

# Mitigation Effects of Vitamin C and E Against Copper Toxicity in Broiler Chickens, With Reference to Biochemical, Genotoxicity and Pathological Studies

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## Research Article

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# Abstract

Copper (Cu) is necessary for biological utility, nevertheless when existing in abundance; it can produce plentiful injurious impacts. This enquiry was carried out to explore the efficiency of individual or combined doses of vitamin C (Vit C) and vitamin E (Vit E) in ameliorating some biochemical, genotoxicity and pathological changes in the liver of copper sulphate ( $\text{CuSO}_4$ )-intoxicated chickens. One hundred-one day old broiler chicks were haphazardly divided into 5 groups of 20 chicks each. The broilers were fed on basal diet only (control, gp.1) or supplemented with 300 mg  $\text{CuSO}_4$  /kg diet (Cu, gp.2),  $\text{CuSO}_4$  + 250 mg Vit C /kg diet (Cu+ Vit C, gp.3),  $\text{CuSO}_4$  +250 mg Vit E /kg diet (Cu+ Vit E, gp.4) and both vitamins C + E (Cu+ Vit C+ Vit E, gp.5) for six weeks. The results displayed that  $\text{CuSO}_4$  - intoxicated birds (gp.2) had significantly ( $p < 0.05$ ) dwindled body weight, gain and feed consumption with increased feed conversion rate from week 2 till the 6th week compared with control group. Serum aminotransferases (ALT, AST), and alkaline phosphatase (ALP) were significantly ( $p < 0.05$ ) augmented in  $\text{CuSO}_4$ - exposed group (gp.2) with significantly ( $p < 0.05$ ) drop in serum total protein (TP), albumin, globulins, triglycerides (TG), total cholesterol (TC), low density lipoprotein-cholesterol (LDL-C), very low density lipoprotein-cholesterol (VLDL-C), and high density lipoprotein-cholesterol (HDL-C) levels compared to control. Concomitantly, histopathological and DNA changes were perceived in liver of  $\text{CuSO}_4$  -intoxicated birds. Co-supplementation of Vit C, and Vit E single-handedly or incorporation to  $\text{CuSO}_4$  -intoxicated chickens displayed an enhancement in performance traits and abovementioned changes, especially with those given combination of vitamins. From the extant enquiry, it could be established that supplementation of vitamin C and E were beneficial for alleviation the harmful effects of  $\text{CuSO}_4$  on performance and other studied parameters in broiler chickens.

# Introduction

The proper activities of iron metabolism-related metalloenzymes are maintained by copper (Cu), an important microelement involved in poultry diets (Samanta et al. 2011). Despite the need for unlike metabolic processes and enzyme activities, chronic over-exposure to copper caused some adverse effects (Spatari et al. 2002). In poultry, the extreme type of Cu poisoning is long-term ingestion of Cu compounds from different sources. Metabolism of Cu is regulated primarily by the liver, where it can be released into the circulatory system or excreted via the bile (Linder and Hazegh-Azam 1996). It accumulates steadily in the liver during chronic Cu toxicity without causing any noticeable signs or symptoms. It can lead to hepatocellular lesions when the hepatic Cu storage capacity is surpassed, and thus, the release of Cu from the liver into the blood stream causes hemolysis, jaundice, and renal disease (Liu et al. 2020). Aforementioned studies have designated that excessive exposure to Cu can induce oxidative stress in the brain tissue of chickens (Li et al. 2010), reduce the activities of copper-zinc superoxide dismutase and glutathione peroxidase, and increase the contents of malondialdehyde and hydroxyl radical in the liver of ducklings (Zhao et al. 2008). An discrepancy between reactive oxygen species (ROS) production and the body's ability to detoxify these intermediate species is considered to indicate oxidative stress. Supplementation with Cu up to 200 ppm is used for growth promotion

(Thompson 2007), but excess amounts of Cu in the diet depress indices of performance as it result in low digestibility of Cu salt which does not exceed 20% and absorption in poultry which led to amplified elimination of fecal matter and environment pollution (Świątkiewicz et al. 2014).

Few reports have displayed that supplementation of antioxidants such as vitamin E, vitamin C, zinc, beta-carotene, alpha-lipoic acid and polyphenols has a protective impact against the toxicity of Cu (Gaetke and Chow 2003; Ajuwon and Idowu 2010). However, only restricted studies have been achieved on vitamin C effects on Cu-induced oxidative stress in broilers; no studies have analyzed the effects of vitamin E single-handedly or incorporation with vitamin C on performance, biochemical markers, DNA damage or pathological findings in broilers exposed to Cu. Consequently, the ambition of the existent inquest was to assess the effects of dietary supplementation with Cu on these parameters and then to appraise the protecting effects of vitamins C and/ or E against excess dietary supplementation with Cu, individually or in combination.

## Materials And Methods

### Experimental animals, diet and protocol

One hundred, 1-day old commercial Cobb broiler chickens were attained from Al-Kahira Poultry Company, 10<sup>th</sup> of Ramadan City, Sharkia Governorate, Egypt. The chicks were given starter diet from day 1 until 14 days of age and grower diet (15–28 day) followed by a finishing diet up to 42-days age. Ingredients and chemical composition of diets were demonstrated in Table 1. As designated by the Ross management manual (Aviagen 1999), experimental diets were formulated. Chicks were reared under identical husbandry circumstances (14 h light: 10 h dark, relative humidity 60% and 25-32°C) in standard battery cages with automatic nipple drinkers and standard feeding trough. The diets and water were offered *ad libitum*. All birds were vaccinated at 7 and 18 days old against Newcastle disease and at 15 days old for Gumboro disease (Giambrone and Clay 1986).

The chicks were haphazardly divided into 5 groups of 20 chicks each. The birds were fed on basal diet only (control or negative group), or supplemented with 300 mg CuSO<sub>4</sub>/kg diet only (Cu or positive group), CuSO<sub>4</sub>+ 250 mg Vit C/kg diet (Cu+ VitC group), CuSO<sub>4</sub> +250 mg Vit E/kg diet (Cu+ Vit E group) and CuSO<sub>4</sub>+ Vit C+Vit E (Cu+Vit C +Vit E group).

Copper sulfates (CuSO<sub>4</sub>·5H<sub>2</sub>O), vitamin C (L-ascorbic acid) and vitamin E (α tocopherol acetate) were delivered by marketable companies (El-Gomhoria, EL Nasr Pharmaceutical and Pharco Pharmaceutical Industries respectively), Zagazig, Egypt. The toxic dose of Cu used in this inquest was dogged according to Cinar et al. (2014) while the vitamins C and E doses are used after Sahin et al. (2002).

### Determination of growth performance

The body weight (BW) of each chick and feed consumption (FC) of each group were weekly verified, starting from 1 day-old, and weight was certified to the bordering 1 g. Mortality was recorded and growth performance was appraised in relations of live body weight gain (BWG), feed intake and feed conversion ratio (FCR). The FCR was calculated according to Wagner et al. (1983). The enquiry was ended at the 6<sup>th</sup> week of the experiment.

