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Silver nanoparticles induced oxidative and endoplasmic reticulum stresses in mouse tissues: implications for the development of acute toxicity after intravenous administration†

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Concerns have arisen about the health and environmental impacts of increasing commercial use of silver nanoparticles (AgNPs). However, the toxic mechanisms and target tissues of AgNPs have not been fully defined. In this paper, we investigated the tissue toxicity of mouse after intravenous administration of AgNPs at a single-dose of 0.2, 2 or 5 mg/kg (body weight), respectively. Biodistribution, endoplasmic reticulum stress, oxidative stress were examined in mouse organs at eight hours after exposure. Stress markers, *e.g.* HSP70, BIP, p-IRE1, p-PERK, *chop* and *xbp-1s* proteins/genes were significantly upregulated in a dose-dependent manner. In liver, spleen, lung and kidney, high stress accompanied with apoptosis occurrence. Low stress levels were observed in heart and brain. Thus, it is proposed that liver, spleen, lung and kidney are dominant target tissues of AgNPs exposure. The lower stress and toxicity in heart and brain were in agreement with lower AgNP accumulation. The present results demonstrated that AgNPs exposure eventually resulted in permanent toxic damage by gradually imposing stress impacts on target organs. These findings highlight the potent applications of stress markers in future risk evaluation of silver nanoparticle toxicity.

Introduction

Silver nanoparticles (AgNPs) are important antimicrobial nanomaterial used in medical products like wound dressings, surgical instruments, medical catheters and bone prostheses. The medical applications of AgNPs will result in uptake in the human body and particles will be distributed in different organs by blood circulation. It has shown that AgNPs are captured by reticuloendothelial system and quickly distribute to associated tissues after entering in the blood.[1-3] The single-photon emission computerized tomography (SPECT) imaging evidence shows that liver and spleen are dominant tissues for capturing AgNPs from the blood.[2] The capturing process is usually less than 10 min and a steady state level was achieved as the consequence of systemic blood circulation within 6 h.[4] The retained AgNP usually remains at a steady state with slightly decreasing trend in silver levels at least till to 14 days for single intravenous (*i.v.*) injection exposure.[5] Similarly, other kinetic studies show AgNPs distributed mostly in liver, spleen, lungs, and kidneys, but much less quantities in brain, heart, and testes after *i.v.* exposure.[6, 7] The concentration in the target tissue is normally an important determinant of its toxicity. However, the mechanisms of toxicity development were not clearly defined.

Previous *in vitro* studies indicated that a key event in AgNPs is intracellular oxidative stress resulting in apoptosis and/or necrosis.[5, 7] Endoplasmic reticulum (ER) is an important organelle and has functions in the folding and assembling of cellular proteins, supplements of lipids and sterols. The interruption of these normal functions by oxidative damages will lead to ER stress.[5] ER stress is also known as unfolded protein response (UPR), a conserved cellular self-protection mechanism of the body.[8] Three ER proteins, including inositol requiring protein 1 (IRE1), PKR-like endoplasmic reticulum kinase (PERK), and activating transcription factor-6 (ATF-6) act as stress sensing proteins.[9, 10] Recently, ER-stress related events have been proposed as an early biomarker for nanotoxicological toxicity.[11-13] As a hallmark of cytotoxicity, ER stress had been reported in human cell lines and zebrafish of AgNPs induced toxicity.[14, 15] In a previous study, we investigated the toxicity and ER stress responses in mice after intratracheal instillation exposure to AgNPs.[13] We observed that most AgNPs were accumulated in the lung with leading to significant apoptosis, but had lower adverse effects in the liver. In this paper, the association between the tissue concentrations of AgNP and ER stress was investigated after mice intravenously injected with AgNPs. Thus, this study gives insight on the realistic adverse health effects of AgNPs and the underlying mechanism of the toxicity development.

