

## Acute toxicity and biodistribution of different sized copper nano-particles in rats after oral administration

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### ABSTRACT

In order to compare the detailed toxicity of nano-copper and  $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$  (Cu ions) *in vivo*, the oral toxicity of four differently sized Cu particles (30 nm, 50 nm, 80 nm and 1  $\mu\text{m}$ ) on rats was investigated compared with  $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$  in acute exposure scenarios. We compared the acute  $\text{LD}_{50}$  values and evaluated the kinetics of Cu following a single equivalent dose (200 mg/kg) of five Cu materials. Continuous gavage of nano-copper for 7 days, the mortality rates, relative organ weights, and hematological, biochemical, and histopathologic characteristics of rats were examined. The results showed that the  $\text{LD}_{50}$  values of Cu ions, 30 nm, 50 nm, 80 nm, and 1  $\mu\text{m}$  copper particles were 359.6, 1022, 1750, 2075, and  $> 5000$  mg/kg, respectively. Physiological and biochemical indexes indicated that 80 nm nano-copper (Cu NPs) produced the highest degrees of toxicity in short term. The liver and kidneys were the major organs most affected by Cu NPs, and also the target organs for Cu accumulation. The toxic effects of Cu ions are similar to those of nano-copper, but they were not the same. Therefore, the toxic effect of nano-copper is likely to be the result of the dual action of nano-copper particles and copper ions. Collectively, the acute toxic effects produced by Cu NPs were highly correlated with particle size. Moreover, the toxic effects produced by repeated dosing differed from those produced by a single dose, and this may be due to organ targeting effects that are dependent on the size of the nano-particles.

### 1. Introduction

In recent decades, nano-materials have attracted greater interest from scientists and technologists due to their special chemical properties and biological characteristics [1], and are now being used for industrial, agricultural, environmental, and biomedical applications [2–5]. Nano-materials have unique optical properties, good biocompatibility, and their surfaces are amenable to modification [6,7]. Scientists are also fascinated with their potential biomedical applications in the photothermal treatment of cancer, biosensing, gene and drug delivery, and biological imaging. However, as we are increasingly exposed to nano-materials either directly during manufacturing processes or indirectly via the environment and food chain, greater attention must be paid to their potential adverse effects on health [8–10].

The toxicity of nano-materials is closely related to their dose, size, surface-area, concentration, chemical properties, and crystalline structure [11,12]. When rats were intravenously injected in the tail vein with gold nanoparticles of 10, 50, 100, or 250 nm in diameter, only the 10 nm particles were found in their blood, liver, spleen, kidney, testis,

thymus, heart, lungs, and brain, whereas the larger particles were only detected in their blood, liver, and spleen, suggesting that the tissue distribution of gold nanoparticles was size-dependent [13]. When rats were intravenously injected with Au-198 radio-labelled monodisperse, negatively charged gold nanoparticles of five different sizes (1.4, 5, 18, 80, and 200 nm) or 2.8 nm gold nanoparticles with opposite surface charges, 50% of the 1.4 nm gold nanoparticles and  $> 99\%$  of the 200 nm gold nanoparticles were found in the liver. Moreover, the oppositely charged 2.8 nm gold nano-particles displayed significantly different accumulations in several organs, indicating that the distribution of gold nano-particles depended on both their size and surface charge [14]. Furthermore, the lifespan of the 50 nm gold nano-particles was significantly longer than that of the 10 nm gold nano-particles [15]. When PC12 cells were treated with different concentrations (0, 1, 10, 30, and 100  $\mu\text{g}/\text{mL}$ ) of Cu NPs, the oxidative stress increased in parallel with particle diameter [16], suggesting that the toxicity of the nanoparticles was related to their physical properties.

The toxicity of nano-materials is also related to their physiological absorption, distribution, metabolism, and excretion. The most common

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routes of administration used to test these properties are inhalation, intragastric administration, and intravenous injection, and the different exposure routes can lead to significantly different toxic effects. Rats given a short-term inhalation exposure to 15 nm diameter silver nanoparticles experienced some moderate pulmonary toxicity, which manifested as an influx of neutrophils into their lungs, accompanied by a 2-fold increase in cellular damage markers and increased concentrations of pro-inflammatory cytokines in their lungs [17]. In contrast, rats given an oral gavage dose of 100 nm silver nanoparticles displayed signs of liver and kidney damage [18]. The different types of toxicity may be caused by different degrees of nanoparticle ionization in the lungs and stomach, resulting in different nano-particle kinetic characteristics *in vivo* [19].

Copper nanoparticles have shown great promise as antibacterial materials, livestock and poultry feed additives, and catalytic agents [20–23]. However, there is only limited data concerning how their dose, size, and biological distribution affect their toxicity. This study investigated how the size and dose of Cu NPs affected their tissue distribution and toxicity in rats.

## 2. Materials and methods

### 2.1. Test materials and their characterization

The tested Cu NPs (10–30 nm, lot: D1614131; 50 nm lot: F1616051, 80–100 nm lot: H1605061, Aladdin Industrial Corporation, Shanghai, China) were compared with copper ions ( $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ , lot: F1620012) manufactured by Aladdin Industrial Corporation (Shanghai, China). The size and shape of the Cu NPs were characterized with a scanning electron microscope (Phenom ProX, Nani Scientific Instruments LTD, Shanghai, China).

The Cu NPs were added to purified water to obtain a stock suspension of 10 g/L, which was then shaken and sonicated for 5–6 h in a sonication ice bath. The distribution of particle sizes in this suspension, as indicated by its polydispersity index, was characterized during dynamic light scattering studies performed with Zeta sizer Nano ZS (Malvern Instruments, Malvern, UK) immediately after sonication.

### 2.2. Animals

The protocols for all animal studies were reviewed and approved by the Animal Ethical Committee of Sichuan Agricultural University (No. 20160513). Healthy male Sprague Dawley rats (aged 8 weeks; 100–120 g) were purchased from Chengdu Dossy Biological Technology Co., Ltd. (Chengdu, China), and housed under specific pathogen-free conditions. After a 1-week acclimatization period, the animals were 9 weeks old at the start of the study. The rats were randomly assigned to a control group and various test groups and housed in an air-conditioned room with a 12-hour light - 12-hour dark cycle. The room's temperature ranged from 20 °C to 24 °C, and its humidity ranged from 30% to 70%. During exposure to the test agents, a maximum of 3 animals were housed in each cage, and food and water were available *ad libitum*.

### 2.3. Acute toxicity and the $LD_{50}$ measurements

Experiments performed to determine the  $LD_{50}$  (median lethal dose) values of copper nano-particles, micro-particles, and Cu ions were designed in accordance with guidelines provided by the Organization for Economic Cooperation and Development (OECD TG 420) [24]. After rats becoming acclimated to their environment, the rats were administered a single oral gavage dose of Cu NPs, Cu micro-particles or Cu ions at levels recommended by the OECD. All of the Cu NPs and Cu ions were prepared in a 1% HPMC (Shanghai Ryon Biological Technology Co. Ltd. Shanghai, China) solution prior to use. 1% HPMC was chosen as vehicle for the oral gavage, because Cu NPs have minimal dissolution

indicating a stable dispersion in the vehicle. Individual animals were dosed in a specific sequence. The first animal received a dose one step below the estimated  $LD_{50}$  dose. If the animal survived, the second animal received a higher dose. If the first animal died, the second animal received a lower dose. Copper micro-particles were found to be non-toxic when administered at doses below the regulatory limit (*i.e.*, 5000 mg/kg); hence, they were evaluated at a dose of 5000 mg/kg. The  $LD_{50}$  and 95% profile likelihood values for the copper nano-particles, micro-particles, and ions were obtained by analyzing the experimental data with the AOT<sub>425</sub>StatPgm software program (OECD TG 425) [25].

### 2.4. *In vivo* kinetics of the nano-copper particles

A total of 90 healthy male rats were randomly assigned to 30 groups ( $n = 3$  per group). Twenty-four treatment groups received a single oral dose (200 mg/kg) of three sizes Cu NPs and 1  $\mu\text{m}$  copper, while six other treatment groups received a single oral dose (200 mg/kg) of Cu ions. In the single oral dose toxicity study, Cu ions administered at a dose of 200 mg/kg caused symptoms of hematuria, paleness, piloerection, diarrhea and death. Therefore, we selected the 200 mg/kg dose of Cu NPs and Cu ions to evaluate their absorption, distribution, and excretion characteristics. Three animals in each group were sacrificed at 0 h (control, before the Cu administration), 12 h, 24 h, 48 h, 72 h, and 1 week after treatment. Absorption of Cu by Cu NP- and Cu ion-treated rats were determined by analysis of blood samples. The Cu tissue distribution profiles were determined by weighing samples liver, kidney, spleen, lung, heart, testis, and brain tissue. A QuantiChrom™ Copper Assay Kit (BioAssay Systems, Hayward, CA, USA) was used to analyze all blood and tissue samples for their level of elemental Cu. The linear detection range is 7  $\mu\text{g}/\text{dL}$  to 300  $\mu\text{g}/\text{dL}$  coppers.

### 2.5. Comparative short-term oral toxicity study

Seventy-two healthy male rats were randomly assigned to twelve different treatment groups ( $n = 6$  per group). Nine treatment groups were dosed with 50 mg/kg, 100 mg/kg, or 400 mg/kg of three differently sized Cu NPs, respectively. Two treatment groups received 200 mg/kg of Cu ions and 400 mg/kg of 1  $\mu\text{m}$  copper-particles, control group just received 1% HPMC vehicle alone. Each suspension of Cu NPs in 1% HPMC was freshly prepared before use. All rats that received the 400 mg/kg dose of copper particles displayed severe toxic effects, whereas no treatment-related effects were observed among rats that received the 50 mg/kg dose. Thus, we selected 400 mg/kg as the highest dose level for Cu NPs. The administration volume (10 mL/kg body weight) was calculated based on the most recently recorded body weight of each animal. All groups were dosed once a day for 7 days, and the control rats received an equivalent volume of 1% HPMC vehicle alone. Animals were treated by oral gavage. If the rat died, first calculate the mortality rate and make up 6 samples for each group.

