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# THE EFFECTS OF DIETARY COPPER SUPPLEMENTATION ON OXIDATIVE AND ANTIOXIDANT SYSTEMS IN BROILER CHICKENS

## EFFECTOS DE LA SUPLEMENTACIÓN CON COBRE SOBRE LOS SISTEMAS OXIDATIVO Y ANTIOXIDANTE EN BROILERS

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### ADDITIONAL KEYWORDS

Oxidative damage. Erythrocytes. Liver. Blood.

### PALABRAS CLAVE ADICIONALES

Daños oxidativos. Eritrocitos. Hígado. Sangre.

### SUMMARY

This study was designed to investigate the *in vivo* effects of dietary copper supplementation on the oxidative and antioxidant systems in the erythrocyte and liver of broiler chickens. Birds were exposed to commercially prepared diet (control group, n=20) or commercial diet supplemented with 250 mg CuSO<sub>4</sub>/kg diet (experimental group, n=20) for 6 weeks. The copper burdens, oxidative lipid peroxidation (LPO), glutathione (GSH) concentration, activity of antioxidant enzymes, superoxide dismutase (SOD) and catalase (CAT) were evaluated in the erythrocyte and hepatic tissues using well established techniques. Oral exposure to copper for a period of 6 weeks significantly (p<0.05) increased the copper burden and lipid peroxidation respectively in the blood and liver of treated birds compared with control. Concomitantly, there was a significant reduction (p<0.05) in the activities of SOD, CAT, and the concentration of GSH in the exposed birds compared with control. It is concluded from this study that dietary copper supplementation (250 mg CuSO<sub>4</sub>/kg diet) induced oxidative stress in the erythrocyte and liver of broiler chickens.

### RESUMEN

El estudio fue diseñado para estudiar los efectos *in vivo* de la suplementación dietética con cobre sobre los sistemas oxidativos y antioxidantes

de los eritrocitos y el hígado de broilers. Las aves fueron sometidas a una dieta comercial (grupo control, n=20) o suplementada con 250 mg CuSO<sub>4</sub>/kg dieta (grupo experimental, n=20) durante 6 semanas. En los eritrocitos y tejido hepático, se evaluó, empleando técnicas consolidadas, la sobrecarga de cobre, la peroxidación oxidativa de los lípidos (LPO), concentración de glutatión (GSH), actividad de los enzimas antioxidantes, superóxido dismutasa (SOD) y catalasa (CAT). La exposición oral al cobre, durante 6 semanas, aumentó (p<0,05) la carga de cobre y la peroxidación lipídica respectivamente en sangre e hígado de las aves tratadas respecto del control. Se concluye, que la suplementación dietética con cobre (250 mg CuSO<sub>4</sub>/kg dieta) indujo estrés oxidativo en los eritrocitos e hígado de los broilers.

### INTRODUCTION

Copper is present in all tissues and is a functional constituent of all living cells. Because of its significance in most of the enzymatic reactions and cellular metabolism, Cu is an essential component of human nutrition and the diets of birds, including poultry.

Commercial organic copper sources and reagent-grade inorganic copper salts have

been used for the supplementation of animal feeds (Guo *et al.*, 2001). Copper is routinely supplemented to swine and poultry diets at concentrations above requirement of the animals, because pharmacological concentrations of copper act as a growth stimulant in these species, thereby improving growth performance, including increase in body weight gain and feed intake (Cromwell *et al.*, 1989; Bakalli *et al.*, 1995). Lien *et al.* (2004), found supplemental copper to reduce VLDL-cholesterol and enhance HDL-cholesterol in egg yolk of laying hens. Also  $\text{CuSO}_4$  supplementation has been reported to reduce the saturated fatty acid proportion in abdominal fat and increase PUFA:SFA ratio, as well as reduces the cholesterol content in meat of broiler chickens (Skrivan *et al.*, 2000; Xia *et al.*, 2004). Research evidences have shown that copper regulates cholesterol biosynthesis by reducing hepatic glutathione concentration (Kim *et al.*, 1992). A large decrease in the reducing capacity of the cellular redox couples, such as glutathione has been used as an indication of oxidative stress in rats and other animal models (Schafer and Buettner, 2001).

