Protective Role Of α-Tocopherol And Ascorbic Acid Supplementation On Halofantrine - Induced Hepatotoxicity In Rats

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Abstract
In this study, the antioxidative potential of α-tocopherol and ascorbic acid was assessed in halofantrine-induced hepatotoxic rats. On administration of halofantrine (60mg/kg/day) for 14 days, the activities of serum hepatospecific makers like aspartate transaminase, alanine transaminase, alkaline phosphatase and the level of bilirubin were significantly increased with significant increase in plasma and liver lipid peroxidation marker (malondialdehyde, MDA). The levels of non-enzymic antioxidants; superoxide dismutase (SOD) and catalase (CAT) were also decreased in halofantrine-induced hepatotoxic rats. On administration of vitamin C (20mg/kg/day) vitamin E. (0.6mg/kg/day) and combined vitamin C and E for 14 days in halofantrine-administered rats, the activities of the serum hepatospecific markers significantly decreased. In addition the non-enzymic and enzymic antioxidants increased on treatment with vitamin C, E and (C and E). On the basis of our results we conclude that halofantrine can induce hepatocellular and oxidative damage in rats and that supplementation of vitamin C and E are not only useful in controlling hepatocellular damage but are also useful in controlling the lipid peroxide levels and strengthening the antioxidant potential in this halofantrine-induced hepatotoxic rats.

INTRODUCTION
Malaria is one of the most common parasitic diseases causing morbidity or mortality in the tropic. It is caused by parasites that belong to genus plasmodium and it spends part of its life cycle in the red blood cells. Digestion of the host haemoglobin during maturation of the parasites within the red cell followed by the lyses of such red cells result in the release of reactive oxygen species (ROS) and iron from the haem moiety of the haemoglobin increasing oxidant stress with the cell and thus compromising the integrity of the cell (Huy et al, 2003, George and Nmoka, 2003). Also malaria infection would activate phagocytes to produce H₂O₂ and O₂ radicals as one of the mechanisms to destroy invading bacteria (Halliwel, 1997). All these factors make the red cells of individuals infected with malaria parasites more susceptible to oxidative stress, lysis, and consequently causing anaemia in such individuals (Mohan et al, 1994).

In addition, halofantrine, a lipophilic phenanthrene methanol belonging to aryl-amino alcohol and used in the treatment of acute uncomplicated multi-drug resistant malaria (Philips-Howard and wood, 1996). It has been found to act directly or indirectly and alters antioxidant status. This reduced antioxidant status makes certain organs more susceptible to oxidative stress. Several studies have shown that halofantrine causes increased lipid peroxidation and decreased enzymic and non-enzymic antioxidants (Gibson and Skett, 1992, Murphy et al, 1983).

The potential of dietary antioxidant to detoxify the H₂O₂ and O₂ radicals produced during malaria infection and more so during antimalaria treatment has received increasing attention in recent years (Murugavel and Pari, 2004; Huy et al, 2003; Mohan et al 1994). The nutrients that have shown promise as protective antioxidants are lipid-soluble antioxidants such as vitamins E, A, β-carotene, γ-carotene and lycopene in humans because of their association with membrane lipids (Hortons and Fairhurst, 1987; Metzger et al, 2001). Vitamin E acts as the most potent lipid soluble antioxidant. Vitamin C another nutrient, serves directly as an antioxidant by scavenging aqueous peroxyl radicals and indirectly by regenerating reduced Vitamin E (Frel, 1991).

Pari and Amali (2005) have reported the protective role of tetrahydrocurcumin (HTC) an active principle of turmeric on chloroquine-induced hepatotoxicity in rats. Also George and Nmoka (2003) have reported the effects of vitamin C and E
supplementation in the treatment of malarial infection. However, there is paucity of information regarding the ability of these vitamins (C and E), whether ingested alone or in combination, to protect against halofantrine-induced oxidative stress. It is therefore, the purpose of this work to examine the protective role of vitamins C and E on halofantrine-induced hepatotoxicity in rats.