## Sampling:

Samples of blood were collected from the wing vein of 10 aimlessly selected birds in each group at termination of 3 and 6 weeks post-supplementations and centrifuged (1200 g for 15 min) immediately for separation of serum, which is stored at -20°C in deep freeze until biochemical analysis (Hashem et al. 2019).

The chicks were sacrificed by manual cervical dislocation and two portions of liver tissues were separated, and blotted dry. The first part was put in ice-cold PBS (phosphate buffer saline) for comet assay determination and the 2<sup>nd</sup> part was fixed in ten percent formalin for histopathological inspection.

## Biochemical studies

Serum liver enzymes (ALT, AST & ALP), protein (TP, albumin & globulin) and lipid (TG, TC, LDL-C, VLDL-C & HDL-C) profile were determined using Automatic Biochemistry Analyser (Robonik Prietest ECO- India) with test kits of Spinreact, S.A./S.A.U. (Ctra. Santa Coloma, Spain).

## Detection of DNA damage

The liver DNA damage was measured using a single-cell gel electrophoresis technique (also known as comet assay) as previously defined by Singh et al. (1988). Comet assay is a quick, accurate and simple method for detecting DNA damage. In this method, 0.5 g of crushed samples was transferred to 1 ml ice-cold PBS. This suspension was stirred for 5 min and filtered. The cell suspension (100 µl) was mixed with 600 µl of low melting agarose (0.8% in PBS). A 100 µl of this mixture was spread on pre-coated slides. The coated slides were immersed in lysis buffer [0.045 mol/l Tris/Borate/EDTA (TBE), pH 8.4, containing 2.5% Sodium dodecyl sulphate (SDS)] for 15 min. The electrophoresis conditions were 2 V/cm for 2 min and 100 mA. The slides were then washed 3 times, for 5 minutes each, with neutralization buffer [0.4 Mol/L Tris (pH 7.5)]. Finally, the slides were stained with 50 µl of ethidium bromide (2 mg/ml) and covered with a cover slip. The DNA fragment migration patterns of 100 cells at 400 magnifications with the Optika Axioscope fluorescence microscope were calculated for each dose level. The length of DNA migration (tail length) on PX was calculated for each cell from the center of the nucleus to termination of tail. By calculating the total intensity (fluorescence) in the cells, which was taken as 100%, the DNA% in the tail was determined and deciding what percentage of this total intensity corresponded to the intensity only

measured in the tail. The tail moment was expressed in arbitrary units. Although any image analysis device may be sufficient for SCGE data quantification, Comet 5 image analysis software developed by Kinetic Imaging Ltd (Liverpool, UK) linked to a CCD camera has been used to determine the degree of quantitative and qualitative DNA damage in the cells by measuring the length of DNA migration and the % of migrated DNA. Finally, the program calculated the tail moment. Generally, 50–100 randomly selected cells are analyzed per sample.

## Histopathological investigations

Tissue samples were taken from the liver of euthanized chicks by manual cervical dislocation and fixed in formalin of 10 %. The samples preserved with formalin are dehydrated and embedded in paraffin. Five-micron thick paraffin slices were set and stained with hematoxylin and eosin (H& E) and inspected microscopically (Suvarna et al. 2018).

## Statistical analysis:

The ANOVA analysis for all data was accomplished using SPSS (IBM, 2013) for windows version 22. The data was expressed as mean and the data variability was expressed as standard error of mean (SE). The Duncan's multiple range tests were used to assess the differences in significance between groups,  $p < 0.05$  suggesting a difference in significance.

## Results

### Clinical signs and body performance:

No clinical signs or mortality were found in all of the supplemented birds during the experimental times except CuSO<sub>4</sub>- intoxicated group (2) which showed mild diarrhea (few cases), decrease appetite, and pale comb.

As illustrated in Fig. 1 (a, b, c), the BW, BWG and FC of broilers were significantly ( $p < 0.05$ ) declined in all groups from the 2<sup>nd</sup> week till the termination of experiment (6<sup>th</sup> week) compared to control (Figs. 1-3). However, these changes were minimized in birds supplemented with vitamins (gps. 3-5) matching with gp. (2), but not return to control values. FCR was significantly ( $p < 0.05$ ) high in CuSO<sub>4</sub>- intoxicated broilers (gp.2) at 2<sup>nd</sup> to 6<sup>th</sup> week of age related to control (Fig.1 d). Comparatively with CuSO<sub>4</sub>- intoxicated broilers (gp.2) FCR was insignificantly decreased in those fed with vitamins (gps. 3-5) from the 4<sup>th</sup> week of age and go back to near normal control values at the 6<sup>th</sup> week.

# Effect of CuSO<sub>4</sub> and vitamins on serum levels of liver biomarkers

As shown in Tables 2-6, CuSO<sub>4</sub> induced hepatotoxicity as reflected by statistically ( $p < 0.05$ ) augmented serum activities of ALT, AST and ALP, whereas serum TP, albumin, globulins, TG, TC, LDL-C, VLDL-C and HDL-C levels are reduced ( $p \leq 0.05$ ) after 3 and 6 weeks compared to normal control values. The reduction in serum albumin and HDL-C was only at 6<sup>th</sup> week period.

On the other hand, co-supplementation of copper with either vit. C or vit. E had significantly ( $p \leq 0.05$ ) declined serum enzymes and enlarged serum TP, albumin, globulins, TG, TC, LDL-C, VLDL-C and HDL-C levels in birds of gps. 3&4 matching with CuSO<sub>4</sub>-induced hepatotoxicity group (gp.2). However, co-administration of CuSO<sub>4</sub>+ Vit C+ Vit E (gp.5) restored the changes in liver biomarkers levels back to near the normal control values.

## Effect of CuSO<sub>4</sub> and vitamins on DNA damage

The data in Tables 6&7 and Fig. 2 (a-f) revealed that CuSO<sub>4</sub> intoxication produced a significant ( $p \leq 0.05$ ) elevation in comet %, %DNA in tail, tail length, tail moment and olive tail moment at the end of 3<sup>rd</sup> and 6<sup>th</sup> week parallel to control group (1). On contrary, co-administration of CuSO<sub>4</sub>+Vit C, CuSO<sub>4</sub>+Vit E or their combination (CuSO<sub>4</sub>+ Vit C+ Vit E) to birds ensued in an enhancement in the results of comet assay performance, showing a substantial decrease in previous parameters relative to the CuSO<sub>4</sub>- intoxicated group but not return to values of normal control. It is obvious that the combination regimen produced the most marked reduction as related to CuSO<sub>4</sub>-intoxicated group.

