

Acute toxicological effects of copper nanoparticles in vivo

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Abstract

To assess the toxicity of copper nanoparticles (23.5 nm) in vivo, LD₅₀, morphological changes, pathological examinations and blood biochemical indexes of experimental mice are studied comparatively with micro-copper particles (17 μm) and cupric ions (CuCl₂·2H₂O). The LD₅₀ for the nano-, micro-copper particles and cupric ions exposed to mice via oral gavage are 413, >5000 and 110 mg/kg body weight, respectively. The toxicity classes of nano and ionic copper particles both are class 3 (moderately toxic), and micro-copper is class 5 (practically non-toxic) of Hodge and Sterner Scale. Kidney, liver and spleen are found to be target organs of nano-copper particles. Nanoparticles induce gravely toxicological effects and heavy injuries on kidney, liver and spleen of experimental mice, but micro-copper particles do not, on mass basis. Results indicate a gender dependent feature of nanotoxicity. Several factors such as huge specific surface area, ultrahigh reactivity, exceeding consumption of H⁺, etc. that likely cause the grave nanotoxicity observed in vivo are discussed.

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1. Introduction

Health effects of nanoparticles are attracting considerable and increasing concern of the public and government worldwide. So far, most of the nanotoxicity research focused on respiratory tract exposures for assessing the health effects of nanoparticles. Other expo-

sure routes, e.g., gastrointestinal tract also needs to be considered as potential portals of entry. There are many ways that nanoparticles can be ingested into the gastrointestinal tract. For instance, nanoparticles cleared from the respiratory tract via the mucociliary escalator can subsequently be ingested into the gastrointestinal tract; nanomaterials can be ingested directly via water, food, cosmetics, drugs, drug delivery devices, etc. (Peter et al., 2004; Oberdörster et al., 2005). Uptake of particles of different size via the gastrointestinal tract can also lead to different toxicological effects (Jani et al., 1994; Böckmann et al., 2000). But the reports about

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toxicological research of nanomaterials by the gastrointestinal tract are few.

Nanosized copper particles (herein after refer to as “nano-copper”), one of the manufactured nanoparticles, are now industrially produced and available commercially. Recently, nano-copper particles are used as the additive in lubricants, polymers/plastics, metallic coating and inks, etc. Due to excellent mending effects of nano-copper particles, they are added into lubricant oil as an additive to effectively reduce friction and wear, or to mend a worn surface (Liu et al., 2004). Nano-copper particles are homogeneously deposited on the surface of graphite to improve the charge–discharge property significantly, such as coulombic efficiency, cycle characteristics, and high rate performance as an anode material for lithium ion batteries (Guo et al., 2002). The copper-fluoropolymer nano-composite is employed as bioactive coatings that are capable of inhibiting the growth of target microorganisms such as *Saccharomyces cerevisiae*, *Escherichia coli*, *Staphylococcus aureus*, and *Listeria* (Cioffi et al., 2005). Accordingly, nano-copper particles, similar to any of other nanomaterials, are likely to enter the environment and human body via different paths such as effluent, spillage during shipping and handling, consumer products and disposal, etc.

In human body, copper is maintained in homeostasis (Jesse and Mary, 2004). If the intake of copper exceeds the range of the human tolerance, it would cause toxic effects such as hemolysis, jaundice and even death. Most recently, the study indicates that the overload of common copper in vivo can induce a set of toxicological activities such as hepatocirrhosis (Björn et al., 2003), changes in lipid profile, oxidative stress, renal dysfunction (Galhardi et al., 2004) and stimulation of mucous membrane of alimentary canal, etc. However, recent toxicological investigations of manufactured nanoparticles revealed such a nature that compared with the larger particles of the same chemical composition (on the identical mass basis), nanoparticles tends to exhibit quite different toxicological effects in vivo (for example, Oberdörster, 1994; Donaldson et al., 1998; Warheit et al., 2004). Specifically, for nano-copper particles, compared with the micro-copper, their primary alteration in biochemical property is the higher reactivity originated from a larger specific surface area. How this property can alter the toxicological effects in vivo? In this paper, the mice are exposed to nanoscale and micro-sized particles of copper via gastrointestinal tract, the nanotoxicity in vivo as well as differences in toxicological effects of nano- and micro-copper particles are investigated comparatively on mass basis.

2. Materials and methods

2.1. Tested chemicals

The nano-copper particles (25 nm) were purchased from Shenzhen Junye Nano Material Co., Ltd. Before the use, the nanoparticles were weighed and divided into several ampoules (each weigh 1 g) under an air-free condition of a specially designed glove box that was filling with dry argon gas, where the nano-copper-loaded ampoules were stored until the animal experiments. The size distribution and specific surface area of nano-copper were analyzed by atomic force microscopy (AFM, Nano III a SPM, Digital Instruments Inc. USA 3A). Micro-copper particles (200-mesh) were purchased from Beijing Hao Yun Co. Ltd. Before the experiment, the size was measured using transmission electron microscopy (TEM, Hitachi H-700 electron microscope) techniques. The impurities (e.g. aluminum, barium, calcium, cadmium, chromium, iron, magnesium, manganese, molybdenum, sodium, potassium, nickel, lead, strontium, zinc, etc.) in both nano- and micro-copper particles were analyzed using X-ray fluorescence spectroscopy (XRFS). The results indicate that the purity of nano- and micro-copper both are better than 99.9%. $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, provided from Shanghai Chemicals Co. Ltd. (purity >99.9%), was used as ion-copper in the experiment.

Hydroxypropylmethylcellulose K4M (HPMC, analytical grade), the suspending agent for copper particles, is obtained from Shanghai Colorcon Coating Technology Limited. To insure the non-reaction to oxygen, the nano-copper particles were dispersed into the 1% w/v HPMC solution inside the glove box which was filled with dry argon gas. The suspending solutions containing copper nanoparticles were treated by ultrasound for 10 min and vibrated for 2 min. Then, these solutions in different doses were subsequently exposed to mice via oral gavage. To ensure non-ionization and non-aggregation of nano-copper before administration, the time interval from preparation to oral gavage was strictly limited in less than 20 min. In addition, 20 min after the preparation, the particle size and surface property of nano-copper were analyzed by AFM, and the cupric ion in the solution was measured by the chemical titration method which was used to monitor if copper is transformed into ions in the suspending agent before oral administration.