Although copper is a major essential element, serious toxic effects of this metal has been reported when it is over loaded (Toplan *et al.*, 2005; Zhang *et al.*, 2000; Bremner, 1998). Copper ions are powerful catalysts of free radical damage. Its ability to induce oxidative damage is generally attributed to the formation of highly reactive hydroxyl radical ( $\text{OH}^\cdot$ ) from hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) via the Haber-Weiss reaction (Bremner, 1998; Kadiiska *et al.*, 1993). The  $\text{OH}^\cdot$  radical generated during copper-induced oxidative stress may lead to lipid peroxidation and formation of reactive products which may be involved in severe damage of cell molecules and structures (Videla *et al.*, 2003). Lipid peroxidation as evidenced by increased malondialdehyde content, as well as alteration in membrane integrity and fatty

acid composition have been observed in the erythrocytes and hepatocytes of copper exposed animals (Zhang *et al.*, 2000) and erythrocytes of human (Hochstein *et al.*, 1980). Erythrocytes are particularly sensitive to oxidative stress and like other cells of aerobic organisms, they are supplied with protective antioxidant mechanisms in order to counteract the toxic action of oxygen radicals. Defense is provided by enzyme activities like superoxide dismutase, catalase and glutathione peroxidase. Superoxide dismutase is the enzyme that catalyses the dismutation of the highly reactive superoxide anion to molecular oxygen ( $\text{O}_2$ ) and to the less reactive species, hydrogen peroxide ( $\text{H}_2\text{O}_2$ ). Glutathione (GSH), GSH peroxidase and catalase, under most circumstances eliminate the resulting  $\text{H}_2\text{O}_2$  (Fridovich, 1995; Teixeira *et al.*, 1998).

Although reports of studies on the use of copper as a feed supplement in poultry has been widely published, however to the best of our knowledge, information regarding the toxicity of copper in broiler chickens is still limited. Therefore, prompted by this paucity of data, we evaluated the copper burden, status of lipid peroxidation and antioxidant systems in the erythrocyte and liver of broiler chickens whose diet has been supplemented with copper.

## MATERIALS AND METHODS

### ANIMALS AND EXPERIMENTAL DESIGN

This study protocol was approved by the University of Agriculture, Abeokuta (Nigeria), animal use and welfare committee and all investigation adhered strictly to the statement for the use of animals in research.

Forty unsexed, three-week old broiler chickens (Anak 2000) 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. Feeds and water were given *ad libitum*. The birds were divided into two groups of twenty

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birds each. Group 1 served as the control and was fed on a basal diet purchased from UNAAB-LEVENTIS Agro-Allied Industry. Group 2, the treatment group, were fed on the same basal diet supplemented with 250 mg  $\text{CuSO}_4/\text{kg}$  diet. This feeding protocol continued for a total of 42 days. The basal diet was formulated following the procedure of Idowu *et al.* (2003), and its composition is shown in **table I**.

### SAMPLE PREPARATION

At the end of the feeding period, birds in the two groups were starved overnight for 12 hours. Exactly 10 ml of blood was drawn from the brachial 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  $750 \times g$  for 5 minutes, supernatants discarded and

the erythrocytes washed thrice using 0.9% NaCl for lipid peroxidation, catalase, glutathione and superoxide dismutase activity determination. The birds were sacrificed and liver excised, perfused 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  $\text{Na}_2\text{EDTA}$  (pH 7.8). The soluble fraction was prepared by centrifugation at  $1000 \times g$  for 20 minutes. 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 brought to 25 ml with deionised water (Ademuyiwa, 1995). For liver, 1g was dried to a constant weight at  $85^\circ\text{C}$ . Dried samples were cold digested in 2 ml of nitric acid overnight. They were then hot digested on a block digester at  $120^\circ\text{C}$  until all the organic matter was dissolved. Two milliliters of 30% (w/v) hydrogen peroxide were added during digestion to enhance oxidization. The digests were allowed to cool, and then diluted to 25 ml with deionised water (Alonso *et al.*, 2000). Copper concentrations in the digests were determined by atomic absorption spectrometry (Buck Scientific, Model 210, Connecticut, USA). Values were expressed as  $\mu\text{g}/\text{ml}$  of blood or  $\mu\text{g}/\text{g}$  of tissue.