## Histopathological findings

The livers of control rats displayed the typical histological arrangement of hepatic lobules. In contrast, the livers of chicken from CuSO<sub>4</sub>- intoxicated group showed hyperplastic, and necrotic biliary epithelium with various degenerative and necrotic changes at third week (Fig.3 a). In addition cholestasis, necrotic bile duct epithelia, beside portal lymphocytic aggregation and fibroblast proliferation were encountered on termination of 6<sup>th</sup> week (Fig.3 b). Liver from chicken of Cu+ Vit C supplemented group for 3weeks viewed moderate enlargement of hepatic cells and hyperplastic kupffer cells (Fig.3 c), as well as partially contracted hepatic cells proliferative kupffer cells and dilated sinusoids after six weeks (Fig.3 d). Liver from chicken of Cu+ Vit E supplemented group revealed portal lymphocytic aggregations within apparently normal hepatic parenchyma on termination of third week (Fig.3 e), while intense hyperplasia of kupffer cells and cloudy swelling of hepatic cells with a few lymphocytic aggregations were seen on 6<sup>th</sup> week (Fig.3 f). Liver of Cu+ Vit C+ Vit E supplemented chicken (gp.5) displaying little portal and interstitial lymphocytic aggregations within apparently normal hepatic parenchyma on termination of

third week (Fig.3 g), mild portal lymphocytic aggregation, normal hepatic parenchyma and dilated blood vessels were observed after 6 weeks (Fig.3 h).

## Discussion

Although the level of copper up to 100 to 200 mg/kg as CuSO<sub>4</sub> improves growth performance in the broilers (Lu et al. 2010; Jegede et al. 2011; Scott et al. 2018; Nguyen et al. 2020), incompatible effects such as poor feed intake, decrease in body weight and hemato-biochemical changes at higher doses of copper have been reported (Oguz et al. 2010; Shahzad et al. 2012; Oğuz et al. 2014).

In this research, no clinical signs or mortality was perceived during the experimental period in all bird groups, except Cu –intoxicated birds (gp. 2) which showed mild diarrhea, anorexia, and reduction in weight. This synchronized with Luo et al. (2005) who reported zero mortality in male chicks supplemented with 300 and 450 mg/kg CuSO<sub>4</sub> for 21 days. Other studies reported no significant differences in mortality % of broiler chickens among the control and other groups given 0, 10, 25, 50, 125, 250 and 500 CuSO<sub>4</sub>/ kg diet for 35 days (Wang et al. 2007) or 100, 200 and 400 mg CuSO<sub>4</sub> / kg ration for 42 days (Kumar et al. 2013).

The exiting inquest showed that copper sulphate had a toxic effect, as exhibited by a statistical reduction in growth efficiency parameters (BW, BWG and FC) with increase of the FCR over the entire growth-out period (days 1-42) in CuSO<sub>4</sub>- intoxicated birds (gp.2). These findings may be attributed to falling in the feed intake and utilization as result of GIT disturbance caused by a high toxic dose of Cu or owing to embarrassment of the satiety center by Cu resulting in loss of interest in the feed (Asmatullah et al. 1999). Also the reduction in feed consumption could be due to an anorexic effect of CuSO<sub>4</sub> on chickens (Luo et al. 2005; Kim et al. 2016). From this work our results were in line with Luo et al. (2005), Cinar et al. (2014), Abduljaleel (2016), Scott et al. (2018) and Zhou et al. (2020). Whereas, previous studies recorded different outcomes such as unchanged BW and FCR in chicks fed up to 400 mg Cu/kg (Miles et al. 1998), and positive effect on live weight gain in broiler chick supplemented with 400 mg/kg feed copper sulfate for 6 weeks (Kumar et al. 2013). The reason of variances between our experimental results and others may be concomitant with the diversities in the CuSO<sub>4</sub>- particle size, stain and age of birds, experimental periods and /or dose of copper between previous and present experiments. In addition, the difference between the effects of Cu levels on the growth performance of rising poultry indicates that the bioavailability of Cu can differ. This was supported with other literature who stated copper at sufficient dietary levels is known to have favorable impacts, but severe toxic effects due to excess copper are well established (Funk and Baker 1991; Yigit et al. 2012).

The growth parameters were upgraded meaningfully in Cu+ Vit C and Cu+ Vit E-supplemented groups compared to Cu- intoxicated group, however, better results have been found in birds-taken both Vit C and Vit E (gp.5). Many reports showed better performance with feeding of Vit C or Vit E to broiler chicks (Cinar et al. 2014; Abduljaleel 2016; Zhu et al., 2019) or fish (Hosseini et al. 2016; Azeez and Braimah 2020). The improvements in performance characteristics in vitamins- supplemented groups could be attributable to

their antioxidant effects that protect the bird from the oxidative stress produced by exposure to Cu (Gao 2010; Selim 2013), or owing to the role of vitamins as an immune stimulant (Franchini et al. 1991).

Birds supplemented with excess  $\text{CuSO}_4$  (gp.2) revealed an elevation in serum transaminases (ALT&AST) and ALP activities correlated with diminished serum TP, albumin and globulins levels equated to the control group, suggesting marked liver damage (hepatotoxicity), which was further confirmed by hyperplastic, and necrotic biliary epithelium, hepatic degeneration and necrosis in the histopathological findings. The height transaminases activities in birds of gp. (2) may be associated with the influence of Cu on kidney, and liver consequently liberating their intracellular enzymes to circulation (Attia et al. 2009) or due to the cytotoxic effect of Cu resulting in lipid peroxidation indicating the hepatotoxic effect of Cu (Zhang et al. 2000). Also, Kumar et al. (2013) specified that greater level of Cu accumulation might have damaged the liver to increase these enzymes. Abundant studies designate that copper can be metabolized in hepatic tissue and be transferred by glutathione (GSH) to metallothionein thus the copper overload is reached and depletion of GSH instant results in enhanced cellular toxicity (Letelier et al. 2005). The finding of high serum enzymes in Cu-intoxicated broilers agreed with the findings of Yigit et al. (2012) who stated that Cu caused changes in the liver transaminases of broilers. As a result of  $\text{CuSO}_4$  addition, the upsurge in ALP activity might recognized for liver, intestine, kidney, and to some degree bile duct injuries, especially liver cell membrane, which appears to act as a stimulus to increase the synthesis of this enzyme, or may be due to hepatic or bile duct cholestasis causing enzyme regurgitation back into the bloodstream (Coles 1986).

The reported hypoproteinemia in this enquiry may be due to impairment protein synthesis or the functional deterioration of liver and excessive loss of protein caused by nephrosis (Abdelazeim et al. 2020), or could be explained due to the oxidative stress of copper on liver and/ or kidney tissue (Oğuz et al. 2014; Wang et al. 2017). Since albumin has unique copper ion binding sites and carries dietary copper to the liver (Torki et al. 2014), the distinguished drop in the serum albumin levels might be related to the decreased hepatic albumin synthesis in the  $\text{CuSO}_4$  group. On contrary to our results, Almansour (2006) reported intensification in serum protein levels in copper-fed quail while, Cinar et al. (2014) displayed no alteration in plasma proteins of 300 mg/kg diet copper-fed bird.

