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The dose-dependent toxicological effects and potential perturbation on the neurotransmitter secretion in brain following intranasal instillation of copper nanoparticles

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Abstract

Increasing production and application of metallic nanomaterials are likely to result in the release of these particles into the environment. These released nanoparticles may enter into the lungs and the central nervous system (CNS) directly through inhalation, which therefore poses a potential risk to human health. Herein, we focus on the systemic toxicity and potential influence on the neurotransmitter secretion of intranasally instilled copper nanoparticles (23.5 nm) at three different doses. Copper nanoparticle-exposed mice exhibit pathological lesions at different degrees in certain tissues and especially in lung tissue as revealed by histopathology and transmission electron microscopy (TEM) observations. Inductively-coupled plasma mass spectrometry (ICP-MS) results show that the liver, lung and olfactory bulb are the main tissues in which the copper concentrations increased significantly after exposure to a higher level of Cu nanoparticles (40 mg/kg of body weight). The secretion levels of various neurotransmitters changed as well in some brain regions, especially in the olfactory bulb. Our results indicate that the intranasally instilled copper nanoparticles not only cause the lesions where the copper accumulates, but also affect the neurotransmitter levels in the brain.

Keywords: *Copper nanoparticles, intranasal instillation, systemic toxicity, neurotransmitter, pathological lesion, neurotoxicity, mouse*

Introduction

Nanotechnology presents many opportunities and benefits for new materials with remarkably improved properties as well as revolutionary applications in the fields of energy, environment, medicine, biotechnology, food industry, information technology etc. The rising commercial use and large-scale production of engineered nanoparticles (NPs) may lead to unintended exposure in humans (Colvin 2003; Nel et al. 2006; Panessa-Warren et al. 2006; Stone and Donaldson 2006). The potential risks of nanoparticle exposure are thus of growing concern despite their significant interests and numerous promising applications. To guarantee the sustainable development of nanotechnology, there is an urgent need to understand the impact of NPs on organisms (Zhao et al. 2008).

Nanoparticles inhalation has been the primary focus for health investigation (Tsuji et al. 2006). The nose-to-brain transport of exogenous materials is indeed a potential route for bypassing the blood brain barrier (BBB) (Illum 2000). The research conducted by Oberdörster et al. (2004) indicated that the NPs may be taken up directly to the brain from olfactory epithelium to the olfactory bulb via the olfactory nerves. Hence the central nervous system (CNS) is a potential target of NPs. To date, few studies have specifically examined the direct transfer of NPs from the nasal cavity to the brain. Particulate materials such as gold (De Lorenzo and Darin 1970), carbon-13 (Oberdörster et al. 2004), manganese oxide (Elder et al. 2006), iridium-192 (Semmler et al. 2004), iron (III) oxide (Wang et al. 2007a) have been reported to accumulate in the olfactory

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bulb or even penetrate deeply into the brain and induce damages. Obviously the lower respiratory tract is also vulnerable to these particles that can eventually reach the distal areas of the lung.

At present, large amounts of nano-sized copper particles are typically found in agriculture and industries involving bactericide (Cioffi et al. 2005) but also in air and liquid filtration, metallic coating on integrated circuits and batteries, sensors, catalysts and additives in lubricant oils, brake lining, polymers and plastics (Liu et al. 2004) where they increase the thermal and electrical conductivity. The multiple applications of copper NPs increase the risk of their release in the environment. Brake lining wear has been cited as a possible source of copper found in urban runoff (Garg et al. 2000). Brake lining wear from road traffic vehicles is an important source of atmospheric (particulate) copper concentration in Europe, with a contribution of 2.4 kilo-tonnes per year (Hulskotte et al. 2006). The total emission of copper from brake linings, tires, and street/road paving within 1 km of a main street has been estimated to 1.2 kg per year (Landner and Lindeström 1999). Recently, there have been increasing reports that nano-sized component of particulate matter (PM) can reach the brain and may be associated with neurodegenerative diseases (Block et al. 2004; Peters et al. 2006). At the same time, studies on humans have indicated that the copper dyshomeostasis in the brain is related to neurodegenerative diseases such as Alzheimer's disease (AD), prion diseases, Parkinson's disease (PD) and amyotrophic lateral sclerosis (ALS) (Loeffler et al. 1996; Maurer et al. 2000; Caviedes and Segura-Aguilar 2001; Gaetke and Chow 2003; Barnham et al. 2004; Maynard et al. 2005; Squitti et al. 2009). Some reports indicated that the translocated nanoparticles can influence the balance of neurotransmitter release (Wang et al. 2008a, 2009; Win-Shwe et al. 2008). Abnormal regulations of neurotransmitter release and/or abnormal levels of extracellular neurotransmitter concentration have remained core components of hypotheses on the neuronal foundations of behavioral and cognitive disorders and the symptoms of neuropsychiatric and neurodegenerative disorders (Sarter et al. 2006). Therefore, the effect of copper NPs on the neurotransmitter levels in the brain should arouse our attention.

Intranasal instillation is a simple and quantitative exposure method to mimic inhalation in the study of nanotoxicology, and some scientists have adopted this method to study the interaction between nanoparticles and the CNS (Elder et al. 2006; Wang et al. 2007a, 2008a, 2008b; Win-Shwe et al. 2008). Our previous study suggests that the intranasally instilled copper NPs can induce damage to the viscera and the olfactory bulb (Liu et al. 2009). In this work, both the systemic toxicity and the neurotransmitter level changes caused by the intranasally instilled copper NPs were studied. The body growth and tissue lesions of the tested mice were evaluated. The histopathological changes were examined by hematoxylin-eosin (HE) staining and TEM. The concentrations of Cu in different tissues and brain regions were analyzed by ICP-MS in order to find the NPs accumulative tissues and organs. The determination of the neurotransmitter levels in various brain regions is more informative than evaluating the whole brain, for the reason that the concentration of a given neurotransmitter generally differs for different brain structures, in some cases markedly (Sharma et al. 1986; Cooper et al. 1996; Fuster 2008). Thus, the levels of monoamine neurotransmitters (dopamine and its metabolites 3,4-dihydroxyphenylacetic acid, homovanillic acid, 5-hydroxytryptamine and its metabolite 5-hydroxyindoleacetic acid, and norepinephrine), nitric oxide (NO), acetylcholinesterase (AChE) and glutamic acid in five brain regions (i.e., the olfactory bulb, hippocampus, striatum, cerebral cortex and cerebellum) were analyzed.

Materials and methods

Copper nanoparticles

Copper nanoparticles were purchased from Shenzhen Zunye Nano Material Co., Ltd. Their properties were characterized in our previous work (Chen et al. 2006) and listed as follows: The purity is higher than 99.9%; the average size of copper NPs is 23.5 nm in diameter; the specific surface area is $2.95 \cdot 10^5$ cm^2/g and the particle number is $1.7 \cdot 10^{10}$ per μg mass. A Milli-Q water system (Millipore, Bedford, MA, USA) was used to prepare the ultra pure water (18.2 MΩ·cm at 25°C). The copper particles were dispersed in Milli-Q water by ultrasonication for 10 min and vibration for 1 min before each instillation. The suspensions were freshly prepared before use. Then, mice were exposed to different doses of these suspensions via nasal instillation. All the other reagents were at least of analytical grade.