MATERIALS AND METHODS

ANIMALS

Thirty wistar rats bred in the Central Animal House of College of Medicine and Health Sciences, Imo State University, Owerri, Nigeria were used in this study. They were maintained at a temperature range of 25oC to 30oC and a 12h light 12h dark. They were fed with commercial growers mash products of Tops Feeds Ltd, Sapele, Nigeria. Water and feed were provided ad libitum, this continues until the rats weighed between 200-300g.

DRUGS

Halofantrine (HAL FAN) (100 mg/5ml Suspension) used in this study was the product of SmithKline and French Laboratories, Nanterre, Cedex, France. Vitamin C (100 mg/5ml suspension) used in this study was the product of Emzor Pharmaceutical Industries Limited, Lagos, Nigeria, while Vitamin E (30 mg/ml injection solution of D-L-α-tocopherol was the product of Chemical Works of Gideon Richer Ltd. Budapest, Hungary. All the drugs were purchased from a standard pharmacy shop in Owerri. The drugs were administered orally using oral cannula.

EXPERIMENTAL DESIGNS

Animals were randomly assigned to groups (n=6x4 groups) each with similar body weights.

- Group I(control): Animals in this group received only distilled water
- Group II: Animals in this group were given 60mg/kg/day of the halofantrine suspension orally.
- Group III: Animals in the group in addition to 60mg/kg/day of halofantrine suspension were given 20mg/kg/day of vitamin C.
- Group IV: Animals in the group in addition to 60mg/kg/day of halofantrine suspension were given 0.6 mg/kg/day of vitamin E.
- Group V: Animals in this group in addition to 60 mg/kg/day of halofantrine suspension received 20mg/kg/day of vitamin C and 0.6 mg/kg/day of vitamin E.

The drugs were administered orally for a period of 14 days. All the animals were allowed free access to food and water till the end of the experiment.

BLOOD SAMPLE COLLECTION

Twenty four hours after the last doses were administered the animals were weighed and then anaesthetized with chloroform vapour, quickly brought out of the jar and sacrificed. Whole blood was collected by cardiac puncture from each animal into two different clean dry centrifuge tubes, i.e. one with anticoagulant, for plasma separation and another without anticoagulant to separate serum. The blood without anticoagulant were allowed to stand for about 30minutes to clot, and further centrifuged at 3500 rpm for 5 minutes using Wisperfuge model 1384 centrifuge (Samson, Holland). Serum was separated from clot with Pasteur pipette into sterile serum sample tubes for the measurement of biochemical parameters. The liver from both control and test animals were dissected out. Washed in ice-cold saline, blotted dry, and weighed. Then homogenate was prepared in phosphate buffer 0.1M, pH 7.4 and used for the biochemical analysis.

BIOCHEMICAL ANALYSIS

SERUM LIVER FUNCTION MARKERS

Serum total bilirubin level was estimated based on Van den Berg reaction (Malloy and Everlyn, 1937). In this method, diazotised sulphonic acid (0.5ml) reacts with bilirubin in diluted serum (0.2ml serum + 1.8ml distilled water) and forms purple coloured azobilirubin, which was measured at 540 nm. Activities of serum aspartate transaminase (AST) and Alanine transaminase (ALT) were assayed by the method of Reitman and Frankel (1957). 0.2ml of serum with 1ml of substrate (aspartate and α-ketoglutarate for AST, alanine and α-ketoglutarate for ALT, in phosphate buffer pH 7.4) was incubated for an hour in case of AST and 30 minutes for ALT. 1ml of DNPH solution was added to arrest the reaction and kept for 20 minutes in room temperature. After incubation 1ml of 0.4N NaOH was added and absorbance was read at 540 nm. Activities expressed as IU/L. Based on the method of King and Armstrong (1934) alkaline phosphates activities (ALP) was assayed using disodium phenyl phosphate as substrate. The colour,
developed read at 680 nm after 10 min activities of ALP expressed as IU/L.

