SILVER NANOPARTICLES INDUCE FERROPTOSIS IN MOUSE HIPPOCAMPAL HT22 CELLS

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Abstract - Silver nanoparticles (AgNPs) have excellent physicochemical properties and are widely used in various antibacterial products. There are many opportunities for human body exposure. Ferroptosis is a newly discovered, iron-mediated cell death that is involved in the development and progression of some neurological diseases. The purpose of this study is to explore ferroptosis induced by AgNPs on mouse hippocampal neuron cell line (HT22) and its mechanism. Cell viability was determined by CCK-8 analysis. Total glutathione (GSH) was measured by a commercial GSH kit. Malondialdehyde (MDA) level was measured by a commercial MDA kit. The protein expression levels related ferroptosis was measured by Western blotting assay. The results showed that (1) AgNPs-induced cell death involved ferroptosis. Ferroptosis inhibitors desferrioxamine (DFO) and ferrostatin-1 (Fer-1) could significantly inhibit the cytotoxicity caused by AgNPs; (2) AgNPs could cause iron homeostasis disorder in HT22 cells by affecting the expression levels of ferritin heteropolymers ferritin light chain (FTL), ferritin heavy chain (FTH1) and iron importers transferrin receptor protein 1 (TFRC); (3) AgNPs-triggered ferroptosis was related to the increase of lipid peroxide MDA, the depletion of reduced glutathione (GSH), the down-regulation of glutathione peroxidase 4 (GPx4) and the up-regulation of the system Xc- transporter subunit cystine/glutamate transporter (SLC7A11). Our data provided a new mechanism basis for ferroptosis as a new cell death phenotype induced by AgNPs. The deeper mechanism and its relationship with the neurotoxicity of AgNPs need to be further studied.

Keywords - Nanotoxicity; Silver Nanoparticles; Ferroptosis; Lipid Peroxidation

I. INTRODUCTION

Silver nanoparticles (AgNPs) are increasingly studied and widely used in biomedicine due totheir excellent physicochemical properties[1]. Numerous studies have shown that AgNPs can penetrate the blood-brain barrier and enter brain tissue to produce central neurotoxic effects. Currently, the neurotoxic effects of AgNPs mainly include brain histopathological effects, behavioral changes, and altered neurotransmitter levels in subject animals, and their toxic mechanisms of action are related to inflammatory responses and oxidative stress. AgNPs can induce various forms of cell death in cultured cells or animals, including apoptosis, necrosis, and autophagy. However, whether AgNPs induce ferroptosis in HT22 cells has not been studied. Ferroptosis is an Fe^{2+}dependent oxidative non-apoptotic programmed death[2]. Studies in recent years have revealed that ferroptosis is involved in the development of certain neurological diseases. The mouse hippocampal neuron HT22 cell line is a good model for studying neurodegenerative diseases in vitro. In this study, HT22 cells were used to investigate the ferroptosis effects of AgNPs. Silver nitrate ($AgNO_3$) is used as a silver ion positive control for AgNPs. It provides a theoretical basis for the application and development of AgNPs.

II. METHODS

2.1 Cell culture and exposure to AgNPs

HT22 cells were cultured with complete medium (10 % fetal bovine serum + high sugar DMEM medium) at 37 ° C in a 5 % CO_2 incubator. HT22 cells were exposed to 0, 2, 4, 6, 8, 10, 12 μ g/ml 20 nm Polyvinylpyrrolidone (PVP)-coated AgNPs (YunfuNano Technology Co., Ltd. Shanghai, China) for 24 h.

Cells were pretreated with ferroptosis inhibitors deferoxamine (DFO) and ferrostatin-1 (Fer-1) for 2 h and used to probe the cellular ferroptosis effects.

2.2. Cellular viability

After AgNPs and $AgNO_3$exposure, the AgNPs suspension was aspirated. 100 μ l of diluted 10% CCK-8 solution without fetal bovine serum was added to the cell culture dish per well. The cells were incubated for 0.5-2 h in a cell incubator, and the absorbance was measured at 562 nm with an enzyme marker. The cellular viability (%) = (OD value of experimental group - blank OD value) / (OD value of control group - blank OD value) 100%.

