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The Potential Ameliorative Effect of Nano-Zinc and Zinc Against Copper Hepatorenal Toxicosis in Rats

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ABSTRACT

This study was designed to evaluate the effect of Nano-Zinc (N-Zn) and Zinc (Zn) against copper (Cu) toxicosis in rats. Sixty male albino rats were randomly divided into 6 groups (n=10); control group, Cu-treated group: orally received $CuSo_4$ (100 mg/kg bwt/day), N-Zn-treated group: intraperitoneally (IP) injected with N-ZnO (5 mg/kg bwt/day), Zn-treated group: IP injected with ZnO (5 mg/kg bwt/day), Cu+N-Zn - and Cu+Zn -treated groups received the same previous doses and routes. All the experimental treatments were given 3 times / week and continued for eight weeks. Results showed that Cu- treated group caused a significant decrease in body weight and a significant increase in relative liver and kidney weights. Also, Cu caused a significant decrease in red blood cells (RBCs) count, hemoglobin (Hb) concentration, mean corpuscular hemoglobin concentration (MCHC) and platelets count with a significant increase in mean corpuscular volume (MCV) without any changes in leukocytic count. Biochemical analysis showed a significant elevation in serum liver enzymes, total and indirect bilirubin with a significant decrease in serum levels of total proteins and albumin. Also, there was a significant elevation in serum urea and creatinine levels. Cu induced a significant increase in renal and hepatic malondialdhyde (MDA) and a significant decrease in reduced glutathione (GSH). Histopathologically, Cu revealed severe degenerative and necrotic lesions in the livers and kidneys. N-Zn and Zn- treatments attenuated Cu-induced oxidative damage, alterations in the liver and kidney function tests and histopathology. Moreover, Zn treatment showed better protection against Cu-induced toxicity as compared to N-Zn.

1. INTRODUCTION.

Copper (Cu) is an essential element that is widely distributed in trace amounts in both animal and human tissues. It is required for many enzymatic reactions important to many living cells and for the functions of many proteins (Lutsenko, 2014 and Pal *et al.*, 2015) as cytochrome c oxidase (Johnson and Larry, 2008), lysyl oxidase, tyrosinase and Cu/Zn superoxide dismutase (SOD). Also, it is required for ceruloplasmin which helps to neutralize reactive oxygen species (Linder, 2010) and has the ability to oxidize Fe (II) and regulates iron efflux from certain cells (Vashchenko and MacGillivray, 2013). It was widely used in building materials, electronic industry water pipes, transportation sectors, wood preservatives, intrauterine contraceptive devices (Zhang *et al.*, 2012 and Roychoudhury and Massanyi, 2014). Copper sulphate ($CuSo_4$) is one of the most available salts of Cu that is present as blue, odorless crystals salt and enters in many products such as herbicides, fungicides and insecticides (Blundell *et al.*, 2003). It can be absorbed from the gastrointestinal tract and binds to albumin in portal circulation and then transported to liver where it enters in the synthesis of ceruloplasmin (Hassan *et al.*, 2010). Cu is a catalyst that leads to induction of oxidative stress and formation of reactive oxygen species (ROS) leading to cellular, DNA and lipid damage. The main target organs for Cu toxicity are liver and kidney leading to hepato-renal toxicity (Hashish and Elgaml, 2016). Zinc (Zn) is an essential metal which plays an important role in several biochemical and cellular functions (Fang *et al.*, 2002) as the stabilization of biological membranes and protecting

them from damage , preventing free radical formation, correcting the immunity, replication and transcription of DNA and intercellular signaling (Sandstead *et al.,* 2007 and Shah, 2011). Also, it can compete with Cu^{2+} and Fe^{2+} ions for binding to protein and cell membranes to displace these redox active metals which catalyze the production of OH from H_2O_2. It increases the activation of antioxidant molecules, proteins and enzymes such as reduced glutathione (GSH), catalase, SOD and decreases the activities of the oxidant enzymes (Prasad, 2014). Nano-Zinc Oxide (N-ZnO) is considered the third highest worldwide produced nano metal after nano SiO2 and nano TiO2 (Piccinno *et al.*, 2012) and the first nano scale material used in industrial products (Tan *et al.*, 2013). N-ZnO is commonly used in the food industry as dietary supplements, food additives, food packaging (Sharma *et al.*, 2012 and Seok *et al.*, 2013), fungicide (He *et al.,* 2011). It has one dimintion in the range of 1-100 nm so, this small size inceases the surface area and the permeability into cells more than the permeability of micro-scale Zn (Wang *et al.*, 2008 and Mironava *et al*., 2010). The permeability of N-ZnO helps to avoid the adverse gastrointestinal reactions and improve the absorption of medicine (Lucas, 2010). N-ZnO has antioxidant properties in broilers (Zhao *et al.*, 2014). But, other researches proved that higher usage of N-ZnO can increase recurrent exposure and the risk of toxicity through ingestion, inhalation or dermal contact during use, manufacture, and disposal (Balasubramanian *et* ***al.,*** 2010). Tang *et al.*, (2016) reported that excessive oral administration of N-ZnO to albino rats induced hepato-renal toxicity. Other studies in vivo and in vitro showed that N-ZnO have toxic effects as membrane damage, inflammation, DNA damage and apoptosis (Osman *et al.,* 2010 and Sharma *et al.*, 2011). This study aimed to evaluate the probable protective effect of N-Zn and Zn against Cu toxicosis in rats on the bases of oxidative stress, hematological and biochemical analyses as well as histopathology.

2. MATERIALS AND METHODS

2.1. Chemicals

Copper Sulphate pentahydrate ($CuSo_4.5H_2O$) and Zinc Oxide (ZnO) were purchased from Biotechnology Company, Cairo, Egypt and were manufactured by Alpha Chemika, Mumbai .Nano-Zinc Oxide (N-ZnO) was obtained in the form of dispersion (Sigma-Aldrich, Steinheim, Germany) with a concentration of 50 wt.% in H_2O, the average nanoparticle size was <35 nm, the particle size distribution (hydrodynamic diameter) was <100 nm using dynamic light scattering (DLS) technique, pH was 7±0.1(for aqueous systems) and density was 1.7 g/mL±0.1 g/mL at 25 °C**.**

2.2. Characterization and preparation of N-ZnO

The diameters of nanoparticles were detected less than 35 nm by Transmission electron microscope (TEM) as shown in Fig (1). N-ZnO was suspended in 0.9% NaCl and before usage, it dispersed by ultrasonication in ultrasonic bath for 15 minutes and to avoid the aggregation of particles before the usage (Wang *et al.*, 2006).