Each animal was observed twice daily throughout the test period for any clinical signs of toxicity or mortality. At the scheduled necropsy, blood samples were collected from the vena cava under carbon dioxide ( $\text{CO}_2$ ) anesthesia. Two collected blood samples were placed into separate tubes, one for anticoagulant blood and the other for serum separation and incubated at room temperature within 30 min. Next, the white and red blood cell counts, mean cell volumes, mean corpuscular hemoglobin concentrations, and packed cell volumes were analyzed with an auto hematology analyzer (BC-2800; Shenzhen Mindray Bio-Medical Electronics Co., Ltd., Shenzhen, China). Serum samples were collected by centrifuging blood samples at 3000 rpm for 10 min and were evaluated using a blood chemistry autoanalyzer (BS-180; Shenzhen Mindray Bio-Medical Electronics Co., Ltd., China). After collecting blood samples, the rats were sacrificed by exsanguination from the abdominal aorta and vena cava. Their organs were removed, weighed, and examined macroscopically for visible lesions. The following organs were weighed: brain, heart, lung, liver, spleen, kidneys,

**Table 1**  
Histopathology scoring system for Copper treated rats.

Score	Tissue	Liver	Spleen	Kidney
0	Normal	Normal	Normal	Normal
1	A small number of hepatocytes mild swell. Hepatic sinuses enlarge.	Lymphocytes slightly reduced.		Minority renal tubular epithelial cells are slightly swollen.
2	Massive hepatic cell swelling and degeneration, mainly manifested as vacuolar degeneration. Homogenous red stained serous exudation in central vein.	The intercellular space of the lymphatic sheath around the artery is enlarged and the lymphocyte decreases.		Glomerular epithelial cells are swollen and there is a small amount of tubular epithelial cells that are shed and necrotic.
3	All liver cells are swollen, lightly stained nuclei. Disorganized liver cords. A large number of neutrophils in the sinusoids.	A large number of connective tissue of splenic trabecular bone in the red pulp region. A large decrease in intra-arterial lymphatic sheath lymphocytes.		The glomerular volume increases slightly, the luminal space becomes smaller, and a large number of renal tubular epithelial cells are obviously swollen, filling the entire lumen of the renal tubule.
4	The central vein dilates congestion and has a homogenous red-stained serous exudation. All hepatocyte structures are destroyed. There is no complete cell structure. Nuclei are swollen and light, and a large number of nucleus have disintegrated and necrotic.	No clear demarcation between the red and white pulps, with a large decrease in lymphocytes in the lymphatic sheath around the artery.		The glomerular volume increased significantly and filled the entire cyst. All epithelial cells of the renal tubules were swollen and filled the entire lumen of the renal tubules. Numerous epithelial cells of the tubular epithelial cells necrotically detached and some of the tubular exudates appeared.

and testes, and their relative weights were calculated based on the organ-to-body weight ratio. Histopathological evaluations were performed by fixing tissue specimens in 10% neutral-buffered formalin solution and then staining them with hematoxylin and eosin for subsequent microscopic examination. All observations were made manually in a totally blinded manner using a light microscope equipped with  $\times 5$ ,  $\times 10$ ,  $\times 20$ , and  $\times 40$  objective lenses (Olympus BX53; Olympus Corporation, Tokyo, Japan). Semiquantitative scoring to determine the extent of injury in the liver, spleen and kidneys was done using a four-digit numerical scoring system where 0 indicates no change and 1–4 indicate increasing severity according to Mann et al. [26]. Histopathology was scored individually for each tissue from each animal based on the scoring system detailed in Table 1.

## 2.6. Statistical analysis

All statistical results are expressed as the mean  $\pm$  standard deviation ( $n = 6$  and  $n = 3$  for the kinetic study), and all experimental values were compared with their corresponding control values. Differences between mean values were analyzed by SPSS 19.0 with one-way ANOVA, and differences with a  $P$ -value  $\leq 0.05$  were considered statistically significant.

## 3. Results

### 3.1. Characterization of test materials

The results of a dynamic light scattering analysis (Fig. 1) showed that the spherical Cu NPs suspended in pure ice water had aggregated after being sonicated for 5–6 h. Aggregates with mean sizes of  $27.6 \pm 9.5$  nm (30 nm),  $53.2 \pm 17.6$  nm (50 nm),  $89.5 \pm 33.4$  nm (80 nm), and  $987.4 \pm 436.7$  (1  $\mu$ m) were detected. The polydispersity index (PDI) of nano- (30, 50 and 80 nm) and micro-copper were 0.12, 0.109, 0.14 and 0.20, respectively, according to the laser particle size analyzer test.

### 3.2. Acute toxicity and the $LD_{50}$ dose in vivo

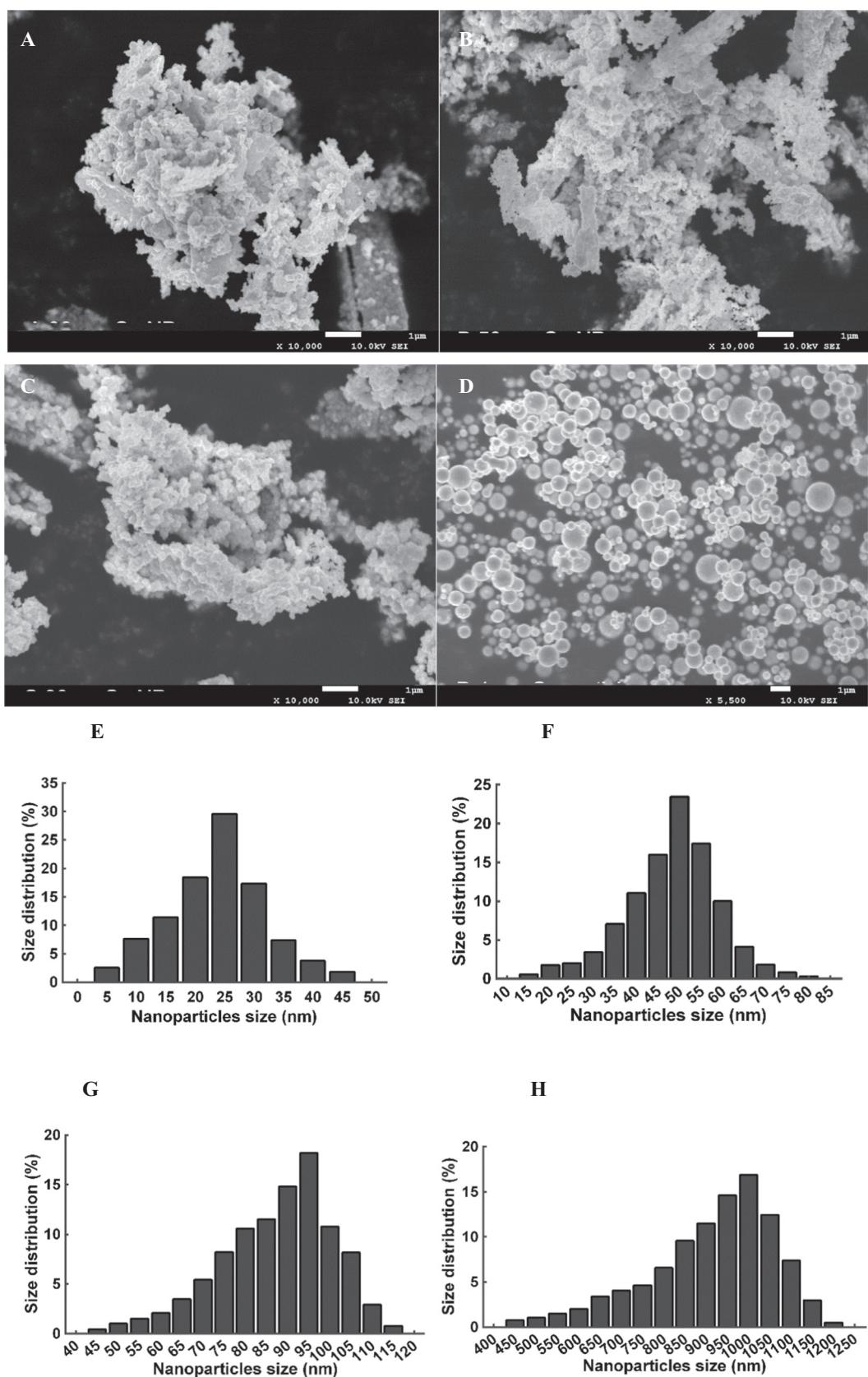
Following oral exposure to test agents, the mortality rate in each dose group was observed and recorded, and the  $LD_{50}$  values of the test samples were calculated using the AOT425 program (OECD guideline 425) (Fig. 2). Based on OECD test guideline 425, the  $LD_{50}$  value of Cu ions (359.6 mg/kg) placed their toxicity in class 3: moderately hazardous. The  $LD_{50}$  values of the 30 nm, 50 nm, and 80 nm Cu NPs were 1022 mg/kg, 1750 mg/kg, and 2075 mg/kg, respectively, which placed

them in class 4: slightly hazardous. In contrast, the  $LD_{50}$  value of the copper micro-particles was  $> 5000$  mg/kg, which placed them in class 5: nearly non-toxic.

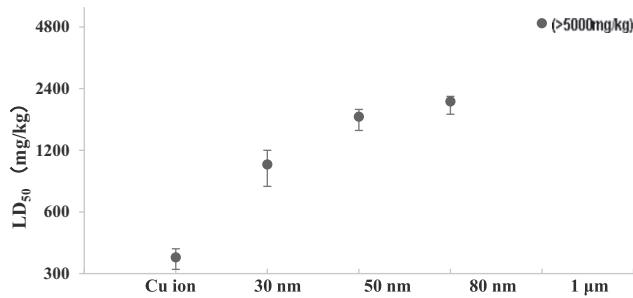
Symptoms exhibited by the experimental animals after exposure to the test particles and ions were observed during the experimental portion of the study. In contrast to rats, treated with Cu micro-particles, among which only a few rats exhibited symptoms of toxicity, all of the rats treated with Cu NPs displayed obvious symptoms of altered alimentary canal function, including loss of appetite, diarrhea and fatigue. Additionally, several rats in the Cu NP and copper ion treatment groups displayed passive behavior, hypopnea, tremor, and arching of the back.