2.3. Intracellular malondialdehyde (MDA) and glutathione (GSH) assays.

HT22 cells were collected at the end of AgNPs and $AgNO_3$ exposure, washed in phosphate buffer saline (PBS) and sonicated in an ice bath to break up the cells. The specific steps in the instructions of the MDA and GSH assay kits were strictly followed. The whole procedure was performed on ice. And the optical density (OD) was measured using an enzyme marker (EPOCH, BIOTEK Corporation, USA). Also, the BCA protein assay was used for total protein quantification. mDA and GSH content was calculated as nmoL/mg protein.

2.4. Western blot

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) separation gels were prepared for protein expression assays. Proteins were first passed through electrophoresis and transferred to PVDF membranes, which were then enclosed in 5% skim milk at room temperature for 2 h. Afterwards, the membranes were washed three times every 10 min using Tris-buffered saline tween (TBST). The membranes were incubated overnight at 4° C with primary antibodies including glutathione peroxidase 4 (GPX4), system Xc- transporter subunit cystine/glutamate transporter (SLC7A11), transferrin receptor protein 1 (TFRC), ferritin heavy chain (FTH1), ferritin light chain (FTL), Cyclooxygenase (COX-2). β-actin was used as an internal control for data normalization. Thereafter, TBST was added twice and Tris-buffered saline (TBS) once, respectively. In the next step, the membranes were washed with TBST as described above and then incubated with goat anti-rabbit secondary antibody (#7074, 1:5000, Cell Signaling Technology, USA) for 2 h at room temperature. Finally, protein bands were displayed using enhanced chemiluminescence (ECL) solution (Thermo, USA).

III. RESULTS

3.1. Cytotoxicity of AgNPs and $AgNO_3$

As shown in Figures 1 and 2, cell viability decreased with increasing staining concentration after 24 h exposure of HT22 cells using AgNPs (2, 4, 6, 8, 10, 12 μg/ml) and $AgNO_3$ (0.2, 0.4, 0.6, 0.8, 1, 1.2 μg/ml).Both AgNPs and $AgNO_3$ exhibited significant toxic effects on HT22 cells.

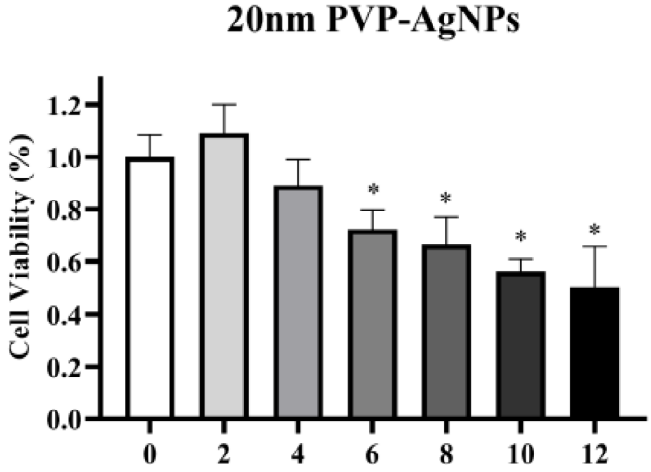


Figure 1Effect of different concentrations (μg/ml) of AgNPs exposure on HT22 cell viability (n=3) (* indicates P<0.05)

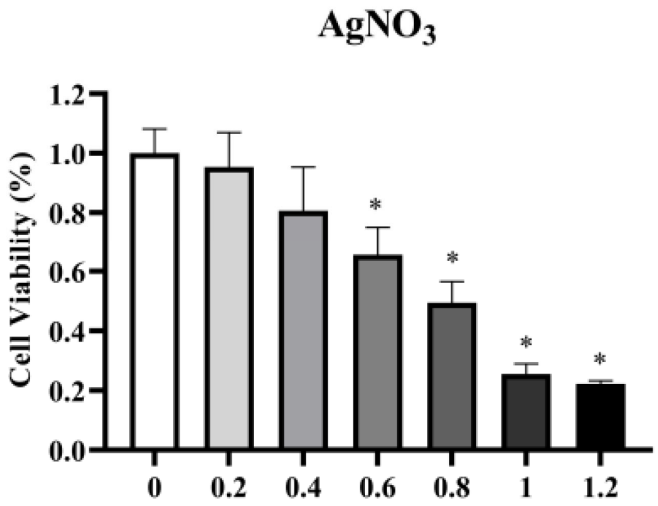


Figure 2Effect of different concentrations (μg/ml) of $AgNO_3$ exposure on HT22 cell viability (n=3) (* indicates P<0.05)

