Vitamin C attenuates copper-induced oxidative damage in broiler chickens

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This study was conducted to evaluate the protective effects of vitamin C on copper-induced oxidative damage in the erythrocyte and liver of broiler chickens. Three week old birds were fed a basal diet (n = 40), or basal diet supplemented with 250 mg CuSO₄/kg diet (n = 40) for 56 days. On the 57th day, the birds of the two groups were further subdivided and fed for 14 days as follows: Group 1A (Control, n = 20), fed with basal diet only. Group 1B (Vitamin C, n = 20), fed with basal diet and 100 mg/kg diet vitamin C. Group 2A (CuSO₄, n = 20), fed with basal diet and 250 mg CuSO₄/kg diet. Group 2B (CuSO₄ + Vitamin C, n = 20), fed with basal diet, 250 mg CuSO₄/kg diet and 100 mg/kg diet vitamin C. Copper supplementation for eight weeks caused oxidative damage as evidenced by a significant (p < 0.05) increase in copper level and lipid peroxidation as well as decreased antioxidant enzymes [superoxide dismutase (SOD) and catalase (CAT)] activities and reduced glutathione (GSH) level. Vitamin C supplementation for 14 days resulted in a significant (p < 0.05) reduction in copper level in exposed birds. Erythrocyte and liver lipid peroxidation were reduced by 48 and 52%, respectively. SOD and CAT activities also increased significantly (p < 0.05), and the decreased level of GSH that was observed in the copper exposed birds was reversed. Our findings indicate that vitamin C may be beneficial in preventing copper-induced oxidative damage in poultry, and shows potential for veterinary use.

Key words: Antioxidant systems, broiler chickens, catalase, copper, glutathione, oxidative damage, superoxide dismutase, vitamin C.

INTRODUCTION

Copper (Cu) is an essential trace element found in small amounts in a variety of cells and tissues with the highest concentrations in the liver (Turnlund, 1998). It functions as a cofactor and is required for structural and catalytic properties of a variety of important enzymes, including cytochrome c oxidase, tyrosinase, p-hydroxyphenyl pyruvate hydrodrolase, dopamine beta hydroxylase, lysyl oxidase and Cu-Zn superoxide dismutase (Cu, Zn-SOD) (Gaetke and Chow, 2003; Turnlund, 1999; Uauy et al., 1998). These enzymes are involved in an array of biological processes required for growth, development and maintenance (Turnlund, 1999).

The nutritional requirement of copper in broiler is about 4 to 5 mg/kg (National Research Council, USA, 1994; Franchini and Bertuzzi, 1991). However, the need to achieve a reduced cholesterol content and decreased lipid percentage in meat and meat products has led to the supplementation of poultry feed with copper above the nutritional requirement. Koh et al. (1996) reported an improved broiler performance, increased animal growth and improved food efficiency as a result of pharmacological supplementation of copper in feed. Also, copper supplementation (250 mg/kg) to broiler diets has been reported to decrease breast muscle and plasma cholesterol concentrations (Pesti and Bakalli, 1996; Bakalli et al., 1995). Research evidences have shown that copper regulates cholesterol biosynthesis by reducing hepatic glutathione concentration (Kim et al., 1992). Depletion of glutathione from cellular pool (either as a result of a disease condition or an experimental administration of thiol inhibitors), has been shown to render cells and living...
Table 1. Composition of the basal diet.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maize (%)</td>
<td>44.00</td>
</tr>
<tr>
<td>Soya bean full fat (%)</td>
<td>36.00</td>
</tr>
<tr>
<td>Rice husk (%)</td>
<td>12.33</td>
</tr>
<tr>
<td>Palm oil (%)</td>
<td>4.00</td>
</tr>
<tr>
<td>Bone meal (%)</td>
<td>2.50</td>
</tr>
<tr>
<td>Oyster shell (%)</td>
<td>0.50</td>
</tr>
<tr>
<td>Vitamin/mineral premix</td>
<td>0.25</td>
</tr>
<tr>
<td>Salt (NaCl) (%)</td>
<td>0.25</td>
</tr>
<tr>
<td>Methionine (%)</td>
<td>0.17</td>
</tr>
</tbody>
</table>