### 3.3. In vivo kinetics of copper nano-particles

The in vivo kinetics of Cu NPs (200 mg/kg) and Cu ions (200 mg/kg, equivalent to 75.3 mg/kg based on Cu mass) were assessed using a Copper Assay Kit at 0 h, 12 h, 24 h, 48 h, 72 h, 96 h, and 1 week after single oral administration. While the distribution patterns of Cu in the blood and organs of rats treated with Cu NPs and Cu ions showed similar trends, there were many differences in the Cu levels that were related to particle size. The liver (Fig. 3B) and kidney (Fig. 3E) were the main organs which accumulated Cu NPs. Cu content in the heart reached its peak level at 48 h in all of the experimental animals (Fig. 3A), and the Cu levels in the Cu ion treatment group rapidly decreased after reaching their peak. The liver had the highest copper content, and Cu NPs reached their highest accumulations at 24 post-administration. Moreover, the Cu NPs were cleared at rates that were significantly slower than those of Cu ions or 1  $\mu$ m copper particles. Cu accumulations in the lungs of rats treated with Cu ions and 50 nm nano-copper particles (Fig. 3D) reached their peak levels at 24 h, while the accumulations of 30 nm and 80 nm Cu NPs and 1  $\mu$ m copper particles peaked at 48 h after exposure. While the spleen (Fig. 3C) and brain (Fig. 3D) showed similar trends of copper content, the 50 nm copper particles showed higher accumulations in the spleen, and the 30 nm copper particles showed higher accumulations in the brain at 24 h after administration. The contents of Cu in the kidney and serum were only slightly different from those in the other tested organs; however, the levels of 30 nm copper particles in the kidney remained significantly increased at more than one week after dosing. The Cu ion and 1  $\mu$ m copper particle groups had higher levels of Cu in the testis, and those levels peaked at 24 h after dosing.



**Fig. 1.** Characterization of the Cu nanoparticles by scanning electron microscopy (A–D), and the nanoparticle size distributions (E–H). A and E 10–30 nm nano-copper, B and F 50 nm nano-copper, C and G 80–100 nm nano-copper, D and H 1 μm copper particle.

Fig. 2. LD<sub>50</sub> values of differently sized Cu NPs and the Cu ions.

### 3.4. Short-term oral toxicity study

#### 3.4.1. Survival rates and clinical signs

In short-term oral toxicity study, the *in vivo* toxicity of orally administered Cu NPs was assessed at several dose levels ranging from 50 mg/mL to 400 mg/kg, and then compared with the toxicity of Cu ions administered at a dose of 200 mg/kg. We found that rats treated with 80 nm Cu NPs displayed more severe clinical symptoms and gained less body weight than rats in the other treatment groups. A significant amount of lethal toxicity was observed when a 400 mg/kg dose of any sized Cu NP was administered, and a single dose of 50 nm Cu particles produced the highest rate of mortality (Fig. 4B). While rats dosed with the 30 nm (Fig. 4A) and 80 nm (Fig. 4C) Cu NPs had similar rates of mortality at the end of the study, rats dosed with the 80 nm

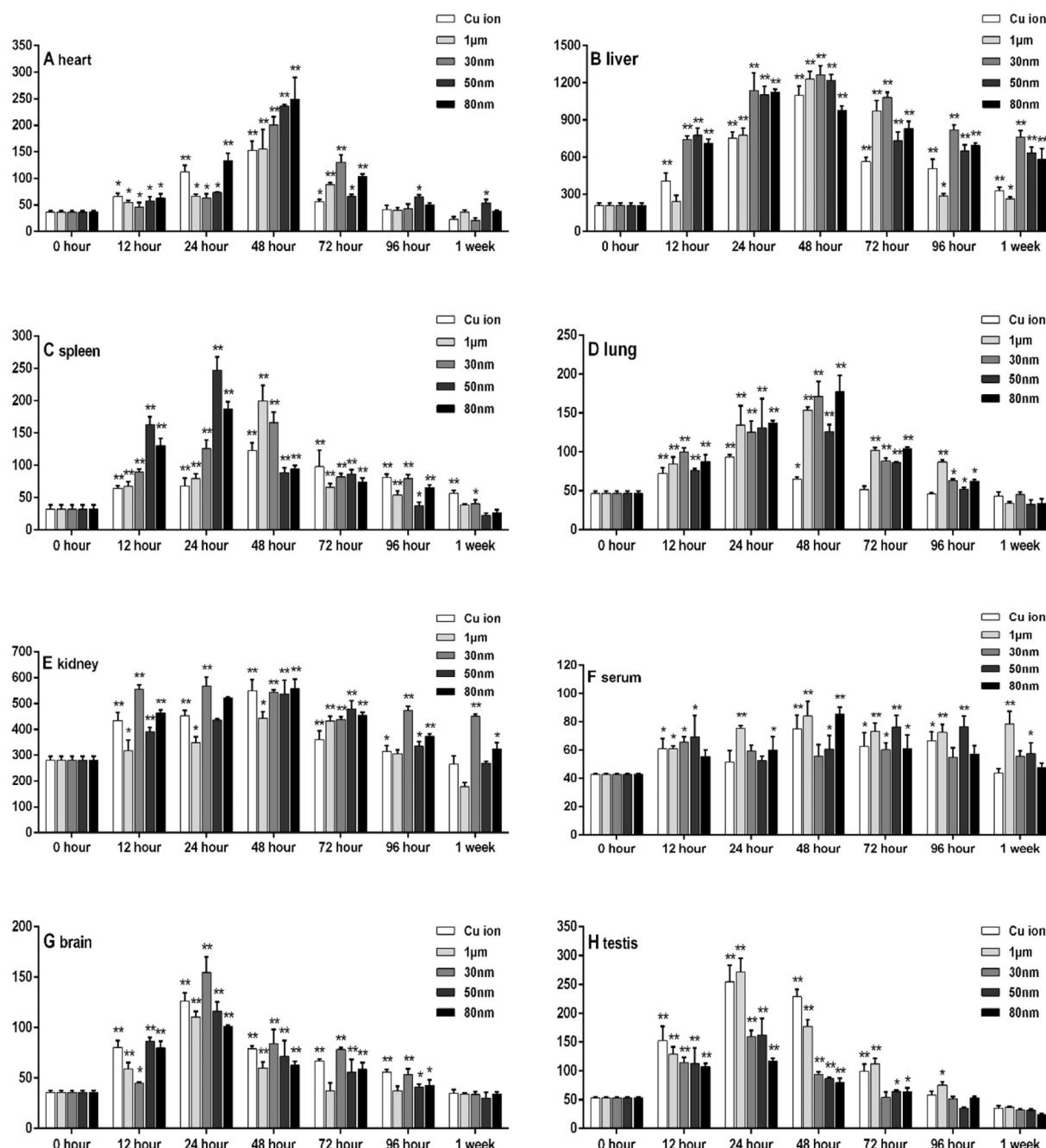
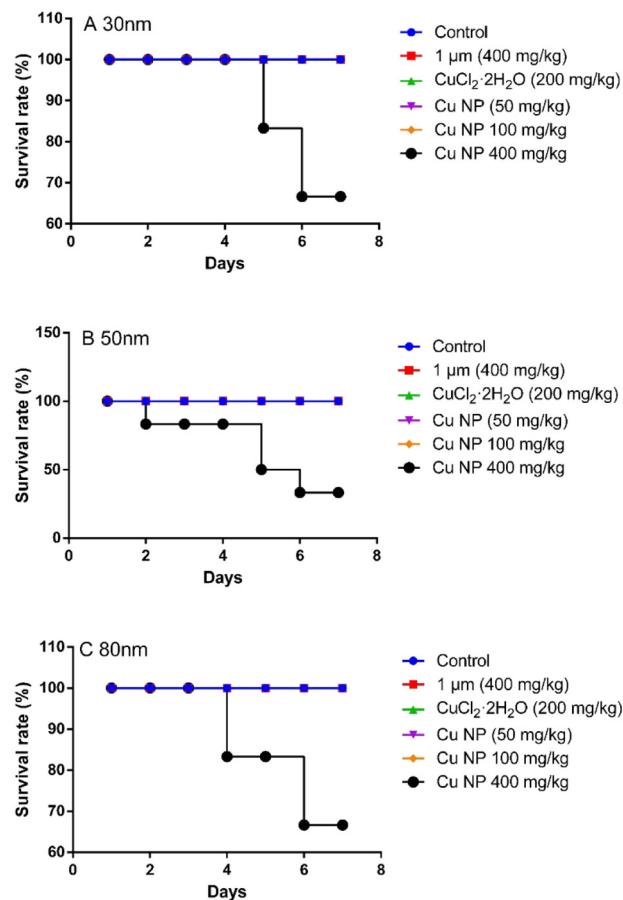


Fig. 3. The kinetics of Cu NPs in rats. Significant difference compared to hour 0. \*P &lt; 0.05 and \*\*P &lt; 0.01. A heart, B liver, C spleen, D lung, E kidney, F serum, G brain, H testis.



**Fig. 4.** Mortality differences between the Cu NP- and Cu ion-treated rats in short term. A Survival of rats after oral administration of 30 nm Cu NPs, B Survival of rats after oral administration of 50 nm Cu NPs, C Survival of rats after oral administration of 80 nm Cu NPs.

copper particles died at earlier time points. Rats in the 400 mg/kg dose group displayed treatment-related adverse events which included soft feces, diarrhea, piloerection, hematuria, depression, paleness, prone position, nasal discharge, and death, while rats in the 1  $\mu$ m copper particle group experienced only slight diarrhea that was non-lethal.

#### 3.4.2. Relative organ weights

For  $\text{CuCl}_2$  (200 mg/kg b.w.) only the relative lung weight decreased, while for the testes it was an increase. For the 1  $\mu$ m Cu particles at 400 mg/kg a decrease in relative organ weight was noted for the liver, spleen and lung. For 30 nm at the highest dose investigated a decrease in body weight and relative organ weights for heart, spleen, lung, was observed while for the liver, kidney, and testes an increase in relative organ weight was observed. At lower dose the 50 mg/kg dose induced a decrease in relative organ weight in the liver, and the dose of 100 mg/kg a decrease was present in the lung. Overall the highest dose of 400 mg/kg induced most changes in organ weights resulting in both an increase and decrease of relative organ weight. Cu NPs at 50 and 80 nm produces the similar effect to 30 nm. The results showed in Table 2.

#### 3.4.3. Hematology analysis

The effects of Cu NPs and Cu ions on hematologic parameters are shown in Table 3. The numbers of WBCs, and Grans were significantly higher in each of the three groups dosed with 400 mg/kg of Cu NPs, and those increases were greatest in the 80 nm Cu NP group. The increase in WBC was mainly due to the increased numbers of neutrophilic granulocytes, because the numbers of Lymphs were significantly decreased. There were significant particle size-dependent decreases in RBC

numbers and HGB levels at the Cu NP dose of 400 mg/kg; however, the numbers of RBCs were significantly increased in the groups dosed with 50 mg/kg of 30 nm or 50 nm Cu NPs. All three doses of 50 nm Cu NPs induced significant decreases in the mean corpuscular hemoglobin concentration, and similar decreases were found when 80 nm Cu NPs were administered at a dose of 50 mg/kg and when Cu ions were administered. The blood platelet counts were significantly increased in the groups dosed with 50 mg/kg of 50 nm or 80 nm Cu NPs, with the effect being greater in the 50 nm group.

