Effect of copper nanoparticles on brain cytochrome P450 enzymes in rats

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Abstract. The aim of the present study was to evaluate the long-term effect of copper nanoparticles (CuNPs) on cytochrome P450 (CYP450) enzymes in the rat brain. Rats were repeatedly gavaged with different forms of copper sources for 28 days, and the levels of oxidative stress and CYP450 mRNA and protein expression in the rat brain were subsequently analyzed. The results demonstrated that a high dose of CuNPs (200 mg/kg) induced severe oxidative stress in the rat brain along with a decrease in the levels of total superoxide dismutase and glutathione, and an increase in hydroxyl radicals and malondialdehyde. A medium dose of CuNPs reduced CYP450 2C11 and CYP450 3A1 protein expression in the rat brain, whereas high doses of CuNPs resulted in decreased expression of most CYP450 enzyme proteins, and inhibition of pregnane X receptor and constitutive androstane receptor expression. The results suggested that CuNPs may inhibit CYP450 enzyme expression by increasing the levels of oxidative stress and decreasing the expression of nuclear receptors in the rat brain, which affects the metabolism of drugs and endogenous hormones in the brain.

Introduction

With the rapid development and widespread application of nanomaterials, the health impact of nanomaterial exposure has attracted increasing attention. Copper nanoparticles (CuNPs) have a number of desirable properties, including a large surface area, ductility, and excellent optical, electrical, catalytic and antimicrobial properties. Therefore, they have been widely used in lithium ion batteries (1), lubricant oil, ceramics (2), polymers/plastics, inks, metallics, coatings, osteoporosis treatment drugs, drug delivery, intrauterine contraceptive devices, and additives to livestock and poultry feed (3-6). However, numerous studies have indicated that the gastrointestinal system, liver and kidney are sensitive targets of copper toxicity following oral exposure beyond the range of biological tolerance (7). The symptoms of copper poisoning are drowsiness and anorexia in the early stages, followed by disruption of the epithelial lining of the liver, gastrointestinal distress, hepatocellular necrosis, hemolysis, jaundice and kidney damage (8,9).

Cytochrome P450 (CYP450) enzymes metabolize a number of exogenous and endogenous compounds, such as antidepressants, opiates, steroids, arachidonic acid, dopamine and serotonin (10-12). These enzymes are abundant in the brain, liver and other organs (10-15). Brain CYP450 content is low compared with that in the liver (0.5-2%), which makes it unlikely to affect systemic drug and peripheral metabolite levels (12). However, local brain levels of centrally acting compounds and the resulting therapeutic effects may be regulated by brain CYP450-mediated metabolism independently of peripheral metabolism and systemic drug levels. Variable brain CYP450 activity has substantial potential impact; effects have been demonstrated on behavior, neurotoxicity and drug response (16,17). The expression of a specific CYP450 enzyme within an organ can increase or decrease substantially in response to certain inducers and inhibitors (18,19). A number of CYP450 enzymes have tissue- and cell type-specific expression levels and regulators, and brain tissue expresses a unique set of these enzymes (20). For instance, CYP450 2D (CYP2D) has been identified in the liver and brain, and is involved in the metabolism of numerous centrally acting drugs, but is essentially uninducible in the liver. Brain CYP2D, however, can be induced by nicotine and clozapine (21,22).

Most studies of CuNPs explore their hepatotoxicity and nephrotoxicity (23,24); whether CuNPs affect the expression of brain CYP450 enzymes remains unknown. The present

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Abbreviations: CYP450, cytochrome P450 enzyme; CAR, constitutive androstane receptor; PXR, pregnane X receptor; CuNPs, copper nanoparticles; GSH, glutathione; ·OH, hydroxyl radicals; MDA, malondialdehyde; HPMC, hydroxypropyl methylcellulose; RT-qPCR, reverse-transcriptase polymerase chain reaction

Key words: nano-copper, brain, oxidative stress, cytochrome P450 enzymes

study investigated the effect of CuNPs on CYP450 enzymes in the rat brain by measuring the protein and gene expression of CYP450 isoenzymes in brain tissue. To identify the changes of CYP450s in the neurotoxicity of CuNPs, the effects of CuNPs on the levels of oxidative stress and nuclear receptors in the rat brain were investigated.

