ANTIOXIDANT – MEDIATED HEINZ BODIES LEVELS OF GLUCOSE – 6 – PHOSPHATE DEHYDROGENASE – DEFICIENT ERYTHROCYTES EXPOSED TO ACETYLPHENYLHYDRAZINE.

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ABSTRACT

The effects of two antioxidants, ascorbic acid and α-tocopherol, on the levels of Heinz bodies (Hzbs) induced with acetylphenylhydrazine (APHZ), a classical inducer of oxidative stress, were studied in glucose-6-phosphate dehydrogenase (G6PD)-deficient erythrocytes. In both the control and G6PD-deficient erythrocytes, no Hzbs were present pre-APHZ treatment but post – APHZ, the Hzbs levels rose significantly (p<0.05) to 2.40±0.05% and 62.50±2.99% in the control and G6PD-deficient red cells respectively. Upon treatment with ascorbic acid, their respective Hzbs levels were significantly reduced to 1.60±0.05% and 48.60±4.35%, while α-tocopherol was more effective as it reduced the post-APHZ Hzbs levels to 0.98±0.02% and 41.80±5.83% respectively. It was concluded that the red cells did not bear Hzbs pre- APHZ treatment most probably because of an efficient system of removal of those offending inclusions (Hzbs) in the absence of exogenous oxidant challenge, but upon...
exposure to APHZ, both erythrocyte types formed significant levels of Hzbs, although more in G6PD-deficient cells. Both ascorbic acid and α-tocopherol (antioxidants), tested in this study could reduce the post-APHZ Hzbs levels significantly but the latter was more effective. None of the two antioxidants tested could reduce the Hzbs of the erythrocytes to their pre-APHZ treatment levels in both the control and G6PD-deficient erythrocytes. In spite of this, it is advisable to co-administer, or follow, oxidant drugs with an antioxidant, especially α-tocopherol which, in this study, was found to be very effective in counteracting APHZ-induced Heinz bodies formation.

**KEYWORDS:** Antioxidants, Heinz bodies, acatylphenylhydrazine, glucose -6- phosphate dehydrogenase.

**INTRODUCTION**

Heinz bodies are inclusions of precipitated, oxidatively denatured haemoglobin (Howanitz & Howanitz, 1999; Walter & Talbot, 1990). They are continually formed in all people in the absence of drug/oxidant challenge, but are rapidly cleared by the spleen (Desnoyers et al., 2000). If removal from circulation is impaired, its presence becomes one of the principal causes of haemolysis in G6PD-deficient individuals, in unstable haemoglobin disease, and in the thalassaemias (Walter & Talbot, 1990).

Protein precipitation leading to Heinz bodies formation is effected by reactive oxygen species (ROS) - hydrogen peroxide ($\text{H}_2\text{O}_2$), singlet oxygen ($\text{O}_1$), and hydroxyl radical (OH) (Desnoyers et al., 2000). These ROS damage red cell haemoglobin (Hb) by oxidising its reactive sulphhydryl (SH) groups to form bonds that change the conformation of globin protein chains, resulting in the precipitation of Hb molecule (Tanford, 2000). This oxidative assault on Hb converts its ferrous ion ($\text{Fe}^{2+}$) to a permanent ferric state ($\text{Fe}^{3+}$) found in methaemoglobin (MetHb) which does not bind $\text{O}_2$ for transport to the tissues (Duncan et al., 2004). It is this MetHb that presents microscopically as Heinz bodies. Because Heinz-bodied red cells are less deformable on account of altered intracellular fluidity, there is increased entrapment in the narrow splenic sinusoids as blood is filtered through the spleen (Hasegawa et al., 1993), and this leads to intravascular haemolysis if they are produced in large numbers (Desnoyers et al., 2000).

In susceptible erythrocytes, such as G6PD-deficient ones, there is an accelerated breakdown of the enzyme protein even in the absence of oxidant drugs (Goldberg & Rock, 1992). The
resultant low level of NADPH in turn, precipitates the biochemical defects associated with G6PD deficiency: glutathione (GSH) deficiency and instability, diminished MetHb reduction, precipitation of protein globules (Heinz bodies formation), etc. (Mehta et al., 2000). However, the human body uses two lines of defence to counteract free-radical-mediated oxidative stress: a system of enzymes as the primary line of defence (e.g. glutathione peroxidise, superoxide dismutase and catalase), and antioxidants or free radical scavengers (e.g. vitamins A and E, GSH, carotenoids, ubiquinol, arginine, etc.) as the secondary defence mechanism (Bilgin-karabulut et al., 2001; van –Acker et al., 2000).

In this study, oxidative stress was induced in G6PD-deficient erythrocytes with 5mg of acetylphenylhydrazine (APHZ), a classical inducer of oxidative stress (Beutler et al., 1955), per ml of whole blood. Heinz bodies levels were monitored pre-APHZ, post-APHZ, post-APHZ + ascorbic acid, and post-APHZ + α-tocopherol.

**MATERIALS AND METHODS**

**Selection of Subjects/Volunteers:** The consents of the volunteers were obtained to collect 30 blood samples from G6PD-deficient and 30 from non-deficient (control) subjects. G6PD screening procedure was used to separate the deficient from the non-deficient (control) red cells. The anticoagulant used was EDTA.