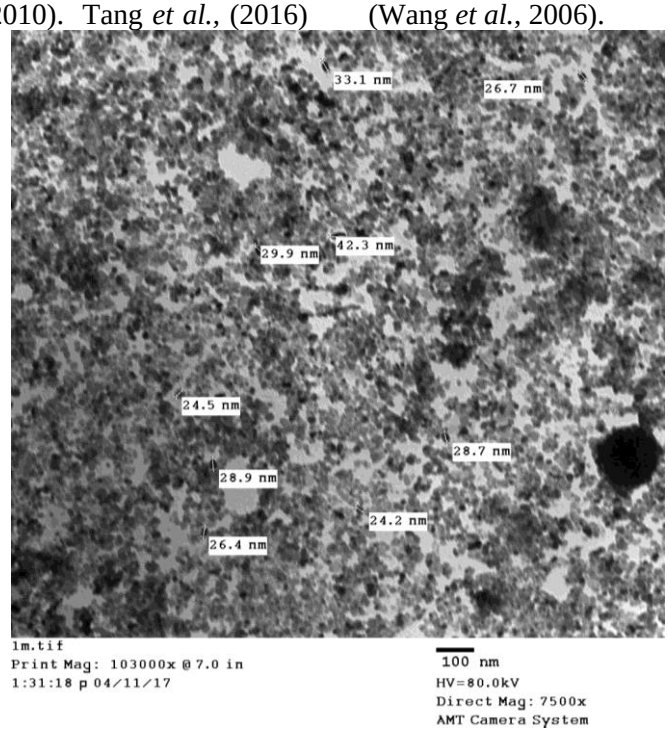

Fig. (1): Transmission electron microscope (TEM) image of N-ZnO dispersion showing particles size less than 35 nm.

2.3.Animals and experimental design

Sixty apparently healthy adult male albino rats (140 – 160 g bwt, 10-12 weeks of age), were purchased from a closed random bred colony at the Medical Research Institute of Alexandria University, Egypt. Rats were kept in separated clean metal cages and kept under constant environmental conditions. The rats received water and food *ad libitum.* All rats were acclimatized for three weeks before the beginning of the experiment for adaptation and make sure normal growth and behavior. Rats were randomly divided to 6 groups (10 rats each): the 1st group was kept as control group: received 0.5 ml/rat of distilled water (vehicle of $CuSo_4$) orally by gastric gavage and IP injected with 0.5 ml/rat saline. The 2nd group received $CuSo_4$ orally by stomach tube (100 mg/kg b.wt) (Akomolafe *et al*., 2014). The 3rd group rats were IP injected with N-Zn (5 mg/kg b.wt dispersed in 0.9% saline) (El-Masry *et al.*, 2015). The 4th group rats were IP injected with Zn (5 mg/kg b.wt) (Torabi *et al*., 2014). The 5th and the 6th groups treated with Cu+N-Zn and Cu+Zn, respectively at the same previous doses and routes. All experimental treatments were given 3 times /week and were continued for eight weeks.

2.4. Body Weight and relative organ weight ratio

Rats of each group were weighed weekly and the body weights were recorded. The liver and kidney weights were recorded after euthanasia and relative organ weights (ROW) ratio was calculated as follows: ROW= absolute organ weight (g)/final body weight (g) x 100 (Khatoon *et al*., 2016).

2.5. Blood sampling

Blood samples were collected from the retro-orbital venous plexus of each rat before euthanasia under light ether anesthesia. Two blood samples were collected from each animal. The first sample was collected on K_2 salt of EDTA as anticoagulant and used for estimation of hemogram and the second part of blood was placed in plain tubes, left in slope position at room temperature to clot and centrifuged for 15 minutes at 3000 rpm for separation of serum. The clear serum was separated carefully and collected into clean dry epindorffs and kept frozen at –20°C for biochemical analysis.

2.6. Hematological examination

The estimated parameters were: red blood cells (RBCs) count, hemoglobin (Hb) concentration, packed cell volume % (PCV), leukocytic count, and platelet count (Feldman *et al.,* 2000). The erythrocytic indices as mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were calculated according to Coles (1986).

2.7. Serum biochemical parameters

Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were measured colorimetrically according to the method described by Reitman and Frankel (1957) using kits from Bio-labo, France. Serum alkaline phosphatase (ALP) activity was measured kinetically according to Belfield and Goldberry (1971). Serum gamma glutamyle transferase (GGT) activity was estimated kinetically according to Szasz (1974). The level of serum total proteins (TP), serum albumin, serum total and indirect bilirubin, serum urea and creatinine were determined according to the methods of Doumas *et al*. (1981); Doumas (1971); Walter and Gerade (1970); Rock *et al*. (1987) and Fabiny and Ertingshausen (1971), respectively using commercial diagnostic kits supplied by Bio-diagnostic Co. Egypt .

2.8. Antioxidant status and oxidative stress assays

Small specimens of livers and kidneys of each rat was kept frozen at – 70°C for assessment of hepatic lipid peroxidation (LPO) contents and reduced glutathione (GSH) concentration. LPO was quantified as malondialdehyde (MDA) according to the method of Ohkawa *et al*. (1979). Moreover, assessment of GSH level spectrophotometrical was performed according to the method recorded by Sedlak and Lindsay (1968) using kits supplied by Bio-diagnostic co. Egypt.

2.9. Histopathologic studies

After necropsy, small specimens of liver and kidney were collected from all rat groups and rapidly fixed in 10% neutral formalin for at least 24 hrs. After fixation, tissue specimens were processed through the conventional paraffin embedding technique. Five µm thick sections were obtained from paraffin blocks, stained with hematoxylin and eosin (Bancroft *et al*., 2013) and examined under light microscope.

