Mitochondria dysfunction was involved in copper-induced toxicity in MES23.5 cells

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Abstract: Objective To investigate the toxicity of copper on MES23.5 dopaminergic cells and the probable mechanisms involved in this process. **Methods** MES23.5 dopaminergic cells were selected as our experimental model. [3-(4, 5-dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide] (MTT) assay was used to detect the influence of copper on the cell viability. The semi-quantitative reverse transcription polymerase chain reaction (RT-PCR), Western blotting and the high performance liquid chromatography-electrochemical detection (HPLC-ECD) have been used to detect the tyrosine hydroxylase (TH) mRNA and protein expression and the dopamine content in MES23.5 cells. The flow cytometry have been used to detect the changes of mitochondrial transmembrane potential. **Results** 100 and 200 $\mu\text{mol/L}$ copper had no effect on the MES23.5 cell viability, whereas 400 and 800 $\mu\text{mol/L}$ of copper could decrease the cell viability ($P < 0.01$). Treating cells with 200 $\mu\text{mol/L}$ copper for 24 h decreased the TH mRNA expression, the TH expression and the dopamine content compared with the control ($P < 0.01$, $P < 0.01$, $P < 0.05$, respectively). Besides, the mitochondrial transmembrane potential also decreased with the treatment of 200 $\mu\text{mol/L}$ copper for 24 h ($P < 0.01$). **Conclusion** Copper could exert the toxic effects on MES23.5 dopaminergic cells and decrease the cell function. The dysfunction of mitochondria may be the mechanism of this toxicity effect.

Keywords: copper; dopamine; mitochondria

1 Introduction

Copper is an essential trace element in the body and plays important roles in many physiological processes, such as growth, red and white blood cell maturation, normal host defense mechanisms, bone strength, iron transport, cholesterol and glucose metabolism, myocardial contractility and brain development^[1]. In the brain particularly, copper-dependent enzymes, such as dopamine- β hydroxylase and Cu-monoamine oxidase catalyse the formation and metabolism of neuronally active substances. Brain contains high levels of copper, which enhances the importance of copper for the central nervous system^[2].

On the other hand, altered homeostasis of copper (either deficiency or excess) seems to be toxic and can result in serious consequences to the organism^[3]. As a transition metal ion, copper could cycle between oxidized Cu^{2+} and reduced Cu^+ forms. Cu^+ catalyzes the formation of hydroxyl radicals in the presence of hydrogen peroxide through Fenton reaction^[4]. Hydroxyl radicals constitute some of the most harmful free radicals, and induce cellular damage by reacting with lipids, amino acids, thiol groups, RNA and DNA^[5].

The dysfunction of copper metabolism has been suggested in many diseases such as Menkes disease, Wilson disease, and some neurodegenerative diseases including Alzheimer disease and Prion disease^[3,6]. Recently, high concentration of copper was found in the substantia nigra (SN) of 6-hydroxydopamine (6-OHDA)-induced Parkinson's disease (PD) rat models^[7]. But whether this increased level of copper has toxic effects on the dopaminergic neurons is still unknown. In the present study, we selected MES23.5 dopaminergic cells as a model to detect the effects of copper and to elucidate the probable mechanisms of copper toxicity.

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2 Materials and Methods

2.1 Materials CuSO₄ and the primary antibody against tyrosine hydroxylase (TH) were purchased from Sigma. MES23.5 cell was kindly provided by Dr Wei-Dong LE (Departments of Neurology and Molecular Physiology and Biophysics, Baylor College of Medicine, Houston, Texas). Dulbecco's modified Eagle's F12 medium (DMEM/F12) was purchased from Gibco. TRIzol reagent and the reverse-transcription kit were purchased from Promega. Taq polymerase and DNA Marker DL2000 were purchased from TaKaRa.

2.2 Cell cultures The MES23.5 cell line, which was derived from somatic cell fusion of rat embryonic mesencephalon cells and the murine neuroblastoma-glioma cell line N18TG2, contains TH and synthesizes dopamine, but no other catecholamine^[8]. MES23.5 cells were cultured in DMEM/F12 growth medium supplemented with 5% fetal bovine serum (FBS), 2% Sato's components, 1% L-glutamine, 100 units/mL penicillin and 100 µg/mL streptomycin at 37 °C, in a humid 5% CO₂, 95% air environment. The culture medium was changed every 2 d and the cells were subcultured approximately every 4 d at a ratio of 1:3.