Materials and methods

Materials. The tested CuNPs (cat. no. H1605061), copper microparticles (cat. no. A1711069) and copper ions ($CuCl_2 \cdot 2H_2O$, cat. no. F1620012) were obtained from Aladdin Industrial Co., Ltd. The sizes of the CuNPs and copper microparticles were 80 nm and 1 μm, respectively. Western blotting and SDS-PAGE preparation kits were purchased from Chengdu Baihe Technology Co., Ltd. Other molecular biology reagents were purchased from Bio-Rad Laboratories, Inc. Antibodies were purchased from Abcam. All analytical commercial kits were purchased from Nanjing Jiancheng Bioengineering Institute.

Particle characterization. The sizes of the CuNPs and copper microparticles were confirmed using a Phenom ProX scanning electron microscope (Phenom Scientific Instruments Co., Ltd.). The CuNPs were dispersed in purified water, shaken and sonicated in an ice bath to avoid aggregation. The distribution of particle sizes in the suspension was characterized by dynamic light scattering studies performed using a Zetasizer Nano ZS (Malvern Panalytical, Ltd.) immediately following sonication.

Animals and treatments. A total of 60 specific pathogen-free (SPF) male rats (100-120 g, 6 weeks old) were purchased from Chengdu Dossy Biological Technology Co., Ltd. Male rats were chosen as the subjects of the study due to differences in the expression of CYP450 between female and male rats (25,26). Rats were housed in plastic cages under SPF conditions at 25±2˚C and 70±10% relative humidity, under a 12-h light/dark cycle. Water and food were provided *ad libitum*. Copper particles were suspended in 1% hydroxypropyl methylcellulose (HPMC) solution (w/v) (Shanghai Ryon Biological Technology Co., Ltd.) every day prior to use. Following 7 days of acclimatization, rats were randomly divided into a control group, which was administered with 1% HPMC, and five test groups that were administered with different concentrations of copper by gavage for 28 days: i) 200 mg/kg 1 μm copper; ii) 200 mg/kg $CuCl_2 \cdot 2H_2O$ (Cu^{2+}); iii) 50 mg/kg CuNPs (low dose); iv) 100 mg/kg CuNPs (medium dose); v) 200 mg/kg CuNPs (high dose) (n=10 per group). The study was approved by Sichuan Agricultural University (Chengdu, China), and the protocols for animal care and treatment were in accordance with their guidelines for animal experiments (approval no. 20170314). All possible efforts were made to relieve unnecessary suffering of the experimental animals.

Sample collection. On day 28 of the experiment, following an overnight fast, the rats were anesthetized by gas anesthesia with diethyl ether at the rate of 0.2 l/min. Anesthesia was confirmed by righting reflex, and the animals were rapidly taken out of the anesthesia machine and sacrificed by cervical dislocation. Brain tissues were snap-frozen and stored at -80˚C for oxidative stress and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analyses.

Brain microsomes were used to analyze the protein expression of the nuclear receptors pregnane X receptor (PXR) and constitutive androstane receptor (CAR) and CYP450 enzymes, which were prepared by differential centrifugation as previously described (27). The tissue was homogenized in a 0.05 mM Tris/KCl buffer (pH 7.4; Boster Biological Technology), centrifuged at 10,000 x g at 4˚C for 30 min, and the supernatant was centrifuged at 105,000 x g at 4˚C for 60 min. Subsequently, the brain protein settlement was re-suspended with 0.05 mM Tris/KCl buffer (pH 7.4) and stored at -80˚C until western blot analyses were performed. The protein content in the brain microsomes was determined using the Bicinchoninic Acid Protein Assay kit (Beyotime Biological Technology Co., Ltd.) with the Thermo Scientific™ Multiskan™ GO Microplate reader (Thermo Fisher Scientific, Inc.).

Oxidative stress. The levels of total superoxide dismutase (T-SOD), glutathione (GSH), hydroxyl radicals (·OH) and malondialdehyde (MDA) in the rat brain were determined to evaluate oxidative stress and damage. This was performed using commercial assay kits from Nanjing Jiancheng Bioengineering Institute, which were: Total Superoxide Dismutase (T-SOD) assay kit (Hydroxylamine method); Malondialdehyde (MDA) assay kit (TBA method); Reduced glutathione (GSH) assay kit (Spectrophotometric method); and Hydroxyl Free Radical assay kit. All assays were performed according to the manufacturer's instructions.

