

VITAMIN ANTIOXIDANTS MAY PREVENT DRUG-INDUCED HAEMOLYSIS OF G6PD-DEFICIENT ERYTHROCYTES

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Summary

Objectives: The effects of oxidative stress-causing drugs on G6PD-deficient erythrocytes is a disturbing one. In most cases, it makes the choice of antimalarial drugs and antibiotics for the deficient patients a daunting one for the clinicians. We investigated the effects of vitamin antioxidants – Vitamins C and E, on the actions of a sulphonamide (co-trimoxazole) on G6PD-deficient human erythrocytes. This is to determine whether co-administration of these antioxidants and the sulphonamides can reduce or ameliorate their haemolytic effects on the G6PD-deficient erythrocytes.

Method: A total of 200 adult males aged between 18 and 40 years within Enugu urban of Enugu State of Nigeria were screened qualitatively for G6PD deficiency. The deficient erythrocytes were treated with different concentrations of co-trimoxazole, using non-deficient erythrocytes as control. Again, the deficient erythrocytes were treated with two different concentrations of vitamins C and E (separately and in combination) and the co-trimoxazole. The extent of haemolysis was assessed by the level of Heinz body formation.

Results: Our results showed that (1) haemolysis of G6PD-deficient erythrocytes increased with increasing concentration of co-trimoxazole and time of incubation, (2) at an optimum concentration of 50ug/ml, the oxidative haemolytic effect of co-trimoxazole was drastically reduced by vitamin C (2.0mg/ml and 2.5mg/ml) and

vitamin E (1.5mg/ml and 2.0mg/ml), (3) the combination of the two antioxidants had no synergistic effect on the action of co-trimoxazole and (4) the effects of these antioxidants reduced as time of incubation increased to 6 hours.

Conclusion: We concluded that vitamin antioxidants can reduce and may even prevent haemolytic effects of sulphonamides (and possibly other oxidative stress-causing drugs) on G6PD-deficient erythrocytes. However, it is not yet clear whether such action may affect the efficacy of the drugs.

Key words: Antioxidants, Sulphonamides, G6PD-deficiency, Haemolysis.

Glucose-6-phosphate dehydrogenase (G6PD) is an initiation and rate-limiting enzyme of the pentose phosphate pathway of glycolysis. It catalyses the first step in the pathway to produce NADPH – a reducing agent essential in biosynthetic pathways (1). It's inheritance is an X-linked recessive trait. The enzyme has many variants with varying activities, electrophoretic mobilities and other kinetic properties or characteristics. G6PD mediates the reversible transfer of hydrogen atom from β -glucose-6-phosphate to co-enzyme II – NADP to form 6-phosphogluconate and NADPH. The NADPH so produced is used for the sustenance of high cellular concentration of reduced glutathione (GSH) necessary for the maintenance of the integrity of red cell walls. Hence G6PD deficiency undermines the concentration of cellular GSH and therefore the integrity of red cell walls. G6PD deficiency is the most common metabolic disorder of the red blood cells which affects about 100 to 400 million people world-wide with higher frequency in the tropical and subtropical zones of the eastern hemisphere including Nigeria (1, 2). The basic disorder of G6PD deficiency is mild to severe haemolysis. Most G6PD-deficient individuals are asymptomatic; only the combination of G6PD deficiency and certain environmental factors produces the clinical manifestations (1). Haemolytic anemia due to G6PD deficiency therefore results from a hereditary, sex-linked enzyme defect and occurs mostly when the person is exposed to stress of infections and certain drugs. Since newly produced erythrocytes have normal G6PD activity (3), the haemolytic episodes due to G6PD deficiency is usually brief. This may be because of rapid response and subsequent erythropoiesis. Drugs that have been implicated in induction of

haemolysis of G6PD-deficient erythrocytes are mainly sulphonamide-containing antimalarials and antimicrobials, non-steroid anti-inflammatory drugs (NSAID) and quinines. Hypersensitivity to sulphonamide antimicrobials range in severity from relatively mild cutaneous manifestations like maculopapular rash and fever, to serious and potentially life threatening reactions like Stevens-Johnson Syndrome, toxic epidermal necrosis and anaphylaxis (4).

In response to a variety of pathophysiological conditions such as inflammation, immunological disorders, hypoxia, hyperoxia, drugs and alcohol metabolism, and even deficiency of some vitamins, a lot of reactive oxygen species (ROS) are generated (3). These ROS are highly active intermediates that carry unpaired electrons and readily interact with other molecules in human tissues resulting in oxidative destruction. Under normal circumstances, they are generated as a steady-state cellular event in respiring cells. However, uncontrolled production of these reactive molecules often lead to oxidative damage of cellular macromolecules like DNA, Proteins, Lipids etc. G6PD deficiency, like other hereditary disorders (e.g sickle cell anaemia, thalasaemia etc) has high potential for oxidative damage, probably due mainly to iron overload. The role of antioxidants is to protect cells from oxidation by the generated ROS and other free radicals (5). Common and more well known antioxidants such as vitamins C and E work by interrupting chain reactions that would otherwise result in oxidation of cells (6). We studied the possibility of using these common antioxidants (vitamin C and E) in preventing or ameliorating the oxidative reactions exerted by sulphonamide drug – cotrimoxazole, on G6PD-deficient human erythrocytes. Ethical clearance was obtained from the Ethical Clearance Committee of University of Nigeria Teaching Hospital Enugu – Nigeria, while additional consents were sought from the subjects.

