Copper (Cu^{2+}) induces degeneration of dopaminergic neurons in the nigrostriatal system of rats

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Abstract: Objective To study the effects of intranigral injection of different doses of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ on dopaminergic neuron in the nigrostriatal system of rats. **Methods** Wistar rats were divided into four groups, including control group, 10 nmol, 50 nmol and 200 nmol copper injected into left substantia nigra (SN) groups. Seven days after the intranigral injection of copper, dopamine (DA) contents in the striatum (Str) were measured by high performance liquid chromatography (HPLC); the density of tyrosine hydroxylase (TH) positive axons in the Str was measured by TH staining method; TH and Caspase-3 mRNA expression in the SN were measured by semi-quantitative RT-PCR. We detected the activity of superoxide dismutase (SOD) in the lesioned midbrain of rats using biochemical methods. **Results** DA and its metabolites contents had no significant difference between control group and low dose (10 nmol) copper group. But from 50 nmol copper group, DA contents in the lesioned sides were reduced with the increase in the copper doses injected, showing a significant linear correlation ($F = 34.16$, $P < 0.01$). In the 50 nmol copper group, TH positive axons in the Str decreased compared with those of the control and unlesioned sides ($F = 121.9$, $P < 0.01$). In the 50 nmol copper group, TH mRNA expression decreased ($t = 3.12$, $P < 0.01$) while Caspase-3 mRNA expression increased ($t = 8.96$, $P < 0.01$) in the SN compared with the control. SOD activity decreased in the midbrain of rats treated with 50 nmol copper compared with that of the control ($t = 2.33$, $P < 0.01$). **Conclusion** Copper could induce damage of dopaminergic neurons in the SN of rats through destroying antioxidant defenses and promoting apoptosis.

Keywords: copper; substantia nigra; dopamine; rats

1 Introduction

The trace element copper is essential to normal central nervous system development and function. It is needed at the catalytic site of many critical enzymes including the free radical scavenger superoxide dismutase (Cu, Zn-SOD), dopamine β -monooxygenase, cytochrome oxidase, lysyl oxidase and ceruloplasmin. Thus, copper ions are essential in cellular respiration, antioxidant defence, neurotransmitter function and connective tissue biosynthesis^[1]. Although these functions of copper are important, copper is also a

highly toxic, redox-active metal that has been associated with several neurodegenerative disorders^[2,3].

Ions of copper, in its free state are toxic when injected intracerebrally or after addition to neuronal cell cultures^[1,4,5,6]. It has been reported that redox cycling between Cu^{2+} and Cu^+ catalyzes production of hydroxy radical ($\cdot\text{OH}$). Oxidative stress causes molecular damage that can lead to a critical failure of biological functions and ultimately cell death^[7]. The basal ganglia is a region of the brain particularly susceptible to copper-mediated damage. This is likely due to high concentration of dopamine. This catecholamine is highly susceptible to oxidative damage and its oxidation products are neurotoxic. Furthermore, copper-mediated oxidation converts the dopamine metabolite salsolinal into a neurotoxic compound that in part mediates neuronal death^[3]. These findings suggest that copper might be involved in the degeneration

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tion of dopaminergic neurons in the substantia nigra (SN). However, there is still no *in vivo* evidence to demonstrate the possible mechanisms of copper-induced dopaminergic neurons degeneration. In this study, we investigated the neurotoxic effect and possible mechanisms of copper on dopaminergic neurons in the nigrostriatal system.

2 Materials and Methods

2.1 Animals All procedures were carried out in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and were approved by the Animal Ethics Committee of Qingdao University. Adult male Wistar rats weighing 200–220 g were housed for 1 week with a 12 h light-dark cycle and free access to food and water. Forty-eight adult Wistar rats were randomly divided into one of the following four groups: (1) control group, $n = 18$; (2) 10 nmol copper injected into the left SN group, $n = 6$; (3) 50 nmol copper group, $n = 18$; (4) 200 nmol copper group, $n = 6$.

