ANTIOXIDANT–MEDIATED AMELIORATION OF DRUG – INDUCED OXIDATIVE STRESS AND METHAEMOGLOBINAEMIA IN G6PD – DEFICIENT AND SICKLE ERYTHROCYTES.


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Article Received on 26/12/2014 Article Revised on 17/01/2015 Article Accepted on 08/02/2015

ABSTRACT

This study assessed the effects of two antioxidants – ascorbic acid and α-tocopherol – on the methaemoglobinemia induced by an oxidant drug, acetylphenylhydrazine (APHZ) on three erythrocyte types. Oxidative stress was induced in the control, glucose-6-phosphate dehydrogenase – deficient (G6PD –deficient) and sickle erythrocytes (SEs) with 5mg of APHZ per ml of whole venous blood. Prior to APHZ treatment, the mean methaemoglobin (MetHb) levels were 0.34 ± 0.06 mgMetHb, 0.42 ± 0.03 mgMetHb, and 0.66 ± 0.04 mgMetHb in the control, G6PD – deficient and SEs respectively. After APHZ treatments, the mean MetHb level of the control erythrocytes did not change, while those of the G6PD – deficient and SEs rose significantly (p < 0.05) to 0.66 ± 0.01 mgMetHb and 0.76 ± 0.08 mgMetHb respectively. Post – APHZ + ascorbic acid in the reaction mixture, the mean MetHb levels were significantly (p < 0.05) scaled down to 0.33 ± 0.09 mgMetHb in the control, 0.64± 0.09 mgMetHb in the G6PD – deficient and 0.67 ± 0.04 mgMetHb in the SEs respectively. α-
Tocopherol had a more profound effect as the mean MetHb levels went down to $0.29 \pm 0.08$ mgMetHb, $0.42 \pm 0.02$ mgMetHb and $0.40 \pm 0.05$ mgMetHb respectively. This study indicates that MetHb is present in red cells even in the absence of exogenous oxidant challenge, and that its level rises significantly in the G6PD–deficient and sickle erythrocytes when subjected to oxidative stress. α-Tocopherol was found to be more effective in counteracting drug–induced oxidative stress and the attendant methaemoglobinaemia than ascorbic acid. Based on the results of this study, co–administration of, or following, oxidant drugs with an effective antioxidant, such as α-tocopherol, is advisable as it will reduce oxidative stress, the attendant methaemoglobinaemia, and its consequences.

**KEYWORDS:** Antioxidants, methaemoglobin, oxidative stress, acetylphenylhydrazine.

**INTRODUCTION**

It has long been established (Synder *et al*., 1982), that oxidative stress is exerted by free radicals (FRs), species which peculiarly possess an unpaired or odd number of electrons whose charges may be positive, negative or zero. FRs are formed continually in cells as a consequence of both enzymatic and non–enzymatic reactions, and may be either of organic or inorganic origin (Bagchi & Puri, 1998).

Oxidative stress engendered by the imbalance between the rates of production and scavenging of FRs has been implicated in the pathogenesis of many diseases, ageing and disease complications (Ho *et al*., 2007). FR–mediated disorders / diseases are due to the damages they cause in functional cellular components, including proteins, carbohydrates, lipids and nucleic acids (Lam *et al*., 2002).

Both glucose – 6 – phosphate dehydrogenase (G6PD) deficiency (an enzymopathy), and sickle cell disease /syndrome (a haemoglobinopathy) are adversely affected by metabolically generated free radicals (Switzer *et al*., 2006). The low activity or deficiency of G6PD in the erythrocytes of susceptible individuals which is due to the accelerated breakdown of the mutant enzyme protein, results in low levels of NADPH which it generates through the pentose phosphate pathway (Goldberg & Rock, 1992). This, in turn, precipitates directly and otherwise, the myriad of biochemical haematological defects associated with G6PD deficiency, including diminished methaemoglobin (MetHb) reduction and Heinz bodies formation leading to intravascular haemolysis (Mehta *et al*., 2000). MetHb has the iron in its haemoglobin in the permanent ferric state and does not bind oxygen for transport to the
Sickle cell syndrome is clinically associated with such FR – induced complications as vasoocclusive crises, acute chest syndrome, stroke, acute splenic sequestration, priapism, among others (Satyen et al., 2006). The human body has two mainlines of defence against free radical damages: a system of enzymes, such as glutathione peroxidise (GPx), superoxide peroxidise (SOD) and catalase (Uday et al.,1999), and the presence of antioxidants (free radical scavengers), including glutathione, ascorbic acid and α-tocopherol among others (Bilgin – Karabulut et al., 2001).