Animals

CD-1 (ICR) female mice (Beijing Vitalriver Experimental Animal Technology Co. Ltd, bodyweights of 19–22 g) were housed in stainless steel cages. The standard conditions (20 ± 2°C room temperature, 60 ± 10% relative humidity) were maintained with a 12-h light/dark cycle for mice. Distilled water and

sterilized food for mice were available *ad libitum*. Animals were acclimated to this environment for five days prior to treatment. All procedures used in this experiment were compliant with the local ethics committee.

Intranasal instillation exposure

Animals were randomly divided into four groups to form a control group and three experimental groups consisting of a low-dose group (L-Dose), a middle-dose group (M-Dose) and a high-dose group (H-Dose). The low dose was 1 mg/kg body weight while the middle and high dose was 10 and 40 mg/kg body weight, respectively. Mice without anesthesia were held in a supine position, then 10 μL of suspension was instilled gently and slowly into the nasal cavity using a micro-syringe every other day. To avoid the loss caused by sneezing and maximize the dose inside the nasal cavity, the suspension was instilled in two or three times to ensure the particles were instilled into the nasal cavity as completely as possible. The time interval from preparation to nasal instillation was limited to less than 5 min. An equal volume of Milli-Q water was taken as control. After 15 and 21 days, the experimental mice were instilled 7 and 10 times, respectively.

Evaluation of the physiological condition of the tested animals

The mice were sacrificed 48 h after the last instillation. The tissues and organs such as heart, liver, spleen, kidneys, lung and brain were excised and weighed accurately. After weighing the body and tissues, the coefficients of heart, liver, spleen, lung and kidneys to body weight were calculated as the ratio of tissue (wet weight, mg) to body weight (g).

Histopathological examination

After exposure for 15 and 21 days, the tissues/organs (liver, lung and kidneys) were collected and immediately fixed in 10% neutral buffered formalin. The histopathological tests were performed using standard laboratory procedures. Briefly, the tissues were embedded in paraffin blocks, then sectioned into 4 μm slices and mounted onto the glass slides. After HE staining, the sections were photographed using an optical microscope (Leica DM4000M, Germany). The identity and analysis of the pathology sections were blind to the pathologist.

Ultrastructure of lung tissue by transmission electron microscopy

The fresh lung samples were immersed in 2.5% glutaraldehyde at 4°C. After washing with phosphate buffer solution, they were fixed with 1% osmium tetroxide, dehydrated in a graded series of ethanol, embedded in araldite and polymerized for 24 h at 37°C. Ultrathin sections (50 nm) were cut with an ultramicrotome (LKB-V, Sweden), contrasted with uranyl acetate and lead citrate, and finally observed by TEM (H-600, Hitachi) by an independent pathologist.

Determination of Cu contents in tissues/organs

The frozen tissues/organs were taken out and digested using concentrated nitric acid (ultrapure grade) and 30% H_2O_2 in a microwave accelerated reaction system (CEM MARS, USA). These mixtures were heated at about 170°C using a high-pressure reaction container in the chamber until the samples were completely digested. Then the solutions were heated at 170°C to remove the remaining nitric acid until the solutions became colorless and clear. Finally, the remaining solutions were diluted to 3 mL with 2% nitric acid. Inductively coupled plasma-mass spectrometry (ICP-MS, Thermo Elemental X7, Thermo Electron Co.) was used to analyze the copper concentration in the samples. Indium at 20 ng/mL was chosen as an internal standard element. Data are expressed as micrograms per gram of wet tissue.

Evaluation of the neurotransmitter secretion changes in different brain regions

Different brain regions, including the olfactory bulb, hippocampus, striatum, cerebral cortex and cerebellum, were separated and collected. Half of the different brain regions were kept in dark and homogenized by an ultrasonic cell disruptor (Sonics vibra cell, VCX105) in cold 0.4 mol/L $HClO_4$ solution for 8 s × 4 times at 4°C. The homogenates were centrifuged at 14,000 *g* for 20 min at 4°C. 50 μL of supernatant was submitted to a high performance liquid chromatography with electrochemical detection to determine the concentrations of monoamine neurotransmitters and their metabolites, including norepinephrine (NE), dopamine (DA) with its metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), and 5-hydroxytryptamine (5-HT) with its metabolite 5-hydroxyindoleacetic acid (5-HIAA). The HPLC system was

equipped with an ESA 542 Plus pump, a 5 μm analytical column (Agilent, Zorbax SB-C18, 150 mm length × 4.6 mm diameter) and an ESA Coularray 5600A detector. A citric acid buffer system composed of 70 mmol/L citric acid-sodium citrate, 97 μmol/L Na_2EDTA and 832 μmol/L 1-octanesulfonic acid sodium salt in 10% methanol was used as mobile phase. Its pH was adjusted to 4.1. NE, DA, DOPAC, HVA, 5-HT and 5-HIAA standards were purchased from Sigma. The analytical run time for each sample was 20 min at an isocratic flow rate of 1 mL/min at room temperature.

The olfactory bulb, hippocampus, striatum, cerebral cortex and cerebellum from the second half of the brain regions collected were weighed and transferred into centrifuge tubes. The 1:9 (w/v) volume of cold 0.1 mol/L phosphate buffer (0.1 mol/L Na_2HPO_4, 0.1 mol/L KH_2PO_4, 0.1 mmol/L PMSF, pH 7.4) was added, and the mixtures were homogenized by an ultrasonic cell disruptor for 8 s × 4 times at 4°C. The homogenates were centrifuged at 825 *g* for 10 min or 100 *g* for 5 min at 4°C. The supernatants were collected to assay NO, AChE and glutamic acid levels. NO level in the homogenates was measured using the method of nitric reductase (Green et al. 1982). The activity of AChE was measured according to the method developed by Ellman et al. (1961), which employed acetythiocholine iodine (ATChl) as a synthetic substrate. One unit is defined as the amount of ATChl broken down by AChE per 6 min. Results were expressed as one unit per milligram protein. The content of glutamic acid was determined spectrophotometrically by detecting the changes of absorbance at 340 nm when the coenzyme NAD^+ was oxidized to NADH with the glutamic acid being hydrolyzed to α-ketoglutarate by glutamate dehydrogenase. Protein concentrations were determined according to the Bradford's method (1976). For above assays, absorbance was measured with a TECAN Infinite M200 microplate reader (Tecan, Durham, USA).

Statistical analysis

Metabolite/monoamine ratios (DOPAC/DA, HVA/DA, and 5-HIAA/5-HT) are widely used as markers of turnover of DA and 5-HT in brain neurons. Amine ratios (DOPAC/DA, HVA/DA, 5-HIAA/5-HT) were calculated for each mice and the mean value of these ratios were obtained for each group. Data are presented as mean plus standard deviation of the mean (mean ± SD) of each experimental group. The SPSS v13.0 statistical package was used for the statistical analyses. The comparison between the control and exposure groups and differences within exposure groups at different doses were analyzed using a one-way ANOVA followed by Least-significant difference and Student-Newman-Keuls tests. Differences between groups were considered significant when $p < 0.05$.