2.2 Surgery and reagent infusion Rats were anesthetized with 8% chloral hydrate (400 mg/kg, i.p.) and placed in a stereotaxic apparatus with rectal temperature maintained at $(37 \pm 1)^\circ\text{C}$. After skin incision and exposure of the parietal bone, holes were drilled above the cortical surface for intranigral infusion of drugs. Two microliter (μL) solution of different concentrations of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ solution (5, 25, 100 mmol/L) or normal saline (NS) was infused stereotactically into the left side of SN (lesioned side) according to the coordinates of Paxinos and Watson (AP: -5.3 mm, ML: 1.9 mm, DV: -8.2 mm) at a rate of $0.5 \mu\text{L}/\text{min}$. The microinjection needle was held in place for an additional 10 min following drug infusion. After surgery, rats were allowed to recover from anesthesia and were placed in their home cages for 7 d.

2.3 High-performance liquid chromatography with electrochemical detection (HPLC-ECD) Both sides of the Str were isolated and transferred to liquid nitrogen for storage. Samples were weighed and then homogenized in 0.3 mL liquid A (0.4 mol/L perchloric acid). After being centrifuged once ($120\,000$ rpm for 20 min at 4°C), $80 \mu\text{L}$ of the supernatant was transferred to Eppendorf tubes with $40 \mu\text{L}$ liquid B (20 mmol/L citromalic acid-potassium, 300 mmol/L dipotassium phosphate, 2 mmol/L EDTA·2Na) added. After being centrifuged again ($120\,000$ rpm for 20 min at 4°C), $100 \mu\text{L}$ samples of the supernatant were assayed for dopamine and its metabolites by HPLC-ECD. Separation was achieved on a

PE C_{18} reversed-phase column. The mobile phase consisted of 20 mmol/L citromalic acid, 50 mmol/L sodium caproate, 0.134 mmol/L EDTA·2Na, 3.75 mmol/L sodium octane sulphonic acid, 1 mmol/L di-sec-butylamine and contained 5% (v/v) methanol; the flow-rate was 1 mL/min. A 2465 electrochemical detector (Waters, USA) was operated in screen mode. Results were expressed as ng/mg wet weight of brain tissue.

2.4 Tyrosine hydroxylase (TH) staining Animals were anesthetized (chloral hydrate, 400 mg/kg, i.p.) and killed by perfusion with 0.9% saline followed by 4% paraformaldehyde. Brains were removed and stored in 25% sucrose until sectioning. Sections ($16 \mu\text{m}$) through the Str were cut on a freezing microtome and alternate sections were stained for TH. The sections were warmed to room temperature and washed in 0.01 mol/L phosphate buffered saline (PBS) for 10 min and then placed in 99.7% methanol containing 0.3% hydrogen peroxide for 30 min to eliminate endogenous peroxidase. After washing 3 times in PBS, sections were incubated in PBS containing 10% normal goat serum for 15 min at 37°C , and then incubated overnight with mouse anti-rat TH (1:3 000, Sigma) at 4°C in a humidified chamber. The sections were washed in PBS and incubated with biotinylated goat anti-mouse IgG for 30 min at 37°C , followed by amplification with streptavidinperoxidase for 30 min at 37°C . Next, sections were rinsed with PBS and incubated for 10–15 min with diaminobenzidine (DAB). Staining was absent in sections where the primary antibodies were omitted. After TH-staining, the images of the sections from the Str were digitalized with a camera, connected to the microscope and a computer. Density in both sides of the Str was measured using the Simple PCI Image Analysis software. A background measurement (taken in the cortex) was subtracted from each measurement in the Str. Three sections from the Str of each rat were selected. The mean was calculated.

2.5 Isolation of total RNA and RT-PCR Both sides of SN were isolated and put into liquid nitrogen for further experiments. Total RNA was extracted from each sample using TRIzol reagent (Gibco Invitrogen). The primers were constructed on the basis of the published cDNA sequence of rat from GenBank. The primers used for amplifying TH, Caspase-3 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene were showed in Tab. 1. The PCR products were resolved by 1.5% agarose gel electrophoresis stained with ethidium bromide, visualized by an ultraviolet transilluminator,

photographed, and analyzed by Gis-1000 analysis software (Tanon, Shanghai). The intensities of the bands of TH and caspase-3 were normalized to that of GAPDH, which was amplified simultaneously.

2.6 Assay of superoxide dismutase (SOD) activity Both sides of midbrain were isolated immediately and put into normal saline (NS), homogenated. After determining the amount of total protein in the supernatants, we detected the activity of SOD using biochemical methods following the instructions for reagent kits (Nanjing Institute of Jiancheng Biological Engineering). The assay for SOD was based on its ability to inhibit the oxidation of oxyamine by the xanthine-xanthineoxidase system. The red product (nitrite) produced by the oxidation of oxyamine had absorbance at 550 nm. One unit of SOD activity was defined as the amount that the absorbance was reduced at 550 nm by 50%.