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Materials and methods

Materials

AgNPs (NM-300K) and their stabilizing dispersant (NM-300K-DIS) were provided by the European Commission Joint Research Center (Ispra, Italy). The NPs were maintained in the same stabilizing agent solution, comprising 4% each of polyoxyethylene glycerol trioleate and Tween 20. Its dispersant (NM-300K-DIS) was used in all following tests as the parallel control sample. The characterization was described in detail previously. [13, 16]

Animal exposure

Balb/c mice (Beijing Vital River Experimental Animal Technology Co. Ltd., bodyweights of 20–22 g) were housed in macrolon cages in an isolated animal room with water and rodent food supplement. Animals were acclimated to the environment for at least one week prior to experiment. All procedures were approved by the Ethics Committee of Animal Care and Experimentation of the National Institute for Environmental Studies, China. Mice were randomly divided into four groups, a control group, 0.2, 2, 5 mg/kg body weight (bw) AgNPs (NM-300K) treatment groups. The tail *i.v.* injection was executed for the exposure. Animals were sacrificed at 8 hours to assess the stress and toxic responses after AgNP exposure as illustrated in Fig. 1. At necropsy, animals were anesthetized with 40 mg/kg intraperitoneal sodium pentobarbital and terminated by exsanguination via the abdominal aorta. The tissues and organs such as heart, liver, spleen, kidneys and lung were excised and weighed accurately. Blood samples were collected following the standard operation procedures for routine blood draw.

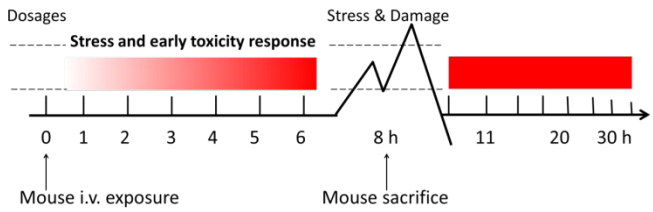

Fig. 1 Schematic diagram of the experimental design.

Blood assay

Totally 1 ml of blood was drawn by removing the right eyeball of mouse before necropsy. About 100 µl whole blood was directly put into blood collection tubes containing EDTA for hematological analysis. Others about 900 µl blood was allowed to clot for serum separation and biochemical analyses. IL-6/TNF-α levels were analyzed by using mouse IL-6/TNF-α specific ELISA kit (eBioscience, San Diego, CA, USA) following the manufacturer's instruction. Results were expressed in pg/mL, and three independent experiments were performed.

RNA isolation, reverse-transcription PCR, and quantitative real-time PCR

Total RNA was isolated from mouse livers and spleens with TRIzol (Invitrogen) extraction. After quantification of the extracted RNA pellets, first-strand complementary DNA synthesis was performed by Superscript First-Strand Synthesis kit (Invitrogen). All samples were analyzed by quantitative real-time PCR (Eppendorf, Germany) using the SYBR Green contained PCR Master Mix with reaction volumes of 25 µl. The primer sequences were listed in ESI Table 1. †

Western blotting analysis

The frozen tissues were homogenized in a lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton-X100 and protease inhibitor, pH 7.4) and then centrifuged for 15 min at 10,000g to discard tissue debris at 4℃. The supernatants were collected and the protein concentrations were determined using a Bio-Rad kit (USA). Proteins were separated on SDS-PAGE gels and subjected to desired antibodies after transfer to nitrocellulose membranes. The antibody against CHOP was purchased from Cell Signaling Technology (USA), other antibodies including Actin, HSP70, BIP, CHOP, PERK, p-PERK, IRE-1, p-IRE-1 were from Santa Cruz (USA). The blots were developed using HRP-conjugated secondary antibodies and ECL (enhanced chemiluminescence) solution (Thermo Scientific, USA).

TUNEL assay

The terminal deoxynucleotidyl transferase-mediated dUTP nick endlabelling (TUNEL) assay could specifically detect the fragmented genomic DNA usually caused from sequential activation of Caspases and endonucleases in apoptosis. The detailed method was described previously.[13]

Histopathological examination

The histopathological test was performed using routine procedures as previously described.[17]

ICP-MS

Ag element analysis was performed by ICP-MS test. Briefly, samples (about 100 mg) were predigested overnight with 5.0 mL concentrated nitric acid (MOS grade), then mixed with 1.0 mL 30 % H_2O_2 (MOS grade) and digested for 2 h in open vessels on a hot plate at 150 ℃. At last, the remaining solution about 0.5 mL was cooled, and then diluted to 3.0 g with 2% HNO_3. Ag^{107} was used as a standard material to drawing the standard curve by measurement of a series of dilution samples. Indium (In) was used as an internal standard throughout the test. Both standard and test solutions were measured for three times by ICP-MS (PerkinElmer, Waltham, MA, U.S.A.).