Gene expression. The expression levels of *CYP450 1A2*, *2D22*, *2E1* and *3A11* in the brain were analyzed using RT-qPCR as previously described (28). Total RNA was extracted using an OMGA total RNA kit II (Omega Bio-Tek, Inc.) and cDNA was synthesized using PrimeScript™ RT reagent kit with gDNA Eraser (Takara Bio, Inc.). The qPCR was performed using iQ SYBR® Premix Ex Taq™ II (Tli RNaseH Plus; cat. no. RR820A; Takara Bio, Inc.). The qPCR was performed under the following conditions: 45 cycles, each involving 5 sec of denaturation at 95˚C, and 40 sec of amplification at 60˚C. The housekeeping gene GAPDH was used as an internal control. All primers were designed with Primer premier v 5.0 software (Premier Biosoft International) and commercially produced (BGI Tech Solutions Co., Ltd.; Table I) based on the target gene. Melting curves and PCR efficiency were used as standard quality criteria for each qPCR run. The target gene mRNA expression was normalized to GAPDH expression, and were analyzed using the $2^{-\Delta\Delta Cq}$ method (29).

Western blot analysis. The protein levels of CYP450 1A1, CYP450 2C11, CYP450 2D6, CYP450 3A1, CAR and PXR in the brain microsome of rats were estimated using western blot analysis as previously described (30,31). Microsomal proteins (10 μg) were separated by 10% SDS-PAGE and transferred to PVDF membranes (Pall Corporation). The membranes were blocked with skimmed milk (Beijing Solarbio Science & Technology Co., Ltd.) and incubated for 12 h at 4˚C with primary antibodies against CYP450 1A1 (cat. no. ab22717; 1:1,000; Abcam), CYP450 2C11 (cat. no. ab3571; 1:1,000; Abcam), CYP450 2D6 (cat. no. 73867S; 1:1,000; Cell

Table I. Reverse transcription-quantitative polymerase chain reaction primers.

Target	Primer sequence (5'-3')
CYP450 1A1	F: GGGAGGTTACTGGTTCTGG R: ATGAGGCTGTCTGTGATGTC
CYP450 2C11	F: AATCCGCAGTCTGAGTTTACCC R: GGTTTCTGCCAATTACACGTTCT
CYP450 2D6	F: AGCTTCAACACCGCTATGGT R: CAGCAGTGTCCTCTCCATGA
CYP450 3A1	F: TGCCATCACGGACACAGA R: ATCTCTTCCACTCCTCATCCTTAG
CAR	F: CCACGGGCTATCATTTCCAT R: CCCAGCAAACGGACAGATG
PXR	F: TGGACAAACTCTCCGTTCTAAGG R: GATTTTAATGCAACATCAAAGAA GCT
GAPDH	F: GATGGTGAAGGTCGGTGTG R: ATGAAGGGGTCGTTGATGG

CYP450, cytochrome P450 enzyme; CAR, constitutive receptor; PXR, pregnane X receptor.

Signaling Technology, Inc.), CYP450 3A1 (cat. no. ab22724; 1:1,500; Abcam), PXR (cat. no. ab118336; 1:500; Abcam), CAR (cat. no. ab62590; 1:1,500; Abcam), and β-actin (cat. no. bs-0061R; 1:10,000; Beijing Biosynthesis Biotechnology Co., Ltd.). Following incubation with primary antibody, the blots were incubated with a horseradish peroxidase-labeled secondary antibody for 1 h at room temperature (cat. no. bs-0295G; 1:10,000; Beijing Biosynthesis Biotechnology Co., Ltd.). β-actin was used as an internal loading control. The bands were visualized using enhanced chemiluminescence (ECL Western Blotting Substrate; Beijing Solarbio Science & Technology Co., Ltd.) and densitometric analysis was performed using ImageJ software version 1.48u (National Institutes of Health).

Statistical analysis. The assays were performed in triplicate. All data were expressed as the mean ± standard deviation. Statistical analysis was performed by one-way ANOVA in SPSS version 19.0 (IBM Corp.), and the least significant difference test was used following comparison of the mean values with the control group. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Physiochemical characterization of CuNPs and copper microparticles. Physiochemical characteristics of CuNPs and copper microparticles were evaluated using scanning electron microscopy and a laser particle size analyzer (Fig. 1). CuNPs and copper microparticles exhibited spherical morphology (Fig. 1A and B), and the size distribution is presented in Fig. 1C and D. The most common sizes of the CuNPs and copper microparticles were 80 nm (average size: 82.5±33.4 nm) and 1 μm (average size: 987.4±436.7 nm), respectively.