This study therefore set out to investigate the effects of two antioxidants, ascorbic acid and α-tocopherol, on the methaemoglobin profiles of G6PD-deficient and sickle erythrocytes in which oxidative stress was induced with 5mg of acetylphenylhydrazine (APHZ) per ml of whole venous blood.

**MATERIALS AND METHODS**

**Selection of Subjects/Patients**

**Subjects/Patients**

With the consents of the relevant authorities and volunteers, G6PD screening test and reliance on hospital records were used to select 30 individuals in each category for the study as follows: (a) Control, non-G6PD – deficient, non-HbSS subjects (b) G6PD – deficient, non-HbSS subjects, and (c) HbSS, non-G6PD – deficient patients.

**Blood Samples**

About 5ml of venous blood was collected from each subject / patient using a disposable syringe and transferred into venoject tubes containing ethylenediamine tetraacetate anticoagulant.

**Methaemoglobin Reduction Test**

This was carried out according to the method of George et al.,(1960) as modified by Stott & Lewis (1995). Drabkin’s neutral diluting fluid, pH7.40, was constituted with potassium ferricyanide (200mg), potassium cyanide (50mg), potassium dihydrogen phosphate (140mg), Triton – X – 100, and made up to 1 litre with distilled water. Before assaying for MetHb in the blood samples listed in (a), (b) (c) above, commercial, ready – to – use cyanmethaemoglobin (CNMetHb) standards equivalent to 30g/l, 115g/l and 180g/l were used to plot a standard graph according to the procedure hereby described, except that CNMetHb
replaces venous blood. Exactly 0.02ml (20µl) of venous blood was added to 4.0ml of Drabkin’s neutral diluting fluid to make a 1:20 dilution. A supplementary 0.10ml of 0.28M glucose, and 0.10ml of 0.0004M methylene blue chloride (mol. wt. 373.92) were added to the reaction mixture. The tube was stoppered, mixed, and left at room temperature, protected from sunlight, for 4 – 5 minutes to convert Hb to MetHb. For the effect APHZ, 5mg/ml of blood was added and the above procedure carried out. To assess the effect of ascorbic acid on the drug/APHZ – Induced oxidative stress / methaemoglobinemia, 0.08mg of ascorbic acid per ml of blood was added to the reaction mixture after adding the 5mg of APHZ per of blood to the reaction mixture and the procedure continued. Similarly, to assess the effect of α-tocopherol on APHZ - induced oxidative stress / methaemoglobinemia, 0.01mg of α-tocopherol was added at that stage to a separate reaction mixture and the procedure continued. The spectrophotometer wavelength was set at 540nm and its reading zeroed by using the neutral diluting fluid as blank. The optical densities (ODs) of the samples were then read and subsequently extrapolated on the standard graph as methaemoglobin units.

RESULTS
The MetHb levels were subjected to a t – Test at p < 0.05 to compare the differences in their means.

Figure 1: Paired comparison of mean MetHb levels of control (0.34mgMetHb), G6PD-deficient (0.42mgMetHb) and sickle erythrocytes (0.66mgMetHb) before APHZ treatment
Figure 2: Paired comparison of mean MetHb levels of control (1B, 0.34mgMetHb), G6PD deficient (2B, 0.67mgMetHb) and sickle cell patients (3B, 0.67mgMetHb) after APHZ treatment.