**Determined analysis**

<p>| | |</p>
<table>
<thead>
<tr>
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</thead>
<tbody>
<tr>
<td>Crude protein (%)</td>
<td>19.90</td>
</tr>
<tr>
<td>Fat (%)</td>
<td>11.00</td>
</tr>
<tr>
<td>Ash (%)</td>
<td>14.31</td>
</tr>
<tr>
<td>Fibre (%)</td>
<td>4.78</td>
</tr>
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</table>

**Calculated analysis**

<p>| | |</p>
<table>
<thead>
<tr>
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<tbody>
<tr>
<td>Energy (ME) (Kcal/kg)</td>
<td>3203.76</td>
</tr>
<tr>
<td>Energy/protein ratio</td>
<td>160.20</td>
</tr>
<tr>
<td>Methionine (%)</td>
<td>0.45</td>
</tr>
<tr>
<td>Calcium (%)</td>
<td>1.33</td>
</tr>
<tr>
<td>Phosphorus (%)</td>
<td>0.69</td>
</tr>
<tr>
<td>Copper (mg/kg)</td>
<td>5.90</td>
</tr>
</tbody>
</table>

organisms more susceptible to the effects of oxidants (Rahman et al., 1999). Therefore, glutathione is important in conferring protection and preserving the integrity of living organisms (Suntres, 2002; Rahman et al., 1999; Anderson and Luo, 1998; Anderson, 1997).

Although, copper is a major essential element, the serious toxic effects of this metal have been reported when it is overloaded in rats (Toplan et al., 2005; Zhang et al., 2000). One of the best known consequences of excess copper is the peroxidative damage to membrane lipids (Ferretti et al., 2004). Lipid peroxidation occurs by the reaction of lipid radicals and oxygen to form peroxo radicals. Lipid peroxo radicals may damage cells by changing the cell membrane’s fluidity and permeability or by directly attacking DNA and other intracellular molecules such as proteins (Mattie and Freedman, 2001). In our previous study (Ajuwon et al., 2009), we have demonstrated that dietary copper supplementation (250 mg/kg diet) induces oxidative damage by increasing lipid peroxidation and reducing concentration of superoxide dismutase (SOD), catalase (CAT) and glutathione (GSH) in both erythrocyte and the liver of broiler chickens.

The potential role of oxidative stress in the injury associated with copper poisoning suggests that antioxidants may enhance the efficacy of treatment protocols designed to mitigate copper-induced toxicity in poultry. Recently, various reports have suggested the protective role of antioxidant vitamins against toxic injury. Vitamin C (Kadirvel et al., 2007; Farombi and Onyema, 2006; Ademuyiwa et al., 2005, 1994; Patra et al., 2001; Vismara et al., 2001; Kang et al., 1998) and Vitamin E (Kadirvel et al., 2007; Kalendar et al., 2005; Patra et al., 2001; Hsu et al., 1998; Omara and Blakley, 1993; Ademuyiwa et al., 1990) have been reported to protect against toxic injury from xenobiotics and those resulting from metal as well. For these reasons, we evaluated the copper burden, status of lipid peroxidation and antioxidant systems in the erythrocyte and liver of broiler chickens whose diet were supplemented with copper. We also tested the hypothesis, whether vitamin C, as an antioxidant vitamin, would attenuate copper effects on the aforementioned biochemical parameters.

**MATERIALS AND METHODS**

**Animals and experimental design**

All the animals received humane care and the principles outlined in the Helsinki declaration were adhered to strictly. Eighty unsexed broiler chickens (Anak 2000), three weeks of age were purchased from the UNAAB-LEVENTIS Agro-Allied Industry, Abeokuta, Nigeria. The birds were kept in standard battery cages with automatic nipple drinkers and standard feeding trough. Feed and water were given ad libitum. The birds were divided into two groups of 40 birds each. Group 1 served as the control and was fed on a basal diet purchased from UNAAB-LFN Agro-Allied Industry. Group 2, the treatment group, were fed on the same basal diet supplemented with 250 mg CuSO$_4$/kg diet. This feeding protocol continued for 56 days. On the 57th day, the birds in the two groups were further subdivided and fed for 14 days as follows: Group 1A (control group) - fed with the basal diet only (n = 20); Group 1B (vitamin C group) - fed with the basal diet + vitamin C (100 mg/kg diet) (n = 20); Group 2A (copper group) - fed with the basal diet + CuSO$_4$ (250 mg/kg diet) (n = 20); and Group 2B (copper + vitamin C group) - fed with the basal diet + CuSO$_4$ (250 mg/kg diet) + vitamin C (100 mg/kg diet) (n = 20).