Oxidative stress. The levels of oxidative stress markers in the rat brains were determined using commercial assay kits (Fig. 2). The levels of T-SOD were significantly decreased compared with those of the control following all treatments, with the exception of Cu^{2+}. GSH content was decreased significantly in the 1 μm, Cu^{2+} and high-dose CuNPs groups compared with that in the control group. The levels of ·OH were increased in the 1 μm, Cu^{2+} and high-dose CuNPs groups compared with that in the control group. The level of MDA was increased in the Cu^{2+} and high-dose CuNPs groups compared with that in the control.

mRNA expression of nuclear receptors and CYP450s. RT-qPCR was performed to determine the mRNA expression levels of different CYP450s (Fig. 3). CYP450 1A1 mRNA expression levels were significantly decreased in the 1 μm and Cu^{2+} groups and significantly increased in the low-dose CuNPs group compared with that in the control group. The mRNA expression levels of CYP 2C11 were significantly decreased in rats treated with high-dose CuNPs compared with that in the control. The mRNA expression levels of CYP450 2D6 were significantly decreased in the Cu^{2+} group, but significantly increased in the low- and medium-dose CuNPs groups compared with that in the control group. The mRNA expression levels of CYP450 3A1 were increased in the 1 μm, Cu^{2+} and low-dose groups, but significantly suppressed in the high-dose CuNPs group compared with that in the control. The mRNA expression levels of CAR were reduced in the low and medium CuNPs groups, and significantly reduced in the high CuNPs groups, whereas PXR mRNA expression levels of were reduced significantly in the medium and high dose CuNPs groups, and significantly increased in the 1 μm, Cu^{2+} and low-dose CuNPs groups compared with that in the control group.

Protein expression of nuclear receptors and CYP450 enzymes. Western blot analysis was performed to determine the protein expression levels of CYP450 enzymes (Fig. 4). Protein expression levels of CYP450 1A1 were decreased significantly in the high-dose CuNPs group compared with that in the control. The levels of CYP450 2C11 were significantly decreased in the medium- and high-dose CuNPs groups, but increased in the 1 μm group compared with that in the control group. The protein expression levels of CYP450 2D6 were suppressed in the medium- and high-dose CuNPs groups compared with that in the control. The activity of CYP450 3A1 was suppressed in all treatment groups compared with that in the control group. The CAR protein expression levels did not change under any treatment, whereas the protein levels of PXR were decreased in the Cu^{2+}, and the low-, medium- and high-dose CuNPs groups compared with that in the control.

Discussion

Brain CYP450 is expressed in glial cells in the barrier regions and in neurons throughout the brain, and certain endogenous compounds, as well as central nervous system