Results

Influence on mice growth and tissue coefficients

Table I shows the coefficients for the heart, liver, spleen, lung and kidneys to body weight 15- and 21-day post-exposure. In both L-Dose groups there were no differences in the body weight compared with

Table I. The body weight and coefficients of various tissues to body weight after nasal instillation of copper nanoparticles at different doses for 15 days ($n = 14$) and 21 days ($n = 12$). Each value represents the mean ± SD.

		Organ coefficients (mg/g)				
Groups	Body weight (g)	Heart	Liver	Spleen	Lung	Kidneys
15 days						
Control	21.75 ± 1.21	6.24 ± 0.82	54.13 ± 3.12	4.25 ± 0.65	8.63 ± 1.03	15.13 ± 1.18
L-Dose	21.72 ± 1.52	6.27 ± 0.56	47.87 ± 2.13*	3.85 ± 0.49*	8.22 ± 0.64	13.99 ± 0.94*
M-Dose	19.39 ± 1.64*	6.40 ± 0.60	50.46 ± 4.19*	3.64 ± 0.52*	9.51 ± 0.96*	14.78 ± 0.97
H-Dose	17.60 ± 1.77*	6.72 ± 0.84	51.57 ± 4.22	3.90 ± 0.48*	10.56 ± 0.99*	15.65 ± 1.23
21 days						
Control	25.82 ± 2.19	5.84 ± 0.77	53.77 ± 3.53	4.48 ± 0.76	9.25 ± 1.09	12.85 ± 0.67
L-Dose	25.08 ± 1.67	5.71 ± 0.57	53.00 ± 5.11	4.48 ± 0.53	9.09 ± 0.93	13.34 ± 1.07
M-Dose	22.52 ± 2.02*	5.67 ± 0.51	52.24 ± 4.09	4.07 ± 0.69	10.26 ± 1.05*	12.83 ± 1.04
H-Dose	18.93 ± 1.94*	6.19 ± 1.02	50.31 ± 4.74	3.80 ± 0.47*	11.31 ± 0.55*	14.03 ± 1.23*

*Represents significant difference ($p < 0.05$) compared to the control group.

the corresponding control group. By increasing the exposure dose (M-Dose and H-Dose groups), the body weight both at 15- and 21-day post-exposure was significantly decreased, compared with the control ($p < 0.05$). There was no change in heart coefficients at both 15 and 21 days. The coefficient of liver significantly decreased in L- and M-Dose groups at 15 days, but it returned to normal level at 21 days. The nano-sized copper particles significantly decreased the coefficient of spleen in all exposed groups ($p < 0.05$) at 15 days. When the exposure time was prolonged, it returned to normal level in L- and M-Dose groups, while the NPs still induced damage to spleen in the H-Dose group. Cu NPs significantly decreased the coefficient of kidneys in the L-Dose group at 15 days, while NPs increased it remarkably in the H-Dose group at 21 days. The increased coefficient of lung indicates that a congestion and swelling might be induced and still existed in M-Dose and H-Dose groups, which were further confirmed by the following histological examination.

Histopathological changes in tissues

The histopathological micrographs of liver, lung and kidneys sections are shown in Figures 1–3, respectively. In liver tissues, spotty necrosis and slight hepatocyte steatosis were found in the L-Dose group (Figure 1B). The degree of hepatocyte steatosis was more elevated and the liver sinusoids were expanded when increasing the dose (Figure 1C and 1D). In the lung tissues, hyperaemia in pulmonary vessel, thickened alveolar wall, alveolar shrinkage and inflammatory cell infiltration were found in all copper-treated groups (Figure 2). Thinned and broken alveolar wall, and the formation of compensatory pulmonary bullae were observed in the H-Dose group (Figure 2D). The injury degree was in proportion to the dose. Swelling, shrinkage and necrosis in the renal glomerulus of the kidneys were found in all 15-day treated groups (Figure 3A). At 21 days, Cu NPs still caused renal glomerulus swelling and necrosis (Figure 3B) but the lesions in the kidneys were not dose-dependent.

Subsequently, the ultrastructure of the lung tissues was observed by TEM (Figure 4). The alveolar epithelial type I cells were intact in the control group (Figure 4A and 4B). With the increasing exposure dose, the alveolar walls thickened significantly because of the interstitial connective tissue proliferation; the intumescent endothelial cells caused the alveolus cavity to narrow or even close completely (Figure 4C, 4E and 4G). The mitochondria became varicose, frothy and even appeared as vacuoles (Figure 4D, 4F and 4H). Thin alveolar walls were observed in the H-Dose group (Figure 4G). These

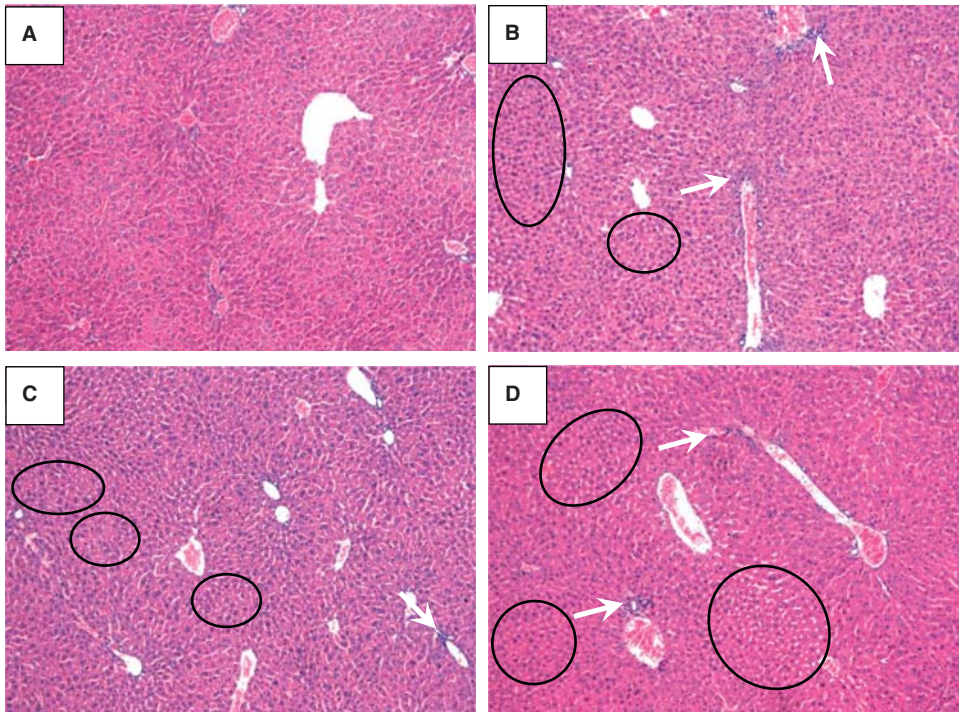

Figure 1. Histopathological images of liver tissues after intranasal instillation of copper nanoparticles for 15 days. (A) control group (×100); (B) L-Dose group (×100); (C) M-Dose group (×100); (D) H-Dose group (×100). Arrows indicate the spotty necrosis of hepatocytes; Circles indicate hepatocyte steatosis.