2.3 [3-(4, 5-dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide] (MTT) assay MES23.5 cells were seeded at a density of 2×10^4 onto 96-well plates. After attachment, 100-800 µmol/L copper was added to DMEM/F12 without serum supplement for the subsequent 24 h. Then 20 µL of 5 mg/mL MTT was added to each well, plates were incubated at 37 °C for 4 h and the medium was replaced with 100 µL of DMSO. The absorbance in control and copper-treated wells was measured at 570 nm using a colorimetric assay (TECAN, Austria). Each experimental data point represented the average value obtained from six replicates, and each experiment was performed six times.

2.4 Semi-quantitative RT-PCR Total RNA was isolated from MES23.5 cells using TRIzol reagent according to the manufacturer's instructions, and then 5 µg RNA was reverse-transcribed in a 20 µL reaction with oligodT primers using a reverse-transcription system (Promega). The following PCR amplification reaction utilized Taq polymerase and specific primers. The primers were constructed on the basis of the published cDNA sequence of mice from GenBank. The two primers used for amplifying glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene were sense, 5'-GAC CAC AGT CCATGC CAT CAC-3' and antisense, 5'-GCT GTT GAA GTC

GCA GGA GAC-3', which generated a 340 base pair (bp) PCR product. The two primers used for amplifying TH mRNA was sense, 5'-AAA ATC CAC CAC TTA GAG ACC-3' and antisense, 5'-TAG CCA CAG TAC CGT TCC-3', which generated a 467 bp product. The conditions for PCRs were denaturation at 94 °C for 30 s; annealing at 62 °C for GAPDH and 50 °C for TH for 30 s; and extension at 72 °C for 45 s with 32 cycles for GAPDH and 34 cycles for TH. The PCR products were analyzed using 1.5% agarose gel electrophoresis, stained with ethidium bromide, and visualized in an UV-illuminated imager (Shanghai Kelong Corp., Shanghai, China). The intensities of the bands of TH were normalized to that of GAPDH, which was amplified simultaneously.

2.5 Western blotting Cells were seeded in 6-well plates and incubated with 200 µmol/L copper for 24 h as described above. After washing with cold PBS three times, cells were lysated directly on culture plates with lysis buffer containing 50 mmol/L TrisHCl, 150 mmol/L NaCl, 1% Nonidet-40, 0.5% sodium deoxycholate, 1 mmol/L EDTA, 1 mmol/L PMSF and protease inhibitors (pepstatin 1 µg/mL, aprotinin 1 µg/mL, leupeptin 1 µg/mL). The protein concentration was determined by the Bradford assay kit (Bio-Rad Laboratories, Hercules, CA). Sixty microgramme total protein was separated using 8% SDS-polyacrylamide gels and then transferred by electroblotting into PVDF membranes. After overnight blocking with 10% non-fat milk at 4 °C, the membranes were incubated with mouse-anti-serum TH antibody of 1:20 000 for 2 h at room temperature. Goat-anti-mouse secondary antibody conjugated to horseradish peroxidase was used at 1:500. Cross-reactivity was visualized using ECL Western blotting detection reagents and then analyzed through scanning densitometry by a Tanon Image System. β-actin was detected by mouse-anti-β-actin monoclonal antibody (1:500) using a similar procedure to ensure equal samples of protein.

2.6 High-performance liquid chromatography electrochemical detection (HPLC-ECD) Cells were seeded in 6-well plates, treated with copper for 24 h and then the samples were prepared using previous described techniques in our laboratory^[9]. Separation was achieved on a PE C₁₈ 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 sulphonate, 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 oper-

ated in screen mode. Results were expressed as ng/10⁶ cells.

2.7 Detection of mitochondrial transmembrane potential

Changes in the mitochondrial transmembrane potential with various treatment in MES23.5 cells were measured by rhodamine123 using flow cytometry (Becton Dickinson, USA) as described before^[10,11]. The uptake of rhodamine123 into mitochondria is an indicator of the mitochondrial transmembrane potential ($\Delta\psi_m$). Cells were seeded in 6-well plates and treated with copper for 24 h as described above, and then incubated with rhodamine 123 in a final concentration of 5 $\mu\text{mol/L}$ for 30 min at 37 °C. After washing twice with HBS, fluorescence was recorded at 488 nm excitation and 525 nm emission wavelengths.