Dietary addition of Vit C and Vit E alone or in combination to  $\text{CuSO}_4$  - intoxicated broilers significantly ameliorated the previous changes in liver enzymes, indicating that these vitamins have potential protective effects on cell membranes, thereby preventing enzyme leakage into the blood (Prabu et al. 2011; Cinar et al. 2014). A better results was clear with both vitamins supplementation than each alone because of some liver biomarkers were returned back to near the normal control values. These results were in synchronization with those obtained by Idowu et al. (2011), Abou-Kassem et al. (2016), Mashkoor et al. (2016) and Hashem et al. (2019). The hepato-protective influence of vitamin C or E are associated with their antioxidant properties (Bhattacharyya and Mehta 2012). Also, ascorbic acid causes its binding with heavy metals, ensuing in a falling in the oxidative stress at the tissue level and restoration of AST, ALT, ALP, and LDH levels (Rana et al. 2010). Beside, Vit C and Vit E may preserve the hepatic cellular membrane and protect hepatocyte from copper's toxic effects, which may minimize the enzymes leakage

into the blood stream (Prabu et al. 2011). In dissimilarity, supplementation broilers- exposed to toxic levels of cadmium with vitamins C and E did not improve transaminases activity (Cinar et al. 2011).

Co-supplementation of copper with Vit C and Vit E alone or in combination had significantly ( $p < 0.05$ ) elevated serum TP, albumin, globulins. The enhancement in the protein profile in Cu- exposed birds fed with Vit C and Vit E alone or in combination may possibly due to the immunostimulant effect (El-Bahr et al. 2017) or due to impairments of the copper uptake and utilization (Cinar et al. 2014) by dietary supplement of Vit C or Vit E. This was in consistent with Imik et al. (2009) who reported a high total protein concentration in blood of quail supplemented vitamin E and C in diet. Therefore, only, the co-administration of copper with both vitamins offered a protective effect against hepatotoxicity induced by  $\text{CuSO}_4$ .

Regarding lipogram analysis,  $\text{CuSO}_4$  administration causes dyslipidemia evidenced by numerical reduction in serum TG, TC, LDL-C, and VLDL-C levels, however serum HDL-C did not display any change at third week but significantly dropped at 6<sup>th</sup> week in chickens of gp. (2) matching with the control. Our findings were approved with the results of Bakalli et al. (1995) and Idowu et al. (2011). Moreover, Wu et al. (2020) noticed significantly lessened serum cholesterol and LDL-C levels in broilers fed 3 sources of copper (copper sulfate, tribasic copper chloride, and copper methionate) in the diet. The reduction in plasma cholesterol and triacylglycerol in blood of Cu-exposed chickens is owing to fall cholesterol synthesis, high degradation or excretion rates (Konjufca et al. 1997). The excess level of Cu supplementation to diet either decline GSH that diminished stimulation of HMG-CoA reductase activity resulted in the diminished synthesis of cholesterol (Paik et al., 1999), or lead to changes in lipid metabolism, which result in decreasing plasma lipid, 17 beta estradiol and hepatic lipogenic enzyme activity (Pearce et al. 1983). The reduction in LDL level was related to copper toxicity because Cu is an effective catalyst of LDL-C oxidation to an atherogenic form (Steinberg 1997) or to alkoxy radicals (Burkitt 2001). The reduction in HDL-C is due to hypocholesterolemia and hypoproteinemia in this enquiry as more than 40% of HDL-C value represents cholesterol value and the remaining proteins (Ganong 2005). The same findings were obtained by El-Ghalid et al. (2019) in broiler chickens with dietary supplementation of  $\text{CuSO}_4$  at levels 50 and 100 ppm for 5 weeks. However, earlier studies exposed dissimilar outcomes, for example rises of total lipid, cholesterol and LDL-C with no variation HDL-C level in Cu-exposed quail (Almansour 2006) or no alteration in plasma total cholesterol levels in broilers (Cinar et al. 2014). Also, Jegede et al. (2011) reported a reduction in plasma triglycerides and cholesterol in Arbor-Acre unsexed broilers fed  $\text{CuSO}_4$  or copper proteinate at concentrations of 50, 100 or 150 mg/kg diet for 56 days. The strain, dietary ingredients, and also the experimental design and methodology can explain these differences.

Dietary supplementations of  $\text{CuSO}_4$ -intoxicated birds with Vit C and Vit E alone or in combination reduce the opposing effects of copper on lipid profile. The treatment of Cu toxicity with Vit C and Vit E combination is more effective than the use of each alone. This can be contributed to capacity of Vit C to regenerate Vit E by removing free radicals bound to it (Niki, 1987). A combination of the two vitamins protects lipid structures against peroxidation (Prabu et al., 2011). Vit C guards against Cu toxicity by

preventing excess Cu absorption by minimizing copper absorption from the intestine by reducing soluble Cu- levels in the small intestine (Kies and Harms 1989; Van den Berg et al. 1994). In addition, Vit E can prevent cholesterol-related endothelial dysfunction, preventing functional impairment induced by ROS (Gaetke and Chow 2003).

Although the micronucleus assay was informative and its usefulness should be considered (Corona-Rivera et al. 2007), comet assay is a more sensitive method to assess genotoxicity (Zhong et al. 2001; Andrade et al. 2004). The realistic comet assay has been used to test genotoxic agents (Andrighetti-Fröhner et al. 2006). In collaboration with others, it is a sensitive and fast method for detecting DNA damage caused by trace metals, such as copper (Urbina-Cano et al. 2006).

Data from this research showed that CuSO<sub>4</sub> has the genotoxic ability to interact with DNA and induce mammalian cell alterations that was specified by elevation in comet %, %DNA in tail, tail length, tail moment and olive tail moment. Similarly, Saleha Banu et al. (2004) indicated a significant DNA damage with decrease in mean comet tail-length after adding CuSO<sub>4</sub>. DNA damage by Cu might be limited or not evident at low copper concentrations, as it is connected firmly bound in storage or transport proteins (like ceruloplasmin) and is subsequently not available for oxidation reactions (Nair et al. 2005), but at high concentrations free Cu can have an enormous genotoxic effect (Corona-Rivera et al. 2007). Free Cu causes ROS and many forms of DNA damage to be generated, such as base alteration and DNA strand breaks, which can cause massive cell death (Hayashi et al., 2000). A copper-induced high ROS production consequence in oxidative destruction to a single DNA base and sugar phosphate and breaks DNA strands (Bjelland and Seeberg 2003). In addition, copper reduces DNA-binding cell viability, resulting in cell death (Tchounwou et al. 2008).