2.2. Animals

ICR mice of either sex (provided by Weitong-Lihua Experimental Animal Center), aged 8 weeks and weighing 20–22 g, were used in the experiments. Every five mice with same sex were housed in stainless steel cages containing sterile paddy husk as bedding in ventilated animal rooms. They were acclimated in the controlled environment (temperature: $22 \pm 1^\circ\text{C}$; humidity: $60 \pm 10\%$ and light: 12 h light/dark cycle) with free access to water and a commercial laboratory complete food. All animal experiments were performed in compliance with the local ethics committee.

Table 1
Exposed doses for micro-, nano- and ion-copper used in the animal experiment

Micro-group	Dose (mg/kg)	Nano-group	Dose (mg/kg)	Ion-group	Dose (mg/kg)
M1	500	N1	108	I1	24
M2	734	N2	158	I2	35
M3	1077	N3	232	I3	51
M4	1851	N4	341	I4	75
M5	2320	N5	501	I5	110
M6	3406	N6	736	I6	162
M7	5000	N7	1080	I7	237

Mice of the control group were treated with HPMC (1%) solution.

2.3. Acute toxicity and the LD₅₀ measurement

To process the toxicological studies of micro- (M), nano- (N) and ion- (I) copper, a series of doses are set (the dose of CuCl₂·2H₂O is calculated on copper mass), which are labeled as M1, M2, . . . , M7; N1, N2, . . . , N7; I1, I2, . . . , I7 groups (Table 1).

To obtain the LD₅₀ of micro-, nano- and ion-copper, the experiments and its intervals are designed in accordance with the method provided by the Organization for Economic Cooperation and Development (OECD). After acclimatization of the environment, mice were exposed to nano- and ion-copper with doses listed in Table 1 by a single oral gavage. Single animal was dosed in sequence (N1–N7; I1–I7). The first animal receives a dose one step below the assumed estimate of the LD₅₀. If the animal survives, the second animal receives a higher dose. If the first animal dies, the second animal receives a lower dose. Micro-copper showed nontoxic below regulatory limit doses (i.e., 5000 mg/kg); hence, the micro-copper is evaluated by limit test at 5000 mg/kg. The LD₅₀ and 95% profile likelihood (PL) of nano-, micro- and ion-copper are obtained from the experimental data analysis by AOT425 program (OECD guideline 425) (Whitehead and Stallard, 2004). Methodologically, as the data of the conventional method (Karber's Method, Gené and Robles, 1987) is gained based on a stricter measurement, its LD₅₀ test was also applied to determine the LD₅₀ of these particles in mice, and the results are together presented for a comparison.

2.4. Morphological and pathological examinations

To examine the morphological and pathological changes, 90 mice of either sex were exposed to micro- and nano-copper particles with the same doses of M1–M7, N1–N7 and the control (1% HPMC). They were anesthetized by i.p. pentobarbital at 48 h and autopsied. The organs such as lung, liver, kidney, heart, spleen, brain, testes (male) and ovary (female) were stripped out and weighed accurately. All of the organs were immediately fixed in 10% formalin and subject to further histopathological examinations. The tissues of organ samples were embedded in paraffin blocks, then sliced and placed onto glass slides.

After histological H–E staining, the slides were observed and the photos were taken using optical microscope (Nikon U-III multi-point sensor system), and the identity and analysis of the pathology slides were blind to the pathologist.

2.5. Blood biochemical assay

To analyze the changes of biochemical indexes, another 90 mice of either sex (divided into 15 group, each group contains three male plus three female) were exposed by the oral gavage with the same doses of M1–M7, N1–N7, and the control (1% HPMC). After 72 h of postexposure, blood samples were collected via the ocular vein (about 0.8–1 ml each mouse). Then, the blood samples were centrifuged twice at 3000 rpm for 10 min in order to separate serum. The total bile acid (TBA), alkaline phosphatase (ALP), blood urea nitrogen (BUN) and creatinine (Cr) levels of serum were measured by biochemical autoanalyzer (Type 7170, Hitachi, Japan). The statistical significance of the changes between tested groups and the control group were analyzed by multiple comparison test method (*t*-test) using SAS 6.12 (SAS Institute, Inc., Cary, NC).

3. Results

3.1. Nanoparticle characterizations

The average size of nano-copper particles is 23.5 nm in diameter, determined by a high solution atomic force microscopy (Fig. 1). The average size of micro-copper is 17 μm measured by the transmission electronic microscopy method. The specific surface areas of the nano- and micro-copper particles are 2.95×10^5 and 3.99×10^2 cm²/g, respectively. In per unit mass (1 μg), the particle number for nano-copper is 1.7×10^{10} μg⁻¹ and that for micro-copper is only 44 μg⁻¹. The results for nano-copper both with and without 1% HPMC indicate that the influence of the inert suspending agent on the particle size and surface property of nanoparticles is little. In addition, the results of the chemical titration indicate that neither micro- nor nano-copper particles in the HPMC suspending solution could be transformed into copper ion before the oral administration of animal experiments.