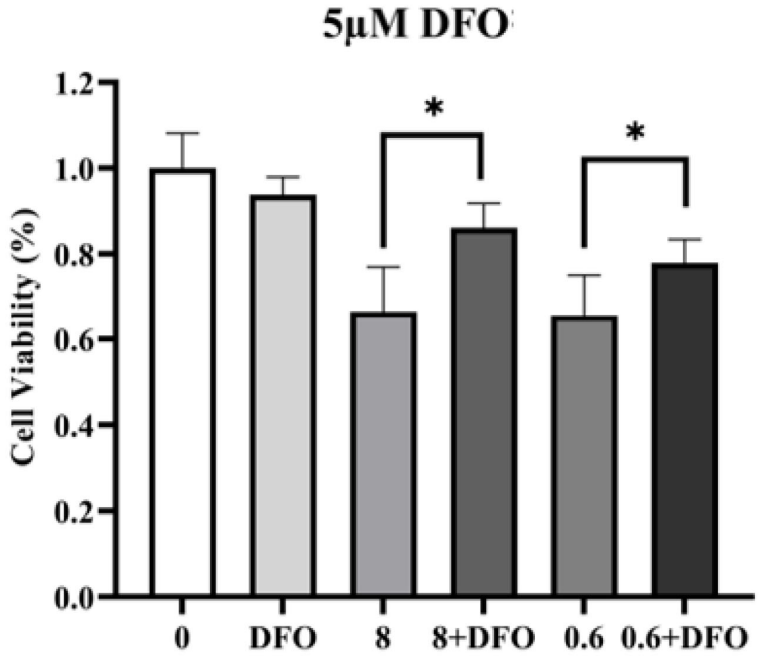


Figure 3. Effect of 5μM DFO inhibitor pretreatment on the viability of high-dose AgNPs and $AgNO_3$exposed HT22 cells (n=3) (* indicates P<0.05)

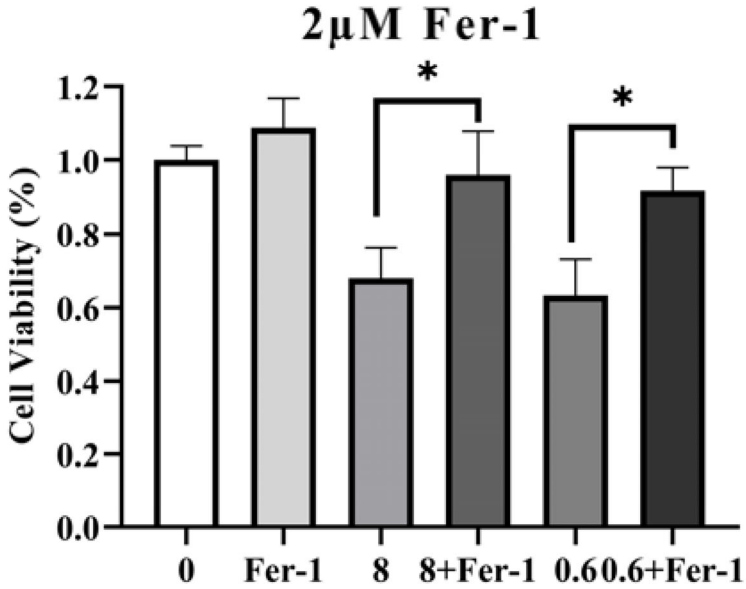


Figure 4. Effect of 2μM Fer-1 inhibitor pretreatment on the viability of high-dose AgNPs and $AgNO_3$exposed HT22 cells (n=3) (* indicates P<0.05)

3.3. AgNPs and $AgNO_3$ induced lipid peroxidation in HT22 cells

The results of the MDA test showed a significant increase in the intracellular MDA content of HT22 cells in the 4, 8 μg/ml AgNPs groupsand 0.3, 0.6 μg/ml $AgNO_3$ groups. Moreover, the intracellular GSH content was significantly decreased in the 4, 8 μg/ml AgNPs and 0.6 μg/ml AgNO3 groups after 24h exposure of HT22 cells. These results suggest that both AgNPs and $AgNO_3$ can induce lipid peroxidation effects in HT22.