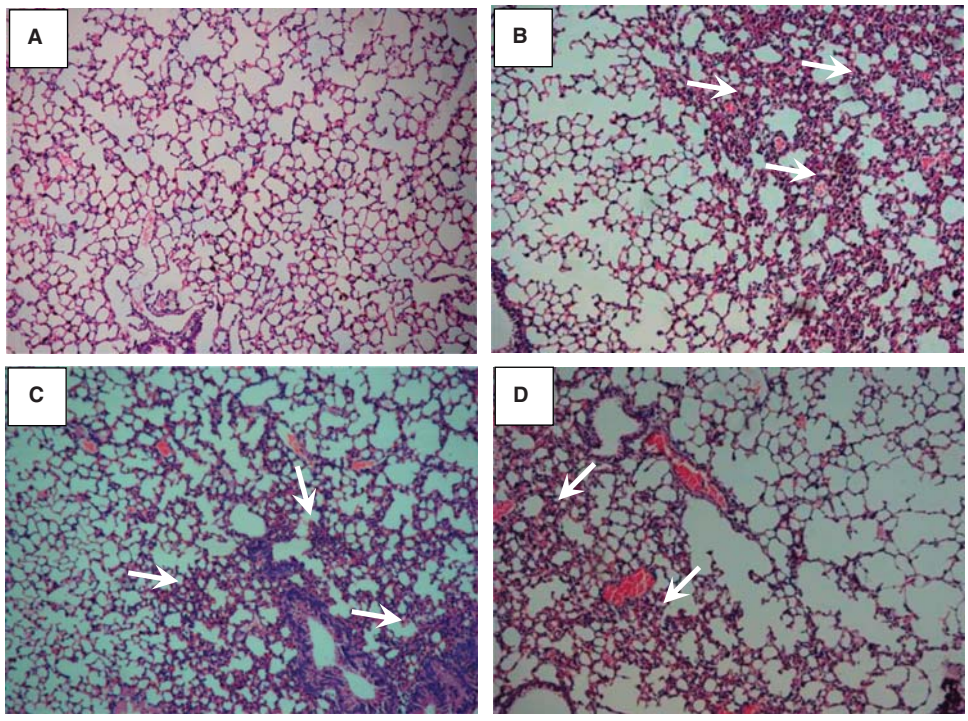

Figure 2. Histopathological images of lung tissues after intranasal instillation of copper nanoparticles for 15 days. (A) control group (×100); (B) L-Dose group (×100); (C) M-Dose group (×100); (D) H-Dose group (×100). Arrows indicate inflammatory cell infiltration.

changes are in agreement with the corresponding HE staining results and are also clearly dependent on the exposure dose.

Copper contents in different tissues

The results for copper concentrations by ICP-MS analysis are shown in Figure 5. After intranasal instillation of copper NPs for 15 days, the Cu concentrations were significantly higher in the liver and lung tissues of the H-Dose group (Figure 5A). In the olfactory bulb, the Cu content increased gradually with the increased dose, however, no obvious changes were found in other brain regions (Figure 5B). The above result indicates that the intranasally instilled copper NPs are mainly accumulated in the liver, lung and olfactory bulb.

Modulation of copper nanoparticles on neurotransmitter secretion in various brain regions

Changes in norepinephrine levels after intranasal instillation of copper NPs at different doses for 15 days are shown in Figure 6. Exposure significantly increased the NE level in the olfactory bulb, cerebral cortex and striatum, while there was no such effect on the hippocampus and cerebellum.

Figures 7–11 show the secretion and turnover of DA and 5-HT as well as their metabolites in five brain regions. In the olfactory bulb, the levels of DA and HVA were decreased in all the exposure groups, while DOPAC only increased in the M-Dose group (Figure 7A); the level of 5-HT increased in the M- and H-Dose groups, and 5-HIAA increased in all the exposure groups (Figure 7B). The DA level increased in the striatum, cerebral cortex and cerebellum of H-Dose group, and the hippocampus of M- and H-Dose groups. The DOPAC level increased in the hippocampus, cerebral cortex and cerebellum of all exposed groups. The HVA level increased in the hippocampus (M- and H-Dose groups) and cerebellum (H-Dose groups), while decreased in the cerebral cortex (L-Dose group) and cerebellum (L- and M-Dose groups). The intranasally instilled Cu NPs stimulated the 5-HT secretion in hippocampus and cerebral cortex of M-Dose group and striatum of L-Dose group. Significant up-regulation of 5-HIAA was observed in the cerebral cortex of H-Dose group, while its down-regulation was found in the cerebellum in all the exposure groups. With regard to neurotransmitter turnover, an index of neuronal activity was calculated as a ratio of metabolite to transmitter. Up-regulation of DOPAC/DA was found in the olfactory bulb and cerebellum in all the exposure groups, hippocampus (L- and M-Dose groups) and cerebellum (L-Dose group), while down-regulation was only