3.2. Acute toxicity and LD₅₀ in vivo

To determine LD₅₀, experimental animals are dosed at different levels (Table 1). After exposure, the mortality in each dose group was observed and recorded. LD₅₀ values of the test samples were hence calculated by AOT425 program (OECD guideline 425) and Karber method (Table 2). The LD₅₀ of 413 mg/kg determined by OECD test guideline 425 with statistical software

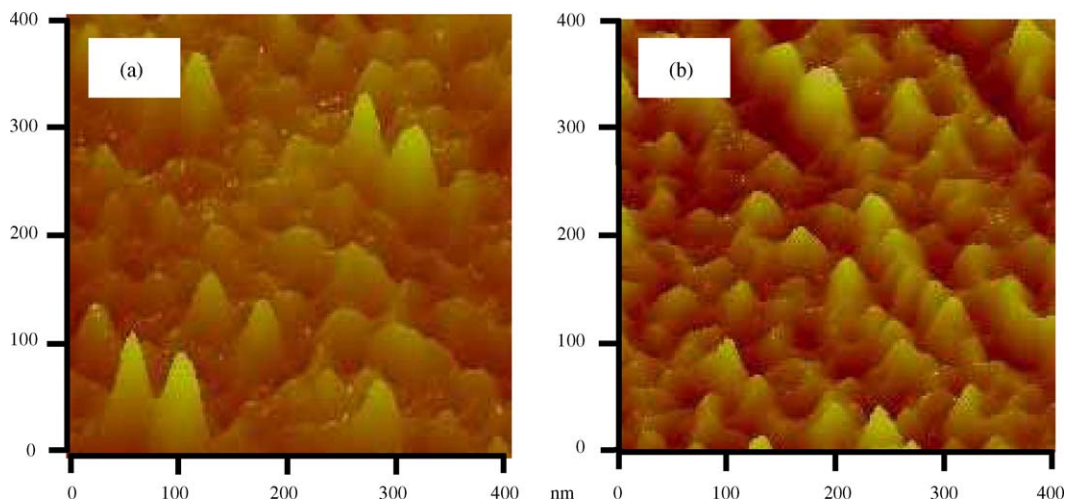


Fig. 1. An atomic force microscopic image for nano-copper particles without suspending agent (a) and in suspending agent of 1% HPMC (b).

(AOT425) and the same value (413 mg/kg) by the conventional method suggest that toxicity of nano-copper particles belongs to class 3 (moderately toxic) of Hodge and Sterner Scale. The LD₅₀ of cupric ions, 110 mg/kg by OECD test guideline 425 and 118.9 mg/kg determined by the conventional method, belongs to the class 3, moderate toxicity. While for the micro-copper particle, the LD₅₀ is greater than 5000 mg/kg determined by OECD test guideline 425 and 5610 mg/kg determined by the conventional method, indicating practically non-toxic, class 5.

Symptoms exhibited by the experimental animals after exposure to the test particles were observed during the experimental period. In contrast to micro-copper treated mice among which only few mice exhibited symptoms of poisoning, all mice treated by nano-copper appeared obviously symptoms of alimentary canal function disorder such as loss of appetite, diarrhea and vomiting, etc. In addition, some mice in nano- and ion-group

showed passive behaviour, hypopnea, tremor and arching of back.

3.3. Morphological changes in mice

Photos in Fig. 2 show morphological changes of kidney of experimental mice exposed to micro-copper (M3, 1077 mg/kg), nano-copper (N7, 1080 mg/kg) and the control. The kidney appearance of micro-copper exposed mice (Fig. 2(a)) is almost same with the control (Fig. 2(c)), but that of nano-copper exposed mice (Fig. 2(b)) exhibits dramatic changes in color and became bronze-colored.

Fig. 3 shows photos of the spleen of experimental mice in M3, N7 and the control groups. Micro-copper particles (Fig. 3(a)) cause a slight change as compared with the control (Fig. 3(c)), but nano-copper particles (of the similar mass) cause severe atrophy and color changes (Fig. 3(b)) of spleens. To

Table 2

The median lethal dose (LD₅₀), 95% confidence interval and toxicity class for micro-, nano- and ion-copper particles, obtained by OECD test guideline 425 and the conventional (Karber) methods, respectively

Particles	Specific surface area (cm ² /g)	Particle number (μg ⁻¹)	LD ₅₀ (mg/kg)	95% PL (mg/kg) ^a 95% FL (mg/kg) ^b	Toxicity Class (Hodge and Sterner scale)
Micro-copper (17 μm)	3.99 × 10 ²	44	>5000 ^a 5610 ^b	N/A ^a 5075–6202 ^b	Non-toxic Class 5
Nano-copper (23.5 nm)	2.95 × 10 ⁵	1.7 × 10 ¹⁰	413 ^a 413 ^b	305–560 ^a 328–522 ^b	Moderately toxic Class 3
Ion-copper (0.072 nm)	6.1 × 10 ⁵	9.4 × 10 ¹⁵	110 ^a 119 ^b	93–145 ^a 102–139 ^b	Moderately toxic Class 3

^a Data obtained by OECD test guideline 425.

^b Data obtained by the conventional (Karber) method.

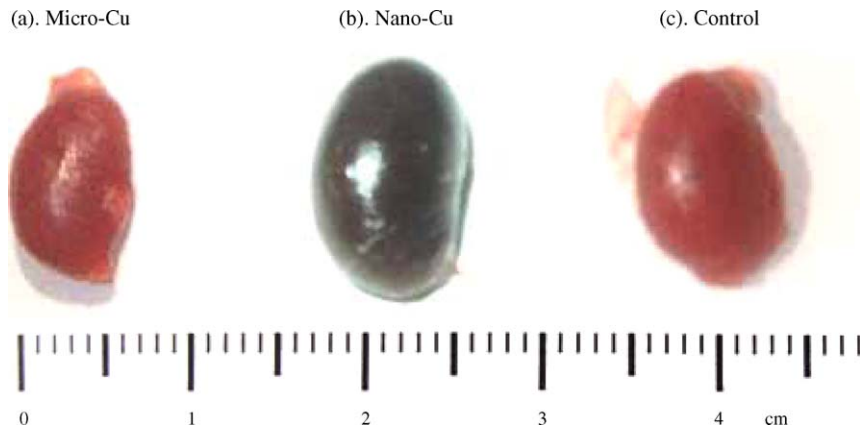


Fig. 2. The appearance of kidneys of experimental mice in dose groups M3 (1077 mg/kg) (a); N7 (1080 mg/kg) (b) and the control (c).

explicitly examine the grade of changes caused by the copper particles, spleen index (S_x) is defined as:

$$S_x = \frac{\text{weight of experimental spleen/weight of the experimental animal}}{\text{weight of the control spleen/weight of the control animal}}$$

The average value of S_x in micro-copper treated mice groups, is 0.97 ± 0.17 , closed to 1.00 ± 0.08 , the normal parameter obtained from the control mice. But in nano-copper treated mice groups the spleen index declines to be as small as 0.58 ± 0.12 , indicating that nano-copper particles induced a dramatic atrophy of spleen. This implies that spleen is one of the target organs for nanoscale copper particles. In addition, the cholecysts of some experimental mice exposed to nano-copper particles show a blackish color at necropsy. The other organs

do not show obvious macroscopic morphological abnormalities.