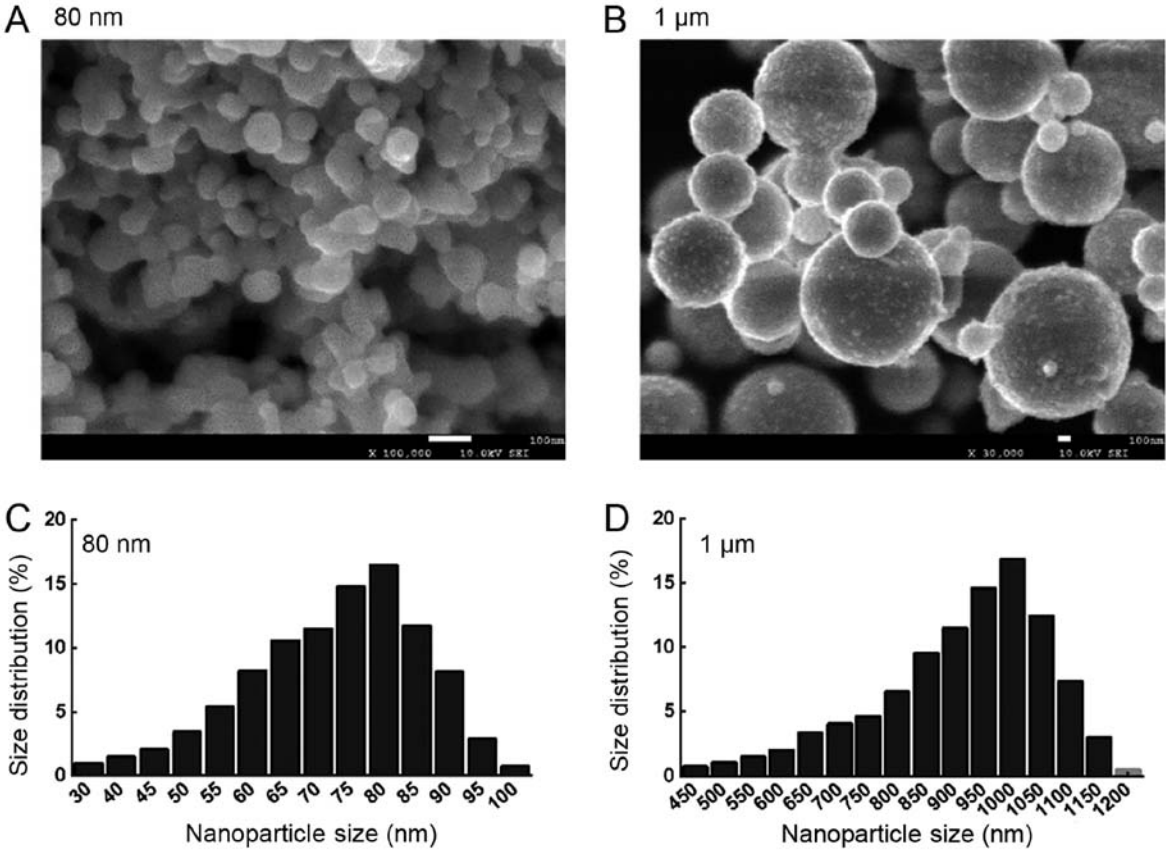

Figure 1. Physiochemical characterization of CuNPs and copper microparticles. (A and B) The scanning electron microscopy results demonstrated that the CuNPs were aggregated spherical particles (image magnification: A, x100,000; B, x30,000). Mean sizes of the particles were (C) 82.5±33.4 nm in the 80 nm group and (D) 987.4±436.7 in the 1 μm group, as determined by a laser particle size analyzer. CuNPs, copper nanoparticles.