2.10. Statistical analysis

Data were statistically analysed using the Statistical Analysis System software (SAS, 2011). Effect of treatments on biochemical was performed by the analysis of variance. Means were compared using Duncan's Multiple Range test at a significance level of $P \leq 0.05$. Values are represented as means $\pm$standard errors.

3. RESULTS

3.1. Body weight and relative organ weight ratio

As shown in Table (1), there was a significant decline in body weights of Cu-treated rats followed by Cu+N-Zn- , Cu+Zn- and N-Zn- treated ones as compared to the control. Relative liver weight (RLW) ratio showed a significant increase in Cu-treated group followed by Cu+Zn- ,
N-Zn- and Cu+N-Zn- treated ones, respectively compared to the control. Relative kidney weight (RKW) ratio showed a significant increase in Cu-treated rats followed by Cu+N-Zn- treated ones as compared to control group. Other treated groups did not show any significant changes in body weight, RLW and RKW, comparatively to the control.

3.2. Hematological Examination:

As shown in Table (2), Cu- treated rats showed a significant decrease in RBCs count, Hb concentration, MCHC and platelets count with a significant increase in MCV if compared to control ones indicating macrocytic hypochromic anemia. However, other treated groups showed non-significant changes in RBCs count, Hb concentration, PCV %, erythrocytic indices and platelets count if compared to control group. All treated groups did not show any marked changes in WBCs count as compared to control one.

3.3. Serum biochemical parameters

As illustrated in Table (3), The serum ALT, AST, GGT and ALP activities were significantly increased in Cu-followed by N-Zn- then Cu+ N-Zn- and Cu+Zn- treated groups as compared to control one. Other treated groups showed non-significant changes in serum liver enzymes activities comparatively to control group. However, the serum TP showed a significant decrease in Cu-followed by N-Zn- and Cu+ N-Zn- treated groups if compared to control group. Also, the albumin level showed a significant decrease in Cu- and N-Zn- treated rats as compared to the control one. Other treated rats did not show any marked changes in TP and albumin levels as compared to control.

Table 1: Effect of copper sulphate ($CuSo_4$), Nano Zinc Oxide (N-ZnO) and Zinc Oxide (ZnO) administration alone or in combination for eight weeks on the body weight and relative organ weight ratio in male albino rats.

	Groups					
Parameter	control	$CuSo_4$	N-ZnO	ZnO	$CuSo_4$+ N-ZnO	$CuSo_4$+ ZnO
Body weight (gm)	322 ± 4.06a	197 ± 3.74d	277 ± 4.36b	299 ± 15.5ab	259 ± 3.32c	272 ± 5.83b
Relative liver weight (%)	2.50 ± 0.09c	3.80 ± 0.20a	3.06 ± 0.16b	2.74 ± 0.06bc	3.00 ± 0.23b	3.07 ± 0.15b
Relative kidney weight (%)	0.52 ± 0.04c	0.76 ± 0.05a	0.63 ± 0.02bc	0.59 ± 0.03bc	0.64 ± 0.05b	0.60 ± 0.02bc

$CuSo_4$= copper sulphate; N-ZnO = Nano Zinc Oxide; ZnO = Zinc Oxide.
Values are means ± standard errors. Means with different letter within the same row differ significantly. ($P<0.05$).

Table 2: Effect of copper sulphate ($CuSo_4$), Nano Zinc Oxide (N-ZnO) and Zinc Oxide (ZnO) administration alone or in combination for eight weeks on the hematological parameters in male albino rats.

	Groups					
Parameter	control	$CuSo_4$	N-ZnO	ZnO	$CuSo_4$+ N-ZnO	$CuSo_4$+ ZnO
RBCs (10^6/ µl)	8.07 ± 0.26a	6.79 ± 0.20b	7.72 ± 0.06a	7.78 ± 0.08a	7.85 ± 0.10a	8.00 ± 0.13a
Hemoglobin (g/dl)	14.5 ± 0.11a	12.0 ± 0.32b	13.9 ± 0.22a	14.5 ± 0.18a	14.0 ± 0.25a	14.4 ± 0.14a
PCV (%)	40.4 ± 0.51a	41.0 ± 1.05a	40.3 ± 0.77a	40.0 ± 0.71ab	40.8 ± 0.56a	40.6 ± 0.51a
MCV (fl)	50.2 ± 1.23b	60.8 ± 2.79a	48.9 ± 1.50b	51.4 ± 0.67b	52.0 ± 0.75b	50.8 ± 0.85b
MCH (pg)	18.1 ± 0.49a	17.7 ± 0.82a	18.1 ± 0.17a	18.6 ± 0.16a	17.9 ± 0.34a	18.0 ± 0.22a
MCHC (g/dl)	36.0 ± 0.26a	29.2 ± 0.92b	37.0 ± 1.29a	36.2 ± 0.25a	34.3 ± 0.36ab	35.6 ± 0.43ab
WBCs (10^3/µl)	12.9 ± 1.61a	14.1 ± 0.60a	12.3 ± 1.23a	14.3 ± 1.26a	13.1 ± 2.04a	15.7 ± 1.51a
Platelets (10^3/µl)	600 ± 11.22a	550 ± 10.00b	584 ± 5.78a	588 ± 3.28a	585 ± 4.63a	588 ± 3.51a

$CuSo_4$= copper sulphate; N-ZnO = Nano Zinc Oxide; ZnO= Zinc Oxide; RBCs, Red blood cells; PCV, Packed cell volume; MCV, Mean corpuscular volume; MCH, Mean corpuscular hemoglobin; MCHC, Mean corpuscular hemoglobin concentration; WBCs, White blood cells. Values are means ± standard errors. Means with different letter within the same row differ significantly ($P<0.05$).

Also, serum total bilirubin and indirect bilirubin levels showed a significant increase in Cu- followed by Cu+N-Zn- then Cu+Zn- and N-Zn- treated rats if compared to the control. The serum urea level showed a significant increase in Cu-followed by Cu+N-Zn- and N-Zn- and treated groups as compared to control one. The serum creatinine level showed a significant increase in Cu-followed by Cu+N-Zn and Cu+Zn- treated rats, comparatively to the control. Other N-Zn-and Zn- treated group did not show any significant alteration in the levels of serum urea and creatinine if compared to control ones.