#### 3.4.4. Serum biochemistry analysis

Serum biochemistry results for the rats treated with Cu micro-particles, Cu NPs or Cu ions are presented in Table 4. Rats treated with 1  $\mu$ m copper particles had significantly decreased serum ALP,  $\gamma$ -GT, TP, GLB, GLU, and TG levels, while their AST, ALB/GLB (A/G), and CK levels were significantly increased when compared with control rats. Rats treated with copper ions had significantly increased levels of A/G and CK, while their TP, GLB, and GLU levels were significantly lower than those in the control group. The serum levels of AST, ALT/ALT, and UREA among rats treated with any of the three doses of 30 nm copper particles were significantly higher than those levels in the control group, whereas the GLB levels in those rats were significantly lower than the corresponding levels in the control group. The serum levels of A/G increased significantly in rats treated with either 50 mg/kg or 100 mg/kg of 30 nm copper particles, whereas the serum levels of TP in those rats showed significant decreases. The serum CK levels in rats treated with 100 mg/kg and 400 mg/kg of 30 nm copper particles were significantly higher than those levels in control rats, while the serum ALB levels were significantly lower than those in control rats. The serum levels of ALT and TG were significantly increased, while the serum levels of TC were significantly decreased in rats treated with 400 mg/kg of 30 nm copper particles when compared with those levels in the control group. The CK levels among rats which received three doses of 50 nm copper particles were significantly increased, while the serum levels of A/G and GLB were significantly decreased when compared with their levels in the control group. The serum levels of ALP and urea were significantly increased, while the serum levels of ALB were significantly decreased in rats treated with either 100 mg/kg or 400 mg/kg of 50 nm copper particles. The serum levels of creatine were significantly increased, and the serum levels of TG were significantly decreased in rats treated with 400 mg/kg of 50 nm copper particles when compared with those levels in the control group. The serum levels of GLB, ALB, and GLU were significantly decreased in all three of the 80 nm copper particle dose groups, and the serum levels of ALP, TP, and A/G were significantly decreased in the 50 mg/kg and 100 mg/kg 80 nm particle dose groups. The serum levels of ALT, AST, and CK were significantly increased, and levels of urea and TG were significantly decreased in rats dosed with either 100 mg/kg or 400 mg/kg of 80 nm copper particles. The serum levels of  $\gamma$ -GT and creatine were significantly increased, and the levels of TG were significantly decreased in rats treated with 400 mg/kg of 400 nm copper particles when compared with those levels in the control group.

#### 3.4.5. Histopathological analysis

Histopathological examination of different organs, namely the liver, spleen and kidneys showed in Fig. 5 and Table 5.

There were no obvious pathological changes in the livers or spleens of rats treated with 200 mg/kg of Cu ions; however, some renal tubular epithelial cell swelling and evidence of renal tubular interstitial light hemorrhage were observed. There were also no obvious pathological changes in the livers of rats treated with 400 mg/kg of 1  $\mu$ m Cu particles. A small amount of spleen cell necrosis was present in the boundary of the spleen between the red and white pulp.

The livers of rats treated with 50 mg/kg of Cu NPs showed some slight swelling of the liver cells and partial swelling of the hepatic sinus. Rats treated with 30 nm or 50 nm Cu NPs showed slight neutrophil

**Table 2**  
Mean relative organ weights (g) in rats treated with Cu ions, Cu micro-particles, and Cu NPs.

Items	Cu NPs 50 nm (mg/kg)			Cu NPs 80 nm (mg/kg)			
	Control	1% HPMC	200	400	50	100	400
Number of rats	6	6	6	6	6	6	6
Body weight at term (g)	230.05 ± 5.81	211.28 ± 15.63	214.43 ± 3.95	237.80 ± 6.50	219.00 ± 10.79	200.40 ± 15.80**	
Heart	0.38 ± 0.01	0.42 ± 0.01	0.38 ± 0.01	0.38 ± 0.01	0.36 ± 0.02	0.32 ± 0.02**	
Liver	3.75 ± 0.05	3.69 ± 0.05	3.30 ± 0.04*	3.08 ± 0.24**	4.09 ± 0.13	4.88 ± 0.13*	
Spleen	0.30 ± 0.01	0.34 ± 0.04	0.24 ± 0.04*	0.26 ± 0.02	0.25 ± 0.03	0.22 ± 0.08*	
Lung	0.63 ± 0.03	0.53 ± 0.01**	0.52 ± 0.02**	0.60 ± 0.01	0.56 ± 0.03	0.50 ± 0.02**	
Kidneys	0.85 ± 0.03	0.94 ± 0.02	0.86 ± 0.04	0.82 ± 0.02	0.85 ± 0.05	0.97 ± 0.04*	
Testes	1.04 ± 0.01	1.16 ± 0.02*	1.06 ± 0.01	1.14 ± 0.03	1.13 ± 0.05	1.17 ± 0.07*	
Brain	0.60 ± 0.01	0.65 ± 0.03	0.59 ± 0.02	0.62 ± 0.04	0.55 ± 0.04	0.60 ± 0.06	

Notes: Data presented as relative organ weight compared to body weight. Data presented as mean ± SD ( $n = 6$ ).

Abbreviations: Cu NPs, copper nanoparticles; bw, body weight; SD, standard deviation.

\*  $P \leq 0.05$ .

\*\*  $P \leq 0.01$  vs. control.

**Table 3**  
Effect of Cu ions, Cu micro-particles, and Cu NPs on hematologic parameters.

Items	Control		Cu ions (mg/kg)		1 μm Cu micro-particles (mg/kg)		Cu NPs 30 nm (mg/kg)	
	1% HPMC	200	400	50	100	400		
Number of rats								
WBC	6	6	6	6	6	6	6	6
WBC	8.93 ± 0.47	8.50 ± 3.05	9.10 ± 1.93	13.00 ± 1.30	13.10 ± 1.28	17.77 ± 0.90 <sup>**</sup>	17.77 ± 0.90 <sup>**</sup>	17.77 ± 0.90 <sup>**</sup>
Lym	74.43 ± 1.37	76.27 ± 3.06	79.57 ± 0.51	80.63 ± 1.43	70.20 ± 3.54	62.13 ± 7.22 <sup>**</sup>	62.13 ± 7.22 <sup>**</sup>	62.13 ± 7.22 <sup>**</sup>
Mon	2.33 ± 0.06	11.23 ± 3.50 <sup>**</sup>	2.40 ± 0.00	2.03 ± 0.23	3.23 ± 0.35	4.70 ± 1.28 <sup>**</sup>	4.70 ± 1.28 <sup>**</sup>	4.70 ± 1.28 <sup>**</sup>
Gran	23.33 ± 1.26	25.00 ± 3.99	20.50 ± 1.81	18.37 ± 0.90	30.30 ± 1.08 <sup>*</sup>	33.67 ± 8.47 <sup>**</sup>	33.67 ± 8.47 <sup>**</sup>	33.67 ± 8.47 <sup>**</sup>
RBC	4.73 ± 0.04	4.58 ± 0.18	5.41 ± 0.04	5.87 ± 0.15 <sup>*</sup>	4.81 ± 0.28	3.07 ± 0.92 <sup>**</sup>	3.07 ± 0.92 <sup>**</sup>	3.07 ± 0.92 <sup>**</sup>
HGB	126.00 ± 2.65	117.67 ± 2.89	137.33 ± 2.89	130.33 ± 5.03	123.67 ± 6.43	77.67 ± 19.30 <sup>**</sup>	77.67 ± 19.30 <sup>**</sup>	77.67 ± 19.30 <sup>**</sup>
HCT	30.70 ± 1.15	29.10 ± 0.90	32.43 ± 0.40	35.97 ± 1.16	35.60 ± 1.83	20.30 ± 4.90 <sup>**</sup>	20.30 ± 4.90 <sup>**</sup>	20.30 ± 4.90 <sup>**</sup>
MCV	63.63 ± 0.58	64.87 ± 0.61	63.30 ± 0.82	66.90 ± 0.20	64.73 ± 1.05	63.73 ± 1.26	63.73 ± 1.26	63.73 ± 1.26
MCH	26.40 ± 0.27	25.63 ± 0.42	25.33 ± 0.47	21.47 ± 0.15	22.47 ± 0.45	24.97 ± 0.80	24.97 ± 0.80	24.97 ± 0.80
MCHC	419.00 ± 3.00	402.00 ± 1.73 <sup>*</sup>	417.33 ± 5.03	343.00 ± 1.00 <sup>*</sup>	347.00 ± 6.00 <sup>*</sup>	386.33 ± 4.93 <sup>**</sup>	386.33 ± 4.93 <sup>**</sup>	386.33 ± 4.93 <sup>**</sup>
RDW	11.67 ± 0.81	14.30 ± 0.10 <sup>*</sup>	13.50 ± 0.59	12.90 ± 0.10	13.63 ± 1.02	13.37 ± 1.94	13.37 ± 1.94	13.37 ± 1.94
PLT	1470.33 ± 96.08	1770.00 ± 52.37 <sup>*</sup>	1660.33 ± 22.72	1570.67 ± 179.53	1463.33 ± 66.58	1249.00 ± 505.02	1249.00 ± 505.02	1249.00 ± 505.02
MPV	6.23 ± 0.06	6.30 ± 0.10	6.33 ± 0.06	5.90 ± 0.10	6.33 ± 0.06	6.43 ± 0.35	6.43 ± 0.35	6.43 ± 0.35
Cu NPs 50 nm (mg/kg)								
Items	50	100	400	50	100	400		
Number of rats								
WBC	6	6	6	6	6	6	6	6
WBC	8.67 ± 0.93	11.03 ± 0.81	16.58 ± 0.83 <sup>**</sup>	7.40 ± 0.44	8.50 ± 2.71	20.17 ± 4.41 <sup>**</sup>	20.17 ± 4.41 <sup>**</sup>	20.17 ± 4.41 <sup>**</sup>
Lym	78.40 ± 2.23	76.70 ± 0.95	58.17 ± 4.41 <sup>**</sup>	78.43 ± 3.51	75.77 ± 2.03	59.50 ± 3.94 <sup>**</sup>	59.50 ± 3.94 <sup>**</sup>	59.50 ± 3.94 <sup>**</sup>
Mon	2.27 ± 0.12	2.80 ± 0.17	3.93 ± 0.64	2.70 ± 0.10	2.27 ± 0.15	2.37 ± 0.15	2.37 ± 0.15	2.37 ± 0.15
Gran	17.40 ± 2.29	20.77 ± 1.24	40.43 ± 2.82 <sup>**</sup>	18.70 ± 3.45	24.40 ± 2.34	34.50 ± 4.25 <sup>**</sup>	34.50 ± 4.25 <sup>**</sup>	34.50 ± 4.25 <sup>**</sup>
RBC	5.85 ± 0.11 <sup>*</sup>	4.44 ± 0.22	3.31 ± 0.26 <sup>**</sup>	5.10 ± 0.14	4.30 ± 0.10	2.25 ± 1.37 <sup>**</sup>	2.25 ± 1.37 <sup>**</sup>	2.25 ± 1.37 <sup>**</sup>
HGB	146.33 ± 2.08 <sup>*</sup>	123.67 ± 2.52	100.00 ± 16.09 <sup>*</sup>	134.00 ± 4.58	115.00 ± 6.08	79.67 ± 3.51 <sup>**</sup>	79.67 ± 3.51 <sup>**</sup>	79.67 ± 3.51 <sup>**</sup>
HCT	38.63 ± 0.15 <sup>*</sup>	31.10 ± 0.44	25.70 ± 7.23	33.42 ± 0.33	28.13 ± 1.11	14.23 ± 2.17 <sup>**</sup>	14.23 ± 2.17 <sup>**</sup>	14.23 ± 2.17 <sup>**</sup>
MCV	66.00 ± 0.17	64.07 ± 1.80	63.77 ± 0.81	66.37 ± 1.86	65.00 ± 0.76	62.43 ± 0.83	62.43 ± 0.83	62.43 ± 0.83
MCH	26.13 ± 0.90	25.47 ± 0.95	23.23 ± 1.42	24.43 ± 0.81	25.27 ± 1.42	26.50 ± 0.78	26.50 ± 0.78	26.50 ± 0.78
MCHC	380.67 ± 0.60 <sup>**</sup>	394.00 ± 1.00 <sup>*</sup>	404.33 ± 11.06	390.33 ± 3.06 <sup>*</sup>	402.67 ± 5.03 <sup>*</sup>	419.33 ± 26.41	419.33 ± 26.41	419.33 ± 26.41
RDW	13.20 ± 0.10	14.73 ± 0.64	15.93 ± 0.95 <sup>*</sup>	14.77 ± 0.31 <sup>*</sup>	15.10 ± 0.72 <sup>*</sup>	16.07 ± 0.50 <sup>*</sup>	16.07 ± 0.50 <sup>*</sup>	16.07 ± 0.50 <sup>*</sup>
PLT	2076.00 ± 155.16 <sup>**</sup>	1759.67 ± 118.15	1416.33 ± 99.32	1910.33 ± 12.06 <sup>*</sup>	1616.33 ± 37.31	1222.67 ± 139.43	1222.67 ± 139.43	1222.67 ± 139.43
MPV	6.07 ± 0.12	6.13 ± 0.15	6.43 ± 0.21	6.30 ± 0.10	6.50 ± 0.10	6.73 ± 0.21	6.73 ± 0.21	6.73 ± 0.21