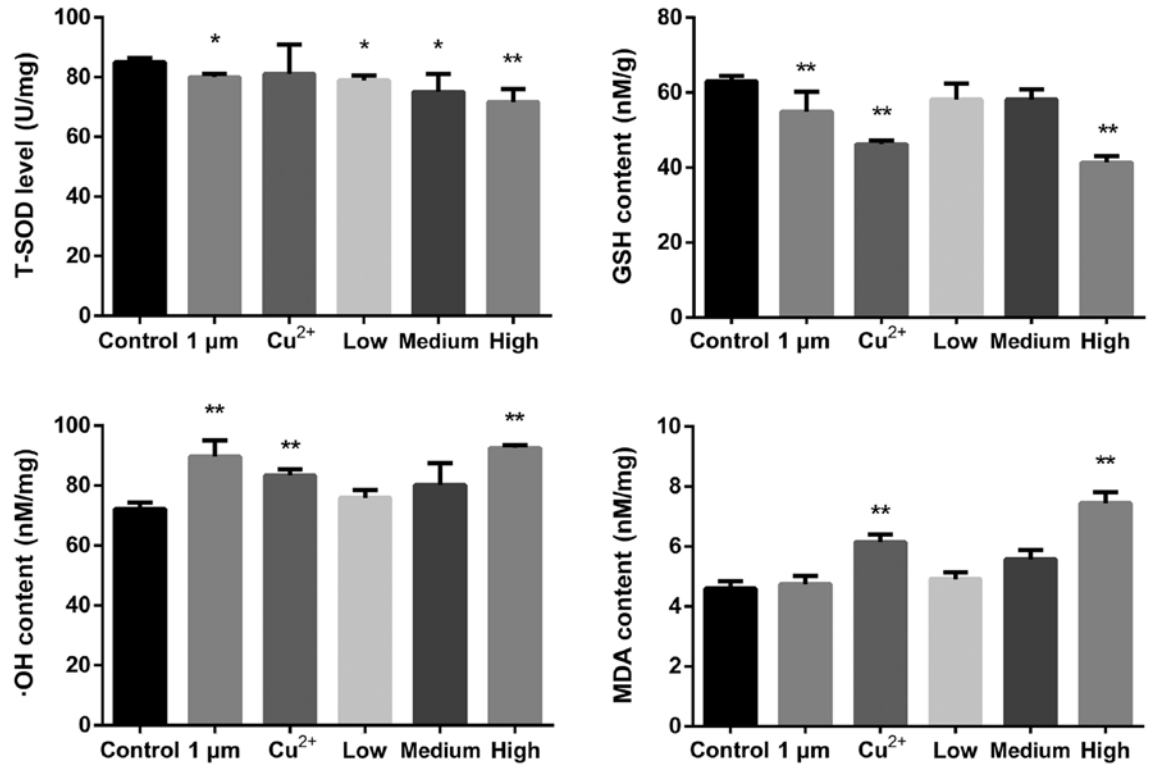

Figure 2. Effects of CuNPs on brain T-SOD, GSH, ·OH and MDA levels in the brain. T-SOD levels were significantly decreased by CuNPs and 1 μm. GSH content was decreased significantly in the 1 μm, Cu^{2+} and high-dose CuNPs groups. The ·OH levels were increased in the 1 μm, Cu^{2+} and high-dose CuNPs groups. MDA was increased in the Cu^{2+} and high-dose CuNPs groups. *P<0.05 and **P<0.01 vs. control. CuNPs, copper nanoparticles; GSH, glutathione; MDA, malondialdehyde; ·OH, hydroxyl radicals; T-SOD, total superoxide dismutase.

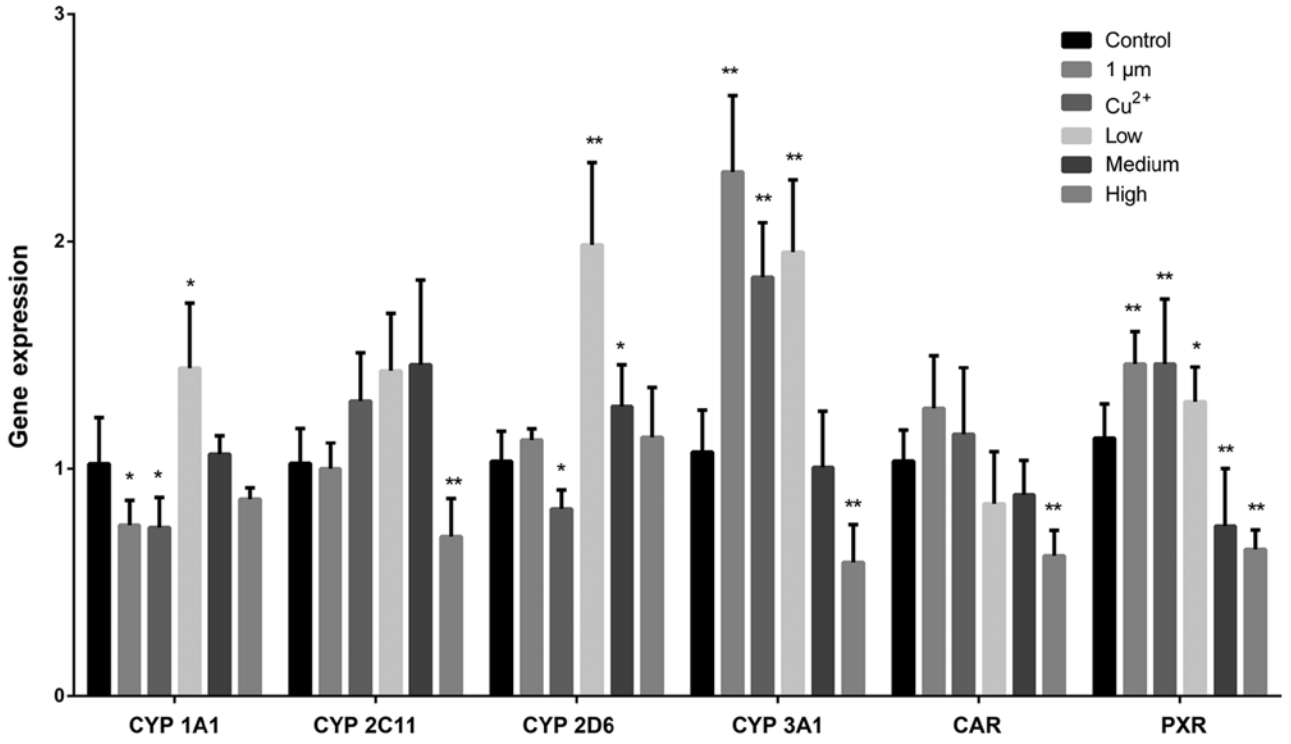

Figure 3. mRNA expression levels of CYP450 1A1, 2C11, 2D6, 3A1, CAR and PXR in the rat brain. Different sources of copper had a different impact on the mRNA expression of brain CYP450s. A high dose of CuNPs decreased the expression levels of CAR, PXR, CYP450 2C11 and CYP450 3A1. A low-dose of CuNPs increased the expression of CYP 450 enzymes (except for CYP450 2C11) and *PXR*. *P<0.05 and **P<0.01 vs. control. CYP450, cytochrome P450; CAR, constitutive receptor; PXR, pregnane X receptor; CuNPs, copper nanoparticles; Low, low-dose CuNPs; Medium, medium-dose CuNPs; High, high-dose CuNPs.

drugs, are metabolized by CYP450s in the brain (32). The function of brain CYP450 and the associated changes may be important for the development of drugs that act and are metabolized locally in the brain, as well as therapeutics that directly target brain CYPs (33). CuNPs not only cause lesions and blood-brain barrier breakdown where copper accumulates, but also affect neurotransmitter levels in the brain; it is not clear whether these changes depend on CYP450s (34).