Statistics

The data were presented as mean ± standard deviation (mean ± SD). Statistical analyses were performed using Student's t-test for comparison of two groups. *P* values < 0.05 were considered to be statistically significant.

Results

Characterization of AgNPs

Transmission electron microscope (TEM) test shows the primary size of AgNPs was about 20 nm and no obvious agglomerates/aggregates were observed when dispersed in saline solution (Fig. 2). The hydrodynamic size of AgNPs was 38.2 ± 2.1 nm as determined by dynamic light scattering in pure water and the zeta potential was 0.3 ± 0.1 mV. [13] Other related characters had been described in detail in our previous reports. [13, 18]

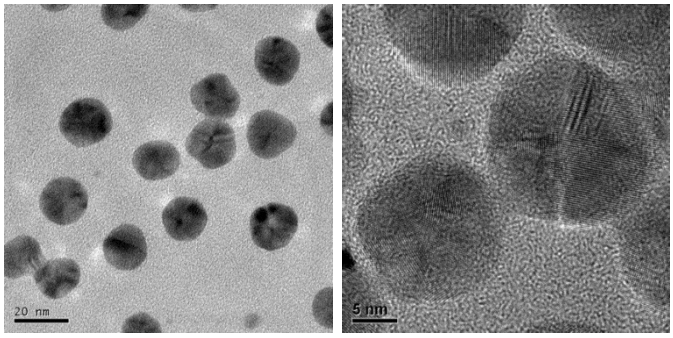

Fig. 2 TEM images of silver nanoparticles.

Tissue distributions of AgNPs after intravenous injection

Silver concentrations were measured in various organs and tissues at 8 hours after one single tail intravenous injection. No significant difference was found in the body weight and organ weight after AgNP exposure (Data not shown). Table 1 shows the results of mean silver concentration per gram organ tissue. Silver mostly accumulated in liver and spleen, followed by lung, kidney, heart and brain. After treatment by AgNPs, the Ag element distributions were dose dependent in a range of 34.7 % to 73.2 % in liver, 5.6 % to 23.5 % in spleen of total administrated silver NPs. The quantity of Ag element in other organs was less than 1 % of the total administration. Silver levels in these organs could be overestimated due to silver contributions from the blood residue in heart, kidneys and brain. The realistic organ stress and following damages were judged by the metrics of concentration (per organ tissue weight). By this metrics, the AgNP has an impact to the organ tissues of following order: Spleen > Liver > Lung >> Kidneys > Heart > Brain.

Evaluation on the stress levels

AgNPs were mostly distributed in liver and spleen. Based on this finding, it was suspected these organs would be the mostly sensitive target organs after *i.v.* exposure. ER stress marker levels were evaluated at the protein levels in liver and spleen (Fig. 3). HSP70, BIP, p-IRE1, p-PERK and CHOP were significantly up-regulated with the increase of exposure doses of AgNPs. Endoplasmic reticulum stress related genes *xbp-1s* and *chop* showed significant higher expression comparing to the control in liver of high dose group, but not in spleen (Fig. 4 A and B). With happening of ER stress in liver, the equilibrium of redox was disturbed as illustrated from significantly increase of oxidative stress related genes of heme oxygenase 1 (*HO1*), glutathione peroxidase (*GPX*) and superoxide dismutase 1 (*SOD1*) (Fig. 4 C, D and E). High expression of cytokine genes like *IL-6* and *TNF-α* indicates that AgNPs induced immune responses in liver tissues (Fig. 4 F and G).

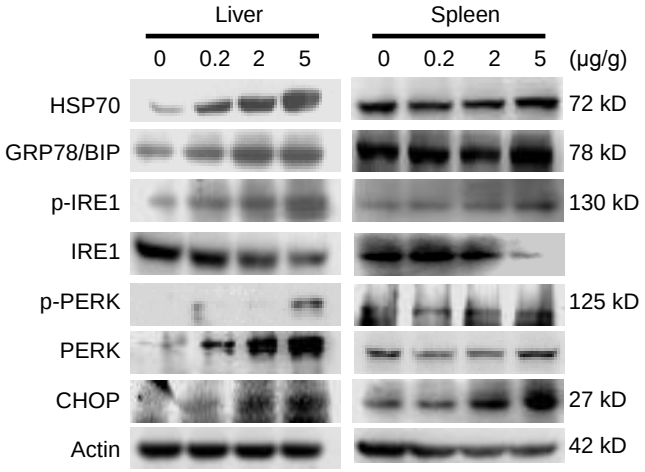

Fig. 3 Western blotting results of earlier stress marker proteins in liver and spleen tissues after *i.v.* injection of AgNPs for 8 h.