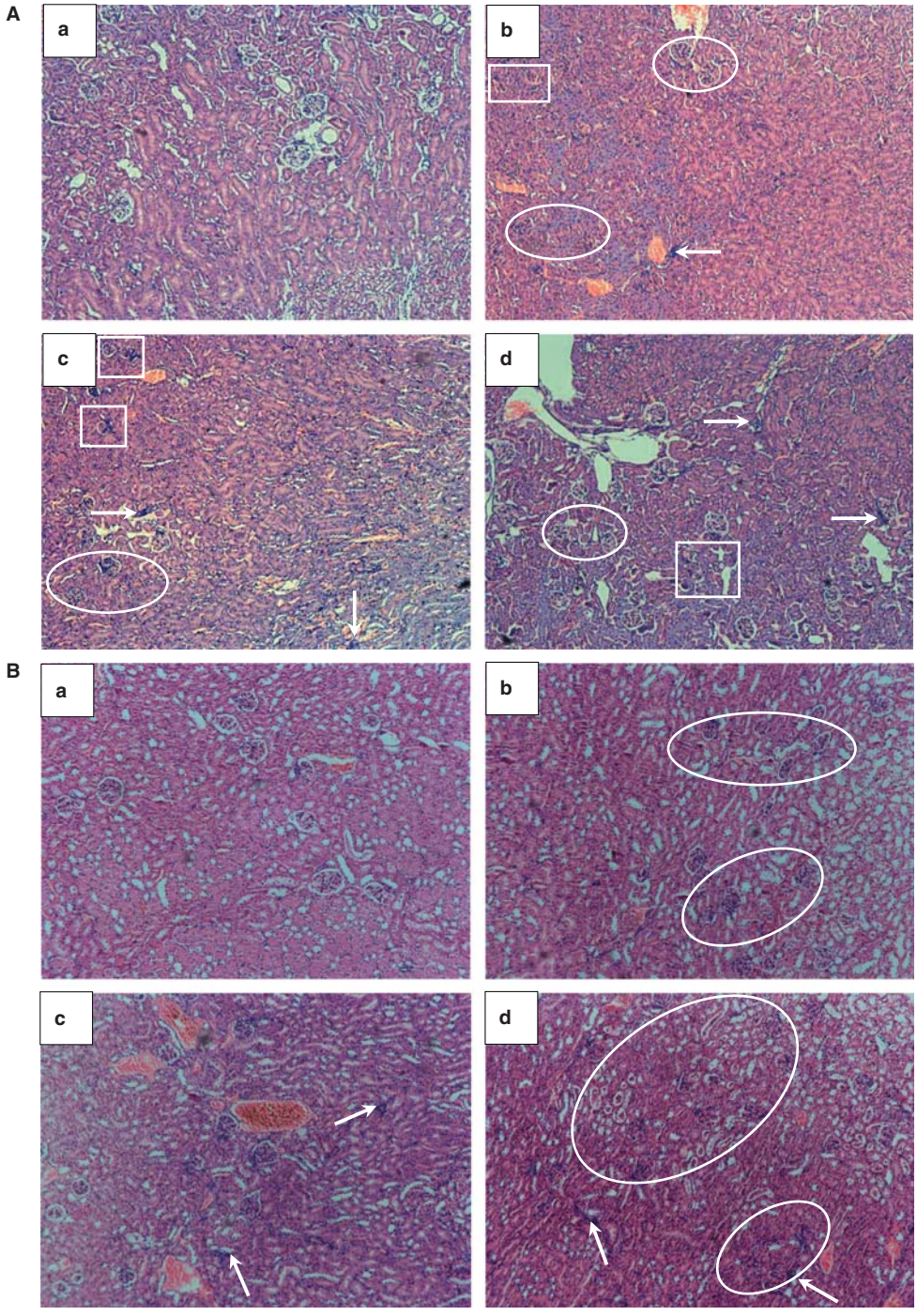

Figure 3. Histopathological images of (A) kidney tissues after intranasal instillation of copper nanoparticles for 15 days. (a) control group (×100); (b) L-Dose group (×100); (c) M-Dose group (×100); (d) H-Dose group (×100). (B) kidney tissues after intranasal instillation of copper nanoparticles for 21 days. (a) control group (×100); (b) L-Dose group (×100); (c) M-Dose group (×100); (d) H-Dose group (×100). Circles indicate the swelling, squares indicate shrinkage and arrows indicate the necrosis in the renal glomerulus or tubular.

found in the hippocampus of H-Dose group. The ratio of HVA/DA decreased in the olfactory bulb of all the exposure groups, cerebellum of M- and H-Dose groups, hippocampus of H-Dose group, and striatum of M-Dose group. The ratio of 5-HIAA/5-HT had a little change and only down-regulated in the hippocampus and cerebral cortex of M-Dose group.

Other neurotransmitter activities/levels are illustrated in Figure 12. Nitric oxide levels increased significantly in the striatum (L-Dose and M-Dose treated groups) compared with the control, but

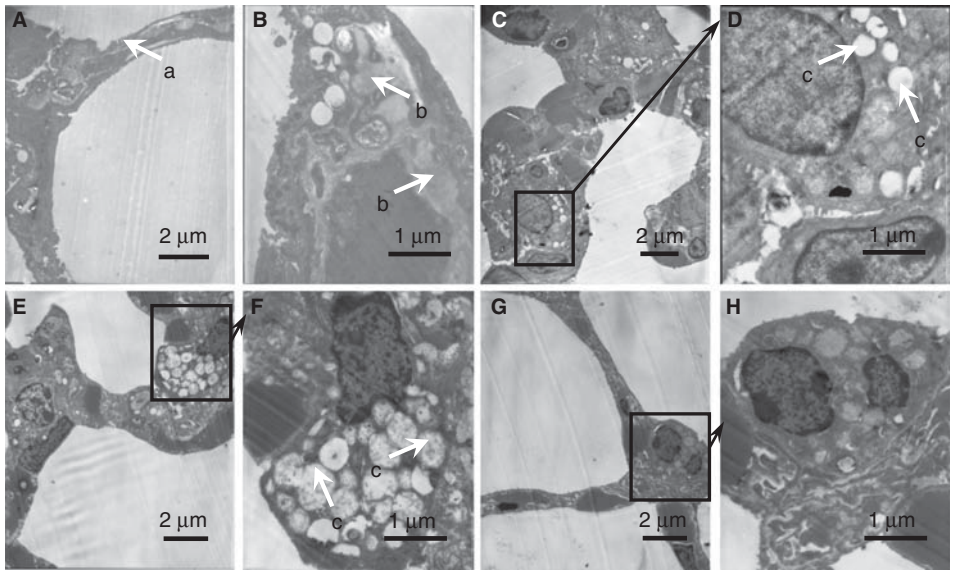

Figure 4. Ultrastructure of lung sections after intranasal instillation of copper nanoparticles for 15 days. Control group (A, B); L-Dose group (C, D); M-Dose group (E, F); H-Dose group (G, H). Arrows a, alveolar epithelial type I cells; b, laminated bodies; c, mitochondria vacuoles.

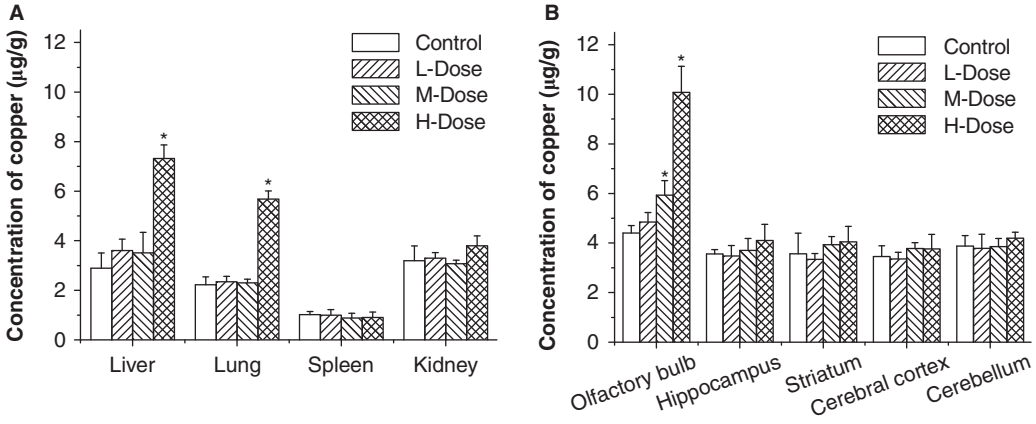

Figure 5. Copper concentrations in different tissues after intranasal instillation of copper nanoparticles at different doses for 15 days ($n = 6$). (A) different tissues; (B) different brain regions. *Significantly different ($p < 0.05$) from the control group.

decreased remarkably in the olfactory bulb of the H-Dose treated group (Figure 12A). Significant up-regulation of AChE was only observed in the olfactory bulb of M-Dose treated group after exposure for 15 days (Figure 12B), which might not be induced by the intranasally instilled particles. Glutamic acid, the most abundant excitatory neurotransmitter in the brain, decreased significantly in the olfactory bulb, striatum and cerebral cortex of H-Dose treated group (Figure 12C) after intranasal instillation of nano-sized copper particles for 15 days. However, in the hippocampus, a decreased secretion of glutamic acid was found in M- and H-Dose treated groups, but without statistical significance.