The basal diet was formulated following the procedure of Idowu et al. (2003), and its composition is shown in Table 1.

**Sample preparation**

At the end of the feeding period, birds in the four groups were starved overnight for 12 h. Exactly 10 ml of blood was drawn from the wing vein of each bird into heparinized tubes and 2 ml aliquot of blood sample was transferred into another set of tubes for copper content determination. The remaining blood was centrifuged at 3000 rpm for 5 min; supernatants were discarded and the erythrocytes were washed thrice using 0.9% NaCl solution. The birds were sacrificed and liver was excised, and washed with ice-cold 0.9% NaCl solution to remove residual blood. Half of the liver was homogenized in ice-cold 50 mM sodium phosphate buffer and 0.1 mM Na$_2$EDTA (pH 7.8). The soluble fraction was prepared by centrifugation at 4000 rpm for 10 min. The remaining half was stored frozen for copper content determination.

**Copper content determination**

Exactly 1 ml of blood was digested with 10 ml concentrated nitric acid and digests were brought to a constant volume of 25 ml with deionised water (Ademuyiwa, 1995). For liver, 1 g of sample was dried to a constant weight at 85°C. Dried samples were cold digested in 2 ml of nitric acid overnight. They were then hot digested on a block digester at 120°C until all the organic matter was
described by Clairborne (1986), in which the disappearance of H activity of catalase was determined by following the method (MDA) as standard by the method of Beuge and Aust (1978). The bituric acid reactive substances (TBARS), using malondialdehyde (MDA).

Measurement of indices of oxidative stress

The extent of lipid peroxidation was estimated in terms of thiobarbituric acid reactive substances (TBARS), using malondialdehyde (MDA) as standard by the method of Beuge and Aust (1978). The activity of catalase was determined by following the method described by Clairborne (1986), in which the disappearance of H2O2 was monitored spectrophotometrically at 240 nm. Superoxide dismutase was assayed according to the procedure of Das et al. (2000). Glutathione concentration was determined in samples according to the method of Boyne and Ellman (1972). Protein content was determined according to the method of Bradford (1976) using bovine serum albumin as standard. All chemicals used in the enzymatic activity were of analytical purity and were obtained from Sigma Chemical, Germany.

Statistical analysis

The values were presented as mean ± standard deviation (SD). Differences between group means were estimated using one-way analysis of variance (ANOVA) followed by Duncan test for multiple comparisons. Results were considered statistically significant when "p < 0.05". All the statistics were performed using SAS (The SAS system for windows, v8; SAS Institute Inc., Cary, NC).

RESULTS

Tables 2 and 3 illustrate the effects of vitamin C on copper level, lipid peroxidation, activities of SOD, CAT as well as concentrations of GSH in all groups in both erythrocyte and liver, respectively.

The mean copper level in the blood of copper exposed birds was 1.77 times higher than that of control birds. Similarly, in the liver, there was a 1.95 fold increase in copper level of exposed birds compared with control group. Two weeks vitamin C supplementation brought about a significant reduction (p < 0.05) in the copper level, both in the blood and liver of exposed birds. A 35% reduction in the blood and a 30% reduction in the liver were observed.

Lipid peroxidation as determined by MDA formation, amounted to 48% in both tissues, which amounted to 48% in the erythrocyte and 52% in the liver (Tables 2 and 3).

Table 2. Effects of vitamin C (100 mg/kg diet) on blood copper levels, erythrocyte lipid peroxidation, SOD, CAT and GSH concentrations of broilers exposed to copper.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control (n = 20)</th>
<th>CuSO4 (n = 20)</th>
<th>Vitamin C (n = 20)</th>
<th>Cu + Vitamin C (n = 20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Copper burden (µg/ml)</td>
<td>2.75 ± 0.45</td>
<td>4.88 ± 0.69</td>
<td>2.62 ± 0.37</td>
<td>3.18 ± 0.22</td>
</tr>
<tr>
<td>Lipid peroxidation (µmol MDA/g Hb)</td>
<td>0.37 ± 0.09</td>
<td>1.26 ± 0.15</td>
<td>0.28 ± 0.04</td>
<td>0.65 ± 0.07</td>
</tr>
<tr>
<td>GSH (mmol/mg protein)</td>
<td>1.07 ± 0.13</td>
<td>0.41 ± 0.04</td>
<td>1.10 ± 0.30</td>
<td>0.84 ± 0.33</td>
</tr>
<tr>
<td>SOD (Unit/g Hb)</td>
<td>1.96 ± 0.08</td>
<td>1.02 ± 0.11</td>
<td>1.89 ± 0.05</td>
<td>1.46 ± 0.08</td>
</tr>
<tr>
<td>CAT (Units/mg Hb)</td>
<td>4.12 ± 0.43</td>
<td>2.14 ± 0.30</td>
<td>3.96 ± 0.12</td>
<td>2.71 ± 0.24</td>
</tr>
</tbody>
</table>