3.4. Pathological changes in mice

In mice exposed to micro-copper at almost all dose levels, necropsy and pathological examinations of the experimental animals do not show observably pathological changes with viscera. Only in the highest dose group (M7), 1 male and 1 female mice died and whose intestines show ileus. Unlike these observations, viscera (e.g., kidney, spleen and liver) of all mice (N1–N7) exposed to nano-copper particles were gravely harmed. In Table 3, we summarize and compare the pathologi-

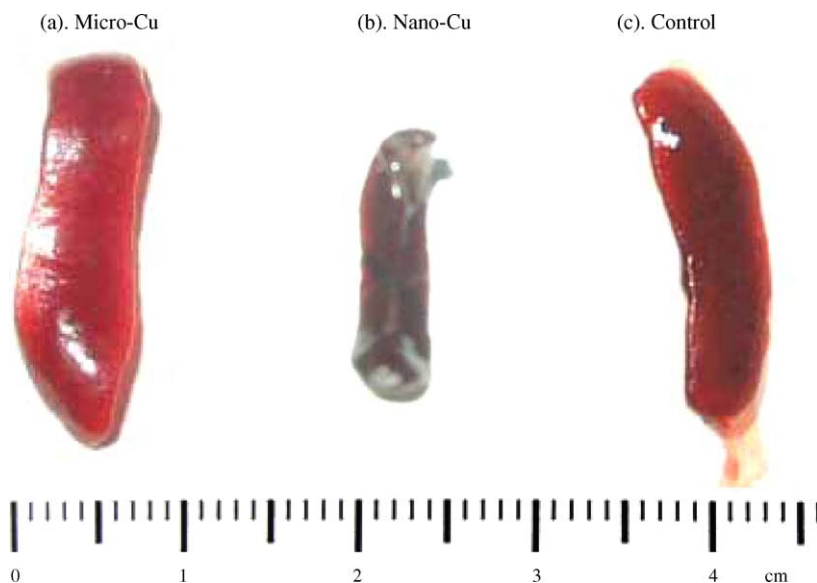


Fig. 3. The appearance of spleens of experimental mice in dose groups M3 (1077 mg/kg) (a); N7(1080 mg/kg) (b) and the control (c).

Table 3

Pathological changes and their severeness in experimental mice exposed to nano-copper (N1–N7), micro-copper (M1–M7) and the control (C)

	I	II	III	IV	V	VI	VII
N1	–	–	–	–	–	–	–
N2	–	–	+	–	–	–	–
N3	+	–	++	–	–	+	+
N4	+	++	+++	+	+	+	++
N5	++	+++	+++	+	+	++	++
N6	+++	++++	+++	++	+	++	++
N7	+++	++++	+++	+++	++	++	+++
M1	–	–	–	–	–	–	–
M2	–	–	–	–	–	–	–
M3	–	–	–	–	–	–	–
M4	–	–	–	–	–	–	–
M5	–	–	–	–	–	–	–
M6	–	–	–	–	–	–	–
M7	–	–	+	–	–	–	–
C	–	–	–	–	–	–	–

I: Glomerulitis; II: degeneration and necrobiosis of renal tubule; III: proteinic liquid in renal tubule; IV: purple deposition; V: steatosis of hepatic tissue; VI: atrophy of spleen; VII: dwindling of splenic units and fibrosis; +: degrees of pathological changes; –: no pathological changes; +: faintness; ++: median; +++: severity and ++++: deadly severity.

cal changes and their severeness observed from all mice groups exposed to nano-copper (N1–N7), micro-copper (M1–M7) and the control (C). In addition, nanoparticles-induced tissue damages show an explicit dose-dependent trend: the higher the exposed dose of nanoparticles is, the severer the damage to the viscera of experimental mice is. However, as one sees from Table 3, all the mice exposed to micro-copper particles (even at much higher dose levels) do not show such pathological changes and tissue damages as observed from mice of nano-copper groups. The photomicrographs show pathological examinations in kidney (Figs. 4 and 5), liver (Fig. 6) and spleen (Fig. 7): (a) represents the control; (b–d) correspond to the results from experimental mice of N1 (108 mg/kg, the lower dose group), N4 (341 mg/kg, the medium dose group) and N7 (1080 mg/kg, the higher dose group), respectively. Damages of renal proximal tubular cells are clearly observed in mice exposed to nano-copper particles. In renal tissues (Fig. 4), glomerulus are swollen and dwindle in the lumen of Bowman's capsules, being signs of glomerulonephritis. In Fig. 5, further pathological changes within the renal tubule are visible: (1) epithelial cells of renal proximal convoluted