**Procedure:** (Beutler et al., 1955). A buffered acetylphenylhydrazine (APHZ) solution was prepared by dissolving 100mg of APHZ in 100ml of 0.07M phosphate buffer (pH 7.60) containing 200mg of glucose per 100ml. Then, 0.1ml of whole gently agitated venous blood was added with a “blow-out” pipette to 2ml of the APHZ solution in a test tube of 12mm inner diameter. The suspension was mixed immediately and aerated two or three times by drawing about 0.1ml of it into a pipette and blowing it and a small quantity of air back into the suspension. The suspension was incubated at 37°C in a water bath and the mixing procedure repeated after 2 hours. After 4 hours, a drop of the suspension was placed on a cover-slip and inverted on a microscope slide that contained a drop of half-saturated crystal violet in 0.73% NaCl solution. An area in which the structure of the red cells was well preserved (least-destroyed) was selected for microscopic examination. One hundred cells were examined and classified as containing either FIVE OR MORE, or FOUR or LESS Heinz bodies. To assess the effect of ascorbic acid on the drug/APHZ-induced Heinz bodies formation, 0.08mg of ascorbic acid per ml of blood was added to the reaction mixture after adding the 0.1ml of blood to APHZ reaction mixture and the procedure continued. Similarly,
to assess the effect of α-tocopherol on APHZ - induced Heinz bodies formation, 0.01mg of α-tocopherol was added at that stage to a separate reaction mixture and the procedure continued. Between 45 – 92% of susceptible red cells should bear five or more Heinz bodies, while less than 30% of the non – susceptible ones contain this many Heinz bodies as part of normal metabolism.

RESULTS AND DISCUSSION
The means of Heinz bodies levels were subjected to a t-Test at p<0.05 to compare their differences.

Table 1: Mean Heinz bodies levels of control and G6PD-deficient erythrocytes pre-APHZ, post-APHZ, post-APHZ + ascorbic acid, and post – APHZ + α- tocopherol treatments.

<table>
<thead>
<tr>
<th>Erythrocyte type</th>
<th>Pre- APHZ (%)</th>
<th>Post – APHZ (%)</th>
<th>Post – APHZ + Ascorbic acid (%)</th>
<th>Post – APHZ + α-tocopherol (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>2.40 ± 0.05</td>
<td>1.60 ± 0.05</td>
<td>0.98 ± 0.02</td>
</tr>
<tr>
<td>G6PD - deficient</td>
<td>0</td>
<td>62.5 ± 2.99</td>
<td>48.60 ± 4.35</td>
<td>41.48 ± 5.83</td>
</tr>
</tbody>
</table>

It is known (Desnoyers et al., 2000) that ROS- induced oxidative damage to haemoglobin produces methaemoglobin (MetHb) that presents microscopically as Heinz bodies (Hzbs) but are rapidly cleared from the circulation by the spleen. This should account, at least in part, for the zero – presence of Heinz bodies in both the control and G6PD- deficient erythrocytes before APHZ treatment (Table 1). In other words, although MetHb might have been produced in those erythrocytes before APHZ treatment, it was either not enough to cause Hbzs formation or, according to Giger et al., (2000), the Hbzs- bearing erythrocytes were promptly cleared as they filtered through the splenic sinusoids. Upon exposure to the oxidant stressor (APHZ), there was a less drastic elevation in the mean Hbzs level of the control than the G6PD-deficient erythrocytes (0→2.40 %vs 0 →62.50). The significant drops in the mean Hbzs levels of the control red cells from 2.40% post-APHZ to 1.60% and 0.98% when treated with ascorbic acid and α-tocopherol respectively, could be attributed to the existence in these cells, oan efficient system for counteracting the effects of ROS, which includes elevated levels of antioxidants (GSH, ascorbic acid, α – tocopherol, etc., ). MetHb reductase activity, or macrophage – mediated splenic destruction and phagocytosis (Duncan et al., 2004). The presence of these anti-ROS mechanisms explains why there is usually no anaemia in non-G6PD – deficient subjects (or control in this study) when placed on oxidant therapeutic agents because the rate of formation, size, number of Hzbs, and concurrent membrane
damage do not outstrip the rate of removal from circulation (Desnoyers et al., 2000). APHZ treatment of the G6PD-deficient erythrocytes also caused a significant (p<0.05) elevation of their mean Hbzs level. From the foregoing, it is reasonable to infer that this drastic elevation of the mean Hbzs level of these cells was due to a defective ROS-countering system in these erythrocytes with a concomitant overwhelming levels of the offending oxidant species. Although both ascorbic acid and α-tocopherol treatments significantly lowered the post-APHZ mean Hbzs contents of the cells, they could not be restored to their pre-APHZ treatment levels. α-Tocopherol was however more effective in countering the effect of APHZ in inducing Hbzs in the red cells studied. Defective ROS-countering system could therefore explain the massive haemolysis that occurs in G6PD-deficient subjects placed on oxidant antimalarials (Mehta et al., 2000).

It can be concluded, from the results of this study, that, oxidant drugs including APHZ, induce significant Hbzs formation in both control (“normal”) and G6PD-deficient erythrocytes. It was also found that ascorbic acid and α-tocopherol, both antioxidants, could significantly (p<0.05) reduce the levels of Hbzs induced in the erythrocytes by such oxidant drugs, and that, although both were effective in ameliorating this effect, α-tocopherol was more effective. Based on these findings, it is hereby advised that oxidant drugs be co-administered or followed with antioxidants, especially α-tocopherol which was found to be more effective in this study.

REFERENCES