Discussion

Copper, as a trace element in organism, plays an important role in cell physiology as a cofactor of several enzymes (Linder and Hazegh-Azam 1996; MacPherson and Murphy 2007). Though acute toxicity resulting from excessive uptake of copper is not common in humans and animals, chronic overexposure can damage the liver and kidneys. The WHO Provisional Maximum Tolerable Daily Intake (PMTDI) upper limit of copper is 0.5 mg/kg per day (Goldhaber 2003), based on the fact that copper does not appear to be a cumulative toxic hazard for human (International Programme on Chemical

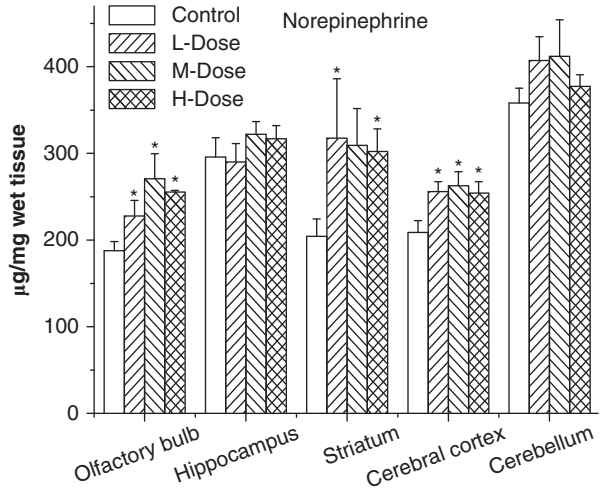

Figure 6. Levels of norepinephrine (NE) in different brain regions after intranasal instillation of copper nanoparticles at different doses for 15 days (n = 6). *Significantly different (p < 0.05) from the control group.

Safety [IPCS] 1982). Taking into account that the PMTDI upper limit of copper was calculated based on oral intake, while the intranasally instilled particles can be exhaled partly, the lowest dose of copper nanoparticles used in the present study was 1 mg/kg of body weight. Previously, *in vivo* studies administered nano-copper suspension at doses of 30 mg/kg body weight or even higher to 200 mg/kg to the animals in order to study the adverse effect of Cu NPs (Lei et al. 2008; Sharma et al. 2010). A dose-dependent toxicity caused by copper NPs was observed in some *in vivo* and *in vitro* studies (Lei et al. 2008; Prabhu et al. 2010; Jose et al. 2011). To further observe whether the copper NPs used in the present study possess a dose-dependent neurotoxicity, we chose the middle and the maximal doses about one fortieth (10 mg/kg of body weight) and one tenth (40 mg/kg of body weight) of the LD50 (i.e., 413 mg/kg body weight) of copper NPs (23.5 nm), respectively (Chen et al. 2006).

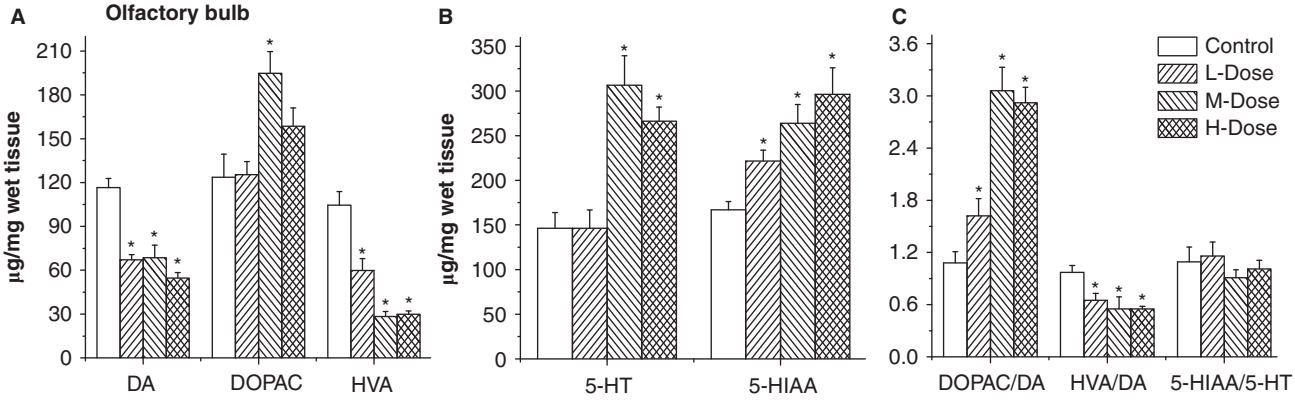

Figure 7. Levels of (A) dopamine (DA) and its metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA); (B) 5-hydroxytryptamine (5-HT) and its metabolite 5-hydroxyindoleacetic acid (5-HIAA); (C) DOPAC/DA, HVA/DA and 5-HIAA/5-HT in the olfactory bulb after exposure to copper nanoparticles at different doses for 15 days (n = 6). *Significantly different (p < 0.05) from control group.

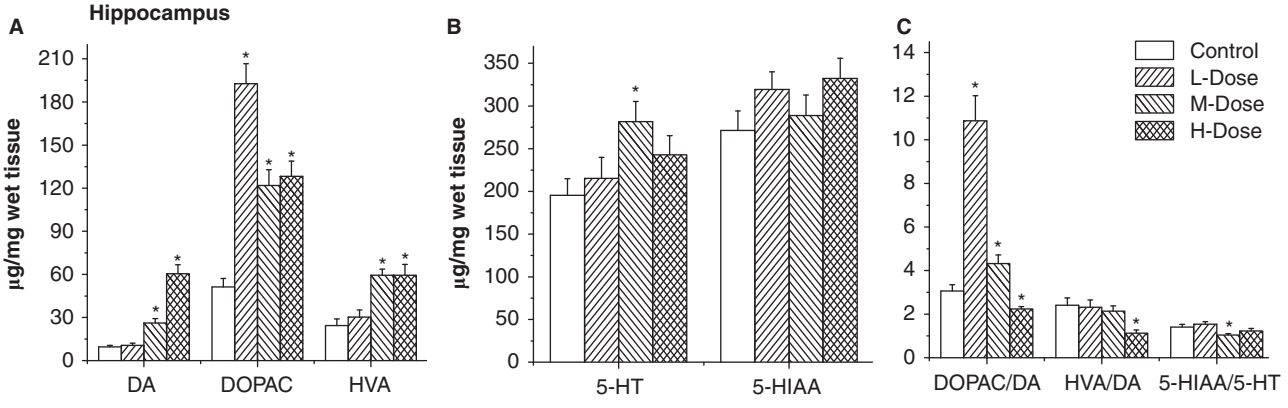

Figure 8. Levels of (A) dopamine (DA) and its metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA); (B) 5-hydroxytryptamine (5-HT) and its metabolite 5-hydroxyindoleacetic acid (5-HIAA); (C) DOPAC/DA, HVA/DA and 5-HIAA/5-HT in the hippocampus after exposure to copper nanoparticles at different doses for 15 days (n = 6). *Significantly different (p < 0.05) from control group.