3.4. Antioxidant status and oxidative stress assays

As recorded in Table (4), The concentration of hepatic MDA displayed a significant increase in Cu-followed by N-Zn- and Cu+N-Zn –treated groups as compared to the control. But the levels of renal MDA showed a significant increase in Cu- then N-Zn– treated rats. However, there were a significant decrease in the hepatic and renal GSH in Cu-followed by N-Zn- and Cu+N-Zn –treated groups if compared to control one. While, the other treated groups showed non- significant changes in the level of hepatic and renal MDA and the concentration of GSH.

Table 3: Effect of copper sulphate ($CuSo_4$), Nano Zinc Oxide (N-ZnO) and Zinc Oxide (ZnO) administration alone or in combination for eight weeks on serum biochemical parameters in male albino rats.

	Groups					
Parameter	control	$CuSo_4$	N-ZnO	ZnO	$CuSo_4$+ N-ZnO	$CuSo_4$+ ZnO
ALT (U/L)	30.7 ± 1.68d	56.8 ± 1.46a	40.4 ± 1.21c	28.5 ± 1.36d	49.2 ± 2.27b	40.0 ± 1.37c
AST (U/L)	105 ± 2.00e	175 ± 1.80a	146 ± 1.43d	103 ± 1.84e	164 ± 1.71b	152 ± 2.08c
GGT (U/L)	19.7 ± 0.82d	40.2 ± 1.71a	30.0 ± 1.33b	19.6 ± 0.85d	31.3 ± 1.86b	24.0 ± 1.30c
ALP (U/L)	130 ± 1.62e	206 ± 1.83a	146 ± 1.72d	128 ± 1.03e	196 ± 1.89b	187 ± 2.13c
Total protein (g/dl)	8.58 ± 0.14a	6.24 ± 0.22d	7.12 ± 0.24c	8.07 ± 0.18ab	7.72 ± 0.23b	8.08 ± 0.18ab
Albumin (g/dl)	4.14 ± 0.09a	2.86 ± 0.16b	3.26 ± 0.15b	3.78 ± 0.12ab	3.60 ± 0.18ab	3.84 ± 0.09ab
Total bilirubin (mg/dl)	0.35 ± 0.018d	0.79 ± 0.016a	0.51 ± 0.024c	0.35 ± 0.036d	0.73 ± 0.042ab	0.67 ± 0.023b
In Direct bilirubin (mg/dl)	0.20 ± 0.007c	0.43 ± 0.011a	0.30 ± 0.016b	0.23 ± 0.024c	0.43 ± 0.022a	0.40 ± 0.017a
Urea (mg/dl)	27.1 ± 2.07c	47.8 ± 1.47a	36.2 ± 1.21b	27.2 ± 0.99c	37.6 ± 1.07b	28.6 ± 1.16c
Creatinine (mg/dl)	0.41 ± 0.014d	0.74 ± 0.014a	0.43 ± 0.015d	0.41 ± 0.012d	0.69 ± 0.012b	0.60 ± 0.027c

$CuSo_4$= copper sulphate; N-ZnO = Nano Zinc Oxide; Zinc Oxide; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; GGT, Gamma glutamyle transferase.
Values are means ± standard errors. Means with different letter within the same row differ significantly ($P<0.05$).

Table 4: Effect of copper sulphate ($CuSo_4$), Nano Zinc Oxide (N-ZnO) and Zinc Oxide (ZnO) administration alone or in combination for eight weeks on **hepatic and renal lipid peroxidation** and **antioxidant biomarkers** in male albino rats.

	Groups					
Parameter	control	$CuSo_4$	N-ZnO	ZnO	$CuSo_4$+ N-ZnO	$CuSo_4$+ ZnO
Hepatic MDA (nmol/g wet tissue)	9.54 ± 0.71c	19.3 ± 0.89a	15.8 ± 0.95b	9.30 ± 0.43c	13.0 ± 1.07b	8.03 ± 1.25c
Hepatic GSH (µmol/g wet tissue)	4.83 ± 0.17a	1.33 ± 0.17d	2.17 ± 0.17c	4.83 ± 0.33a	3.00 ± 0.29b	4.47 ± 0.29a
Renal MDA (nmol/g wet tissue)	12.9 ± 1.35b	24.2 ± 2.11a	22.6 ± 1.98a	12.6 ± 1.37b	16.5 ± 5.53ab	10.0 ± 0.85b
Renal GSH (µmol/g wet tissue)	3.67 ± 0.17a	1.33 ± 0.17c	2.17 ± 0.17b	3.17 ± 0.17a	2.40 ± 0.32b	3.43 ± 0.23a

$CuSo_4$= copper sulphate; N-ZnO = Nano Zinc Oxide; ZnO = Zinc Oxide; MDA= Malondialdhyde GSH=Reduced glutathione, SOD= Superoxide dismutase .Values are means ± standard errors. Means with different letter within the same row differ significantly ($P<0.05$).

3.5. Histopathological results:

Liver

Livers of control rats and Zn- treated ones exhibited normal histological appearance of the blood vessels and hepatocyte which arranged in radiating rows around the central veins (Fig.2a). The liver of Cu-treated rats showed wide spread of degenerated hepatocytes with severe cytoplasmic vacuolation, periportal and mid-zonal necrosis associated with severe mononuclear cell infiltration and severe vascular congestion (Fig. 2b). Furthermore, the portal areas appeared thickened with obvious connective tissue proliferation, formation of newly formed bile ducts and intensive mononuclear inflammatory cell infiltration mainly lymphocytes. Portal to portal bridging necrosis was evident with absence of hepatocytic vacuolation. The co-treatment with N-Zn showed some hepato-protective effects as there were medium-sized areas of hepatic necrosis associated with moderate mononuclear cell infiltrates with absence of hepatic vacuolation (Fig. 2c). But, the co-treatment with Zn showed more hepato-protective than those of N-Zn treated as there was periportal hepatocytic vacuolation besides minute areas of hepatocytic necrosis that was associated with mild mononuclear cell infiltrate. (Fig.1d). N-Zn treatment revealed mild hepatocytic necrosis with mild mononuclear cell infiltration. Also, thickening in portal area with newly formed bile ducts was evident (Fig. 2e).