### MEASUREMENT OF INDICES OF OXIDATIVE STRESS

**Lipid peroxidation:** The extent of lipid peroxidation was estimated in terms of thiobarbituric acid reactive substances (TBARS), using malondialdehyde (MDA) as standard by method of Beuge and Aust (1978). Briefly, 1 ml of erythrocyte or liver homogenate was added with 2 ml of the TCA-TBA-HCl reagent [15% (w/v) TCA, 0.375% (w/v) TBA and 0.25N HCl]. The contents were boiled for 15 minutes, cooled and centrifuged at  $1000 \times g$  to remove the

**Table I.** Composition of the basal diet. (Composición de la dieta basal).

Ingredients	
Maize %	44.00
Soya bean full fat %	36.00
Rice husk %	12.33
Palm oil %	4.00
Bone meal %	2.50
Oyster shell %	0.50
Vitamin/mineral premix %	0.25
Salt (NaCl) %	0.25
Methionine %	0.17
Determined analysis	
Crude protein %	19.90
Fat %	11.00
Ash %	14.31
Fibre %	4.78
Calculated analysis	
Energy (ME) kcal/kg	3203.76
Energy/protein ratio	160.20
Methionine %	0.45
Calcium %	1.33
Phosphorus %	0.69
Copper mg/kg	5.90

precipitate. The absorbance was read at 535 nm and the MDA concentration of the sample calculated using extinction coefficient of  $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$  and expressed as  $\mu\text{mol MDA/g Hb}$  for erythrocyte and  $\mu\text{mol MDA/g tissue}$  for liver.

Catalase determination was carried out following the method described by Clairborne (1986), in which the disappearance of  $\text{H}_2\text{O}_2$  was monitored spectrophotometrically at 240 nm. Briefly, 20  $\mu\text{l}$  of erythrocyte or liver homogenate was added to 1 ml of 0.05M phosphate buffer pH 7.0. Then, 0.5 ml of 30%  $\text{H}_2\text{O}_2$  was added to the buffered sample to initiate the reaction. The change in absorbance for 1 minute at 10 seconds interval was recorded. One unit activity is defined as the amount of enzyme catalyzing the formation of water and oxygen from hydrogen peroxide under assay condition.

Superoxide dismutase was assayed according to a modified procedure of Das *et al.* (2000). In this method, 1.4 ml aliquot of the reaction mixture (comprising 1.11 ml of 50 mM phosphate buffer, pH 7.4, 0.075 ml of 20 mM L-Methionine, 0.04 ml of 1% (v/v) Triton X-100, 0.075 ml of 10 mM hydroxylamine hydrochloride and 0.1 ml of 50 mM EDTA) was added to 100  $\mu\text{l}$  of the erythrocyte or liver homogenate and incubated at 30°C for 5 minutes. 80  $\mu\text{l}$  of riboflavin was then added and the tubes were exposed to 20W-Philips fluorescent lamps for 10 minutes. After the exposure time, 1 ml of Greiss reagent (mixture of equal volume of 1% sulphanilamide in 5% phosphoric acid) was added and absorbance of the colour formed measured at 543 nm. One unit of enzyme activity was measured as the amount of SOD capable of inhibiting 50% of nitrite formation under assay condition.

Glutathione concentration was determined in samples according to the method of Boyne and Ellman (1972). Briefly, 1 ml of erythrocyte or liver homogenate was treated with 4.0 ml of metaphosphoric acid precipitating solution (1.67 g of glacial metaphosphoric acid, 0.2 g EDTA and 30 g NaCl

dissolved in 100 ml of distilled water). After centrifugation, 2 ml of the supernatant was mixed with 0.2 ml of 0.4 M  $\text{Na}_2\text{HPO}_4$  and 1 ml of DTNB (5, 5'-Dithio-bis-(2-nitrobenzoic acid) reagent (40 mg DTNB in 100 ml of aqueous 1% trisodium citrate). Absorbance was read at 412 nm within 2 minutes. GSH concentration was expressed as mmol/g Hb or mmol/g tissue.

Protein content was determined according to the method of Bradford (1976) using bovine serum albumin as standard. All chemical used in the enzymatic activity determination were of analytical purity and were obtained from Sigma Chemical.