Table 1 The silver concentrations in various tissues (expressed as ng per gram wet weight) at 8 h time point after a single intravenous injection of AgNPs at 0.2 (Low), 2 (Middle) or 5 (High) mg/kg, respectively. Data were expressed as Mean±SD, n=3

Groups	Brain	Heart	Lung	Liver	Spleen	Kidneys
Vehicle	7.1±1.6	17.0±7.6	9.5±0.7	27.1±4.7	15.9±1.0	6.7±0.4
Low	8.6±1.0	63.5±3.2	149.2±1.9	1022.0±33.9	2254.6±138.2	48.8±3.6
Middle	49.3±3.1	192.6±7.1	1051.5±50.4	17527.8±4108.1	28419.0±2837.6	262.9±23.5
High	84.6±10.6	425.1±1.6	4663.2±27.1	53872.8±1217.4	234688.3±32057.9	644.2±18.1

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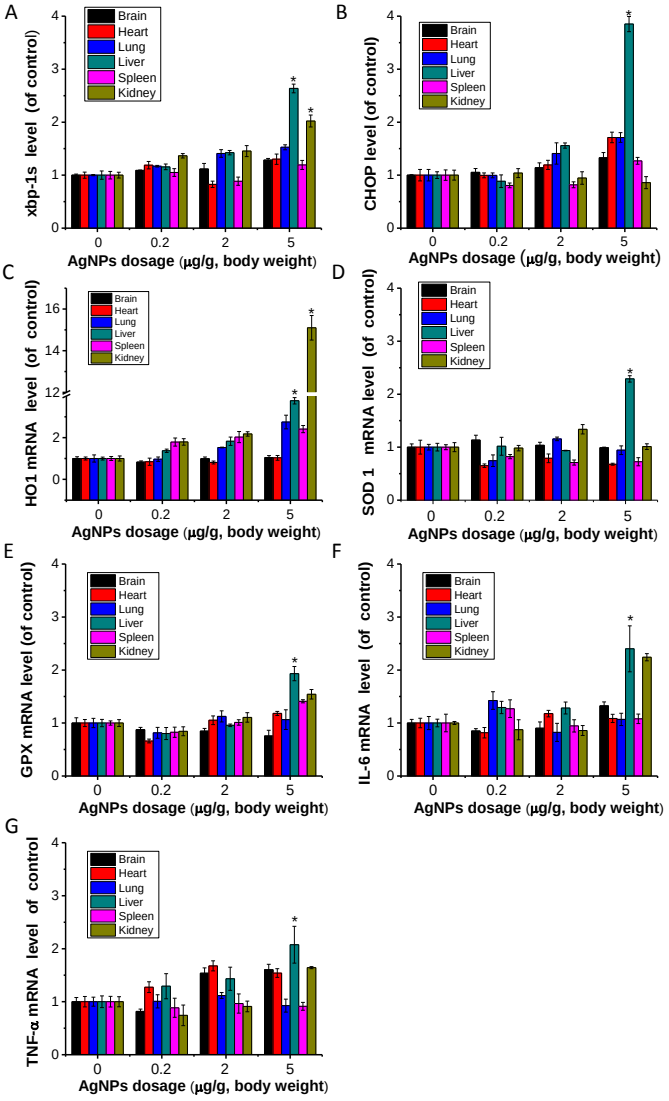

Fig. 4 Gene expression levels of mice organ tissues after exposure to one single injection of AgNPs at 0.2 (L, Low), 2 (M, Middle) and 5 (H, High) mg/kg dosages for 8 h. (A) *xbp-1s*; (B) *chop*; (C) *HO-1*; (D) *SOD1*; (E) *GPX*; (F) *IL-6*; (G) *TNF-α*. Data represent mean ± SD from three independent experiments. * P< 0.05, compared with the control group.