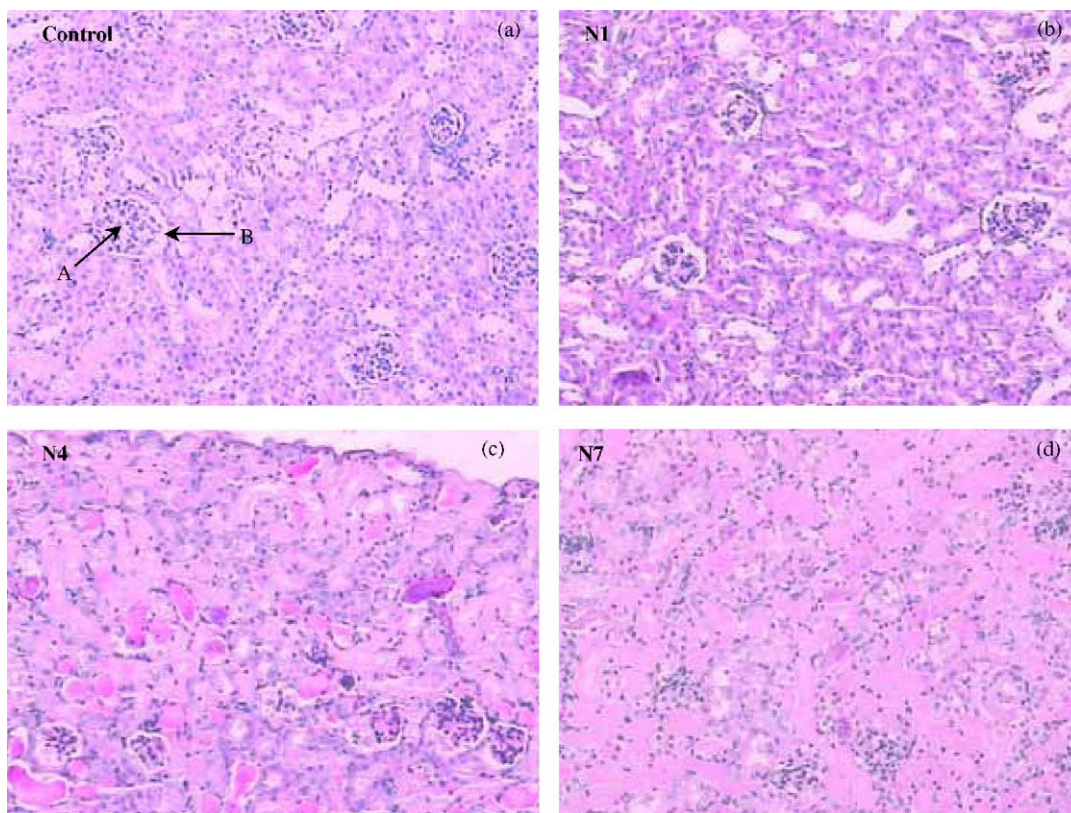


Fig. 4. The microscopic pictures ($\times 100$) show the pathological changes in kidney tissues of experimental mice of the control (a); lower dose group N1 (b); medium dose group N4 (c) and higher dose group N7 (d). A: renal glomerulus and B: Bowman's capsule.

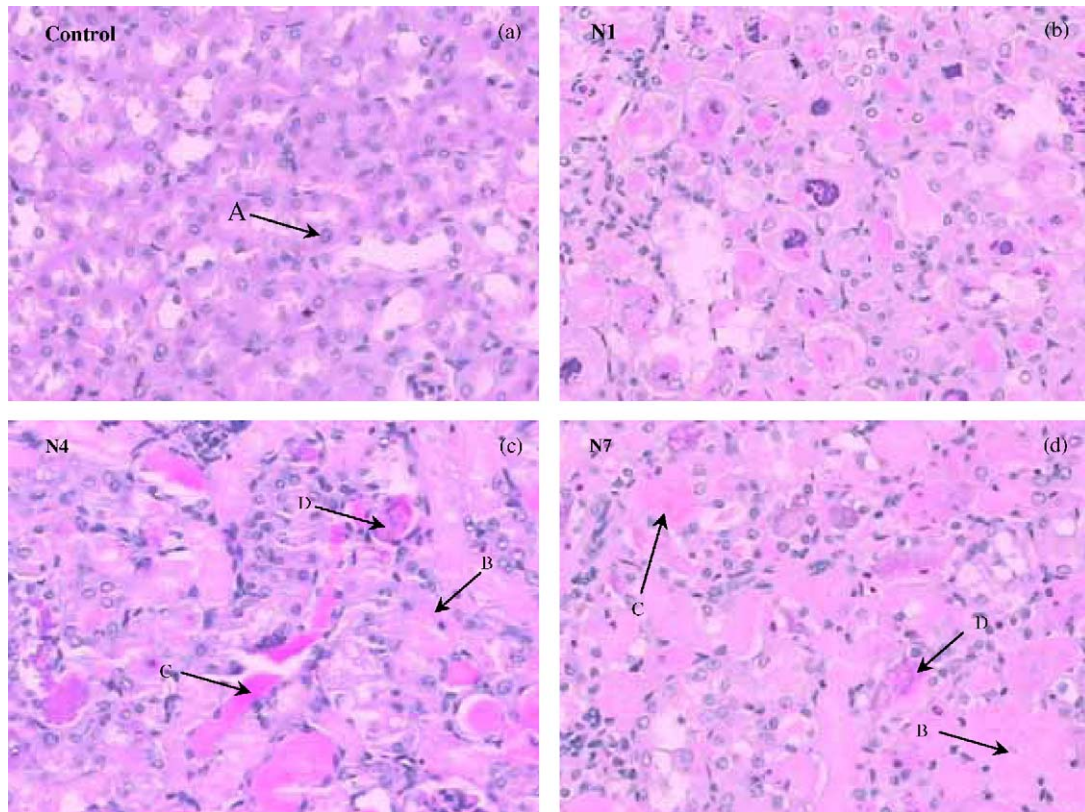


Fig. 5. The same with Fig. 4 but with magnification $\times 200$. A: the alive epithelial cells of renal tubule; B: the necrobiotic epithelial cells of renal tubule; C: the proteinic liquid and D: the purple deposition.

tubules degenerate in the mice exposed to nano-copper of the medium dose N4 (5(c)); (2) irreversibly massive necrobiosis occurs in mice exposed to nano-copper of higher dose N7 (5(d)); (3) karyons of the epithelial cells of renal tubules are clearly seen in mice of N1 (5(b)) and the control (5(a)), but they become less in N4 (5(c)) and nearly vanish in N7 (5(d)) group; (4) proteinic liquid filled in renal tubules is seen, in which purple deposition is further found in the kidney tissues of mice of the medium and higher dose groups, e.g., N4 and N7 (5(c) and 5(d)), but not found in those of the lower dose groups, e.g. N1 (5(b)). These observations explicitly indicate a dose-dependent feature in the nano-copper particles induced damages to renal tissues, with increasing dose the damage to kidneys of the exposed mice quickly becomes heavier.