**LIPID PEROXIDATION MARKER**

Lipid peroxidation in plasma and liver was estimated colorimetrically by measuring malondialdehyde (MDA) by a thiobarbituric acid assay procedure (Albro et al, 1986). In brief, 0.1 ml of plasma/liver homogenate was treated with 2 ml of (1:1:1 ratio) TBA-TCA-HCl reagent (TBA, 0.37%, 0.25N HCl and 15% TCA) and placed in water bath for 15 minutes, cooled and centrifuged and then clear supernatant was measured at 535 nm against blank. MDA was calibrated using 1,1,3,3- tetraethoxypropane (Sigma Chemical, St Louis, MO) as a standard. Results were expressed as nanomoles of MDA per millimetre of plasma/liver homogenate.

**ANTIOXIDANT STATUS**

Plasma/liver homogenate vitamin C (ascorbic acid) was assayed by Omaye et al. (1979) method. To 0.5 ml of plasma/liver homogenate 1.5 ml of 6% TCA was added and centrifuged at 3500 g for 20 minutes using Wisperfuge model 1384 centrifuge (Samson Holland). To 5 ml of supernatant, 0.5 ml of DNPH reagent (2% DNPH and 4% thiourea in 9N Sulphuric acid) was added and incubated for 3 hours at room temperature. After incubation 2.5 ml of 85% sulphuric acid was added and colour developed was read at 530 nm after 30 minutes.

Plasma/liver homogenate vitamin E (α-tocopherol) was determined by the method of Desai (1984). Vitamin E was extracted from plasma/liver homogenate by addition of 1.6 ml ethanol and 2.0 ml petroleum ether to 0.5 ml plasma/liver homogenate and centrifuged. The supernatant was separated and evaporated. To the residue, 0.2 ml of 0.2% 2,2-dipyridyl, 0.2 ml of 0.5% ferric chloride was added and kept in dark for 5 minutes. An intense red coloured layer obtained on addition of 4 ml butanol was read at 520 nm.

Catalase (CAT) was assayed colorimetrically as described by Sinha (1972) using diachromate-acetic acid reagent (5% potassium dichromate and glacial acetic acid were mixed in 1:3 ratio). The intensity was measured at 620 nm and the amount of hydrogen peroxide hydrolysed was calculated for the catalase activity.

Superoxide dismutase (SOD) activity was determined by the modified method of NADH- Phenazinemethosulphate-nitroblue tetrazolium formazan inhibition reaction spectrophotometrically at 560 nm (Kakkar et al, 1984). A single unit of enzyme was expressed as 50% inhibition of NBT (Nitroblue tetrazolium) reduction/min/mg protein.

**STATISTICAL ANALYSIS**

All values were expressed as mean ± S.D. The statistical analysis was carried out using one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT) (Duncan, 1957). Test with P<0.05 were considered significantly different.

**RESULT**

The body weight changes and the relative liver weights in both normal and experimental animals are shown in table 1. There was a slight gain in body weight of animals in group I, III, IV and V whereas the group II showed a slight loss of weight. There was a significant increase in the relative weight of the liver in group II when compared with the group I (control) which significantly reversed to near normal in group III, IV and V (Table I).

Table 1: The mean values of body weight change and mean relative weight of the liver in normal and experimental animals.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Mean initial weight (g)</th>
<th>Mean final weight (g)</th>
<th>Mean weight change (g)</th>
<th>Relative liver weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>148.01 ± 4.2</td>
<td>152.32 ± 5.0</td>
<td>4.27 ± 0.30</td>
<td>4.85 ± 0.31</td>
</tr>
<tr>
<td>II</td>
<td>154.52 ± 4.4</td>
<td>151.93 ± 6.02</td>
<td>-2.59 ± 0.61</td>
<td>7.55 ± 0.50</td>
</tr>
<tr>
<td>III</td>
<td>150.08 ± 6.23</td>
<td>152.82 ± 5.22</td>
<td>2.74 ± 0.44</td>
<td>5.41 ± 0.22</td>
</tr>
<tr>
<td>IV</td>
<td>147.46 ± 3.88</td>
<td>150.23 ± 4.68</td>
<td>2.77 ± 0.25</td>
<td>5.46 ± 0.42</td>
</tr>
<tr>
<td>V</td>
<td>149.83 ± 4.25</td>
<td>151.32 ± 4.24</td>
<td>1.44 ± 0.19</td>
<td>5.62 ± 0.38</td>
</tr>
</tbody>
</table>