2.7 Statistics The results were represented as mean $\pm$ SEM. One-way analysis of variance followed by Student-Newman-Keuls test was performed using GraphPad 3.0 software. $P < 0.05$ was considered to be statistically significant.

3 Results

3.1 Copper reduces the DA and its metabolites contents in the Str of rats After 7 d of copper infusion, the DA and its

metabolite 3, 4-dihydroxyphenylacetic acid (DOPAC) levels decreased dose-dependently in the lesioned Str compared with that of NS-infusion group. The homovanillic acid (HVA) levels also showed a tendency of decrease, however, no significant difference can be detected (Tab. 2). No changes were observed in the unlesioned side of Str in each group.

3.2 Copper reduces TH positive axons in the Str of rats As shown in Fig. 1B, the TH immunoreactive fibers in the lesioned Str markedly decreased after 7 d of copper infusion (50 nmol). The value of gray scale in the unlesioned side also showed a decrease compared with that of NS-infusion group (Fig. 1A).

3.3 Copper down-regulates TH mRNA expression in the SN of rats As shown in Fig. 2, copper injection (50 nmol) caused a down-regulation of TH mRNA expression in the lesioned SN. No significant changes were detected in the unlesioned side.

3.4 Copper up-regulates caspase-3 mRNA expression in the SN of rats After 50 nmol copper injection, caspase-3 mRNA expression was up-regulated in the lesioned side of SN of rats (Fig. 3). No significant changes were detected in the unlesioned side.

3.5 Copper decreases the activity of SOD in the midbrain of rats After 7 d of 50 nmol copper infusion, the SOD activity decreased in the lesioned side of midbrain compared with that of control group (Fig. 4).

Tab 1. Primers of TH, Caspase-3 and GAPDH genes

Genes	Primer sequences	RT-PCR product
TH	Sense: ACACAGCGGAAGAGATTGCT	400 bp
	Antisense: CCCAGAGATGCA AGTCCAAT	
Caspase-3	Sense: TATGCTTACTCTACCGACCCG	271 bp
	Antisense: CCCACTCCCAGTCATTCCTTA	
GAPDH	Sense: TGTTCAGTATGATTCTACCCA	575 bp
	Antisense: GGTAGGAACACGGAAGGC	

Tab. 2 Effects of intranigral copper injection on the DA contents and its metabolites in the Str of rats (ng/mg wet weight of brain tissue)

Group	<i>n</i>	DA	DOPAC	HVA
control	6	7.65 $\pm$ 0.41	1.43 $\pm$ 0.12	1.48 $\pm$ 0.24
10 nmol	6	6.69 $\pm$ 0.62	1.19 $\pm$ 0.17	1.03 $\pm$ 0.62
50 nmol	6	4.04 $\pm$ 0.51**	0.78 $\pm$ 0.06**	0.74 $\pm$ 0.48
200 nmol	6	3.77 $\pm$ 0.68**	0.37 $\pm$ 0.07**	0.24 $\pm$ 0.02

DA: $F = 34.16$; DOPAC: $F = 19.05$; HVA: $F = 2.26$. ** $P < 0.01$ compared with control; * $P < 0.01$ compared with 10 nmol group.

Fig. 1 Effects of copper on the TH immunoreactive fibers in the Str. The TH positive axons in the lesioned Str (L) decreased markedly (B) compared with that of control (A, $P < 0.01$) and unlesioned side (U, $P < 0.01$) after intranigral infusion of 50 nmol copper. $n = 6$, Bar = 2 mm.

Fig. 2 Effects of copper on the TH mRNA expression in the SN of rats. Copper down-regulated TH mRNA expression in the lesioned SN of rats treated with 50 nmol copper. $^*P < 0.01$ compared with control, $n = 6$.

Fig. 3 Effects of copper on caspase-3 mRNA expression in the SN of rats. Copper up-regulated caspase-3 mRNA expression in the lesioned SN of rats treated with 50 nmol copper. $^*P < 0.01$ compared with control, $n = 6$.

Fig. 4 Changes of SOD activity after copper treatment. Copper decreased the activity of SOD in the lesioned midbrain of rats. $^*P < 0.01$ compared with control, $n = 6$.