At a medium dose level of nano-copper particles, e.g., in N4 group, the steatosis (indicated by arrows in Fig. 6) around venae centrals of hepatic tissue of the experimental mice was observed. Splenic atrophy was observed in mice groups from N3 to N7, but not in N1 and N2, all micro-copper groups and the control. In Fig. 7,

nanoparticles-induced dwindling of splenic units, reducing of lymphocytes and fibrosis of splenic interstitium are observed. Except for those displayed in Figs. 4–7, none observable pathological changes in other organs of the experimental mice were found, they showed similar to those of the control group.

3.5. Blood biochemical parameters

The observations from the pathological examinations and morphological changes indicate that liver and kidney are two of the target organs for nanoparticles of copper via the oral exposure route. Hence, blood biochemical parameters (BUN, Cr, TBA and ALP) that reflect the renal and hepatic functions were further investigated (Table 4). No statistically significant difference between the control and micro-copper treated mice groups is observed, except a very slight increase of TBA in mice of the higher dose groups M6 and M7. But contrarily, in all mice exposed to nanoparticles, these four biochemical indexes became significantly higher than the control. The abnormality of BUN and Cr is particularly obvious.

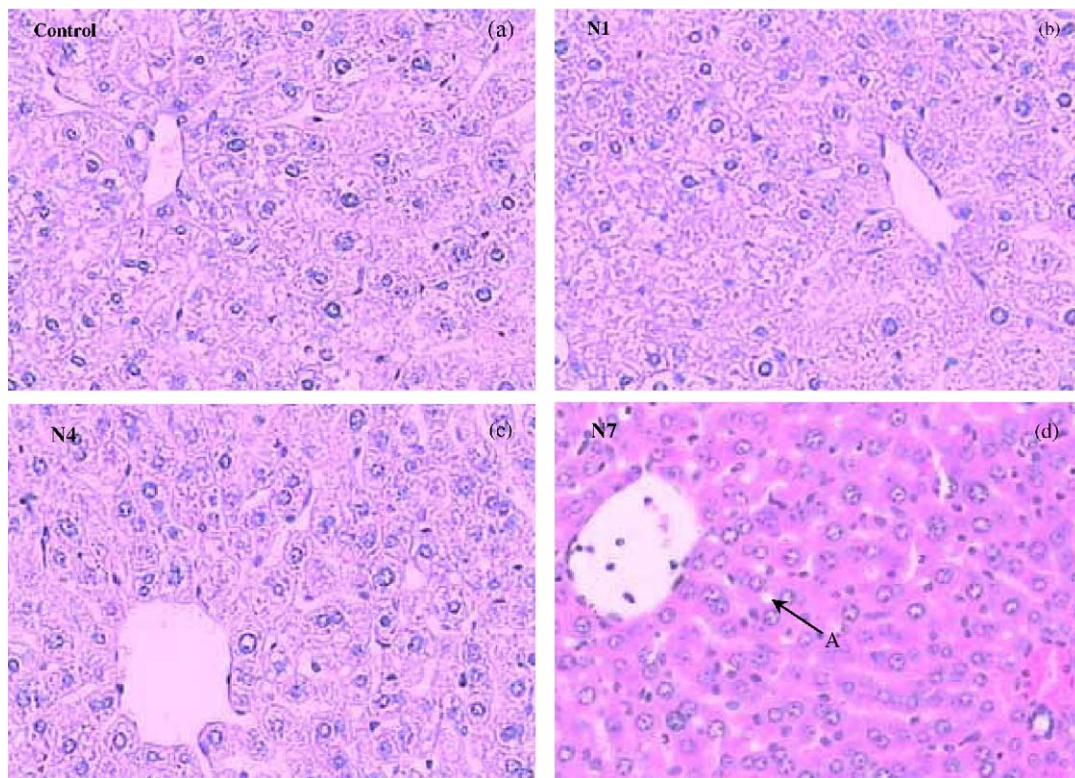


Fig. 6. The microscopic pictures ($\times 200$) show the pathological changes in liver tissues of experimental mice of the control (a); groups N1 (b); N4 (c) and N7 (d). A: steatosis.

Table 4

Statistical results of blood biochemical indexes for experimental mice exposed to nano-copper (N1–N7), micro-copper (M1–M7) and the control (C)

Group	Dose (mg/kg)	BUN (mmol/L)	Cr ($\mu\text{mol/L}$)	TBA ($\mu\text{mol/L}$)	ALP (IU)
N1	108	8.8 ± 1.0	55.8 ± 2.5	1.70 ± 0.94	105 ± 17
N2	158	7.8 ± 0.8	52.3 ± 2.6	1.33 ± 0.38	102 ± 32
N3	232	8.6 ± 1.5	$59.8 \pm 5.0^*$	1.87 ± 1.27	109 ± 24
N4	341	10.5 ± 2.7	$57.7 \pm 6.6^*$	2.20 ± 1.34	110 ± 28
N5	501	$12.7 \pm 1.5^{**}$	$68.2 \pm 5.3^{**}$	$1.97 \pm 0.46^{**}$	133 ± 46
N6	736	$14.3 \pm 2.7^{**}$	$66.0 \pm 5.6^{**}$	$2.08 \pm 0.74^*$	$186 \pm 23^{**}$
N7	1080	–	–	–	–
M1	500	7.8 ± 1.1	48.2 ± 3.9	1.83 ± 0.73	96 ± 20
M2	734	8.0 ± 0.5	49.0 ± 4.8	1.35 ± 0.19	92 ± 19
M3	1077	8.3 ± 1.2	50.7 ± 4.7	1.50 ± 0.71	131 ± 52
M4	1851	7.7 ± 1.3	49.7 ± 6.5	1.20 ± 0.21	$79 \pm 7^*$
M5	2320	9.1 ± 1.4	50.3 ± 2.9	1.47 ± 0.53	105 ± 15
M6	3406	8.3 ± 0.8	50.8 ± 3.9	$1.60 \pm 0.35^*$	113 ± 41
M7	5000	7.6 ± 1.4	49.3 ± 2.9	$1.43 \pm 0.15^*$	110 ± 48
C	0	8.8 ± 1.3	51.8 ± 3.6	1.22 ± 0.24	112 ± 27

All animals in N7 group died within 72 h, for those mice no blood samples and data were obtained.