*Significantly different from control (p < 0.05)

Table 2 showed the changes in the concentration of serum total bilirubin and in the activities of AST, ALT, and ALP in both normal and experimental animals. The significantly increase activities of AST, ALT, ALP and the concentration of bilirubin due to halofantrine treatment (group II) were significantly decreased (P<0.05) on treatment with vitamin C (group III), vitamin E (Group IV) and combined treatment of vitamin C and E (Group V).
The observed increase in the relative weight of the liver in the halofantrine treated group indicates that the drug might have toxic effect on this organ. It has been reported that increase or decrease in either absolute or relative weight of an organ after administering of a chemical or drugs is an indication of the toxic effect of that chemical (Simons et al, 1995; Orisakwe et al, 2003). Upon administration of vitamin C, E, or (C and E) to halofantrine treated rats, the relative weights of the livers reversed to near normal.

The activities of AST, ALT, ALP and the level of bilirubin increased significantly in halofantrine treated group. Liver enzymes are usually raised in acute hepatotoxicity, but tend to decrease with prolonged intoxication due to damage to the liver (Cornelius, 1979). This observation was confirmed by Obi et al, (2004). Other antimalarials such as chloroquine (Pari and Amali, 2005), amodiaquine (Farombi et al, 2000) and quinine (Debra and Megan, 1999) were also reported to induce hepatic damage. Vitamin C, E, (C and E) treatment significantly decreased the activities AST, ALT, ALP and the level of bilirubin in serum of halofantrine treated rats. Thus suggesting that they offer protection by preserving the structural integrity of hepatocellular membrane against halofantrine. This finding could be correlated with previous works, which reported that treatment with tetrahydrocurcumin (an antioxidative substance, which is derived from curcumin, the component of turmeric) significantly reduced the activities of serum hepatotoxicity markers in chloroquine-induced hepatotoxicity (Pari and Amali, 2005) and erythromycin-induced hepatotoxicity (Pari, 2004).

The observed significant high levels of plasma and liver MDA concentrations (end product of lipid peroxidation) and decreased level of some antioxidants like vitamin C, E, SOD and CAT in rats treated with halofantrine reflected the lipid peroxidation as a consequence of oxidative stress. The measurement of lipid peroxidation is a convenient method to monitor oxidative damage (Viani et al, 1991). In addition, non-enzymic antioxidants such as vitamin C and E play excellent role in protecting the cells from oxidative damage (Nwanjo and Ojiako, 2005). It has been shown that vitamin E (α-tocopherol) scavenges peroxyl radicals quicker than vitamin C (ascorbic acid); the α-tocopheroxyl radical derived from tocopherol is reduced back to regenerate α-tocopherol by ascorbic acid (Packer, 1992; Nwanjo and Ojiako, 2005). The enzymic antioxidant defence systems (SOD and CAT) are the natural protector against lipid peroxidation. SOD and CAT are important scavengers of superoxide ion and hydrogen peroxide. These enzymes prevent generation of
hydroxyl radical and protect the cellular constituents from oxidative damage (Ugochukwu et al, 2003). This observed decrease in antioxidants also suggested increased lipid peroxidation.

The decreased level of plasma and liver MDA and increase antioxidants (vitamin C, E, SOD and CAT) in Vitamin C, E, and (C and E) supplemented rats may result from the scavenging of the free radicals generated by halofantrine induced peroxidation by these antioxidant vitamins thereby reducing the utilization of these antioxidant enzymes to reduce the halofantrine induced oxidative stress. It also increases the antioxidant vitamins in these rats.

A number of conclusions are clearly evident from this work. The first is that vitamin C and E exert significant protection against halofantrine-induced toxicity by its ability to ameliorate the lipid peroxidation through their free radicals scavenging ability, which enhanced the levels of antioxidant defense system. This study also showed that combined supplementation of vitamin C and E has greater effect than when taken separately, in halofantrine-induced lipid peroxidation. Such a beneficial combination may therefore be recommended to patients taking antimalarial drugs like halofantrine or other drugs that generate oxidative stress.

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