Materials and Methods

Subjects and Samples: A total of 200 apparently healthy adults aged between 18 and 40 years whose consents were sought were recruited for the study. They were randomly selected from blood donors and undergraduate students of University of Nigeria Teaching Hospital and National Orthopedic Hospital, all in Enugu urban, Enugu State of Nigeria. 6.0ml of whole blood was collected from each subject into a sequestrene bottle and properly mixed. Screening for G6PD status was done within two (2) hours of collection of the sample. Each

G6PD-deficient blood sample was washed several times with physiological saline. The deficient samples were then pooled together and re-washed twice. A 12.5% cell suspension of the pooled sample was made with the same saline. This was aliquotized for easy use without contamination. Equal number of non-deficient blood samples were also washed, pooled together and constituted to serve as control.

Drug Preparation:

- (a) *Co-trimoxazole*: Three different concentrations of the antimicrobial was prepared from the commercially prepared suspension of 40mg/ml (ie 200mg/5ml Septrin by Wellcome Nigeria Ltd) to obtain a final blood concentrations of 40, 50 and 60ug/ml in the reaction volumes. This covers the Cmax of 50ug/ml of co-trimoxazole (7).
- (b) *Antioxidants*: Two concentrations of vitamins C and E each were prepared to get final blood concentrations of 2.0 and 2.5mg/ml for vitamin C and 1.5 and 2.0mg/ml for vitamin E in the reaction volumes. These also cover the peak plasma concentrations of 2.0mg/ml and 1.5mg/ml for vitamins C and E respectively (8, 9).

Analysis: G6PD screening was done using methylene blue reduction method as previously described (10), while heinz body formation was assessed by the method described by French et al (11). The different concentrations of co-trimoxazole were incubated at 37°C with G6PD-deficient red cells and concurrently with non-deficient red cells as control. The quantity of heinz body formed was measured after 1, 2, 4 and 6 hours of incubation. Again, the deficient red cells were incubated with two different concentrations of the individual antioxidant and the optimum concentration (50ug/ml) of the co-trimoxazole. Finally, the deficient red cells were incubated with mixed antioxidants and the co-trimoxazole. In each case, the heinz body formed was assessed at intervals as earlier mentioned.

Results

Out of the 200 blood samples collected, only 21 (10.5%) were G6PD deficient, giving a prevalence of 10.5% in Enugu urban. Table 1 shows effects of different concentrations of co-trimoxazole on both G6PD-deficient and non-deficient erythrocytes, represented by level of heinz body formed. The three drug concentrations (40ug/ml, 50ug/ml and 60ug/ml) showed significant increases (P=0.04; P=0.005

and $P=0.0001$ respectively) in their effects on deficient erythrocytes over their effects on non-deficient erythrocytes. It was also observed that with the exception of 40ug/ml concentration, others showed increasing haemolysis of the erythrocytes as the time of incubation increased. With 50ug/ml co-trimoxazole, the heinz body formation was directly related to the time of incubation while after 2 hours of incubation, 60ug/ml co-trimoxazole did not show further significant increase in heinz body formation. Inversely, there was no appreciable heinz body formation by non-deficient erythrocytes.

Table 1: Effect of different concentrations of cotrimoxazole (COT) on G6PD-deficient and non-deficient erythrocytes.

Conc.of COT (ug/ml)	Def. red cells Mean(SEM)	Non-def. Red cells Mean(SEM)	P – value
40	0.11 (0.030)	0.039 (0.004)	=0.0384
50	0.22 (0.036)	0.055 (0.010)	=0.0047
60	0.269 (0.014)	0.075 (0.013)	=0.0001

The effects of the antioxidants – vitamins C and E, against heinz body formation from deficient erythrocytes by 50ug/ml (Cmax) co-trimoxazole is shown in Table 2. At 2.0mg/ml and 2.5mg/ml vitamin C, the heinz body formation almost ceased. However, slight but non-significant haemolysis occurred after 6 hours of incubation when slight turbidity was noticed. Similar results were obtained with 1.5mg/ml and 2.0mg/ml vitamin E. Combination of vitamins C and E did not exert better effect than the individual vitamins.

Table 2: Effects of vitamin antioxidants on the extent of haemolysis of G6PD-deficient erythrocytes by COT.