## STATISTICAL ANALYSIS

The results of all measurements were expressed as mean  $\pm$  standard deviation. Data collected were subjected to analysis of variance (ANOVA) and means separated using student's t-test with a probability factor of  $p < 0.05$  considered significant.

## RESULTS

### BIOCHEMICAL FINDINGS

Copper burden, lipid peroxidation, SOD, CAT and GSH levels in the erythrocyte and liver for each group are presented in **tables II** and **III** respectively. Copper supplementation in the diet significantly ( $p < 0.05$ ) increased the copper burden in the blood and liver of treated birds compared with control. The mean copper concentration in the blood and liver of treated group were respectively 2.15 and 1.67 times higher than the control. A significant elevation ( $p < 0.05$ ) was noted in lipid peroxidation in the copper exposed group with values being 3.67 and 1.25 times higher than control in both erythrocyte and liver respectively.

Values of SOD activity showed a significant reduction ( $p < 0.05$ ) in the erythrocyte ( $10.9 \pm 1.09$  vs.  $18.3 \pm 1.21$ ) and liver ( $49.8 \pm 14.94$  vs.  $77.4 \pm 13.60$ ) of copper exposed birds compared to the control. Similarly, a significant decrease was noted

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**Table II.** Blood copper burden, erythrocyte lipid peroxidation, SOD, CAT, and GSH concentrations. (Carga de cobre, peroxidación lipídica en los eritrocitos y concentraciones de SOD, CAT y GSH en la sangre).

Parameter	Control (n=20)	Treatment (n=20)
Copper burden <sup>1</sup>	2.0 ± 0.73 <sup>b</sup>	4.3 ± 0.28 <sup>a</sup>
Lipid peroxidation <sup>2</sup>	0.3 ± 0.07 <sup>b</sup>	1.1 ± 0.19 <sup>a</sup>
SOD	18.3 ± 1.21 <sup>a</sup>	10.9 ± 1.09 <sup>b</sup>
CAT	3.8 ± 0.65 <sup>a</sup>	1.8 ± 0.35 <sup>b</sup>
GSH	0.9 ± 0.17 <sup>a</sup>	0.3 ± 0.19 <sup>b</sup>

Values in the same row with different superscript are significantly different at  $p < 0.05$ .

<sup>1</sup>µg/ml; <sup>2</sup>µmol MDA/g Hb (MDA= malondialdehyde); SOD: superoxide dismutase (unit/mg protein); CAT: catalase (unit/mg protein); GSH: glutathione (mmol/g Hb).

**Table III.** Liver copper burden, lipid peroxidation, SOD, CAT and GSH concentrations. (Carga de cobre, peroxidación lipídica en los eritrocitos y concentraciones de SOD, CAT y GSH en el hígado).

Parameter	Control (n=20)	Treatment (n=20)
Copper burden <sup>1</sup>	5.1 ± 0.61 <sup>b</sup>	8.5 ± 0.66 <sup>a</sup>
Lipid peroxidation <sup>2</sup>	0.4 ± 0.02 <sup>b</sup>	0.5 ± 0.08 <sup>a</sup>
SOD	77.4 ± 13.60 <sup>a</sup>	49.8 ± 14.94 <sup>b</sup>
CAT	16.0 ± 1.26 <sup>a</sup>	9.6 ± 2.44 <sup>b</sup>
GSH	3.9 ± 0.17 <sup>a</sup>	2.5 ± 0.43 <sup>b</sup>

Values in the same row with different superscript are significantly different at  $p < 0.05$ .

<sup>1</sup>µg/g tissue; <sup>2</sup>µmol MDA/g tissue (MDA= malondialdehyde); SOD: superoxide dismutase (unit/mg protein); CAT: catalase (unit/mg protein); GSH: glutathione (mmol/g tissue).

in catalase activity after copper exposure in both the erythrocyte and liver (**tables II and III**). The level of glutathione indicated a significant depletion ( $p < 0.05$ ) in both erythrocyte (67% lower) and liver (36% lower) of birds exposed to copper compared to those of the control.