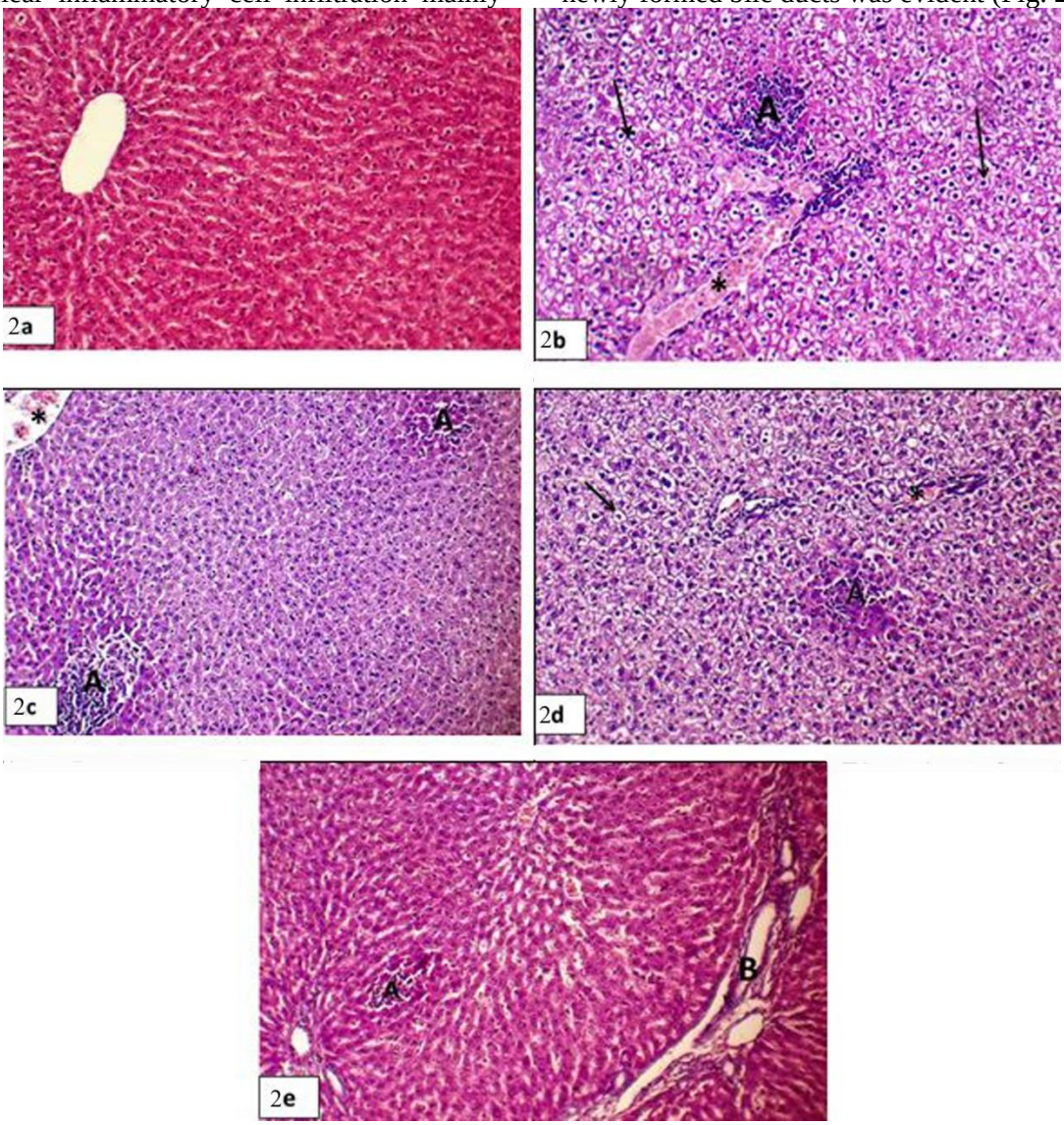

Fig .2. Photomicrograph of rat liver stained with H&E X100: (2a) normal histological structure of liver of control rats. (2b) liver of Cu-treated rats exhibited diffuse cytoplasmic vacuolation(arrows), hepatic necrosis associated with severe mononuclear cell infilterates (A) and severe congestion (asterisk). (2c) liver of Cu+N-Zn- treated rats showed moderate hepatocytic necrosis (A) and congestion (asterisk). (2d) liver of Cu+ Zn treated group showed mild vacuolation (arrow) associated with mild hepatocytic necrosis (A) and mild congestion (asterisk). (2e) liver of N-Zn- treated rats showed mild hepatocytic necrosis and mild inflammatory cell infilterates (A) with biliary hyperplasia (B).

Kidneys

The kidney of control rats and Zn- treated ones exhibited normal histological appearance of renal parenchyma and glomeruli (Fig. 3a). The kidney of Cu-treated rats exhibited severe tubular degeneration represented by cloudy swelling of renal tubular epithelium, besides tubule necrosis that was accompanied by mononuclear cell infiltrates and mild fibroplasia. Sometimes the interstitium showed severe inflammatory cells infiltrates and severe fibroplasia in the renal cortex. Also, there were severe congestion of the inter-tubular blood vessels and perivascular edema (Fig. 3b). Similar picture was evident in kidneys of Cu+N-Zn treated rats but to lesser extent. As, kidneys did not show fibroplasia, but there were cloudy swelling in renal tubules and interstitial nephritis (Fig. 3c). While, the co-treatment with Zn showed marked protection that was indicated by presence of mild congestion of the inter-tubular capillaries and mild perivasculitis with presence of hyaline cast within the cortical tubular lumen (Fig. 2d). N-Zn treatment exhibited mild to moderate tubular necrosis with mononuclear cells infiltration and congestion of inter-tubular blood vessels (Fig. 2e).