Blood analysis

The total white blood cell numbers were unaffected by AgNPs exposure for 8 hours. However, the lymphocyte percentage was significantly decreased in high dosage treatment group (ESI Fig. 1†). Inflammation was evaluated by testing the IL-6 and TNF-α levels in serum (Fig. 5). Significant increase of IL-6 was observed in high dose treatment group of AgNPs exposure.

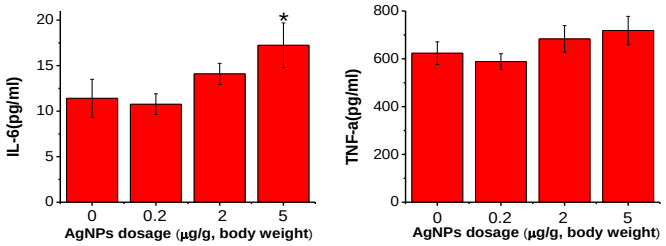

Fig. 5 IL-6 (A) and TNF-α (B) production levels in serum after one single dose AgNPs i.v. exposure. Data were expressed as Mean±SD, n=6, * P < 0.05, *vs*. the control group.

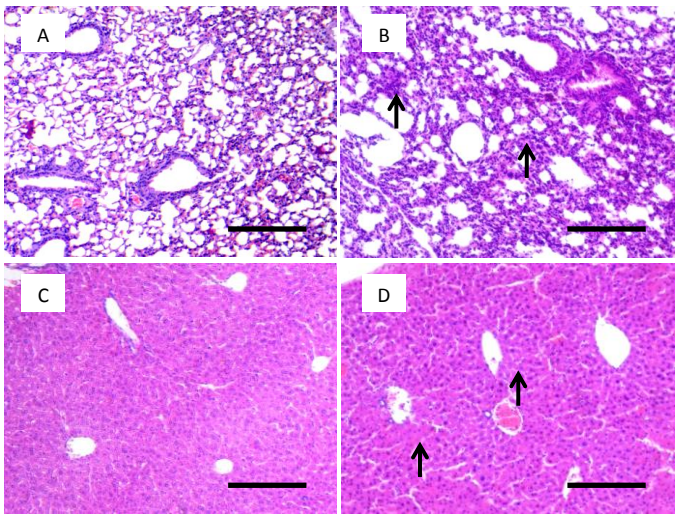

Fig. 6 Histopathological images of tissues after intravenous exposure to AgNPs at 5 mg/kg for 8 h in mice. (A) Lung of saline treatment group; (B) Lung of AgNPs treatment group; (C) Liver of saline treatment group; (D) Liver of AgNPs treatment group. Black arrows show the obvious pathological changes of cell swelling. Scale bar = 200 μm.

Acute toxicity in high dose exposure group

Histopathological observations of mouse tissues from high-dose AgNPs exposure were conducted. Liver and lung tissues showed obvious acute toxicity pathology changes post exposure. Lung demonstrated thickened alveolar walls, multifocal consolidation and infiltration of focal inflammatory cells. Liver showed disorganized hepatic cords, damaged hepatic lobule, edema cytoplasm and ballooning-like tissue changes (Fig. 6). There were no remarkable histopathological changes in brain, heart, spleen and

kidneys (ESI Fig. 2†). Apoptosis was evaluated using fluorescence-conjugated TUNEL assay. Significant higher level of apoptotic cells was detected in lung, liver, spleen, kidneys of high dosage AgNPs-exposed group compared to saline-treated control. No significant apoptosis was observed in heart and brain tissues (Fig. 7 and ESI Fig. 3†). It is interesting to note that high apoptosis rate was mostly found in glomerular tissue parts in kidney (ESI Fig. 3F†).

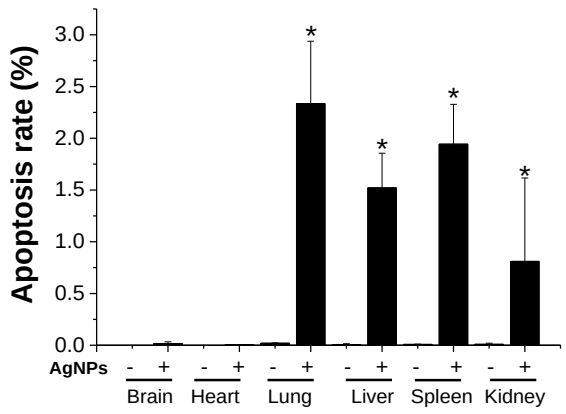

Fig. 7 Apoptosis rate of TUNEL assay after intravenous exposure to AgNPs at 5mg/kg for 8 h in mice. The results are expressed as mean± SD, **P* <0.05 comparing to control of the same tissue.