The existing inquiry revealed that supplementation of CuSO<sub>4</sub>- intoxicated chickens with Vit C and Vit E alone or in combination exert a partial genoprotective effect against DNA damage induced by excessive concentrations of copper which was proved by the decrease of comet%, tail length and moment. A combination of vitamins is more effective in reducing genotoxic effects of copper than use of each vitamin alone. The genoprotective effect of Vit C and Vit E could act as free radical scavengers and antioxidants (Traber and Stevens 2011). These fallouts are analogous to those of Jiraungkoorskul and Sahaphong (2007) who demonstrated that [ascorbic acid](#) reduce the genotoxicity in fish induced by copper, and Assy et al. (2019) who exhibited a defending effect of Vit E (100 mg/kg diet) against DNA damage in rats with CuO NPs (250 mg/kg diet) toxicity. Similarly, Oner et al. (2019) stated that addition of Vit C and/ or Vit C + Vit E in water contained 150 ppm NaF to rats caused a reduction in DNA mutilation and 8-hydroxy-2'-deoxyguanine (8-OHdG) analysis. Also, our outcomes are in settlement with a new study reporting potential anticipatory effects of dietary antioxidants including ascorbic acid, vitamin E, polyphenols, phytosterols, and extracts from medicinal plants on vanadium-induced DNA damage (Zwolak 2020). Another inquiry exposed that exposure of mice to 390 ppm copper induced severe genomic cellular DNA damage that was in turn diminished by curcumin (Corona-Rivera et al. 2007).

Histopathological examination confirmed the aforementioned findings of  $\text{CuSO}_4$ -intoxicated chickens in the existing inquest and is in coordination with the previous report of Oguz et al. (2010), Shahzad et al. (2012), Wang et al. (2017) in chickens and Baruah et al. (2018) in ducks with copper toxicity. Similarly, another studies exhibited a significant hepatocyte vacuolar degeneration and inflammatory cell infiltration (Liu et al. 2018), severe microscopic changes, including vacuolar degeneration, local tissue necrosis, and blurred hepatic lobules in birds- supplemented with 300 mg Cu/kg diet (Zhou et al. 2020). And our results are different from others who displayed that birds fed high level of Cu diets did not change any tissue histologically (Jackson et al. 1979). Similarly, dietary supplementing of Cu (300 and 450 mg Cu/kg) as Cu proteinate resulted in no deviations in the liver of laying hens (Güçlü et al. 2008). These unsuited fallouts may be credited to variation in the types of test materials, experiment animals, and test duration. Long-term exposure of birds to high levels of Cu, hazardous impacts would occur (Zhou et al. 2020).

In contrast, the  $\text{CuSO}_4$ -intoxicated rats supplemented with Vit C and Vit E alone or in combinations reduced the histopathological findings with apparently normal liver architecture in some cases. Our results are supported with other finding presented that addition of vitamins (C&E) in feed has been successful in offsetting arsenite's toxic effects in broiler chicken (Khatun et al. 2020).

## Conclusions

In conclusion, long-term exposure to  $\text{CuSO}_4$  caused significant alterations the liver evaluation biomarkers, genotoxicity and histopathology. Dietary supplementation of Vit C and E showed a fall in the harmful effects induced by  $\text{CuSO}_4$ , especially with their combination which cause an improvement in the growth performance; return the levels of some biochemical parameters in close to normal values, with subsidence the histopathological changes and DNA degeneration. Overall, the protective roles of vitamins C & E and their synergistic action against the toxic effects of Cu are clearly seen in our research, but further studies are still needed to understand the complete potential of vitamins.

## Declarations

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## Author contribution

Mohamed A Hashem: supervision, methodology, investigation, writing (original draft, review, and editing).  
Sahar S. Abd El Hamied: supervision, conceptualization, data curation, methodology, and writing—review

and editing. Eman M.A. Ahmed: conceptualization, formal analysis, methodology, resources, and writing—review and editing.

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## Data Availability

All author stated that all information produced or analyzed during this work is included.

## Compliance with Ethical standards

### Ethical statements

The care and revival of the birds used was in consistent with the strategies in Animal Research Ethics Committee (AREC, Zagazig University, Egypt).

### Consent to participate

Not applicable.

### Consent for publication

Not applicable.

### Competing interests

The authors declare no competing interests.

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## Tables

**Table (1)**

**Composition of the basal experimental broilers diet (%)**

<b>Ingredients:</b>	<b>Starter (0–14 days)</b>	<b>Grower (15–28 days)</b>	<b>Finisher (29–42 days)</b>
Yellow corn	55%	59%	55.03%
* Soya	48%	44%	33.5%
* Soya oil	2.20%	1.7%	7.5%
* Bone meal	1.5 %	1.5 %	1.3 %
* Glycotein	62%	60%	
* Salt	0.42%	0.35%	0.4%
*monocalcium phosphate	1.60%		
*Dicalcium phosphate		1.8%	18%
* DL-Methionine	0.10 %	0.05 %	0.27 %
*L lysine HCL	0.10%	0.10%	0.10%
*Crude fiber	not more than 3.63%	not more than 3.58%	not more than 3.71%
* Crude protein	not less than 23%	not less than 21%	not less than 19%
* Crude fat	not less than 4.63%	not less than 4.52%	not less than 10%
* Calories	not less than 3027 kcal/kg	not less than 2950 kcal/kg	not less than 3228 kcal/kg

**Table (2)**

**Some liver enzyme activities and protein profile in all groups of chickens at the end of 3<sup>rd</sup> week (Mean  $\pm$ SE, n= 10).**

Groups	Parameters					
	ALT (U/L)	AST (U/L)	ALP (U/L)	TP (g/dl)	Albumin (g/dl)	Globulins (g/dl)
Gp.1 (Control)	9.30±0.99 <sup>c</sup>	150.5±10.9 <sup>d</sup>	620±71.2 <sup>c</sup>	3.99±0.20 <sup>a</sup>	1.99±0.13	1.70±0.08 <sup>a</sup>
Gp.2 (Cu)	21.60±6.14 <sup>a</sup>	193.7±9.2 <sup>a</sup>	727±58.3 <sup>a</sup>	3.33±0.18 <sup>b</sup>	1.83±0.15	1.50±0.09 <sup>b</sup>
Gp.3 (Cu+ Vit C)	16.60±4.41 <sup>b</sup>	177.9±5.6 <sup>b</sup>	711±99.2 <sup>a</sup>	3.52±0.07 <sup>b</sup>	1.84±0.15	1.68±0.12 <sup>a</sup>
Gp.4 (Cu+ Vit E)	15.60±4.41 <sup>b</sup>	171.8±7.4 <sup>bc</sup>	702±95.1 <sup>a</sup>	3.65±0.12 <sup>a</sup>	1.94±0.16	1.71±0.19 <sup>a</sup>
Gp.5 (Cu+ Vit C+ Vit E)	13.10±3.29 <sup>bc</sup>	162.6±13.9 <sup>cd</sup>	689±57.5 <sup>b</sup>	3.67±0.32 <sup>ab</sup>	1.95±0.15	1.72±0.13 <sup>a</sup>
F-test	*	*	*	*	NS	*
<p><i>All data having different letters are significantly different at p &lt; 0.05. NS: Non significant difference * : Significant at p &lt; 0.05. ALT: alanine aminotransferase, AST: aspartate aminotransferase, ALP: alkaline phosphatase, TP: Total Proteins.</i></p>						

**Table (3)**

Some liver enzyme activities and protein profile in all groups of chickens at the end of 6<sup>th</sup> week (Mean ±SE, n= 10).

