle cell individuals (USDHHS, 1989), is indicative of subsisting haemolysis that characterizes sickle cell anaemia because GSH is needed for the maintenance of red cell integrity (Pamel *et al.,* 2005). Further depletion of this pre-APHZ mean GSH level to 13 $\pm$ 1.98 mg/100ml post – APHZ was strongly significant ($p < 0.05$). This drastic reduction in GSH level could either be due to low G6PD activity in the erythrocytes of such patient leading to a lower reductant (NADPH) level, or there were high levels of endogenously generated redox species, particularly reactive oxygen and nitrogen species which are known to deplete red cell GSH (H_O, et al, 2007).

If the latter reason is correct, it implies that the reactive species so generated overwhelmed the primary enzymes line of defence for counteracting

Table 1: Mean glutathione (GSH) and malondialadehyde (MDA) levels of erythrocytes before APHZ treatment, post APHZ treatment, and post – APHZ + ascorbic acid and α - tocopherol

Parameter	Pre-APHZ Treatment	Post-APHZ Treatment	Post-APHZ + Ascorbic acid	Post – APHZ + α - tocopherol
GSH (mg/100ml)	26 ± 2.32	13 ± 1.98	16 ± 1.82	22 ± 2.24
MDA (nmo1hr^{-1})	210.91 ± 23.43	284 ± 31.00	266. 70 ± 31.1.00	238. 50 ± 26. 37

free radical damages, especially glutathione peroxidase which catalyzes the reaction of hydroperoxides with GSH to form GSSG and either an alcohol or water which is a further source of free radicals (H_o, et al, 2006). That the GSH depletion of the erythrocytes was free – radical mediated was borne out by the fact that ascorbic acid and α - tocopherol, the two antioxidants which should be secondary lines of defence against free radical damages (Bilgin – Karabulut, et al, 2001) elevated the mean GSH levels from the post – APHZ level of 13 ± 1.98 mg/100ml to 16 ± 1.82 mg / 100ml and 22 ± 2.24 mg/100ml respectively. compared with the known mean GSH level of 40 mg/100ml in non – sickle cell subjects (USDHHS, 1989), the mean levels in the cells studied were still too low to maintain the red cells membrane integrity. Over whelming level of free radicals with a concomitant depletion of red cell GSH level must be a major contributory factor to the massive haemohysis seen in sickle cell anaemia.

At baseline, ie, pre-APHZ, the mean MDA level of the erythrocytes studied was very high (210.91 ± 23.43 nmol/hr) when compared with the level found in non-sickle cell subjects under the same condition, i.e., in the absence of an oxidant stressor (Aloh, 2008). As demonstrated by Klings and Farber (2001), this is likely to be due to the presence of lipid peroxyl radicals which are known to be involved in the pathogenesis of sickle cell complications, especially in the presence of oxidant drugs. This is also consistent with the human studies by Aslan *et al.* (2000), which demonstrated high levels of TBARs in sickle erythrocytes at baseline. The significant ($p < 0.05$) elevation of the mean MDA level to 284 ± 31.00 nmol/hr post – APHZ treatment was consistent with the significant ($p < 0.05$) depletion of the man GSH levels of the red cells post – APHZ. In other words, the free radicals generated by APHZ in addition to the endogenously generated ones markedly depleted the red cell GSH and exacerbated the lipid peroxidation observed as high MDA levels. Although both ascorbic acid and α - tocopherol down-regulated the MDA levels in the APHZ – treated erythrocytes, they (the MDA levels) still remained at life – threatening levels. Hence, as a consequence of free – radical medicated depletion of the red cell GSH levels, lipid peroxidation was aggravated, the iron in haemoglobin was maintained in the ferric (Fe^{3+}) state that could not transport oxygen to the tissues (Stryer, 2000), and haemohysis occurred. This result strongly indicated that much of the haemolysis seen in sickle cell anaemia is intravascular since this study was in vitro.

This study has demonstrated that the GSH and MDA levels of the erythrocytes of sickle cell patients are lower and higher respectively, than those of "normal" (non-sickle cell) subjects even in the absence of oxidative stress. It further demonstrated that oxidant drugs deplete red cell GSH levels and consequently aggravate lipid peroxidation to cause the massive haemolysis which characterizes sickle cell anaemia. It also established that these effects of oxidant drugs on sickle erythrocytes are not significantly reduced by the two antioxidants tried – ascorbic acid and α - tocopherol. Until a more effective free radical scavenger is found however, it is advised, based on the results of this study, to constantly maintain sickle cell patients on a regular supply of α - tocopherol, the more effective of the two antioxidants tried in this study.

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