The underlying molecular mechanism of brain CYP450 regulation remains poorly understood, but a large body of data has demonstrated that CYP450 expression can be regulated by oxidative stress via the activation of nuclear receptor signaling pathways (35-37). Oxidative stress is a state of redox disequilibrium, which occurs when reactive oxygen species (ROS) production exceeds the antioxidant defense capacity of a cell (38). Previous studies have suggested that exposure to CuNPs leads to oxidative stress, as indicated by elevated ROS levels and decreased antioxidant enzyme activity (39,40). ROS, including superoxide anions, hydrogen peroxide and hydroxyl radicals, exhibit higher reactivity than molecular oxygen (41). Exposure to CuNPs increases the production of ROS, which may result in damage to nuclear DNA and alterations of proteins, lipids and carbohydrates when present at a high level (42,43). In the present study, the levels of antioxidants (T-SOD and GSH) were decreased, whereas the levels of lipid peroxidation products (·OH and MDA) were increased upon exposure of the rat brain to CuNPs, when compared with results in untreated rats. Cu^{2+} and high-dose CuNPs induced severe oxidative stress. These results suggested that CuNPs may induce redox disequilibrium and exert negative effects on CYP450 in the rat brain.

Brian CYP450s regulate cellular mechanisms transcriptionally, post-transcriptionally and post-translationally (44-47). Brain CYP450s are sensitive to xenobiotic inducers, which may differ from the induction of liver CYPs. The regulation of brain CYP450s is highly dependent on the isoform and inducer of CYP (19). CuNPs are small (1- to 100-nm) particles that can cross the blood-brain barrier and damage the brain (48). The results of the present study demonstrated that the mRNA expression levels of CYP450s and nuclear receptors were increased or suppressed by different copper treatments compared with the control group, but CYP450 protein expression levels were decreased in the CuNP-treated groups compared with that in the control. The expression of CYP450 3A1 and PXR exhibited similarly trends following treatment with different levels of copper nano-particles, which is in line with a previous study that posited that PXR is a regulator of CYP450 3A enzymes (49). CAR and PXR are thought to be activated in response to exogenous stimuli, and are involved in CYP450 regulation (49-51). The results of the present study indicate that oxidative stress may suppress the expression of PXR expression through CuNPs. Therefore, the toxicity of CuNPs may decrease the expression of CYP450 in the brain, and their main target is CYP450 3A1. The mRNA expression level of CYP450s were either unchanged or reduced following a high dose of CuNPs, but overall higher doses were shown to reduce the protein level of CYP450s. Although an increase in mRNA expression was observed