* Indicate a $P \leq 0.05$ vs. the control.

** Indicate a $P \leq 0.01$ vs. the control.

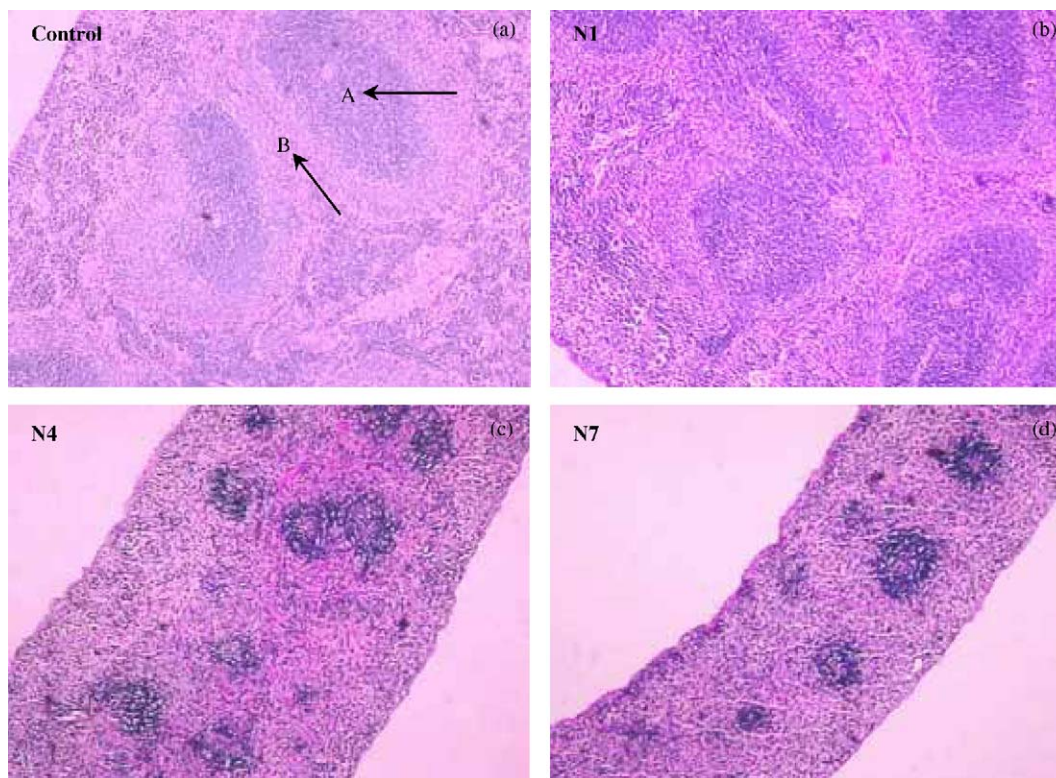


Fig. 7. The microscopic pictures ($\times 40$) show the pathological changes in spleen tissues of experimental mice of the control (a); groups N1 (b); N4 (c) and N7 (d). A: splenic unit and B: lymphocytes.

Note that because all animals in N7 group died within 72 h, no blood samples from these mice were collected. If comparing the results for N6 and M7 groups in Table 4, one can easily find that even though the exposure dose of micro-copper is about seven times higher than that of nano-copper particles, nano-copper do, while micro-copper do not cause any abnormality (relevant to dysfunction of liver and kidney) in the blood biochemical parameters.

4. Discussion

4.1. Nanotoxicity caused by the small size of particles

For insoluble particles, their pathway and extent of uptake through the digestive tract are known to be size-dependent (Hodges et al., 1995; Donaldson et al., 1998). Nanosized particles can cross small intestine by persorption and further distribute into blood, brain, lung, heart, kidney, spleen, liver, intestine and stomach (Hillyer and Albrecht, 2001). The small size copper particles associated with ultrahigh activity likely inter-

act with local tissues and provoke dysfunctions of the organs. Nanoparticles can translocate from the lumen of the intestinal tract via aggregations of intestinal lymphatic tissue (Peyer's patches (PP)) containing M-cells (specialized phagocytic enterocytes). Moreover, they can promote phagocytosis at gastrointestinal mucosa and cause antigen-mediated immune responses (Lomer et al., 2002). Study of nanoparticles of polystyrene latex by oral administration indicates that nanoparticles can be absorbed across the gastrointestinal tract, and pass through the mesentery lymph supply and lymph node to liver and spleen (Jani et al., 1990). Moreover, the drug carrier research suggests that nanoscale substances are easily taken up by the reticuloendothelial system (RES) (Yoshifumi, 2002). As shown in the present results of the pathological, morphological experiments and blood biochemical index assay, in nano-copper treated mice groups, dwindling of splenic units, reduction of lymphocyte numbers, and the sharp decline of splenic index are observed. These toxicological effects may be attributed to the similar processes as suggested in reference mentioned above, though the specific mechanism needs further study.