Groups	Parameters					
	ALT (U/L)	AST (U/L)	ALP (U/L)	TP (g/dl)	Albumin (g/dl)	Globulins (g/dl)
Gp.1 (Control)	11.56±4.86 <sup>c</sup>	160.5±9.1 <sup>d</sup>	580±31.1 <sup>b</sup>	4.33±0.42 <sup>a</sup>	2.48±0.14 <sup>a</sup>	1.85±0.69 <sup>a</sup>
Gp.2 (Cu)	24.42±5.00 <sup>a</sup>	195.7±7.8 <sup>a</sup>	660.0±54 <sup>a</sup>	3.01±0.18 <sup>b</sup>	2.01±0.24 <sup>b</sup>	0.9±0.18 <sup>b</sup>
Gp.3 (Cu+ Vit C)	18.42±3.35 <sup>b</sup>	182.3±5.5 <sup>b</sup>	628±44 <sup>ab</sup>	4.15±0.36 <sup>a</sup>	2.27±0.19 <sup>ab</sup>	1.88±0.25 <sup>a</sup>
Gp.4 (Cu+ Vit E)	16.87±3.72 <sup>b</sup>	177.8 ±5.4 <sup>bc</sup>	616±55 <sup>ab</sup>	4.17±0.40 <sup>a</sup>	2.28±0.18 <sup>ab</sup>	1.89±0.24 <sup>a</sup>
Gp.5 (Cu+ Vit C+ Vit E)	15.25±3.12 <sup>bc</sup>	169.6±7. 6 <sup>cd</sup>	578±30 <sup>b</sup>	4.30±0.33 <sup>a</sup>	2.30±0.20 <sup>a</sup>	2.00±0.32 <sup>a</sup>
F-test	*	*	*	*	*	*
<p><i>All data having different letters are significantly different at p &lt; 0.05. *: Significant at p &lt; 0.05.</i></p> <p><i>ALT: alanine aminotransferase, AST: aspartate aminotransferase, ALP: alkaline phosphatase, TP: Total Proteins.</i></p>						

**Table (4)**

**Lipid profile in all groups of chickens at the end of 3<sup>rd</sup> week (Mean ±SE, n= 10).**

Groups	Parameters				
	TG (mg/dl)	TC (mg/dl)	LDL-C (mg/dl)	VLDL-C (mg/dl)	HDL-C (mg/dl)
Gp.1 (Control)	65.03±4.74 <sup>a</sup>	155.82±2.94 <sup>a</sup>	89.52±3.19 <sup>a</sup>	13.00±0.98 <sup>a</sup>	53.29±3.49
Gp.2 (Cu)	48.14±7.33 <sup>c</sup>	126.79±11.00 <sup>c</sup>	70.65±7.74 <sup>c</sup>	9.62±1.46 <sup>c</sup>	46.51±4.79
Gp.3 (Cu+ Vit C)	50.90±5.57 <sup>bc</sup>	130.48±6.75 <sup>c</sup>	72.86±5.53 <sup>c</sup>	10.17±1.11 <sup>bc</sup>	47.43±2.60
Gp.4 (Cu+ Vit E)	53.64±3.49 <sub>bc</sub>	138.92±5.51 <sup>bc</sup>	78.56±1.95 <sup>bc</sup>	10.72±0.69 <sup>bc</sup>	49.78±5.73
Gp.5 (Cu+ Vit C+ Vit E)	60.12±5.91 <sub>ab</sub>	148.48±5.78 <sup>ab</sup>	84.66±3.94 <sup>ab</sup>	12.02±1.18 <sup>ab</sup>	52.12±7.23
F-test	*	*	*	*	NS
<p><i>All data having different letters are significantly different at p &lt; 0.05. NS: Non significant difference * : Significant at p &lt; 0.05. TG: Triglycerides, TC: Total cholesterol, LDL-C: Low density lipoprotein-cholesterol, VLDL-C: Very low density lipoprotein-cholesterol, HDL-C: High density lipoprotein-cholesterol.</i></p>					

Table (5)

Lipid profile in all groups of chickens at the end of 6<sup>th</sup> week (Mean ±SE, n= 10).

Groups	Parameters				
	TG (mg/dl)	TC (mg/dl)	LDL-C (mg/dl)	VLDL-C (mg/dl)	HDL-C (mg/dl)
Gp.1 (Control)	67.96±2.32 <sup>a</sup>	166.68±7.77 <sup>a</sup>	98.75±6.50 <sup>a</sup>	13.58±0.46 <sup>a</sup>	54.33±1.91 <sup>ab</sup>
Gp.2 (Cu)	50.58±5.42 <sup>c</sup>	130.86±4.15 <sup>b</sup>	74.52±5.36 <sup>c</sup>	10.11±1.08 <sup>c</sup>	46.22±3.1 <sup>b</sup>
Gp.3 (Cu+ Vit C)	57.34±1.85 <sup>bc</sup>	135.95±4.51 <sup>b</sup>	79.38±4.59 <sup>c</sup>	11.46±0.36 <sup>bc</sup>	47.95±4.07 <sup>b</sup>
Gp.4 (Cu+ Vit E)	58.23±3.02 <sub>bc</sub>	141.36±7.65 <sup>b</sup>	81.88 ±4.50 <sup>bc</sup>	11.64±0.60 <sup>bc</sup>	50.16±8.55 <sup>bc</sup>
Gp.5 (Cu+ Vit C+ Vit E)	62.43±6.19 <sup>ab</sup>	157.40±5.92 <sup>a</sup>	91.51±7.34 <sup>ab</sup>	12.48±1.23 <sup>ab</sup>	53.41±4.67 <sup>ab</sup>
F-test	*	*	*	*	*

*All data having different letters are significantly different at p < 0.05. \*: Significant at p < 0.05.*

*TG: Triglycerides, TC: Total cholesterol, LDL-C: Low density lipoprotein-cholesterol, VLDL-C: Very low density lipoprotein-cholesterol, HDL-C: High density lipoprotein-cholesterol.*