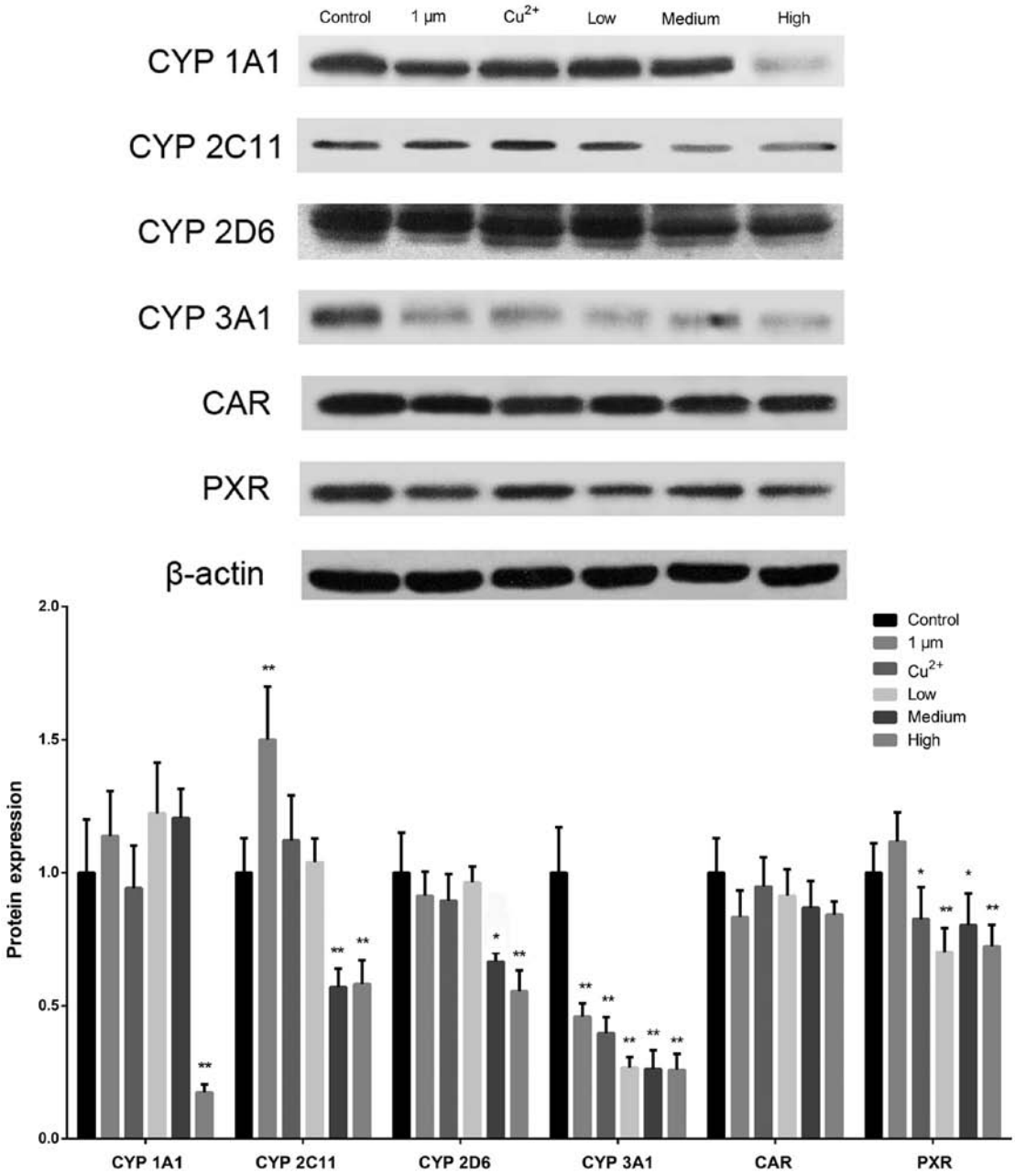

Figure 4. Protein expression levels of CYP450 1A1, 2C11, 2D6, 3A1, CAR and PXR in the rat brain. CYP450 3A1 was the most strongly affected of the tested CYP450s when analyzing all sources of copper. A high dose of CuNPs decreased the protein expression of all CYP450 enzymes and PXR. *P<0.05 and **P<0.01 vs. control. CYP450, cytochrome P450; CAR, constitutive receptor; PXR, pregnane X receptor; CuNPs, copper nanoparticles; Low, low-dose CuNPs; Medium, medium-dose CuNPs; High, high-dose CuNPs.

for both CYP450 2D6 and CYP450 3A1, the western blotting analyses showed a reduction in protein levels following CuNP treatment in a seemingly dose dependent manner. This demonstrated that CuNPs may affect CYP450 enzymes differently depending on whether they act at the post-transcriptional and/or post-translational levels.

CYP450 enzymes of the brain serve an important role in maintaining brain homeostasis, therefore it is of interest to continue researching the role of CYP450s in the metabolism of endogenous neurochemicals, some of which have already been described (52,53). The results of the present study provide evidence that CuNPs may have an impact on rat brain CYP450 enzymes, and unnecessary neurotoxicity and nervous system disorders should be avoided in practical applications.

In conclusion, the present study demonstrates that CuNPs may have an impact on brain CYP450 enzymes through ROS accumulation. The understanding of the roles of CuNPs in the regulation of brain CYP450s may be useful for better prediction, prevention and treatment of the toxicity of copper therapeutics in the brain.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YW, HT and YL made substantial contributions to conception, design, acquisition of data, analysis and interpretation of data, and were major contributors in writing the manuscript. MX and JL helped in experimental operation and data analysis. LZ, FS, GY, and CL contributed to the experimental design. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by Sichuan Agricultural University, and the protocols for animal care and treatment were in accordance with their guidelines for animal experiments (approval no. 20170314).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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