Notes: Data presented as mean ± SD (n = 6).

Abbreviations: WBC, white blood cell count; RBC, red blood cell; Lym, lymphocyte; Mon, monocyte; GRAN, neutrophil granulocyte; RBC, red blood cell; HGB, hemoglobin; HCT, hematocrit; MCV, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RDW, red blood cell volume distribution width; PLT, platelet count/blood platelet count; MPV, mean platelet volume.

\* P ≤ 0.05.

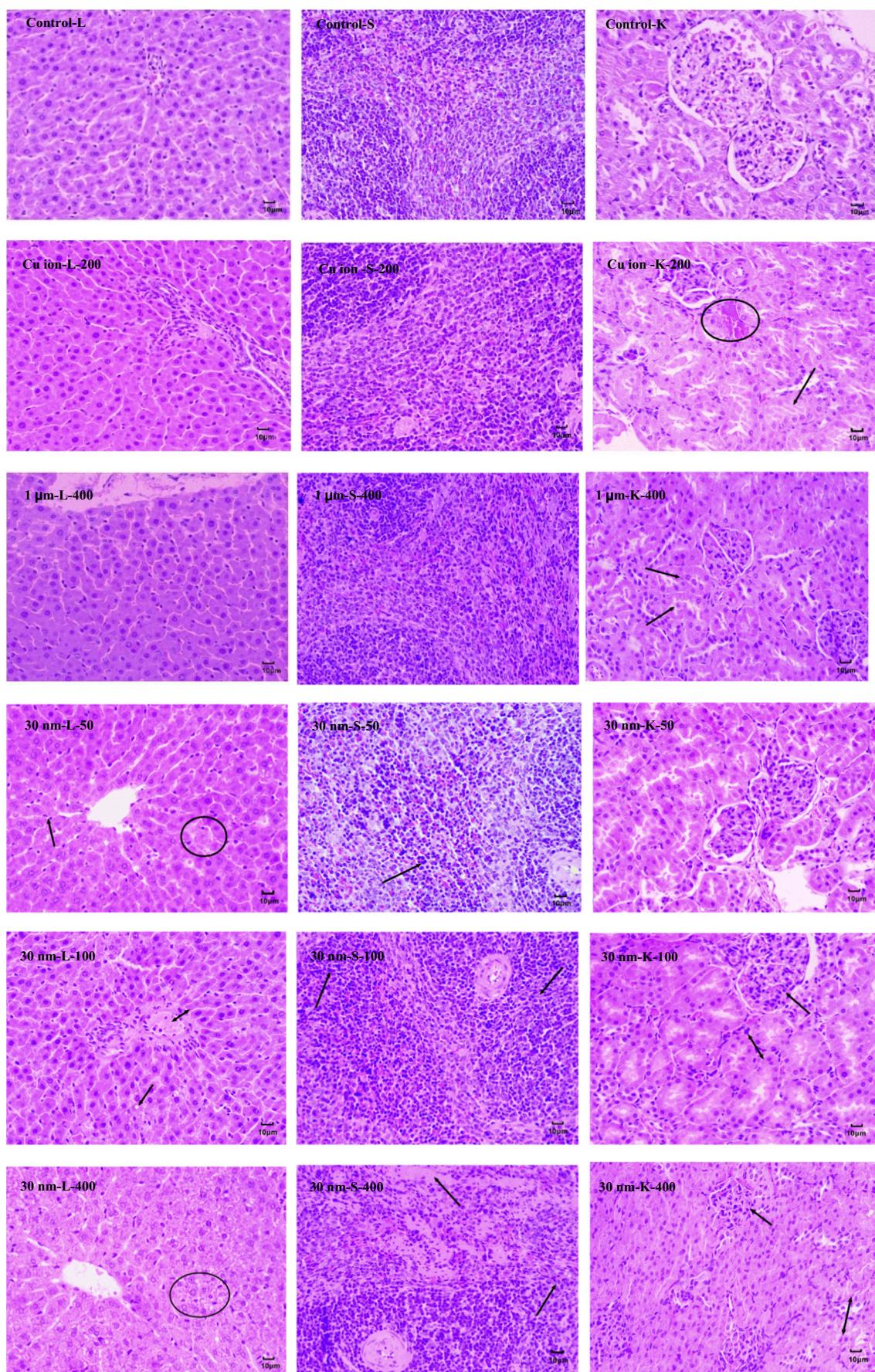
\*\* P ≤ 0.01 vs. control.