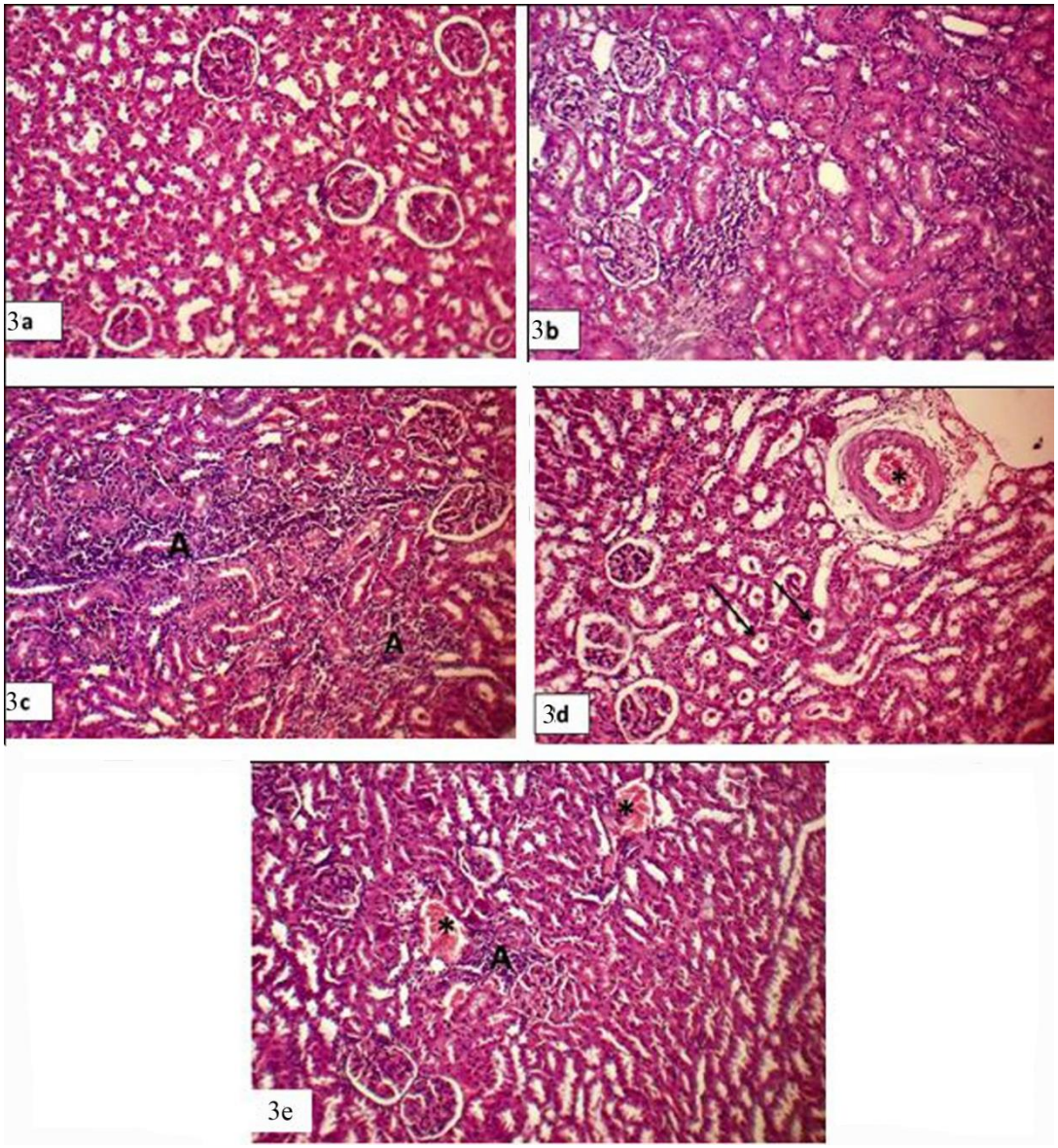

Fig .3. Photomicrograph of rat kidney stained with H&E with X100: (3a) of control group showed normal histological structure of renal tissue. (3b) kidney of Cu- treated rats exhibited cloudy swelling (arrow) and interstitial nephritis with mononuclear cell infiltrates (A). (3c) Kidney of Cu+ N-Zn- treated rats showed interstitial nephritis with mononuclear cell line infiltrates (A). (3d) Kidney of Cu+ Zn-treated rats showed tubular casts (arrows) and severe congestion (asterisk).(3e) kidney of N-Zn- treated rats showed interstitial nephritis associated with mild inflammatory cell infiltrates (A) and mild congestion(asterisks).

5. DISCUSSION.

Cu is very important for normal physiological and biological functions in humans and animals (Uauy *et al.,* 1998). The excessive exposure of cu increased induces adverse toxic effects such as hemolysis, gastrointestinal distress and hepato-renal damage (Björn *et al.,* 2003 and Galhardi *et al.,* 2004). In the present study, administration of $CuSo_4$ showed a significant decline in the body weight. This result comes in agreement with Paumen *et al.* (2008) and Mohammed *et al.* (2014). This reduction may be due to improper metabolism to food due to ulcerative enteritis induced by Cu as $Cuso_4$ is considered a powerful oxidizing irritant to mucous membranes of small intestine and stomach (Oldenquist and Salem, 1999). Also, RLW and RKW exhibited a significant increase in Cu- treated rats and this may be due to the histopathological changes in liver and kidney tissues. This agreed with lee *et al.* (2016) who demonstrated that CuNps (single oral dose, 1250 mg/kg b.wt) resulted in a significant decrease in the body weight and increase liver and kidney weight of male rats. N-Zn-treated rats also showed a significant decrease in the body weight with marked increase in RLW ratio. This came in agreement with Wang *et al.* (2004) who investigated that N-Zn treated mice exhibited significant growth retardation by administration of (single gastrointestinal dose level of 5 gm of N-Zn/kg) due to the toxic effect of N-Zn. The increase in RLW may be due to histopathological changes in liver of N-Zn-treated rats. This came in harmony with Kim et al. (2014) who reported a significant increase in RLW of male rats that orally intoxicated with N-Zn (500mg/kg bwt) for 90 days. This disagreed with El-Sammad *et al.* (2014) who used Zinc Oxide / Ascorbyl Palmitate Nano-Composite orally (25 mg/kg b.wt for 5 days) and showed no effect on RLW of treated rats and this may be due to different nano-structure and dose. Zn plays important roles in nutrients metabolism and as a component of numerous metalloenzymes and transcription factors (Hambidge, 2000. Also, it is essential for normal function of the immune system, growth and bone development (Prasad and Elumalai, 2011). So, the mixed treatment of Cu- treated rats either with Zn or N-Zn partially restored the body weights and relative organ weights but they were significantly differed if compared to the control. These results were in accordance with Babaknejad *et al.* (2015) and Mesquita *et al.* (2016).