Discussion

AgNP is the highest one among commercialized nanomaterials in biomedical application.[18-19] AgNP induces a number of cellular responses in mammalian cells, *e.g.* immune toxicity, tissue stress, inflammation and apoptosis.[1, 20-26] Our recent research has shown that AgNP causes different toxic impacts on peripheral organs from the reason of their various toxic sensitivities to this material after intratracheal instillation exposure.[13] However, the information of *in vivo* toxic mechanisms of AgNP, as an excellent nanomedicine material, is not clearly defined after entering in the circulation. The *in vivo* earlier toxicological study is particularly important for the understanding of the development of AgNPs toxicity, which also forms the crucial link to the following realistic damages. Nanoparticles in medical products can translocate in the bloodstream and distribute systemically to different organs.

Dosage is important for evaluating the different adverse effects of nanomaterials. High dosage absolutely induces the serious acute toxic effects without question, for example, 7.5, 30 or 120 mg/kg ever being used in the *i.v.* exposure to dissect the acute effects in mice.[27] The cytotoxicity and genotoxicity had been found in mouse tissues at the dosages high to a single 25 mg/kg dose or 25 mg/kg/day for 3 consecutive days of 15–100 nm AgNPs, while the dosage range from 0.5 to 20 mg/kg was adopted to disclose the dose-response effect of 5 nm AgNPs.[28] Immunotoxicity of silver nanoparticles was evaluated in rats by adopting 28-day repeated-dose exposure method. It shows that a reduced thymus weight with BMDL (lowest 5% lower confidence bound of the benchmark dose) is 0.76 mg/kg bw/day, and an increased spleen weight, spleen cell number, and spleen cell subsets, with BMDLs between 0.36 and 1.11 mg/kg bw/day by this repetitive exposure manner.[29] In this study, the adverse effects of intravenously administered AgNPs were investigated in Balb/c mice in the quite low dosage range from 0.2 to 5 mg/kg. Then the earlier stress conditions and toxic damages were examined at 8 h post exposure for focusing on the toxicity development post exposure.

Consistent to previous reports, the major fractions of the AgNPs were detected in liver and spleen.[8, 30] However, it should be noted that there is a quite high concentration of AgNPs maintained in the lung, although the relative level in the lung is much less than liver and spleen. The organ distribution could reflect the most important potential organ target which directly exposure to nanoparticles. The depositions of AgNPs in tissue has direct relation to the development of organ toxicity.[31] Previous studies showed ER stress is involved in AgNPs induced apoptosis in cell models.[32, 33] This study shows that ER stress was significantly induced in mice organs including liver and spleen after AgNP *i.v.* exposure. The levels of stress protein marker HSP70 and BIP were induced by a dose-response manner in the liver indicating high cellular stress after AgNP exposure. At the same time, it was accompanied by the activation of ER stress sensor proteins like IRE1 and PERK. CHOP protein, a proapoptotic transcription factor, was significantly induced in a dose-dependent manner in liver and spleen. Further, the consistent upregulation of ER stress maker in the mice liver were found in mRNA levels, which indicates that liver is the dominant target organ for AgNPs exposure. Cha et al reported the significant decrease of liver DNA contents after mice fed with AgNPs, which suggests the possible induction of apoptosis.[34] Using the TUNEL assay, apoptosis was seen in lung, liver, spleen kidney and especially the glomerular region. It is assumed that high apoptosis was due to the high accumulation of AgNPs in glomerular regions than other parts of kidney. Apoptosis was not seen in heart and brain tissues. Histopathological results are in good agreement to the toxic outcomes in liver and lung. It is proposed that the low damage formed in heart and brain is due to low concentrations of AgNPs in these tissues.