Values in the same row with different superscript are significantly different at p < 0.05. MDA, Malondialdehyde; SOD, superoxide dismutase; CAT, catalase; GSH, glutathione.

Table 3. Effects of vitamin C (100 mg/kg diet) on liver copper levels, lipid peroxidation, SOD, CAT and GSH concentrations of broilers exposed to copper.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control (n = 20)</th>
<th>CuSO4 (n = 20)</th>
<th>Vitamin C (n = 20)</th>
<th>Cu + Vitamin C (n = 20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Copper burden (µg/g tissue)</td>
<td>4.56 ± 0.67</td>
<td>8.91 ± 0.35</td>
<td>5.13 ± 0.33</td>
<td>6.26 ± 0.39</td>
</tr>
<tr>
<td>Lipid peroxidation (µmol MDA/g tissue)</td>
<td>0.30 ± 0.03</td>
<td>0.58 ± 0.08</td>
<td>0.27 ± 0.11</td>
<td>0.28 ± 0.06</td>
</tr>
<tr>
<td>GSH (mmol/mg protein)</td>
<td>4.15 ± 0.45</td>
<td>2.43 ± 0.36</td>
<td>3.96 ± 0.25</td>
<td>3.25 ± 0.22</td>
</tr>
<tr>
<td>SOD (Units/mg protein)</td>
<td>7.59 ± 1.12</td>
<td>5.35 ± 0.95</td>
<td>7.79 ± 0.69</td>
<td>6.33 ± 0.45</td>
</tr>
<tr>
<td>CAT (Units/mg protein)</td>
<td>17.02 ± 1.50</td>
<td>10.84 ± 1.78</td>
<td>15.79 ± 0.69</td>
<td>14.24 ± 0.81</td>
</tr>
</tbody>
</table>

Values in the same row with different superscript are significantly different at p < 0.05. MDA, Malondialdehyde; SOD, superoxide dismutase; CAT, catalase; GSH, glutathione.
(51%), while in the liver it was 25%.

Tables 2 and 3 also show the activities of SOD and CAT in the erythrocyte and liver of birds for all groups. Copper exposure decreased SOD and CAT activities significantly (p < 0.05) when compared with the control. The decrease in the erythrocyte was 48% in both SOD and CAT, while in the liver, the decrease was 29% for SOD and 36% for CAT. Vitamin C attenuated the effects of copper on SOD and CAT in the erythrocyte and the liver. The increase in the activity of SOD in the liver was however not statistically significant.

DISCUSSION

The focus of the present study was to provide a better understanding of the effects of copper-induced oxidative stress on macromolecules and the role of vitamin C in alleviating copper-induced oxidative stress in broilers.

Researches have demonstrated the advantageous use of pharmacological concentration of copper (both from organic and inorganic sources) in poultry feeds (Idowu et al., 2006; Pesti and Bakalli, 1996; Bakalli et al., 1995). In spite of all these advantages, toxic effects of copper have been reported when it is overloaded in poultry (Almansour, 2006; Chiou et al., 1997; Jensen et al., 1991). The cytotoxic effects of copper have been related to the ability of copper ions to trigger formation of free radicals which in turn cause cell membrane lipid peroxidation, modification of physicochemical properties and alterations in cell permeability (Luza and Speisky, 1996). Other mechanisms suggested, include a toxic effect of copper on other cell constituents such as proteins, nucleic acids or mitochondria. In fact, using isolated mitochondria, it has been demonstrated that copper induces lipid peroxidation and the collapse of the membrane potential (Saris and Skulskii, 1991).