4.2. Nanotoxicity caused by the massive formation and overload of copper ions in vivo

The LD₅₀ values of nano-copper (23.5 nm) and ion-copper (0.072 nm) particles are 413 and 110 mg/kg body weight, respectively; they are both considered as moderately toxic materials. The LD₅₀ of nano-copper particles lay in rang of toxicity comparable to the toxicity of copper salts. It is implied that the toxicity of nano-copper may correlate with its ionization in vivo. Comparably, the LD₅₀ value of micro-copper (17 μm) is more than 5000 mg/kg, in a non-toxic class. The specific surface areas for the 23.5 nm and 17 μm copper particles are 2.95×10^5 and 3.99×10^2 cm²/g, respectively. The particle number (μg⁻¹) for micro-copper is only 44 μg⁻¹, while that for nano-copper is 1.7×10^{10} μg⁻¹. Accordingly, compared with the micro-copper, nano-copper particles (of the same mass) possess much higher collision probability with bio-substances in vivo. In accordance with the collision theory, the higher the collision probability of particles is, the higher the chemical activity of particles is. For the micro-sized particles, because less can be transformed into ionic states in the gastric acid, the primary reason causing toxicity in vivo should be the obstruction in gastrointestinal tract. This is consistent with the observation that ileus occurred in mice at the dose of 5000 mg/kg. For the nano-sized copper particles, the huge specific surface area leads to the ultrahigh reactivity. When they are taken into stomach, nano-copper particles react drastically with hydrogen ions (H⁺) of gastric juice and can be quickly transformed into ionic states. This chemical processes undoubtedly results in an overload of ionic copper in vivo.

In vivo, homeostasis of copper ions is maintained (Jesse and Mary, 2004), are metabolized in liver (Tao et al., 2004) and evacuated by kidney (Turnlund et al., 1997). If the intake of copper is lower than the need, the excretion of endogenous copper becomes slow; contrarily, the body needs to increase the endogenous excretion (Turnlund, 1998). Previous studies indicate that copper can be metabolized in hepatoma tissue culture (HTC) cells, and be transferred to metallothionein (MT) (where copper is stored) by reduced glutathione (GSH). When a copper overload is reached, depletion of GSH instantaneously results in enhanced cellular toxicity. When MT is depleted, non-MT associated 'free' cytosolic Cu²⁺ is elevated, and HTC cells rapidly lose their resistance to copper toxicity, reflecting in the loss of cell viability (Freedman et al., 1989; Steinebach and Wolterbeek, 1994). With the rapid transformation of nano-copper particles to cupreous ions, the overload of copper ions damage the hepatic cells, which further cause the observed

lipodystrophy and steatosis, and the increase of ALP and TBA in blood. Glomerulitis, degeneration and necrobiosis of renal tubules are observed from mice exposed to nano-copper, but not from mice exposed to micro-copper. These are consistent with the known knowledge that stimulation of copper ions can lead to inflammation in renal tissues. The strong ionization potential of nano-copper particles leads to the copper ion overload. Renal inflammation can result in reduction of glomerular filtration rate (gfr), causing further increases blood Cr, as observed from nano-copper treated mice. On the other hand, the observed proteinic liquid in renal tubule implies occurrence of proteometabolism disorder caused by nano-copper particles. This is related to the increases of BUN observed in the blood biochemical assay.

4.3. Nanotoxicity caused by an exceeding consumption of H⁺ and a massive formation of HCO₃⁻ in vivo

That nano-copper particles react drastically with H⁺ in the gastric juice can lead to a massive formation of HCO₃⁻ which is hardly excreted by kidney because of renal disorder. The overstock of HCO₃⁻ in vivo becomes the direct cause of the observed metabolic alkalosis which further becomes the origin of hypopnea symptom and electrolyte disturbance leading to tremors observed from mice exposed to nano-copper particles. As an alkalescent medium can also reduce the solubility of cupreous salt, this should have a close relation with the purple depositions observed in renal proximal convoluted tubule.

Epidemiologic survey indicates that more males suffer from gastric ulcer than females due to the exceed secretion of acid substance in stomach (Lam, 2000), because males can secrete more H⁺. This means more nano-copper particles should be transformed into copper ions in the male mice than in females. During the experiments, we found that male mice more suffer from nano-copper than females, while no such gender difference is found in micro- and ion-groups. The finding accords with nano-copper particles of the same mass may exert stronger toxicity to males than to females. This is demonstrated by the present observation that the toxicity of nano-copper is sex-dependent: the male mice display more severe toxic symptoms in behavior observation, pathological examination and blood biochemical assay.

5. Conclusion

The toxicity of copper nanoparticles (23.5 nm) exposed to mice by oral gavage was studied com-

paratively with copper micro-particles (17 μm). During the animal experiments, detail characterizations of nanoparticles for the size, surface area and particle number, etc. were conducted using experimental techniques of transmission electron microscopy and atomic force microscopy, etc. Based on animal experiments, LD₅₀ determined for 23.5 nm, 17 μm copper particles and cupric ions are 413, >5000 and 110 mg/kg body weight, respectively. The toxicity classes of both nano- and ion-copper particles are class 3 (moderately toxic), micro-copper particles is class 5 (practically non-toxic) of Hodge and Sterner Scale.

The pathological examinations revealed that kidney, liver and spleen are target organs for nano-copper particles. These were further demonstrated by measurements of the blood biochemical indexes (BUN, Cr, TBA and ALP) reflecting the renal and hepatic functions of experimental mice. Pathological changes and grave injuries on kidney, liver and spleen were observed in mice exposed to 23.5 nm nano-copper particles (e.g., swelling up and dwindling in gap of renal glomerulus, degeneration and irreversibly massive necrobiosis of epithelial cells of renal proximal convoluted tubules, reducing karyons of epithelial cells of renal tubules, proteinic liquid in renal tubules, purple deposition in the proteinic liquid, the steatosis around venae centrals of hepatic tissue, etc.), but they were not found in mice exposed to 17 μm copper particles on mass basis. In addition, toxicity of nano-copper is sex-dependent: male mice exhibit more severe toxic symptoms and suffer more from nano-copper than females after they exposed to the same mass of particles.

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