2.8 Statistical analysis

Each experiment was performed at least three times. The data were analyzed using the SPSS 12.0 software and expressed as mean $\pm$ SEM. One-way analysis of variance (ANOVA) or *t*-test was used to compare the differences between means. $P < 0.05$ was considered to be statistically significant.

3 Results

3.1 Copper decreased the cell viability of MES23.5 cells

MTT assay showed that low dose of copper (100 $\mu\text{mol/L}$) had no effects on the cell viability. When incubating with 200 $\mu\text{mol/L}$ copper, the cell viability showed a tendency to decrease but not significant. A remarkable reduction of cell viability was observed after treating with 400–800 $\mu\text{mol/L}$ copper ($n = 6$, $P < 0.01$, compared with that of control; Fig. 1).

3.2 Copper decreased both the TH mRNA and protein expression in MES23.5 cells

After incubating MES23.5 cells with 200 $\mu\text{mol/L}$ copper for 24 h, both the TH mRNA and the protein expression decreased in copper treated group ($n = 6$,

Fig. 1 Effect of copper on the cell viability. After treatment with various concentrations of copper for 24 h, the changes of cell viability in MES23.5 cells were observed by MTT assay. The cell viability was unchanged up to 200 $\mu\text{mol/L}$ copper treatment. A significant reduction was observed when treated with 400 and 800 $\mu\text{mol/L}$ copper. $n = 6$, ** $P < 0.01$, compared with that of control.

$P < 0.01$, compared with that of control; Fig. 2A and B).

3.3 Copper decreased the dopamine content of MES23.5 cells

After incubating MES23.5 cells with 200 $\mu\text{mol/L}$ copper for 24 h, the dopamine content was investigated using HPLC-ECD. The dopamine content in control and copper-treated group was (10.3 $\pm$ 1.1) ng/10⁶ cells and (6.1 $\pm$ 0.8) ng/10⁶ cells, respectively, which indicated that copper treatment could decrease the dopamine content ($n = 6$, $P < 0.05$, compared with that of control; Fig. 3).

Fig. 2 Effect of copper on the TH mRNA expression and the protein level in MES23.5 cells. After treatment with 200 $\mu\text{mol/L}$ copper for 24 h, TH mRNA expression and the protein levels in MES23.5 cells were observed using RT-PCR and Western blotting. A: The original figure showed the TH mRNA expression in control and 200 $\mu\text{mol/L}$ copper treated groups. Summarized data indicated that TH mRNA expression were down regulated after copper treatment. $n = 6$, ** $P < 0.01$, compared with that of control. B: The original figure showed the TH protein levels in control and 200 $\mu\text{mol/L}$ copper treated groups. Summarized data indicated that TH protein levels were down regulated after copper treatment. $n = 6$, ** $P < 0.01$, compared with that of control.

3.4 Copper decreased the mitochondrial transmembrane potential of MES23.5 cells After incubating MES23.5 cells with 200 $\mu\text{mol/L}$ copper for 24 h, we detected the changes of $\Delta\psi\text{m}$. $\Delta\psi\text{m}$ changes are the marker of mitochondria functions. Data showed that in the control group the percentage of cells in M1 was $23.5\% \pm 1.8\%$, whereas in the copper treated group the percentage of cells in M1 was $35.3\% \pm 2.5\%$, indicating that copper treatment could decrease the $\Delta\psi\text{m}$ ($n = 6$, $P < 0.01$, compared with that of control; Fig. 4).

Fig. 3 Effect of copper on the dopamine content in MES23.5 cells. After treatment with 200 $\mu\text{mol/L}$ copper for 24 h, changes of dopamine content in MES23.5 cells were observed using HPLC-ECD. Summarized data indicated that dopamine content decreased after copper treatment. $n = 6$, $*P < 0.05$, compared with that of control.

Fig. 4 Effect of copper on the mitochondrial transmembrane potential ($\Delta\psi\text{m}$) in MES23.5 cells. After treatment with 200 $\mu\text{mol/L}$ copper for 24 h, changes of $\Delta\psi\text{m}$ in MES23.5 cells were observed using flow cytometry. The original figure showed the fluorometric assay on $\Delta\psi\text{m}$ of control and 200 $\mu\text{mol/L}$ copper treated groups. Summarized data indicated that $\Delta\psi\text{m}$ were down regulated after copper treatment. $n = 6$, $**P < 0.01$, compared with that of control.