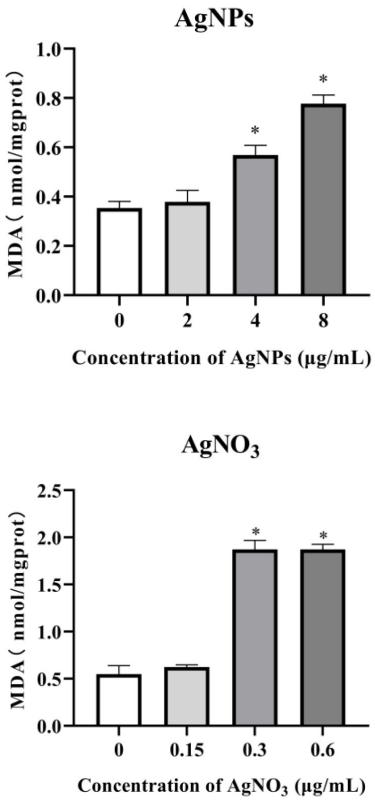


Figure 5. Total MDA content of HT22 cells after 24h exposure to different concentrations of AgNPs and $AgNO_3$(n=3) (* indicates P<0.05)

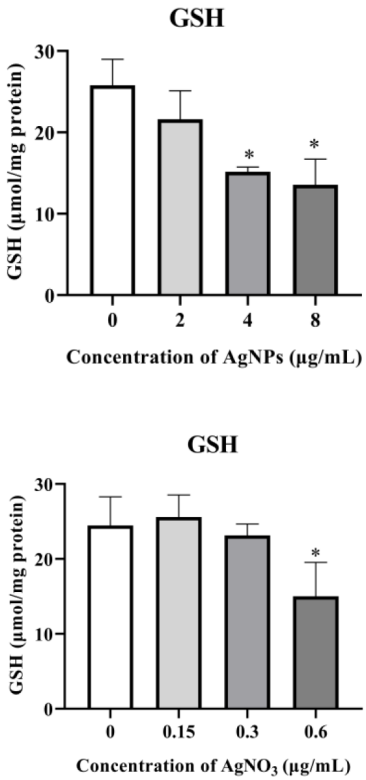


Figure 6. Total GSH content of HT22 cells after 24h exposure to different concentrations of AgNPs and $AgNO_3$(n=3) (* indicates P<0.05)

3.4. Ferroptosis n HT22 cells induced by AgNPs

As shown in Figure 7, the western blot results revealed that AgNPs and $AgNO_3$ exposure induced upregulation of iron homeostasis-related proteins such as TFRC, FTH1 and FTL expression in HT22 cells. Both AgNPs and $AgNO_3$ significantly induced a decrease in the expression of key proteins for ferroptosis in HT22 cells such as GPX4 and an increase in the expression of SLC7A11, and also

induced an increase in the expression of COX-2.