**Table (6)**

**DNA degradation indices in all groups of chickens at the end of 3<sup>rd</sup> week (Mean ±SE).**

Groups	Parameters				
	Comet %	%DNA in tail	Tail length (Pixel)	Tail moment (Arbitrary units)	Olive tail moment
Gp.1 (Control)	13.38±1.17 <sup>c</sup>	1.09±0.03 <sup>e</sup>	1.14±0.02 <sup>c</sup>	0.01±0.006 <sup>e</sup>	0.15±0.05 <sup>c</sup>
Gp.2 (Cu)	21.19±1.13 <sup>a</sup>	3.14±0.06 <sup>a</sup>	2.06±0.09 <sup>a</sup>	0.06±0.002 <sup>a</sup>	0.35±0.06 <sup>a</sup>
Gp.3 (Cu+ Vit C)	19.38±1.05 <sup>ab</sup>	2.47±0.05 <sup>b</sup>	1.92±0.03 <sup>b</sup>	0.04±0.001 <sup>b</sup>	0.31±0.08 <sup>a</sup>
Gp.4 (Cu+ Vit E)	18.96±2.00 <sup>ab</sup>	1.90±0.04 <sup>c</sup>	1.90±0.02 <sup>b</sup>	0.03±0.001 <sup>c</sup>	0.25±0.05 <sup>b</sup>
Gp.5 (Cu+ Vit C+ Vit E)	17.50±2.62 <sup>b</sup>	1.34±0.06 <sup>d</sup>	1.88± 0.01 <sup>b</sup>	0.02±0.003 <sup>d</sup>	0.27±0.03 <sup>b</sup>
F-test	*	*	*	*	*
<i>All data having different letters are significantly different at p &lt; 0.05. *: Significant at p &lt; 0.05. Values are expressed as % of total counts in each assay. Each parameter was done in triplicate.</i>					

**Table (7)**

**DNA degradation indices in all groups of chickens at the end of 6<sup>th</sup> week (Mean ±SE).**

Groups	Parameters				
	Comet %	%DNA in tail	Tail length (Pixel)	Tail moment (Arbitrary units)	Olive tail moment
Gp.1 (Control)	14.56±1.69 <sup>c</sup>	1.25±0.03 <sup>e</sup>	1.99±0.08 <sup>e</sup>	0.02±0.009 <sup>e</sup>	0.21±0.03 <sup>c</sup>
Gp.2 (Cu)	25.90±2.14 <sup>a</sup>	3.47±0.06 <sup>a</sup>	3.00±0.10 <sup>a</sup>	0.10±0.017 <sup>a</sup>	0.42±0.03 <sup>a</sup>
Gp.3 (Cu+ Vit C)	23.95±1.50 <sup>ab</sup>	2.77±0.05 <sup>b</sup>	2.70±0.13 <sup>b</sup>	0.07±0.022 <sup>b</sup>	0.36±0.06 <sup>b</sup>
Gp.4 (Cu+ Vit E)	23.81±3.24 <sup>ab</sup>	2.07±0.04 <sup>c</sup>	2.50±0.10 <sup>bc</sup>	0.05±0.017 <sup>c</sup>	0.31±0.02 <sup>b</sup>
Gp.5 (Cu+ Vit C+ Vit E)	19.95±2.95 <sup>b</sup>	1.38±0.02 <sup>d</sup>	2.27±0.07 <sup>d</sup>	0.03±0.014 <sup>d</sup>	0.29±0.01 <sup>b</sup>
F-test	*	*	*	*	*
<p><i>All data having different letters are significantly different at p &lt; 0.05. *: Significant at p &lt; 0.05.</i></p> <p><i>Values are expressed as % of total counts in each assay. Each parameter was done in triplicate.</i></p>					

## Figures

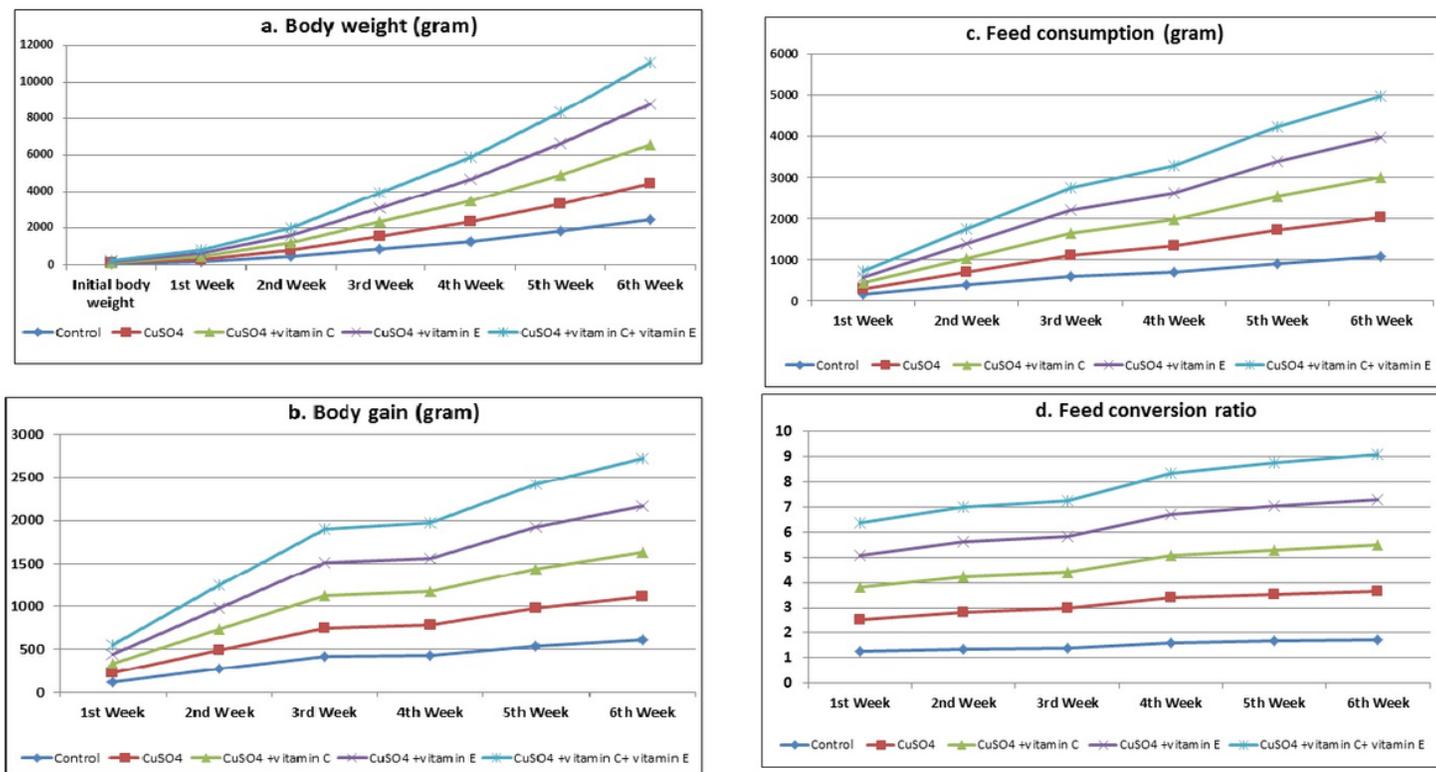


Figure 1

Effect of dietary copper and vitamins supplementation on a: body weight, b: body gain, c: feed consumption, d: feed conversion ratio in chickens of control (gp.1), Cu-intoxicated (gp.2), Cu+ Vit C (gp.3), Cu+ Vit E (gp.4) and Cu+ Vit C +Vit E (gp.5) groups for 6 weeks.