4 Discussion

At present, the function of copper in the degeneration of dopaminergic neurons is still controversial. Clinical studies have indicated that long-time occupational exposure to copper appears to be a risk factor for the degeneration of dopaminergic neurons^[12]. However, Alcaraz-Zubeldia *et al.* has indicated that copper sulfate could against MPP⁺-induced neurotoxicity in mice^[13]. In our present study, we demonstrated that copper may have toxic effects on MES23.5 dopaminergic cells. MES23.5 cells exhibit several properties similar to the primary neurons originated in the SN, such as containing tyrosine hydroxylase and synthesizing dopamine^[8]. Therefore, the results from this cell line will give direct evidence correlated to the degenerated dopaminergic neurons. These results suggested that copper may be toxic to MES23.5 cells, and this toxicity was concentration dependent. Higher concentration of copper (400-800 $\mu\text{mol/L}$) markedly decreased the cell viability, whereas 100 $\mu\text{mol/L}$ copper had no effect on it. According to the results, we selected the concentration of 200 $\mu\text{mol/L}$ copper to do the following experiments. This concentration could slightly decrease the cell viability but at the same time had no statistical significance compared with that of the control. Thus, this concentration could avoid the influence of reduced cell number in the following experiments. Further experiments showed the TH mRNA expression, the TH expression and the dopamine content also decreased by 200 $\mu\text{mol/L}$ copper treatment for 24 h. So it is concluded that copper could decrease the cell function and ultimately lead to the death of dopaminergic neurons.

To further understand the probable mechanism of copper toxicity, we observed the changes of $\Delta\psi\text{m}$. Data showed that copper treatment could decrease the $\Delta\psi\text{m}$ markedly. $\Delta\psi\text{m}$ changes are the marker of mitochondria function. Decrease in $\Delta\psi\text{m}$ stands for the dysfunction of mitochondria. Mitochondria are critical for maintaining neuronal homeostasis. In addition to producing large quantities of ATP required for neuronal function, mitochondria are the important regulator of intracellular calcium, the major site of reactive oxygen species (ROS) production, and are thought to be key activator of programmed cell death. The excess uptake of calcium by mitochondrial and production of ROS may contribute to neuronal injury. Given their central role in both normal and pathological cellular processes, it is not surprising that mitochondrial dysfunction is considered a critical event in various

neurodegenerative schemes^[14,15]. As copper could cycle between oxidized Cu^{2+} and reduced Cu^+ forms, the Cu^+ may catalyze the production of hydroxyl radical through Fenton's reaction. The hydroxyl radical could then lead to the impairment of essential molecules such as lipids, proteins and DNA. So the enhanced level of copper within SN could represent a pro-oxidant condition, which leads to the dysfunction of mitochondria^[5].

This study is the first to illustrate the toxicity of copper on MES23.5 dopaminergic cells and the possible mechanism involved, which provided the new evidence for understanding the complex role of copper in the degeneration of dopaminergic neurons.

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铜离子对 MES23.5 细胞的毒性作用机制与线粒体功能障碍有关

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摘要: 目的 探讨铜离子对 MES23.5 多巴胺能细胞的毒性作用及可能机制。方法 用 MTT 法检测铜离子对细胞存活率的影响; 用半定量逆转录聚合酶链反应 (RT-PCR), Western blotting 以及高效液相色谱电化学检测法 (HPLC-ECD) 检测细胞内酪氨酸羟化酶 (tyrosine hydroxylase, TH) mRNA、蛋白的表达以及多巴胺含量的改变; 用流式细胞仪检测线粒体跨膜电位的改变。结果 100 $\mu\text{mol/L}$ 和 200 $\mu\text{mol/L}$ 铜离子对细胞存活率没有影响, 400 $\mu\text{mol/L}$ 和 800 $\mu\text{mol/L}$ 铜离子可以造成细胞存活率降低 ($P < 0.01$)。200 $\mu\text{mol/L}$ 铜离子孵育细胞 24 h, TH mRNA、蛋白表达量以及多巴胺含量较正常对照组降低 ($P < 0.01$, $P < 0.01$, $P < 0.05$)。200 $\mu\text{mol/L}$ 铜离子孵育细胞 24 h, 线粒体跨膜电位较正常对照组明显降低 ($P < 0.01$)。结论 铜离子对 MES23.5 多巴胺能细胞具有毒性作用, 降低细胞功能的表达, 其机制可能与线粒体功能障碍有关。

关键词: 铜; 多巴胺; 线粒体