Figure 3: Paired comparison of mean MetHb levels of control, G6PD - deficient and sickle cell patients after APHZ + ascorbic acid treatments.

Figure 4: Paired comparison of mean MetHb levels of control, G6PD - deficient and sickle cell patients after APHZ + α - tocopherol treatments.
DISCUSSION
The roles of oxidative stress in the pathogenesis and progression of diseases have been documented by many authors (Introduction supra). In this study, the effects of two scavengers of oxidative stress (antioxidants / anti-stressors), ascorbic acid and α–tocopherol, in the amelioration of oxidative stress and methaemoglobinaemia induced with acetylphenylhydrazine (APHZ), were evaluated before and after stress induction, and after stress induction followed by treating the three erythrocyte types – control, G6PD–deficient and sickle red cells – with the antioxidants.

Fig 1 indicates that “normal” / control red cells do contain some MetHb even in the absence of oxidant challenge, ostensibly as part of normal metabolism and as a consequence of constant exposure of erythrocytes to a highly oxidising environment. Because the MetHb reductase system of the cells is usually effective (Pruijim & de-Meijer, 2002), their mean MetHb level did not change significantly (p > 0.05) even when they were challenged with APHZ. The same effective MetHb reductase system in synergy with the antioxidants should also account for the significant decreases (p < 0.05) in the MetHb levels of these cells when treated with ascorbic acid and α- tocopherol (Figs 3 &4).

When treated with APHZ, the mean MetHb level of the G6PD–deficient erythrocytes rose significantly (p < 0.05) to 0.67mgMetHb (Fig 2). The significant drops in this parameter when treated with ascorbic acid and α- tocopherol respectively, also indicated that their MetHb reductase system was effective. The difference between the post –APHZ + ascorbic acid and post –APHZ + α-tocopherol mean MetHb levels of these cells should therefore be a reflection of the differences in the efficiencies of the two antioxidants in mopping up the offending redox species generated by APHZ. Consequently, the mean MetHb level of these erythrocytes dropped to their pre-APHZ level (0.42 ± 0.03 mgMetHb) when treated with α- tocopherol. It is therefore inferable that either following, or co–administering an oxidant drug with an effective antioxidant, such as α- tocopherol, will forestall oxidative stress and, by extension, methaemoglobinaemia by maintaining haemoglobin in the relaxed conformation which binds and transports O$_2$ to avert tissue hypoxia. Methaemoglobin is known to arise if tyrosine replaces the proximal histidine of haemoglobin thereby stabilising its iron in the permanent ferric state (Fe$^{3+}$) which does not bind O$_2$ for transport to the tissues (Stryer, 2000).

Sickle erythrocytes had a higher baseline (pre-APHZ) mean MetHb level than those of the control and G6PD – deficient subjects (Fig 1). This was most probably due to a subsisting
high level of oxidant species which is characteristic of sickle cell disease (Fang et al., 2005), and which must have oxidised haemoglobin to MetHb. APHZ treatment also significantly (p < 0.05) scaled up the mean level of MetHb of those cells (Fig 2). The introduction of ascorbic acid and α-tocopherol post – APHZ significantly scaled down the mean MetHb level of the sickle erythrocytes. Although α-tocopherol was more effective, the reduction in their level of MetHb was generally less profound than those of the control and G6PD – deficient red cells. This could be accounted for, by either the production of an overwhelming level of reactive oxygen and / or nitrogen species following APHZ treatment, or a deficiency in their MetHb reductase system.

According to this study, there is a subsisting level of methaemoglobin in all red cells with or without oxidative challenge. The higher levels of methaemoglobin seen in the G6PD – deficient and sickle erythrocytes indicated that both defects – enzymopathy and haemoglobinopathy respectively – exacerbated the effect of APHZ on them. Furthermore, drug-induced oxidative stress and methaemoglobinaemia could be ameliorated with ascorbic acid and α- tocopherol, but the latter was more effective.

REFERENCES