Treatment with $CuSo_4$ induced a significant decrease in RBCs count and Hb concentration in male rats. This reduction could be attributed to hemolysis of RBCs (Akomolafe *et al.,* 2014) .When copper concentration elevates, the liver starts to lose its ability to sequester cu. So, the sequestered cu becomes free in the liver cytoplasm leading to hepatic necrosis and escapes to the circulation where it stimulates hemolytic anemia by its oxidant effect which damages cell membranes of RBCs (Mendel *et al*., 2007). The intravascular hemolysis is one of the main hematological changes of $CuSo_4$ toxicity (Sarvu *et al.,* 2007) while, the hemolysis occurred due to the inhibition of Glucose-6-phosphate dehydrogenase enzyme by cu and decreases the level of NADPH in RBCs. Glucose-6-phosphate dehydrogenase plays an important role in maintenance of the level of NADPH (Joshi *et al.,* 2002) that is important for protection of RBCs against any oxidizing agent by maintenance the level of reduced glutathione (Pamila *et al.,* 1991). Our results also, exhibited a significant increase in MCV and a significant decrease in MCHC by Cu and this associated with the hemolytic anemia due to reticulocytosis (Walters and Abelson, 1996). This result is similar to the result of Youssef (2009). Also, the cu toxicity induced a significant decrease in platelets count and production. Thrombopoietin (TPO) is a protein that regulates platelet production, differentiation and maturation and mainly synthesized in the liver (Stoffel *et al.,* 1996). The cu toxicity resulted in liver damage so; the production of TPO decreased leading to thrombocytopenia (Mitchell *et al.,* 2016). In parallel, Atta *et al*. (2011) reported that administration of copper pyrazinate (4 mg/kg b.wt) for 6 weeks caused a significant decrease in platelets count. The co-treatment of Cu intoxicated rats with N-Zn and/ or Zn corrected the anemia induced by cu by restoring RBCs count, Hb concentration, MCV, MCHC and platelets count to normal levels. This agreed with Tizhe *et al.* (2013) who reported that Zn modulated the parameters of anemia induced by an herbicide glyphosate. This could be attributed to that Zn serves as a cofactor of many enzymes and has antioxidant effects (Powell, 2000). Another explanation was documented by Mansour *et al.* (2010) who reported the ability of Zn to regenerate hepatocytes against chlorpyrefos toxicity as increases the efficiency of liver for production of TPO and regulates the platelets production to the normal level.

Regarding to biochemical results, our results showed that the administration of $CuSo_4$ to male rats resulted in a significant increase in serum hepatic enzymes (AST, ALT, ALP and GGT), total and indirect bilirubin with a significant decrease in total protein and albumin. Hepatic marker enzymes increase in hepatic damage due to the increase of the cell membrane permeability and leakage of these enzymes into blood stream. It was reported that $CuSo_4$ can induce hepatic toxicity (El-Masry, 2012),