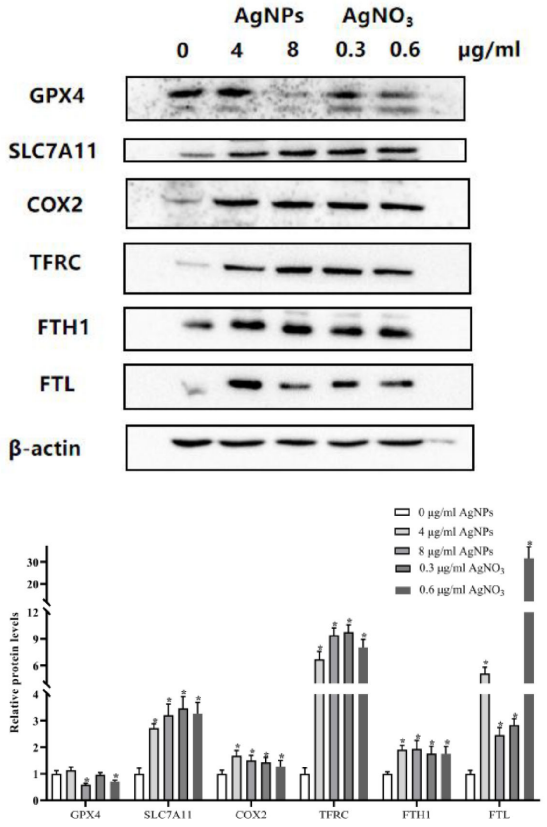


Figure 7. Relative protein levels of HT22 cells after 24h exposure to different concentrations of AgNPs and $AgNO_3$(n=3) (* indicates P<0.05)

IV. DISCUSSION

The widespread use of AgNPs has raised concerns about environmental safety and human health, especially through inhalation exposure to AgNPs. many studies have shown that AgNPs can bypass and cross the BBB, or enter the brain through the olfactory bulb and induce neurotoxicity. Current studies on the mechanisms of AgNPs-induced neurotoxicity have focused on oxidative stress, inflammation, apoptosis, necrosis, mitochondrial dysfunction, and disruption of intracellular calcium homeostasis[3]. However, studies on the adverse effects of AgNPs on hippocampal neurons are very insufficient. Moreover, there is a lack of studies on the mechanisms of iron toxicity induced by AgNPs.

Lipid peroxidation exerts its toxic effects by altering the properties of membranes[4]. Iron toxicity involves cell death induced by lipid peroxidation following iron overload, ROS generation, GSH depletion and reduced GPX4 activity (GSH-dependent antioxidant defense pathway)[5]. In the present study, AgNPs and AgNO3 induced GSH depletion in HT22 cells. According to the process of GSH synthesis and metabolism, the mechanisms of GSH depletion can be divided into four categories: i) cutting off the supply of raw materials for GSH synthesis; ii) inhibiting GSH synthesis or regeneration; iii) depleting GSH reserves; and iv) promoting GSH efflux[6]. We found that AgNPs and AgNO3 inhibited the protein expression of GPX4 in HT22 cells. the SLC7A11 cystine/glutamate reverse transport system Xc- consisted of SLC7A11, suggesting that AgNPs and AgNO3 by affecting system Xc- in order to cut off the supply of raw material for GSH synthesis. In addition, metal nanoparticles can deplete GSH reserves through GSH oxidation[7].GPX4 resists iron- and oxygen-dependent lipid peroxidation by converting lipid peroxides (L-OOH) to non-toxic lipids and acts as a key enzyme in the regulation of iron toxicity.GSH is a cofactor and synthetic substrate of GPX4 and is required for the lipid repair function of GPX4 essential (Song & Long, 2020; Ingold et al., 2018).COX2 is an iron toxicity marker gene[8]. In this study, we found that AgNPs and AgNO3 increased the expression of ACSL4 and COX2.

Iron is an important trace element in the human body. Fe^{2+} can directly catalyze the decomposition of H_2O_2 into hydroxyl radicals via the Fenton reaction and damage cellular proteins, lipids, DNA, etc. The uptake of extracellular Fe^{3+} is mainly mediated by TFRC. Most of the intracellular iron is stored in ferritin (FER) and a small fraction exists in a free state, forming a labile iron pool (LIP)[9].FER consists of FTL and FTH1[10]. In this study, AgNPs and $AgNO_3$ upregulated the protein expression of TFRC, FTH1 and FTL. These results suggest that AgNPs and $AgNO_3$ induce an imbalance of iron homeostasis in HT22 cells.

Conclusion

In summary, both AgNPs and $AgNO_3$ induced ferroptosis in HT22 cells and their mechanisms were similar. It was involved in lipid peroxide accumulation, decrease in GPX4 protein expression, and increase in SLC7A11 and COX2 protein expression. In addition, AgNPs led to the imbalance of iron homeostasis in HT22 cells by inducing increased protein expression of TFRC, FTH1 and FTL. Our findings will provide insight into the neurotoxicity induced by AgNPs.

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