Conc. of vits (mg/ml)	Redcells + COT + Vit	Red cells + COT	P – value
2.0mg/ml vit C	0.045 (0.011)		=0.0047
2.5mg/ml vit C	0.043 (0.015)		=0.0041
1.5mg/ml vit E	0.045 (0.010)	0.22 (0.036)	=0.0036
2.0mg/ml vit E	0.040 (0.012)		=0.0027
2.0mg/ml vit C+ 1.5mg/ml vit E	0.040 (0.013)		=0.0027

Discussion

A 10.5% prevalence of G6PD deficiency in Enugu urban correlates well with the previous studies in Nigeria and America [12,13] but differ significantly from 25% given as its prevalence in tropical Africa and parts of Middle East and Southeast Asia (1). When non-deficient erythrocytes were incubated with co-trimoxazole, there was no appreciable heinz body formation. However, as the concentration of the co-trimoxazole and the time of incubation were increased to 50ug/ml and 6 hours respectively, a slight turbidity occurred, indicating slight haemolysis. But this was significantly lower ($P < 0.05$) than the turbidity formed by the deficient erythrocytes after one hour of incubation with the same concentration of the drug. The extent of heinz body formation by the G6PD-deficient erythrocytes in the presence of co-trimoxazole indicates the level of haemolytic effect of the drug and this is caused by the sulphamethoxazole component of the drug. This is in line with an earlier study (2). With deficient

erythrocytes, the heinz body formed increased with time of incubation particularly with 50ug/ml and 60ug/ml drug concentrations. After 6 hours of incubation, the heinz body formed was about twice the value at 1 hour and this 6 hours is just half of the sulphomethoxazole's half-life in the serum (7), implying that more haemolysis is expected before the drug is finally cleared from the system. This supports the earlier finding that most of the reactions due to oxidative haemolysis of G6PD-deficient cells occur between 24 and 48 hours of administration of the drug (1). Fortunately, oxidative haemolytic episodes are usually brief and self-limiting due to the subsequent stimulation of erythropoiesis, and also due to the fact that newly produced red cells have normal G6PD activity and are relatively resistant to haemolysis (3, 14). It was also observed that haemolysis caused by 60ug/ml co-trimoxazole was relatively but not significantly higher ($P>0.05$) than that caused by 50ug/ml, but both concentrations individually exerted significantly higher ($P<0.05$) oxidation than 40ug/ml. This partly supports an earlier finding that severity of haemolysis caused by another drug – primaquine, was dependent on the dosage (14).

When treated with maximum serum concentrations (C_{max}) of vitamin C (2.0mg/ml) and vitamin E (1.5mg/ml), the oxidative haemolytic action of co-trimoxazole was brought under control. The level of heinz body formed in the presence of these vitamins were not significantly higher ($P>0.05$) than that formed with non-deficient erythrocytes. Vitamin E presumably protects the lipids in erythrocyte membrane from peroxidation – the actual cause of membrane destruction and haemolysis. Hence in animals, the effects of vitamin E deficiency include structural and functional abnormalities of many organs and organ systems (15). Therefore, co-administration of co-trimoxazole and vitamin E will not only protect the erythrocytes from peroxidation but will also prevent other biochemical defects that appear to involve fatty acid metabolism. The action of vitamin C may be seen from the angle of its ability to increase the activities of drug-metabolizing hepatic microsomal enzymes. In other words, the presence of vitamin C is expected to quicken the catabolism and clearance of drugs, probably reducing their half life. For this action alone, vitamin C (ascorbic acid) deficiency is suspected to enhance or prolong the pharmacological actions of some drugs (16), especially those that exert their pharmacological action before they are catabolized.. Its ability to inhibit heinz body formation (haemolysis) correlates with the previous study with another oxidative haemolytic

agent - acetylphenylhydrazine (17). Therefore, co-administration of vitamin C and sulphonamides will not only prevent oxidative haemolysis but will also modulate the pharmacological action of the drug by enhancing the metabolism of the drug through induction of microsomal enzymes. However, it is not known yet whether this modulation will adversely or beneficially affect the efficacy of the drug – COT. Of note is the finding that the two vitamins have no synergistic effect on the action of co-trimoxazole, hence the co-administration of the two with co-trimoxazole has no medical advantage. Fortunately, there is no known documented indication of any limit to which these vitamins can be taken for routine purposes. Though vitamin C is suspected to cause exacerbation of ulceration in prolonged administration (18), the therapeutic regimen is not expected to be too long to initiate such exacerbation.

We are of the opinion that vitamin antioxidants ameliorate and can prevent oxidative haemolysis of G6PD-deficient erythrocytes by sulphonamides and possibly any other drugs capable of causing haemolysis of G6PD-deficient erythrocytes. We therefore suggest that when use of these drugs are absolutely indicated in G6PD-deficient patients, they should be administered with either of these vitamin antioxidants. However, more work is necessary to establish whether the efficacy of the drugs are compromised in the presence of these vitamin antioxidants, particularly vitamin C.

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