Mohammed *et al.* (2014) and Hashish and Elgamal (2016). The elevation in the serum total bilirubin concentration may occur as a result impairment of hepatic bile flow leading to bile retention. Furthermore, the hemolysis of erythrocyte or alteration in bilirubin metabolism may lead to increased production of serum total bilirubin and indirect bilirubin (Hoffmann *et al.,* 1999 and Meyer and Harvey, 2004). Also, returned to the inability of liver to convert the indirect bilirubin to direct one due to Cu toxicity. This came in agreement with Youssef (2009) and Kumar *et al.* (2015).). Liver has an important role in synthesis of most serum proteins particularly albumin and most of the globulins (Thapa and Walia, 2007). Hypoproteinemia may be induced due to hepatocellular injury that was caused by $CuSo_4$ toxicity which leads to the disturbance of protein production and metabolism (El-Masry, 2012). Urea and creatinine are the most sensitive markers for the assessment of renal function because they are mainly excreted by the kidneys. BUN and creatinine were significantly elevated in the rats exposed to $CuSo_4$ suggesting kidney dysfunction. This agreed with Akomolafe *et al.* (2014) and Kumar *et al.* (2015). The obtained serum biochemical results of liver and kidney were confirmed by our histopathological findings in liver and kidney of Cu - intoxicated rats. The liver of intoxicated rats with Cu showed cytoplasmic vacuolar, periportal and mid-zonal necrosis associated with severe mononuclear cell infiltration with vascular congestion. Also, the portal areas exhibited biliary hyperplasia with mononuclear cell infilteration. Histopathologically, the kidneys showed severe tubular necrosis with renal casts and severe interstitial nephritis associated with inflammatory cells infiltration. Similar results were reported by Hashish and Elgaml (2016). The N-Zn treated rats also, showed a significant increase in serum hepatic enzymes (AST, ALT, ALP and GGT), total and indirect bilirubin with a significant decrease in total protein and albumin. This may be due to the hepatic damage induced by N-Zn that increased the cell membrane permeability and leakage of these enzymes into blood stream. The increase in liver enzymes agreed with Esmaeillou *et al.* (2013) and Tang et al. (2016). In our opinion, the increament in bilirubins is associated with the impairment of bile flow due to liver damage caused by N-Zn. These results for bilirubins disagreed with Lee et al. (2016) who recorded a significant decrease in serum levels of bilirubins in pregnant female rats (intravenously injected with N-Zn, 20 mg/kg b.wt). This deviation may be due to variation of species, dose and rout of administration. The role of N-Zn in hepatotoxicity led to decrease the synthesis of TP and albumin. This came in accordance with Hejazy and Koohi (2017) who reported a significant decrease in TP and albumin after daily oral administration of N-Zn (3, 10, and100 mg/kg b.wt for 28 days). Moreover, N-Zn treated rats showed a significant increase in serum urea due to its ability to induce renal damage that was exhibited by histopathological lesion in rat kidneys. These results came in accordance with Faddah *et al.* (2012) and Hejazy and Koohi (2017). The co-treatment of Cu-treated rats with N-Zn and/ or Zn lesser the changes induced by Cu toxicity in liver and kidney. This protection indicated by decreasing the activity of hepatic enzymes, bilirubins, urea, creatinine and increasing the level of TP and albumin if compared to the Cu-treated rats. This may be due to the ability of Zn to regulate the cellular glutathione that is important for cellular antioxidant defence (Parat *et al*., 1997). Also, its ability to interact with cell membranes to stabilize them against various damaging effects (Bettger and O'Dell, 1981). These results came in agreement with Abo- Ghanema *et al.* (2016) who proved that Zn succeeded to decrease the toxic effects of thioacetamide (TAA) by decreasing the levels of (AST, ALT, ALP and GGT) in addition to, increasing the levels of TP and albumin as compared to TAA treated group. Also, Omar *et al.* (2016) recorded that co-treatment of lithium intoxicated rats with Zn returned the plasma levels of urea and creatinine to normal levels as compared to lithium treated group. The protective effects of the co-treatment with N-Zn or Zn were supported by the histopathological examination of liver and kidney. Oxygen free radicals are recognized to be cytotoxic to cells. During oxidative stress, reactive oxygen species such superoxide anion ($O^{-}{}^{2}$), hydroxyl radicals (OH) and hydrogen peroxide (H_2O_2) can prompt extensive cellular damage by lipid peroxidation and producing MDA .This depends on chain breaking antioxidant enzymes SOD and GSH (Novelli *et al.,* 2002; Diniz *et al.,* 2003). GSH catalyzes the conversion H_2O_2 to water. SOD catalyzes the destruction of O_2 and protects various organs from LPO and the damage induced by ROS (Weydert and Cullen, 2010). In the present study, the results exhibited a significant increase in hepatic and renal MDA with a significant decrease in hepatic and renal GSH in Cu -treated group. These results may be due to the ability of copper to form toxic oxygen radicals and induce lipid peroxidation (Weckx. and Clijsters 1996). Also, copper is able to increase the activity of lipooxygenase enzyme that catalyze lipid peroxidation mainly of unsaturated fatty acids leading to formation of various radicals increasing the concentration of MDA. MDA is an indicator of oxidative stress after heavy metals treating and the

increase level is associated with the increase of metal concentrations (Wu *et al.,* 2003). The increased oxygen metabolites and free radicals decrease in the activity of the antioxidant defense enzymes (Kusal *et al.,* 2001). The obtained results came in accordance with Mohammed *et al*. (2014) who reported a significant increase in hepatic MDA and a significant decrease in GSH after excessive administration of $CuSo_4$. Also, came in the same line with Hashish and Elgamal (2016) who recorded a significant increase in hepatic and renal MDA with a significant decrease in GSH. Likewise, our results exhibited that N-Zn treated rats showed a significant increase of renal and hepatic MDA with a significant decrease in GSH and SOD. This may be attributed to cytotoxicity produced by N-ZnO that depends on the extent of their interaction with cellular membranes (Vandebriel and De Jong, 2012). N-ZnO may induce the formation of highly ROS including H_2O_2, OH, and O^2 which can induce oxidative damage to cells (Sharma *et al.*, 2012). This agreed with Faddah *et al.* (2012) and Al Rasheed *et al.* (2012) found a significant decrease in renal GSH after administration of N-ZnO. On the other hand, our results showed that the co-administration of N-Zn or Zn with Cu resulted in a significant decrease in hepatic and renal MDA and a significant increase in GSH. This approved the antioxidant and free radical scavenging properties of both substances. As, Zn has antioxidant effects (Bray and Bettger,1990). In adding, it protects sulphadryle groups against oxidation and prevents ROS production (Gurer and Ercal, 2000). Zinc is also known to induce synthesis of metallothioneins that able to bind to and chelate with toxic metals (Flora *et al.* 1998). Our results came in accordance with El-Sammad *et al.* (2014) who investigated that the pre- and post-treatment with ZnO/ Ascorbyl Palmitate Nano-Composite decreased the cadmium induced oxidative stress by decreasing the hepatic and renal MDA levels and increasing GSH activities. Also, this agreed with Modi *et al.* (2006) who reported that Zn (orally 5 mg/kg b.wt of rats for 3 weeks) increased hepatic GSH that reduced by arsenic toxicity and Omar *et al.* (2016) who proved the antioxidant role of Zn by restoring the levels of renal MDA and the activities of GSH that changed by lithium toxicity to be within normal levels. The co-treatment with Zn showed more protection because it returned the parameters of oxidative stress to normal levels but, the N-Zn caused partial protection. The liver appeared with moderate to mild hepatic necrosis and mild inflammatory cells infiltration. Also, the kidney showed less tubular necrosis and congestion with mild mononuclear cell infiltration when compared to Cu -treated rats. This came in harmony with Omar et al. (2016) who revealed the role of zinc in the improvement of the structure of liver and kidney and restoring the altered hepatic and renal architecture by lithium toxicity. From the previous results, we concluded that Zn is more hepato-renal protective than N-Zn. This may be due to the characters of the nanoparticles (NPs) that are less stable and easily to aggregate in suspension due to high surface area, small size and high surface energy (Moos et al., 2010). Toxicity of N-Zn is higher than their bulk because of dissolution of NPs is more if compared to their bulk and producing many free Zn^{+2}(Chang et al., 2012). Very small particles have the ability to enter, translocate and damage the living organisms. This small size allows them to penetrate physiological barriers and pass to the circulatory systems (Wang et al., 2007) and causing cellular damage.

It could be concluded that Cu induced severe hepato-renal damage in rats. Zn treatment has more protective effects against Cu induced toxicity than N-Zn.

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