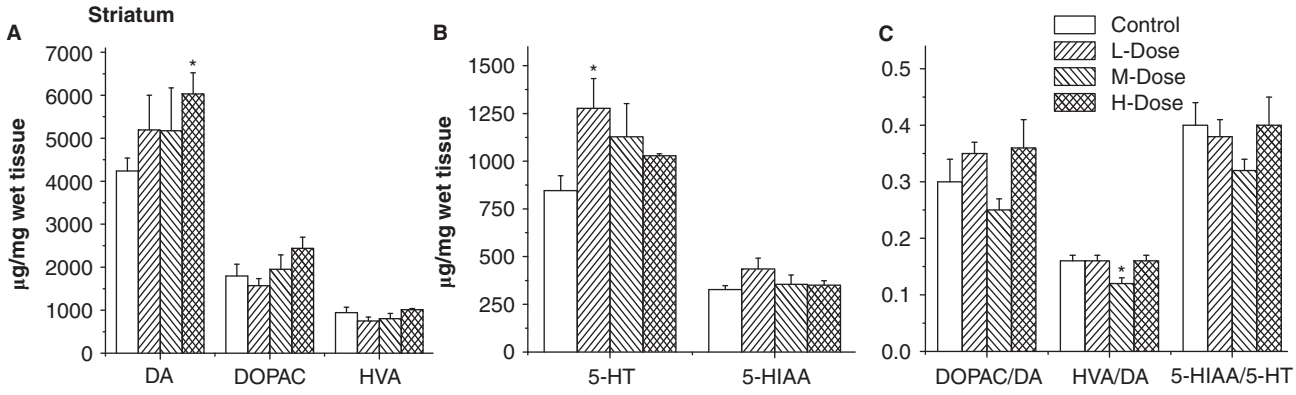

Figure 9. Levels of (A) dopamine (DA) and its metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA); (B) 5-hydroxytryptamine (5-HT) and its metabolite 5-hydroxyindoleacetic acid (5-HIAA); (C) DOPAC/DA, HVA/DA and 5-HIAA/5-HT in the striatum after exposure to copper nanoparticles at different doses for 15 days ($n = 6$). *Significantly different ($p < 0.05$) from control group.

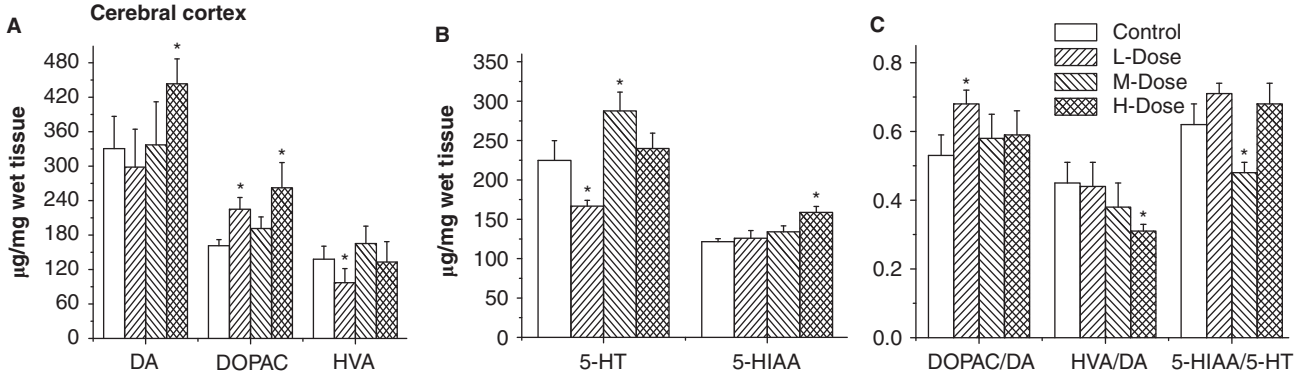

Figure 10. Levels of (A) dopamine (DA) and its metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA); (B) 5-hydroxytryptamine (5-HT) and its metabolite 5-hydroxyindoleacetic acid (5-HIAA); (C) DOPAC/DA, HVA/DA and 5-HIAA/5-HT in the cerebral cortex after exposure to copper nanoparticles at different doses for 15 days ($n = 6$). *Significantly different ($p < 0.05$) from control group.

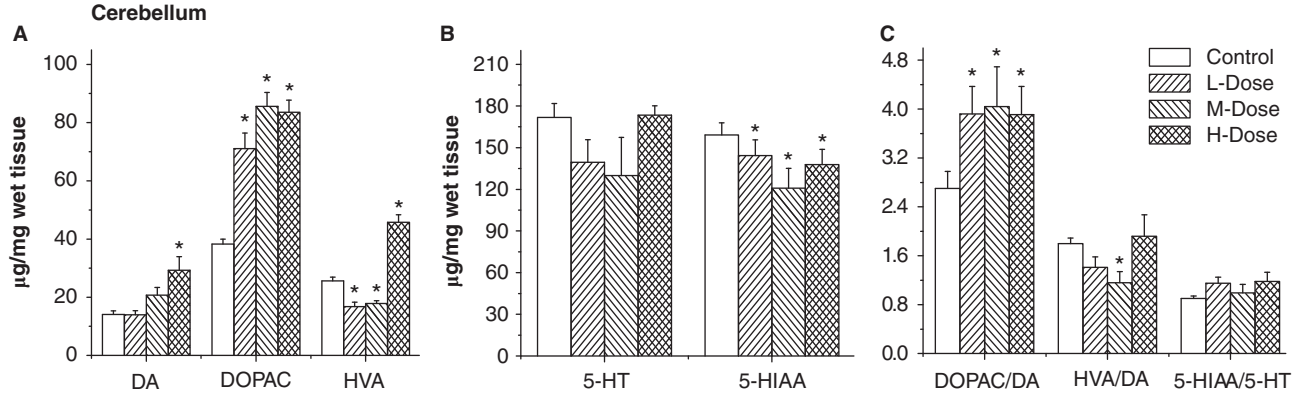

Figure 11. Levels of (A) dopamine (DA) and its metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA); (B) 5-hydroxytryptamine (5-HT) and its metabolite 5-hydroxyindoleacetic acid (5-HIAA); (C) DOPAC/DA, HVA/DA and 5-HIAA/5-HT in the cerebellum after exposure to copper nanoparticles at different doses for 15 days ($n = 6$). *Significantly different ($p < 0.05$) from control group.

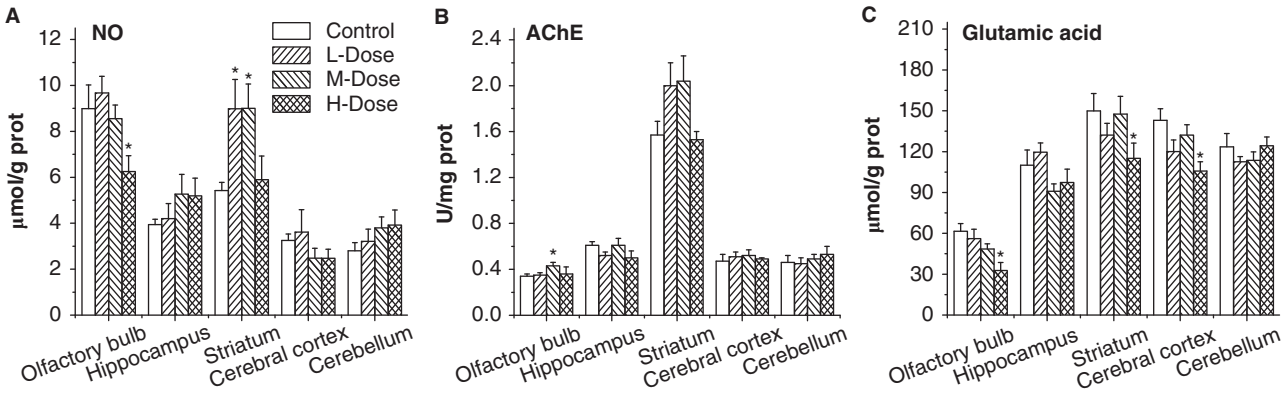

Figure 12. Levels of (A) NO; (B) AChE and (C) glutamic acid in different brain regions after exposure to copper nanoparticles at different doses for 15 days ($n = 6$). *Significantly different ($p < 0.05$) from control group.