**Table 4**  
The effect of Cu ions, Cu micro-particles, and Cu NPs on serum biochemical parameters.

Items	Control	Cu ion (mg/kg)			1 μm Cu micro-particles (mg/kg)			Cu NPs 30 nm (mg/kg)		
		1% HPMC	200	400	50	100	400	6	6	6
Number of rats	6	6	6	6	6	6	6	6	6	6
ALT	35.70 ± 1.74	35.13 ± 3.50	36.70 ± 1.01	34.30 ± 1.05	42.03 ± 1.27	45.43 ± 2.80*	515.33 ± 46.15**	410.80 ± 7.17**	515.33 ± 46.15**	410.80 ± 7.17**
AST	123.80 ± 5.98	102.90 ± 14.73	277.70 ± 4.56**	347.43 ± 20.47**	9.08 ± 0.51**	9.54 ± 0.48**	10.20 ± 0.62**	10.20 ± 0.62**	10.20 ± 0.62**	10.20 ± 0.62**
AST/ALT	4.08 ± 0.09	4.17 ± 0.85	15.95 ± 0.54**	280.03 ± 20.39	279.93 ± 10.99	279.93 ± 10.99	260.0 ± 13.34	260.0 ± 13.34	260.0 ± 13.34	260.0 ± 13.34
ALP	258.27 ± 31.19	302.43 ± 69.96	245.60 ± 25.32	1.13 ± 0.15**	1.20 ± 0.10	1.63 ± 0.23	1.83 ± 0.32	1.83 ± 0.32	1.83 ± 0.32	1.83 ± 0.32
γ-GT	1.53 ± 0.21	1.53 ± 0.31	34.43 ± 1.51**	42.87 ± 5.97**	41.87 ± 6.56**	42.57 ± 5.14**	48.30 ± 3.39	48.30 ± 3.39	48.30 ± 3.39	48.30 ± 3.39
TP	57.30 ± 0.35	13.37 ± 0.29**	9.47 ± 2.30**	8.50 ± 1.41**	12.13 ± 1.53**	15.03 ± 0.47**	15.03 ± 0.47**	15.03 ± 0.47**	15.03 ± 0.47**	15.03 ± 0.47**
GLB	24.13 ± 0.61	30.93 ± 3.36	29.70 ± 1.59	27.80 ± 4.88*	26.40 ± 3.47**	27.80 ± 4.88*	26.40 ± 3.47**	26.40 ± 3.47**	26.40 ± 3.47**	26.40 ± 3.47**
ALB	34.37 ± 0.38	4.16 ± 0.09**	3.27 ± 0.71**	5.28 ± 0.18**	8.06 ± 2.27	9.21 ± 0.22	9.21 ± 0.22	9.21 ± 0.22	9.21 ± 0.22	9.21 ± 0.22
GLU	8.72 ± 0.07	2.37 ± 0.19**	2.83 ± 0.34**	3.41 ± 0.37**	2.18 ± 0.43*	1.95 ± 0.17	1.95 ± 0.17	1.95 ± 0.17	1.95 ± 0.17	1.95 ± 0.17
A/G	1.45 ± 0.03	1.28 ± 0.15	1.83 ± 0.49*	1.40 ± 0.04	1.84 ± 0.11*	1.92 ± 0.10*	2.06 ± 0.53**	2.06 ± 0.53**	2.06 ± 0.53**	2.06 ± 0.53**
UREA	20.10 ± 1.44	20.50 ± 2.82	19.07 ± 1.44	21.47 ± 2.27	25.03 ± 1.25	28.20 ± 6.66**	28.20 ± 6.66**	28.20 ± 6.66**	28.20 ± 6.66**	28.20 ± 6.66**
CREA	1.59 ± 0.03	1.65 ± 0.16	1.28 ± 0.30	1.69 ± 0.37	1.27 ± 0.15	1.24 ± 0.16	1.24 ± 0.16	1.24 ± 0.16	1.24 ± 0.16	1.24 ± 0.16
TC	0.73 ± 0.04	0.63 ± 0.05	0.44 ± 0.10*	0.64 ± 0.09	0.84 ± 0.08	1.09 ± 0.37*	1.09 ± 0.37*	1.09 ± 0.37*	1.09 ± 0.37*	1.09 ± 0.37*
TG	520.97 ± 100.28	710.23 ± 92.32	812.23 ± 111.14**	597.83 ± 129.41	957.30 ± 59.17**	1067.47 ± 95.95**	1067.47 ± 95.95**	1067.47 ± 95.95**	1067.47 ± 95.95**	1067.47 ± 95.95**
CK	0.46 ± 0.06	0.37 ± 0.05	0.39 ± 0.14	0.42 ± 0.08	0.49 ± 0.07	0.51 ± 0.06	0.51 ± 0.06	0.51 ± 0.06	0.51 ± 0.06	0.51 ± 0.06
T-Bil										
Items	Cu NPs 50 nm (mg/kg)			Cu NPs 80 nm (mg/kg)			Cu NPs 30 nm (mg/kg)			
	50	100	400	50	100	400	50	100	400	
Number of rats	6	6	6	6	6	6	6	6	6	
ALT	35.47 ± 3.58	38.03 ± 4.85	50.14 ± 6.93**	33.93 ± 4.31	45.27 ± 6.29*	45.47 ± 7.75*	175.20 ± 16.69	175.20 ± 16.69	175.20 ± 16.69	175.20 ± 16.69
AST	133.87 ± 16.31	151.40 ± 9.75	225.85 ± 37.08**	129.07 ± 16.69	5.67 ± 0.04**	5.28 ± 0.44**	5.67 ± 0.04**	5.67 ± 0.04**	5.67 ± 0.04**	5.67 ± 0.04**
AST/ALT	5.20 ± 0.06**	4.77 ± 0.40	3.51 ± 0.71	154.93 ± 21.84**	180.17 ± 12.50**	180.17 ± 12.50**	180.17 ± 12.50**	180.17 ± 12.50**	180.17 ± 12.50**	180.17 ± 12.50**
ALP	252.23 ± 37.57	372.77 ± 22.23**	510.20 ± 26.06**	1.20 ± 0.10	1.27 ± 0.12	1.93 ± 0.15	1.93 ± 0.15	1.93 ± 0.15	1.93 ± 0.15	1.93 ± 0.15
γ-GT	1.37 ± 0.15	1.40 ± 0.10	1.80 ± 0.30	65.77 ± 5.28	36.37 ± 0.40**	44.43 ± 5.05*	44.43 ± 5.05*	44.43 ± 5.05*	44.43 ± 5.05*	44.43 ± 5.05*
TP	47.83 ± 7.30	58.73 ± 10.47	13.53 ± 1.04**	16.99 ± 7.48**	7.27 ± 0.12**	14.87 ± 0.25**	14.87 ± 0.25**	14.87 ± 0.25**	14.87 ± 0.25**	14.87 ± 0.25**
GLB	8.63 ± 2.16**	26.77 ± 5.84**	22.29 ± 3.31**	28.3 ± 1.42	25.10 ± 1.35**	24.23 ± 1.06**	24.23 ± 1.06**	24.23 ± 1.06**	24.23 ± 1.06**	24.23 ± 1.06**
ALB	35.93 ± 2.85	9.67 ± 0.27	6.24 ± 0.21**	5.96 ± 1.17**	5.85 ± 0.24**	3.81 ± 0.36**	3.81 ± 0.36**	3.81 ± 0.36**	3.81 ± 0.36**	3.81 ± 0.36**
GLU	9.67 ± 0.27	4.37 ± 0.40**	2.28 ± 0.34**	2.16 ± 0.31**	3.58 ± 0.71**	2.63 ± 0.03**	2.63 ± 0.03**	2.63 ± 0.03**	2.63 ± 0.03**	2.63 ± 0.03**
A/G	1.68 ± 0.16	2.37 ± 0.16**	2.75 ± 0.41**	1.20 ± 0.14	1.92 ± 0.36*	3.90 ± 0.40*	3.90 ± 0.40*	3.90 ± 0.40*	3.90 ± 0.40*	3.90 ± 0.40*
UREA	18.40 ± 1.22	20.73 ± 1.27	28.59 ± 4.41**	17.43 ± 0.49	24.33 ± 1.27	27.07 ± 4.25**	27.07 ± 4.25**	27.07 ± 4.25**	27.07 ± 4.25**	27.07 ± 4.25**
CREA	1.71 ± 0.26	1.47 ± 0.28	1.31 ± 0.23	1.43 ± 0.06	1.09 ± 0.09**	0.72 ± 0.02**	0.72 ± 0.02**	0.72 ± 0.02**	0.72 ± 0.02**	0.72 ± 0.02**
TC	0.84 ± 0.07	0.73 ± 0.06	0.49 ± 0.13*	0.75 ± 0.07	0.59 ± 0.16	0.47 ± 0.09	0.47 ± 0.09	0.47 ± 0.09	0.47 ± 0.09	0.47 ± 0.09
TG	821.07 ± 154.07**	890.50 ± 114.29**	1381.77 ± 171.76*	663.3 ± 116.57	879.97 ± 37.06**	1086.53 ± 120.5*	1086.53 ± 120.5*	1086.53 ± 120.5*	1086.53 ± 120.5*	1086.53 ± 120.5*
CK	0.43 ± 0.08	0.43 ± 0.04	0.51 ± 0.07	0.37 ± 0.05	0.51 ± 0.06	0.51 ± 0.07	0.51 ± 0.07	0.51 ± 0.07	0.51 ± 0.07	0.51 ± 0.07
T-Bil										

Notes: Data are presented as mean ± SD.

**Abbreviations:** ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; γ-GT, γ-glutamyl transpeptidase; TP, total protein; GLB, globulin; ALB, albumin; GLU, glucose; TC, total cholesterol; TG, triglyceride; CK, creatine kinase; T-Bil, total bilirubin; A/G, ALB/GLB.\*  $P \leq 0.05$ .\*\*  $P \leq 0.01$  vs. control.



(caption on next page)

**Fig. 5.** Histopathology results for the liver, kidneys, and spleen of rats treated with Cu NPs, and Cu ions (all image showed with  $\times 400$ ).

Notes: The kidneys (K) of rats treated with Cu ions (200 mg/kg) showed renal tubular epithelial cell swelling (Cu-K-200, arrows) and signs of renal tubular interstitial light hemorrhage (Cu-K-200, enclosed). Kidneys of rats treated with 1  $\mu\text{m}$  Cu showed a small amount of tubular epithelial cells shed and necrosis, and the glomerular volume increased (1  $\mu\text{m}$ -K-400). Livers (L) of rats treated with 30 nm Cu NPs showed some slight neutrophil infiltration (30-L-100, arrows), liver cell swelling degeneration (30 nm-L-100, arrows), and a central vein with homogeneous red dye serous effusion (30 nm-L-100, arrows). The spleen (S) red pulp area contained increased numbers of lymphocytes and a lymphatic sheath with decreased numbers of lymphocytes surrounded the arteries (30 nm-S-100, arrows). The renal glomerular volume was slightly increased, and the glomerular and renal small tubes were infiltrated with neutrophils (30 nm-K-100, arrows). Tubular epithelial cell necrosis and disordered renal tubular structures (30 nm-K-400, arrows). The livers of rats treated with 50 nm copper particles had a hepatic sinus that showed small amounts of neutrophil infiltration (50 nm-L-100, arrows), central venous congestion, homogeneous red dye serous effusion, and large numbers of liver cells with a disintegrating nucleus (50 nm-L-400, arrows). Spleen red and white pulp areas and the lymphatic sheath surrounding arteries with intercellular enlargement (50 nm-S-100, arrows). The absence of lymphocytes in the spleen red pulp areas (50 nm-S-400, arrows). The renal tubular epithelial cells were significantly enlarged (50 nm-K-100, arrows). Extensive renal tubular epithelial cell necrosis and an absence of renal tubular structures (50 nm-K-400, arrows). The liver central vein with homogeneous red dye serous effusion (80 nm-L-50, arrows). Liver cells showing granular degeneration and ballooning degeneration (80 nm-L-100). Liver cells showing ballooning degeneration (80 nm-L-400, arrows), a non-obvious hepatic cellular structure, and an enlarged lightly stained disintegrating nucleus (80 nm-L-400, enclosed). A slight reduction in spleen lymphocytes (80 nm-S-100, arrows) and microphage infiltration into spleen tissue (80 nm-S-400, arrows). A slightly increased kidney glomerular volume (80 nm-K-100, arrows) and large numbers of slightly enlarged renal tubular epithelial cells; part of the malpighian tube appeared as a protein type tube in the 80 nm particle group (80 nm-K-80, enclosed).