### DISCUSSION

Numerous studies have reported toxic effects induced when animals and humans are exposed to certain metals (Valko *et al.*, 2005, Stohs and Bagchi, 1995). Several mechanisms have been proposed to explain copper-induced cellular toxicity. Most often the basis for these theories is the ability of free Cu ions to participate in the formation of reactive oxygen species (ROS) and other free radicals (Gaetke and Chow, 2003). The most important consequences of free radical production are lipid peroxidation increase and change in permeability of cell membrane. The significant increase in lipid peroxidation in the experimental group observed in this study could be explained by damage due to

oxidative stress in tissue. Copper produce OH radical from  $H_2O_2$  and this is capable of reacting with practically every biological molecule, initiating oxidative damage by abstracting the hydrogen from an amino bearing carbon to form a carbon centered protein radical and from an unsaturated fatty acid to form a lipid radical (Powell, 2000). Studies by Toplan *et al.* (2005) and Zhang *et al.* (2000) also corroborated our present findings.

Lipid peroxidation is a free radical-mediated chain reaction, since it is self perpetuating. The length of the propagation depends upon chain breaking antioxidants, such as the enzymes SOD, catalase and glutathione peroxidase (Diniz *et al.*, 2003). In this study, supplementation of  $CuSO_4$  (250 mg/kg diet) in the diet of broilers resulted in a reduction in the activities of SOD and catalase compared with birds in the control group. The results of research on copper influence on SOD activity are divergent. Previous studies by Toplan *et al.* (2005), in which rats were given 250 mg/l copper for 9 weeks and Zhang *et al.* (2000)



when rats were overloaded with 500 mg Cu/kg/bw for eight weeks showed that SOD activity was significantly reduced, as also observed in this study. However Ozcelik *et al.* (2003), administered water containing 100 µg/ml copper for 4 weeks to rats and observed an increase in the activity of SOD in experimental animals compared to those of the control. Sansinanea *et al.* (1998), in a study to explain the cytotoxicity of excessive free radical production in the liver of rats, gave water containing 0.2% solution of CuSO<sub>4</sub> and observed SOD activity to be higher in experimental group animals than that of control group animals. SOD catalyses the dismutation of superoxide radical leading to formation of hydrogen peroxide which in turn is detoxified by the enzyme catalase (Fridovich, 1995). Therefore, the decreased SOD and CAT activity observed in this study may be due to the depletion or inactivation of the enzymes by production of free radicals (Kono and Fridovich, 1982) such as superoxide and H<sub>2</sub>O<sub>2</sub> which in turn generate hydroxyl radical resulting in initiation and propagation of lipid peroxidation.

One of the mechanisms by which heavy metals produce effect is through their interaction with cellular sulphhydryl groups in proteins. Sulphydryl groups thus serves as a source of electrons for reduction and also mediate the methylation process. When the availability of free thiol group is low, enhanced expression of toxicity in the form of oxidative stress could occur. Since most metals have high affinity for the sulphhydryl (SH) groups, endogenous SH compounds such as metallothionein (MT) and GSH are known to be involved in metal toxicity. GSH performs a pivotal role in maintaining the metabolic and transport function of cells. It may function as an intracellular chelator and a metal detoxifying agent. The importance of GSH in metal detoxification is also supported by its role in removal of toxic

oxygen species (Ishikawa and Sies, 1989; Seidegard and Ekstrom, 1997). In this study, GSH levels were lower in the copper treated group compared with the control group. The reduction in the GSH levels observed in this study might be due to the reaction of GSH with copper or with free radicals promoted by copper supplementation (Ishikawa and Sies, 1989; Deleve and Kaplowitz, 1991). Also an impairment of GSH synthesis or regenerating enzyme could be responsible for the observed reduction of GSH (Hultberg *et al.*, 1997).

## CONCLUSION

In conclusion, this study has demonstrated that copper intake at high concentration (250 mg CuSO<sub>4</sub>/kg diet) induced adverse effects on the oxidative and antioxidant systems in broiler chickens as shown by the increase in lipid peroxidation and reduction in the activities of SOD and catalase, as well as the concentration of glutathione in both the erythrocyte and the liver. This in turn may be detrimental to the health of the birds since oxidative stress can lead to increased prevalence of infectious diseases via impairment of immune cell functions. Nevertheless, the beneficial effects of Cu supplementation in poultry may be utilized by reducing the copper supplementation level in the diet. Furthermore, dose-response relationship experiments are suggested in order to determine a threshold dose below which there would be no toxicity.

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