In our study, a much severer impact on the animals was observed when exposed to the higher dose of copper NPs. The body weight of the tested animals decreased gradually with the increasing dose and exposure duration. At the same time, severer damages to the viscera were observed at a higher dose, especially in the lung tissues. The pathological examination and TEM results also revealed a similar dose-dependent tendency (Figures 2 and 4). The significantly lowered coefficient of spleen in H-Dose groups (Table I) indicates that a higher dose of copper NPs induced a dramatic atrophy of spleen, which is in accordance with our previous study (Chen et al. 2006). The coefficient of kidneys did not change consistently (Table I), but the instilled particles provoked slight lesions to all the exposed groups without a dose-dependent manner (Figure 3).

Some NPs are characterized by their ability to be translocated from their primary deposition site to other sites through the systemic circulation (Nemmar et al. 2001, 2002; Wang et al. 2007b) or along the olfactory nerve to the olfactory bulb and inner parts of the brain after inhalation (Oberdörster et al. 2004). These pathways can lead to NPs accumulation and potentially adverse effects in critical organs such as the liver, lung and brain. The present results show that the Cu NPs were mainly accumulated in the liver and lung (Figure 5A), which was associated with significant pathological changes (Figures 1, 2 and 4). At the same time, the Cu content increased significantly with the increased exposure doses (Figure 5B). Our previous study reported that serious pathological changes accompanied increases of Cu concentration in the olfactory bulb (Liu et al. 2009). So the liver, lung and olfactory bulb are not only the main accumulative tissues, but also sensitive organs where adverse effects of intranasally instilled copper NPs occur.

At the same time, it should be noted that the intranasally instilled NPs not only induce pathological lesions in brain tissues (Wang et al. 2008a, 2008b, 2009) but also influence the neurotransmitter levels (Win-Shwe et al. 2008; Wang et al. 2009). Neurotransmitters are essential regulators of brain functions. The alteration of neurotransmitters and their metabolites has been used as an indicator of toxicity in the central nervous system (Corsini et al. 1987; Honma et al. 1987; O'Kusky et al. 1988; Tsunoda et al. 1998). Monoamine neurotransmitters in the CNS modulate many physiological processes that are related primarily to central regulation of autonomic functions, motor activity, and the sleep-wake-arousal cycle (Haxhiu et al. 2001). Nitric oxide plays a dual function as a free radical and signal molecule of neurotransmitter in organism. Acetylcholinesterase, as an important enzyme in mammalian nervous systems, can hydrolyze the neurotransmitter acetylcholine in cholinergic synapses and is one of the most crucial enzymes for nerve response and function in higher organisms. Glutamic acid, an excitatory neurotransmitter, is the major 'workhorse' neurotransmitter of the brain. In this study, it should be noted that the biggest changes in these neurochemicals were found in the olfactory bulb. The mechanism responsible for the effects of Cu NPs is not clear; however, these effects appear to be mediated by an uptake through the olfactory bulb following exposure. When the copper NPs were instilled into the murine nasal cavity, the olfactory bulb, as the first target site, received the earliest and highest stimulus. Consistently, the levels of neurotransmitters changed more dramatically in the olfactory bulb. The stimulus can thus be transmitted from the olfactory bulb to the deeper part of the brain (Byrne 2003) where it can induce changes in neurochemical levels. This might partly explain why the other four brain regions did not exhibit similar variation as the olfactory bulb. We also found that among the neurotransmitter levels measured in this study, the monoamines are the

most strongly influenced after exposure to Cu NPs. Changes in the turnover rate of a neurotransmitter in a particular pathway provide a sensitive index of neuronal activity in that pathway (Costa 1970). In the present study, the turnover of DA changed obviously in the olfactory bulb, hippocampus and cerebellum, which indicates that the intranasally instilled Cu NPs might influence the dopaminergic pathway related to these regions, but the detailed mechanism needs further study. Furthermore, the organisms are composed of different systems which not only perform their unique functions, but also coordinate and interact to ensure the normal biological activity. Beside the direct stimuli to the central nervous system through the olfactory bulb, the systemic toxicity caused by the intranasally instilled particles may have influenced the levels of neurotransmitters in the brain.

The toxicological effects of NPs are generally influenced by two aspects (Yamamoto et al. 2004): chemical, corresponding to the release of soluble ions and monomers, and mechanical, a result of the mechanical stimulation produced by the insoluble particles. In order to provide an improved and more quantitative understanding of above effects to the brain, it is necessary to differentiate the dissolution of NPs in the surrounding media and *in vivo*. In our previous study (Liu et al. 2009), we simulated the external dissolution of the Cu NPs in artificial nasal cavity fluid (pH = 5) and cerebrospinal fluid (pH = 7.4) at 37°C with vibration at the speed of 100 rpm. After separating the remaining particles sequentially to a given incubation time, the copper concentration in the supernatant solution was quantitatively measured by ICP-MS. This is allowed calculating the degree and rate of particle dissolution under conditions as close as possible to the nasal cavity's interior. After incubating for 7 days, the Cu NPs only released nearly 0.05% and 0.4% Cu ions of mass in artificial nasal cavity fluid and cerebrospinal fluid, respectively. Lee et al. (2008) found only 0.03% cupric ions released form the Cu NPs after sonication for 1 h. Griffitt et al. (2008) found the dissolution of nanocopper during exposure was relatively low (0.03% of mass 48 h after resuspension), and the observed mortality of zebrafish and *Daphnia pulex* was unlikely to be attributable solely to particle dissolution. Midander et al. (2009) found that the cytotoxic effects related to the released copper fraction from the Cu NPs were significantly lower than the effects related to particles. Based on our previous *in vitro* study and literature data, we believe that contribution of soluble fraction in the suspension to the effects of Cu NPs is negligible. In addition, the solution administrated to the tested mice was freshly prepared before use, so nearly all Cu was solid particles and few ions were distributed into the mice. However, due to the complexity of the organism systems and the limitation of analytical techniques and instruments, currently we have no effective method to track the behavior and metabolism of Cu NPs (including dissolution and valence transformation) *in vivo*. Therefore, we cannot determine these effects were mainly caused by the particulates themselves or by the ions ionized from these particulates. We suggest that the adverse effects of Cu NPs are the outcome of both the particulate nature and the released copper ions.

Conclusion

In conclusion, we have investigated the potential toxicological effect of intranasal instillation copper nanoparticles (23.5 nm) on major organs and the CNS of the tested mice at three different doses. The instilled copper NPs can significantly accumulate in the olfactory bulb, liver and lung tissues. The accumulation is accompanied by a body weight decrease and dose-dependent lesions in the lung and the liver. In addition, the instilled copper NPs can influence the neurotransmitter levels in the brain, especially in the olfactory bulb, and even in regions where particle accumulation was not observed. The present results indicate that substantial respiratory exposure to copper NPs not only poses a risk to pulmonary system but also influences neurotransmitter levels in the CNS. These findings add to our knowledge of the effects of NPs in biological systems and should inspire additional studies. Currently, safety concerns associated with metallic NPs for long-term and high-level exposure may not be sufficiently addressed for human exposure and need further investigation.

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