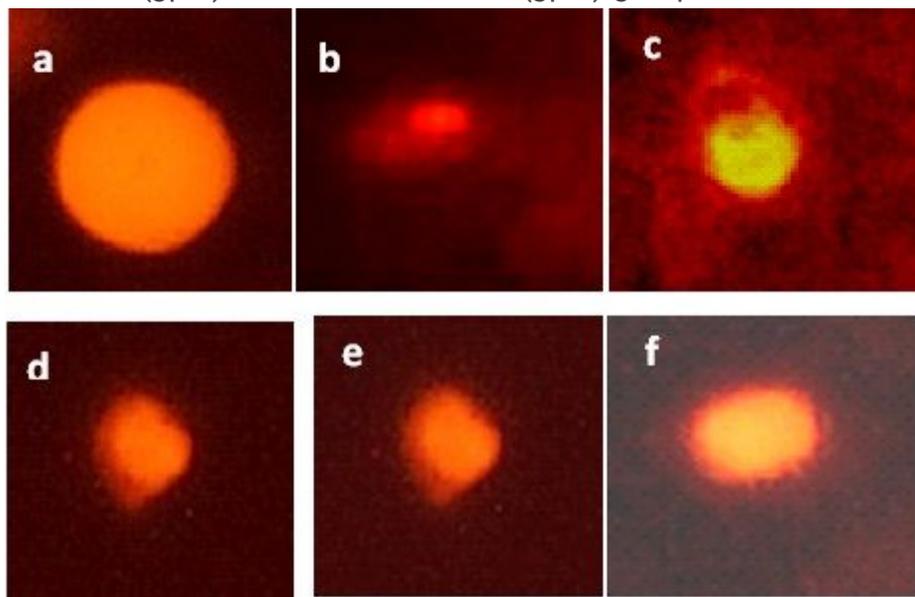
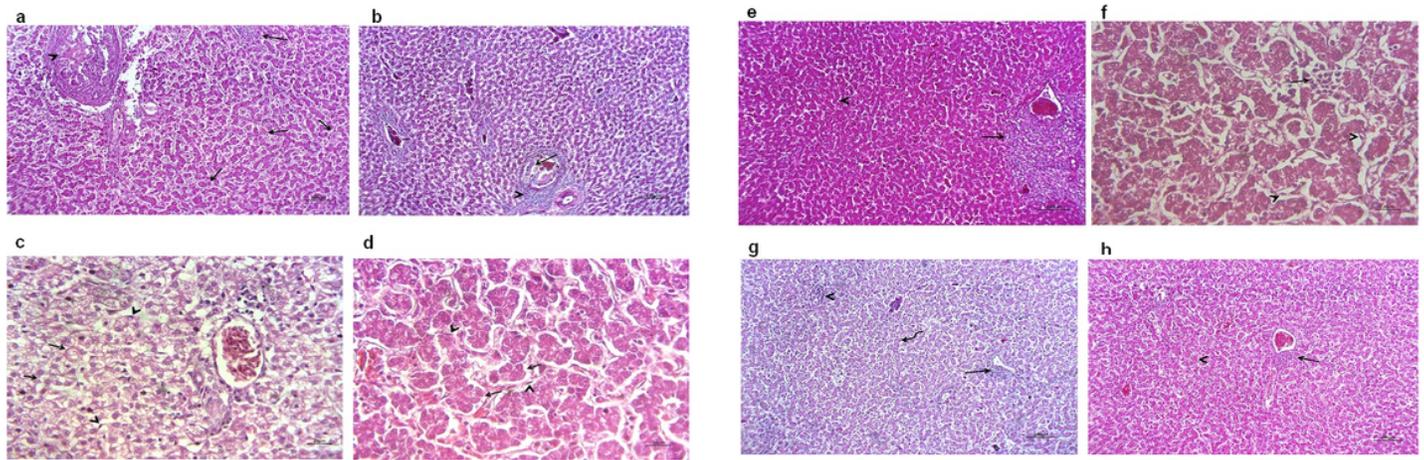


Figure 2

Electrophoresis of hepatic DNA samples of chicken from all experimental groups, using 1.2% ethidium bromide stained agarose (x 400). (a): Control group shows no DNA damage, as demonstrated by the lack

of DNA fragment migration away from the nucleus core. (b, c): Cu group shows a high degree of DNA damage, with a greatly reduced nucleus core and a large cloud of DNA fragments migrating away from the core forming the characteristic comet tail. (d): Cu +Vit C group shows a moderate degree of DNA damage. (e): Cu +Vit E group shows a moderate degree of DNA damage. (f): Cu+Vit C+ Vit E group shows a minimal degree of DNA damage.



**Figure 3**

(a-h):- Photomicrographs of liver sections for chickens of all experimental groups. H&E, x400. (a): Liver of Cu- intoxicated chicken (gp.2) showing hyperplastic, and necrotic biliary epithelium (arrow) with various degenerative and necrotic changes in the hepatic cells (arrow head) at the end of 3rd week. (b): Liver of Cu- intoxicated chicken (gp.2) showing cholestasis, necrotic bile duct epithelia (arrow), beside portal lymphocytic aggregation and fibroblast proliferation (arrow head) at the end of 6th week. (c): Liver of Cu+ Vit C supplemented chicken (gp.3) showing moderate swelling of hepatic cells (arrow) and hyperplastic kupffer cells (arrow head) at the end of 3rd week. (d): Liver of Cu+ Vit C supplemented chicken (gp.3) showing partially contracted hepatic cells (arrow) proliferative kupffer cells and dilated sinusoids (arrow head) at the end of 6th week. (e): Liver of Cu+ Vit E supplemented chicken (gp.4) showing portal lymphocytic aggregation (arrow) within apparently normal hepatic parenchyma (arrow head) at the end of 3rd week. (f): Liver of Cu+ Vit E supplemented chicken (gp.4) showing intense hyperplasia of kupffer cells (arrow head) and cloudy swelling of hepatic cells with a few lymphocytic aggregations (arrow) at the end of 6th week. (g): Liver of Cu+ Vit C+ Vit E supplemented chicken (gp.5) showing little portal (arrow) and interstitial lymphocytic aggregations within apparently normal hepatic parenchyma (arrow head) at the end of 3rd week. (h): Liver of Cu+ Vit C+ Vit E supplemented chicken (gp.5) showing mild portal lymphocytic aggregation (arrow) and normal hepatic parenchyma (arrow head) with dilated blood vessels at the end of 6th week.