This study shows that dietary exposure to 250 mg/kg diet of CuSO₄ for eight weeks induced oxidative injury including increased copper levels and lipid peroxidation, as well as decreased levels of GSH and activities of SOD and CAT in both erythrocyte and liver of exposed birds. The results of this study also indicate that two weeks supplementation of vitamin C (100 mg/kg diet) resulted in a significant decrease in blood and liver copper levels. In addition, alteration induced in the level of lipid peroxidation, GSH, SOD and CAT by copper were reversed by vitamin C. These results suggest a protective effect of vitamin C in copper-induced toxicity. Our findings are in agreement with other investigators who have reported protective effect of vitamin C in metal-induced toxicity (Kardivel et al., 2007; Ademuyiwa et al., 2005; Onunkwor et al., 2004; Chattopadhyay et al., 2001; Messripour and Haddady, 1988). Other antioxidants such as vitamin E (Mattie and Freedman, 2001; Rey and Lopez-Bote, 2001; Sokol, 1996; Kadiiska et al., 1993), lipoic acid (Yamamoto et al., 2001) and selenium (Kadiiska et al., 1993) have been shown to protect against copper over-dose effects.

The reduction in blood and liver copper level as a result of two weeks vitamin C supplementation observed in this study could be due primarily to the decreased copper absorption from the intestine (Kies and Harms, 1989). Vitamin C has been shown to lower copper absorption by decreasing the concentration of soluble copper in the small intestine (Van den Berg et al., 1994).

Studies have shown that superoxide, hydrogen peroxide and hydroxyl radicals are produced after exposure to copper in various cellular systems (Kadiiska et al., 1993; Halliwell and Gutteridge, 1990). The observed elevation in MDA level (index of lipid peroxidation) of birds exposed to copper in this study may be due to either overproduction of reactive oxygen species (ROS) or accumulation of ROS resulting from dysfunction of antioxidants during chronic copper exposure. Vitamin C supplementation for two weeks resulted in a significant reduction in the elevated level of MDA in the exposed birds. Vitamin C is effective in scavenging free radicals, including hydroxyl radicals, aqueous peroxyl radicals and superoxide anions. It acts as a two electron reducing agent and confers protection by contributing an electron to reduce free radicals, thus neutralizing these compounds in the extracellular aqueous environment prior to their reaction with biological molecules (Evans and Halliwell, 2001; Carr and Frei, 1999).

Reduced glutathione (GSH) is an abundant non protein thiol in living organisms and it plays a crucial role in intracellular protection against toxic compounds, such as ROS and other free radicals (Anderson and Luo, 1998; Anderson, 1997). It can function as a nucleophile to form conjugates with many xenobiotics and/or their metabolites and can also serve as a reductant in the metabolism of hydrogen peroxides and other organic hydroperoxides (Suntres, 2002; Deneke, 2000; Rahman et al., 1999; Lu, 1999; Anderson and Luo, 1998; Anderson, 1997). In this study, there was a significant decrease in the level of GSH in both erythrocyte and liver of copper-exposed birds compared with control. Two weeks vitamin C supplementation however, reversed the decrease in the GSH level. The ability of vitamin C to reverse the GSH depletion observed in the copper-exposed birds might be due to its ability to quench ROS (Suntres, 2002), act as an alternative sulphydryl nucleophile to GSH, thereby preventing its oxidation to GSSG in detoxification reaction against free radicals (Onunkwor et al., 2004) and also its ability to regenerate other small molecule antioxidants such as glutathione, α-tocopherol and β-carotene (Evans and Halliwell, 2001; Carr and Frei, 1999; Halliwell, 1996).

One of the most important enzymes that function as a cellular antioxidant is superoxide dismutase, which rapidly converts superoxide radicals into hydrogen peroxide (H₂O₂) that is removed by catalase (Teixeira et al., 1998; Fridovich, 1995). The observation of low activity of SOD and CAT in the erythrocyte and liver of exposed birds in this study could be due to the down regulated synthesis...
or over-utilization of the antioxidant enzymes due to persistent toxicant misuse (Irshad and Chaudhuri, 2002).

Conclusively, vitamin C reduced copper burden and lipid peroxidation, increased GSH levels, and reversed the inhibition of the antioxidant enzymes SOD and CAT observed in copper-exposed birds in this study, suggesting that vitamin C may be beneficial in preventing copper-induced oxidative stress. This study is a contribution to the potential for veterinary use of vitamin C in poultry.

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