Abbreviations: L, liver; S, spleen; K, kidney. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

infiltration, and rats treated with 80 nm Cu NPs showed evidence of mild bleeding in the central vein with red homogeneous serous effusion. Rats treated with 100 mg/kg of 30 nm or 50 nm Cu NPs had large numbers of swollen liver cells that showed signs of vascular degeneration. The liver cells were disorganized, and the central vein showed red dye serous effusion. The hepatic sinus in those animals displayed some slight neutrophil infiltration, and evidence of central vein internal bleeding and partial liver cell granular degeneration. The 80 nm treatment group showed a small amount of hepatocellular ballooning degeneration. Additionally, their liver cells were poorly structured and contained enlarged nuclei, some of which were in the process of disintegrating. Rats treated with 30 nm copper particles at a dose of 400 mg/kg had swollen light colored liver cells in various degrees of degeneration. The cells contained cytolytic blisters of various sizes, enlarged nuclei, and showed signs of vesicular degeneration. Rats treated with 50 nm Cu particles displayed liver central vein blood clots, serous effusion, homogeneous red dye effusion, cellular damage, and poorly structured liver cells in which many of the nuclei showed signs of necrosis. Rats treated with 80 nm Cu particles showed the same pathologic liver characteristics as rats in the 50 nm group, but with the addition of ballooning cellular degeneration.

The spleens of rats dosed with 50 mg/kg of Cu NPs contained a red pulp area with slightly increased numbers of lymphocytes. The group dosed with 100 mg/kg of 30 nm Cu particles contained the same red pulp area with increased numbers of lymphocytes, and also displayed a lymphatic sheath surrounding the arteries and signs of lymphopenia. The 50 nm Cu particle group displayed clear boundaries between the red and white pulp areas, and both the 50 nm and 80 nm groups showed signs of lymphopenia. The spleens of rats treated with 400 mg/kg of 30 nm Cu NPs contained a red pulp area and showed histologic signs of trabecula connective tissue hyperplasia. Those spleens also had a lymphatic sheath that surrounded the arteries with lymphocytes. The spleens of rats treated with 400 mg/mL of 50 nm or 80 nm Cu NPs showed areas of red and white pulp with no obvious demarcation, a lymphatic sheath that surrounded the arteries, and substantially reduced numbers of lymphocytes. While lymphocytes were absent in the red pulp areas, those areas contained large numbers of macrophages.

Kidneys from rats treated with 50 mg/kg of 30 nm or 50 nm Cu NPs showed no obvious pathological changes; however, kidneys from the rats treated with 50 mg/kg of 80 nm particles contained glomerular epithelial cells that were slightly swollen and a small amount renal tubular epithelial cells that were necrotic. Kidneys from rats treated with 100 mg/kg of 30 nm Cu NPs displayed slightly larger glomerular volumes, smaller cavity gaps, smaller Malpighian tubes, glomeruli that were infiltrated with neutrophils, swollen renal tubular epithelial cells, and tubular interstitial regions that were infiltrated with polymorphonuclear cells. Renal tubular epithelial cells in the 50 nm Cu particle

treatment group were significantly enlarged, had a smaller renal tubular lacuna, displayed signs of renal tubular epithelial cell necrosis, and had an enlarged lightly stained nucleus. Renal tubular epithelial cells in the 80 nm treatment group were significantly enlarged and filled the entire kidney small tube cavity. The kidney glomerular volumes in the 30 nm and 50 nm copper particle treatment groups were dramatically increased and filled the entire lumen. Moreover, all of the renal tubular epithelial cells were significantly enlarged and filled the entire kidney small tube cavity. Some of the renal tubular epithelial cells and renal tubular structures showed signs of necrosis. Kidneys from rats in the 80 nm particle treatment group displayed extensive renal tubular epithelial cell necrosis. Rats treated with 400 mg/kg of Cu NPs had The Malpighian tubes that were partially necrotic.

#### 4. Discussion

Copper nanoparticles have been extensively studied for use as antimicrobial (i.e., antiviral, antibacterial, antifouling, and antifungal) agents, antibiotic alternatives, and nanocomposite coatings [27–29]. Most metal nanoparticles dissolve and transfer to an ionic state once the conditions are appropriate. In contrast to copper micro-particles (17  $\mu\text{m}$ ), copper nano-particles (23.5 nm) can rapidly interact with artificial gastric acid juice and be transformed into ionic Cu ions with ultrahigh reactivity; therefore, we generally believed that the relative toxicities of Cu would be Cu ions > copper nano-particles > copper micro-particles [30]. Hence, when performing toxicology studies on metal nanoparticles, it is essential to distinguish between the effects of nano-particles themselves and dissolved metals. A recent study demonstrated that when mice were acutely exposed to copper nano- or micro-particles, only the copper nano-particles induced severe impairment in the kidney, liver, and spleen [31]. Experimental results now indicate that engineered nano-particles such as metal clusters, carbon nanotubes, fullerene, and quantum dots can penetrate cell membranes and be transported into endothelial cells, pulmonary epithelia, intestinal epithelia, alveolar macrophages, and other types of macrophages and neuronal cells *in vitro* [32]. Another study revealed that the toxicity of nano-sized copper particles was highly correlated with their size and specific surface area [11], suggesting that the toxicity of nano-materials may result from both the particles themselves and metal ions [33]. The dissolution rate of copper nano-particles after sonication in 1% HPMC has been calculated as 0.014% [34], thus we chose that vehicle for preparing our copper particle suspensions. In the current study, we compared the acute toxicity of five different Cu materials, and our results indicated that the relative LD<sub>50</sub> values were as follows: Cu ion < 30 nm copper particles < 50 nm copper particles < 80 nm copper particles < 1  $\mu\text{m}$  copper particles. Cu ions caused acute death after 2 h, while copper nano-particles caused significant gastrointestinal

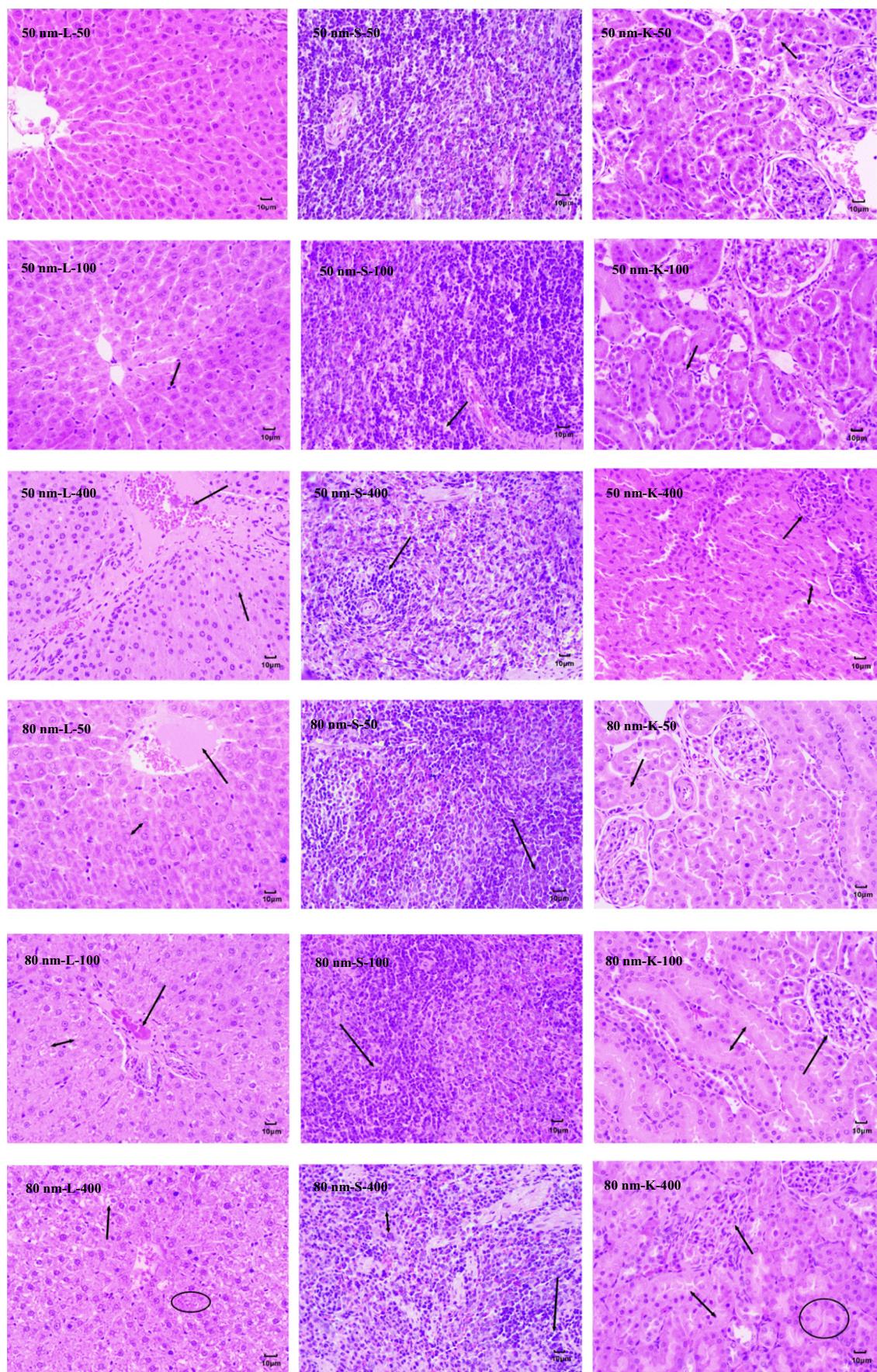


Fig. 5. (continued)