4 Discussion

The present study has illustrated copper-induced damages of dopaminergic neurons in the nigrostriatal system of rats. Our results showed that DA contents in the lesioned Str were reduced with the increase in the copper concentrations injected and had a significant linear correlation. The progressive reduction in DA contents suggested that the copper-induced damage to the dopaminergic neurons might be in proportion to the concentrations of copper injected. To further investigate the harmfulness of copper to dopaminergic neurons, we tested the levels of TH mRNA in the SN and the changes of TH immunoreactive fibers in the Str. The results also showed that copper had neurotoxic effects on dopaminergic neurons.

The mechanisms of copper toxicity on neurodegeneration were considered to attribute to their properties of generating free radical and promoting apoptosis. Oxidative stress, resulting from the increased copper, has been widely believed

to be one of causes of neuronal death in many neurodegenerative diseases^[8]. Oxidative stress can result in disorder of antioxidant system. SOD is an important antioxidant enzyme of antioxidant defenses^[9,10]. The level of this antioxidant enzyme critically influences the susceptibility of the brain to oxidative stress. Our study has shown intranigral infusion of relatively high doses of copper induced SOD activity decreasing in the midbrain of anesthetized rats. This is probably because that the activity of this enzyme was damaged by elevated free radical after copper treatment.

Much of the copper-mediated neuronal death was the result of apoptosis^[11]. However, molecular mechanisms that link copper ions to apoptosis remain largely unclear^[12]. Previous reports suggest that copper catalyze the formation of reactive oxygen species (ROS) through Fenton or Haber-Weiss reactions^[1,13]. As an agent of triggering apoptosis of cells^[14,15], ROS are believed to be responsible for cellular damage, direct oxidation of proteins, and cleavage of DNA and RNA molecules. Moreover, ROS can activate the caspase-3 activity^[16,17] and induce apoptosis mitochondrial oncogene products, Bcl-2 and Bax, to regulate apoptosis^[18]. In this study, we found that caspase-3 mRNA expression was up-regulated in the SN of rats treated with 50 nmol copper. Caspase-3 is a key caspase of the apoptotic cascade and its increase highlights the point of no return in apoptosis^[19].

In summary, the present study showed that copper could induce degeneration of dopaminergic neurons in the SN of rats by destroying antioxidant defenses and promoting apoptosis.

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铜离子引起大鼠黑质多巴胺能神经元的退行性变

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摘要: 目的 探讨黑质(substantia nigra, SN)内注射不同剂量的 $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ 对大鼠黑质纹状体系统多巴胺能神经元的影响。方法 实验用 Wistar 大鼠, 分成对照组和左侧 SN 内分别注射 10 nmol、50 nmol、200 nmol CuSO_4 组, 7 天后采用高效液相色谱法(high performance liquid chromatography, HPLC)检测纹状体内多巴胺(dopamine, DA)及其代谢产物的含量; 酪氨酸羟化酶(tyrosine hydroxylase, TH)免疫组织化学法检测纹状体内 TH 免疫阳性纤维的改变; 半定量 RT-PCR 法检测黑质内 TH、Caspase-3 mRNA 的表达量; 用生化试剂盒分析大鼠中脑内超氧化物歧化酶(superoxide dismutase, SOD)活性的改变。结果 在 10 nmol CuSO_4 注射组中, DA 及其代谢产物的含量与对照组相比没有统计学差别。但是从 50 nmol 组开始, 损毁侧纹状体内 DA 含量随注射 CuSO_4 剂量的增加而逐渐减少, 显示出明显的剂量依赖关系($F = 34.16, P < 0.01$)。注射 50 nmol CuSO_4 组大鼠纹状体内 TH 免疫阳性纤维明显少于对照组和未损毁侧($F = 121.9, P < 0.01$)。注射 50 nmol CuSO_4 组大鼠 SN 内 TH mRNA 的表达与对照组相比下降($t = 3.12, P < 0.01$), 但 Caspase-3 mRNA 的表达量与对照组相比却明显增加($t = 8.96, P < 0.01$)。在注射 50 nmol CuSO_4 组中, 大鼠损伤侧中脑内 SOD 的活性与对照组相比下降($t = 2.33, P < 0.01$)。结论 铜离子可以导致大鼠黑质内多巴胺能神经元的损伤, 该损伤作用可能是通过破坏抗氧化保护系统和促进细胞凋亡而实现的。

关键词: 铜; 黑质; 多巴胺; 大鼠