It had been reported that AgNPs induced toxicity in cells or drosophila melanogaster through generation of ROS.[24, 35,36] In this study, AgNPs induced high oxidative stress responses demonstrated by upregulations at mRNA of *HO1*, *GPX* and *SOD1* in the liver and kidney at high dose treatments. It had been shown that oxidative stress contributing to the progression of inflammation in liver.[34] Inflammation was not quite obvious at the earlier stage after AgNPs exposure in this study. IL-6 was slightly increased in high dose treatment group, while TNF-α level did not show any change in serum. In contrast, the AgNPs significantly induced the expression of IL-6 in liver and kidney tissues, while TNF- α was only induced in liver, at high dosage group. This suggests that immune system may require more than 8 hours to implementing high immune responses represented by serum cytokines.[29]

Conclusion

In summary, we investigated the toxicity and ER stress inducing abilities of AgNPs after intravenous injection exposure for 8 h in mice at doses of 0.2, 2, 5 mg/kg body weight. Our research focused on the organ biodistribution and earlier toxicity forming stage after the exposure. From these results, we concluded that investigating stress markers can be used as a sensitive index of early evaluation of silver nanoparticles in animal experiments. The present results indicate that AgNPs could be quickly distributed in target organs and pose stress/toxicity over time and dosages, presenting a scenario should be further explored and addressed. Our research highlights that stress responses caused by *in vivo* AgNPs exposure has priority to be paid more attention in future toxicity studies.

Acknowledgements

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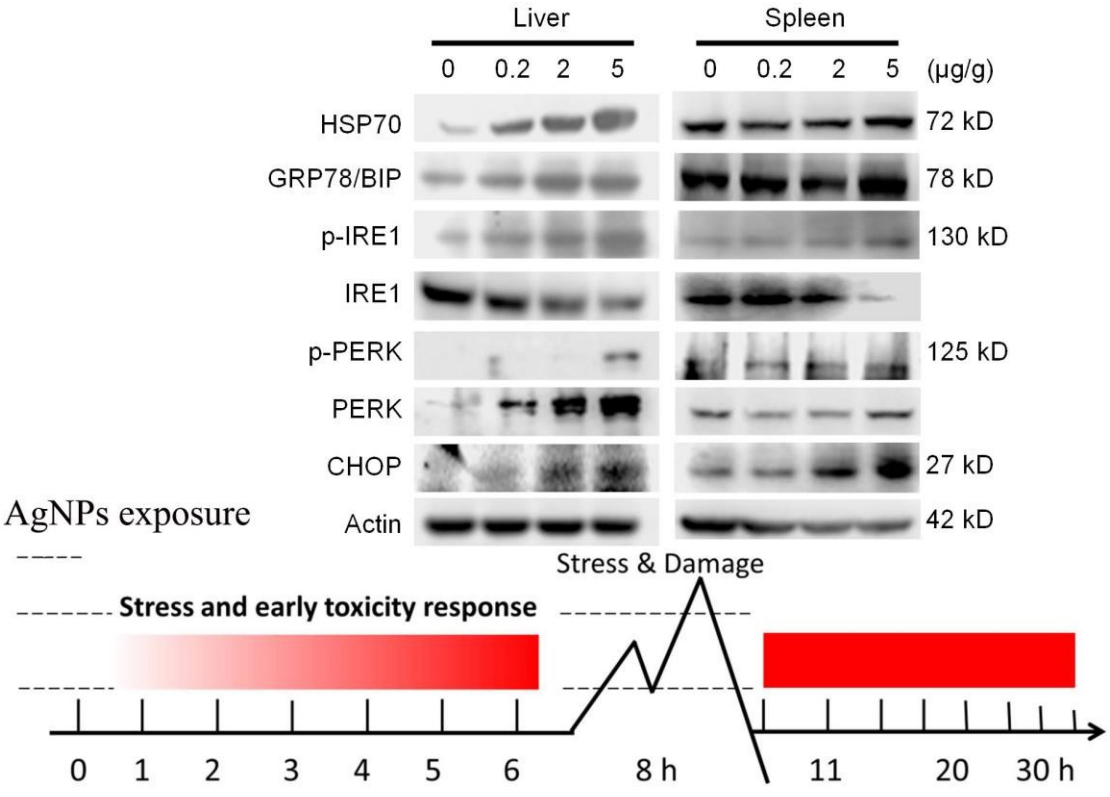

AgNPs form toxic damage by gradually imposing stress impacts on the target organs in mice.