**Table 5**  
Mean  $\pm$  SD scores of lesions in liver, spleen and kidney.

Tissue	Control	Cu ion-200 mg/kg	1 μm-400 mg/kg			30 nm-50 mg/kg			30 nm-100 mg/kg			
			30 nm-400 mg/kg	30 nm-50 mg/kg	30 nm-100 mg/kg	30 nm-400 mg/kg	30 nm-50 mg/kg	30 nm-100 mg/kg	30 nm-400 mg/kg	30 nm-50 mg/kg	30 nm-100 mg/kg	
Liver	0.0 $\pm$ 0.00	0.0 $\pm$ 0.00	0.0 $\pm$ 0.00	0.0 $\pm$ 0.00	1.2 $\pm$ 0.22	1.2 $\pm$ 0.24	1.7 $\pm$ 0.24	3.3 $\pm$ 0.76	2.6 $\pm$ 0.53	3.2 $\pm$ 0.87	3.2 $\pm$ 0.87	
Spleen	0.0 $\pm$ 0.00	0.0 $\pm$ 0.00	0.0 $\pm$ 0.00	1.2 $\pm$ 0.15	1.1 $\pm$ 0.13	2.1 $\pm$ 0.10	2.1 $\pm$ 0.10	2.6 $\pm$ 0.53	3.2 $\pm$ 0.87	3.2 $\pm$ 0.87	3.2 $\pm$ 0.87	
Kidney	0.0 $\pm$ 0.00	1.1 $\pm$ 0.22	1.1 $\pm$ 0.22	1.1 $\pm$ 0.29	1.3 $\pm$ 0.31	1.3 $\pm$ 0.31	2.3 $\pm$ 0.43	3.2 $\pm$ 0.87	3.2 $\pm$ 0.87	3.2 $\pm$ 0.87	3.2 $\pm$ 0.87	
Tissue	50 nm-50 mg/kg	50 nm-100 mg/kg	50 nm-400 mg/kg	50 nm-50 mg/kg	50 nm-100 mg/kg	50 nm-400 mg/kg	80 nm-50 mg/kg	80 nm-100 mg/kg	80 nm-400 mg/kg	80 nm-50 mg/kg	80 nm-100 mg/kg	80 nm-400 mg/kg
	Liver	1.3 $\pm$ 0.32	2.2 $\pm$ 0.13	3.8 $\pm$ 0.91	2.2 $\pm$ 0.61	3.1 $\pm$ 0.69	3.7 $\pm$ 0.56	3.2 $\pm$ 0.45	3.2 $\pm$ 0.45	3.2 $\pm$ 0.45	3.2 $\pm$ 0.45	3.2 $\pm$ 0.45
Spleen	0.0 $\pm$ 0.00	1.3 $\pm$ 0.32	2.8 $\pm$ 0.12	1.1 $\pm$ 0.73	1.9 $\pm$ 0.38	2.4 $\pm$ 0.22	3.5 $\pm$ 0.98	2.4 $\pm$ 0.22	3.5 $\pm$ 0.98	3.5 $\pm$ 0.98	3.5 $\pm$ 0.98	3.5 $\pm$ 0.98
Kidney	1.9 $\pm$ 0.43	2.4 $\pm$ 0.54	3.5 $\pm$ 1.02	1.4 $\pm$ 0.77	2.4 $\pm$ 0.77	2.4 $\pm$ 0.77	3.5 $\pm$ 0.98	2.4 $\pm$ 0.77	3.5 $\pm$ 0.98	3.5 $\pm$ 0.98	3.5 $\pm$ 0.98	3.5 $\pm$ 0.98

symptoms that led to a slow death when the levels of Cu reached a lethal dose. Previous studies reported the oral LD<sub>50</sub> of Cu NPs (23.5 nm) in mice as 413 mg/kg, which places them in the category of “moderately toxic.” In contrast, Cu micro-particles (17  $\mu$ m) did not produce similar effects and were classified as nontoxic, with LD<sub>50</sub> values  $>$  5000 mg/kg [30]. Lee et al. [35] shown that the LD<sub>50</sub> of 33 nm and Cu ion are 1344 mg/kg and 640 mg/kg in male rats, our data shown the similar results and further demonstrated the particle size dependence of acute toxicity of nano copper.

The *in vivo* biodistribution of Cu NPs can provide essential information regarding their absorption, accumulation sites, and clearance. Because there is limited data concerning the biokinetics of different sizes of Cu NPs and Cu ions, we investigated their distribution in the liver, spleen, lung, brain, kidneys, testis, heart, and serum for a period of 1-week after giving a single oral dose to rats. The physico-chemical features and solubility of NPs influence their absorption and physiological/biochemical effects [36,37]. Our results showed that Cu ions were absorbed more rapidly than copper nano- and micro-particles, the liver, kidney, heart, and spleen were the main sites of Cu accumulation. However, because CuNPs are not easily eliminated from the body, it can stay in the body of rat longer than Cu ions. We found that the content of Cu in the target organs had increased 5 to 6-fold at 48 h after dosing. The Cu NPs showed the highest Cu accumulations in the liver and kidneys, and the concentrations of Cu originating from Cu NPs were higher than Cu originated from Cu ions and copper micro-particles at both 96 h and 1 week after dosing. Both Cu ion and Cu NPs administration resulted in Cu increase in testes, and the concentration reached their peak at 24 h in the testes before the peak of copper were reached in the liver, heart, lung, and kidneys. The smaller sized copper nano-particles showed the highest accumulations in the brain, kidneys, and liver, while the larger Cu nano-particles accumulated more readily in the heart and lungs. The spleen tended to accumulate Cu from 50 nm copper particles, and the content of Cu originating for those particles at 12 h and 24 h after dosing was higher than the content of copper which originated from the other sources. In most organs, Cu NPs were cleared more slowly than copper micro-particles and ions; thus, we speculated that the early phase of Cu absorption might be facilitated by dissolution of the nanoparticles. Further studies are needed to investigate whether copper nano-particles are absorbed as an entity in the intestine, and then presented as copper ions to organ tissue at a later time. Ingested Cu ions are mainly metabolized in the liver, and then excreted in bile [38,39]. Aggregation of NPs in gastrointestinal tract can lead to decreased absorption [40,41]. The smaller the nanoparticles are easier to agglomerate in stomach and the larger nanoparticles are easily dissolved under the effect of gastric acid. This will also affect its absorption and tissue distribution. Therefore, the absorption and distribution characteristics of nanoparticles need further study.

Based on the LD<sub>50</sub> values of copper nano-particles, micro-particles and ions, we chose 200 mg/kg as the dose of Cu ions, 400 mg/kg as the dose of copper micro-particles, and 50 mg/kg, 100 mg/kg, and 400 mg/kg, respectively, as dose of Cu NPs in our short-term exposure experiment. Our data indicated that a 400 mg/kg dose of three sizes of copper nano-particles could cause obvious toxic effects even induced death in rats, while the other doses had no influence on mortality. Administration of the 30 nm and 80 nm Cu NPs produced the same rates of mortality by day 7; however, the 80 nm particles produced 20% mortality rate four days sooner than the 30 nm particles did. Administration of the 50 nm copper particles caused 20% mortality by day 2, and higher mortality rates by day 7 than did the other size particles. The weights of the copper-treated rats decreased significantly, and the effect on weight at the dose of 400 mg/kg was dependent on particle size. The larger copper nano-particles caused more significant decreases in animal weight. There was an obvious difference in the relative organ weights of rats treated with the different copper materials, and the copper nano-particles induced the most significant changes in organ weight. All of the copper materials caused significant

decreases in lung weight, and Cu NPs produced that effect at doses of 100 mg/kg and 400 mg/kg. When administered at a dose of 400 mg/kg, Cu NPs induced significant increases in the weights of the heart, kidney, and testes, and a significant decrease in the weight of the spleen. A 400 mg/kg dose of both 30 nm and 50 nm copper particles induced significant increases in relative liver weight. In contrast, the same dose of 80 nm copper particles had no effect on liver weights, and micro-copper particles induced significant decreases in relative liver weight. The higher toxicity of nanosized particles compared to the released metal fraction is that these small particles were observed, in contrast to most metal ions, nano-particles can more easily pass the cell membrane [42,43]. Nano-copper of different particle sizes have different penetrating and accumulating ability to tissues and organs, which may be the reason that causes the difference of target organ toxicity caused by nano-copper and copper ions.

To our understanding the acute toxicity of Cu NPs, we performed a 7-day study which evaluated various hematologic and histopathologic changes which occurred in the treated rats. Previous studies have indicated that Cu NPs produce hepatotoxic and nephrotoxic effects [33]. Our data indicated that the liver and kidney toxicities of Cu NPs were both size- and dose-dependent. The increased ALT and AST levels indicated significant liver damage, which induced a protein synthesis disorder and resulted in decreased serum TP, GLB, HGB, and ALB levels. Kidney damage resulted in the increase of urea and creatinine. Kidney damage leads to a decrease in erythropoietin production, which indirectly leads to a decrease in the number of RBC cells. Cu NPs also induced significant reductions in blood glucose levels, which may have been a result of Cu NP-induced intestinal damage and resultant malabsorption. Because we found significant increases in inflammatory cells, we speculated Cu NPs caused liver and kidney inflammation. Our histopathology studies showed that Cu NPs can cause both morphological and histological changes in the liver, kidneys and spleen. Repeated oral administration of Cu NPs to rats at a dose of 200 mg/kg/d for 7 days has been shown to cause necrosis of hepatocytes and renal proximal tubules, accompanied by hepatic and renal dysfunction [44]. The 80 nm copper particles administered at a dose of 400 mg/kg produced the most obvious toxic effects as judged by physiological and biochemical indexes. At the same time, it has a strong correlation with different sizes of nano-copper in different organs showing different accumulation. Oral exposure to nano-copper is the most common exposure route, but the toxicity of oral exposure is not only related to its own physical and chemical properties, but also affected by the body's gastrointestinal system and internal environmental conditions. Therefore, our experimental results provide a reference to toxicity data, but its in-depth toxicity mechanism needs further study.

## 5. Conclusions

In summary, our study demonstrated both the toxicity and the damage of Cu NPs in rats. Although the ranking of LD<sub>50</sub> values for Cu is Cu ions < 30 nm copper particles < 50 nm copper particles < 80 nm copper particles < 1 μm copper particles, our physiological and biochemical data indicated that 80 nm copper particles were the most toxic in rats when the animals were repeatedly short-term treated with copper particles with oral. Although all of the Cu NPs produced significant toxic effects in the kidney, liver and spleen, the three differently sized nano-particles had different target organs for their Cu distribution. The size of 30 nm Cu NP has higher content in kidney and brain, 50 nm Cu NP has higher content in liver and spleen and 80 nm Cu NP has higher content in heart and lung. Cu ion and 1 μm Cu more easily increase the level of Cu in testis. This implies that the size of the Cu NPs not only determined its relative order of toxicity, but also its effect on target organs.

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## Author contributions

“Min Xu, Huaqiao Tang, and Yinglun Li conceived and designed the experiments; Min Xu, Huaqiao Tang, XueRong Zhou, Qian Rong, and Yuanli Zhang performed the experiments; Min Xu, Huaqiao Tang, Fei Shi, Cheng Lv, and Yinglun Li analyzed the data; Ling Zhao and Gang Ye contributed reagents/materials/analysis tools; Min Xu and Huaqiao Tang wrote the paper.”

## Conflict of interest

The authors have no conflicts of interest to report, and are fully responsible for the content and writing of this manuscript.

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