Autophagy and autophagy dysfunction contribute to apoptosis in HepG2 cells exposed to nanosilica†

Yongbo Yu,a,b Junchao Duan,b,c Yang Yu,b,c Yang Li,b,c Yang Zou,b,c Yumei Yang,b,c Lizhen Jiang,b,c Qiuling Li,b,c and Zhiwei Sun*a,b,c

Great concerns have led to the evaluation of the potential hazards of nanosilica to human health and the environment. However, there still exists persistent debates on the biological effects and toxic consequences induced by nanosilica. The present study investigated both autophagy and apoptosis in ICR mice and Human hepatocellular carcinoma cells (HepG2), and then explored the interactive mechanism between these two distinct cell death modalities in HepG2 cells. Mice liver injuries seen by hematoxylin and eosin (HE) staining indicated the hepatotoxic effects of nanosilica. The TUNEL assay and immunohistochemistry results confirmed that nanosilica could induce both apoptosis and autophagy in vivo. Flow cytometry analysis demonstrated apoptosis induction in vitro, while autophagic ultrastructures, LC3-II expression and immunofluorescence clarified autophagy activation by nanosilica. Apoptosis suppression by the autophagy inhibitor of 3-methyladenine (3-MA) implied that autophagy was involved in apoptotic cell death. A mechanistic study verified that nanosilica induced autophagy via negative regulation of mammalian target of rapamycin (mTOR) signaling but not the Beclin-1 associated pathway. The enhancement of p62 accumulation and mTOR down-regulation might account for the molecular mechanism in contribution of autophagy to apoptosis. As an emerging new mechanism of nanomaterial toxicity, autophagy might be a more susceptible indicator for toxicological consequence evaluation in nanoparticle toxicity. The present study provides novel evidence to elucidate the toxicity mechanisms and may be beneficial to more rational applications of nanosilica in the future.

Introduction

Nanosilica is one of the most commonly used engineered nanomaterials (ENMs).1 Particles of nanosilica were already found in various commercial and biomedical products such as cosmetics and food ingredients as well as for drug delivery, diagnostic and medical imaging and engineering.2–5 It is predicted that the 10–50 nm of amorphous nanosilica was 2.74–14.45 μg g⁻¹ in food products containing the commercial additive E551.6,7 For biomedical applications, nanosilica has been used in combination with DNA and dendrimers to develop a delivery system for therapeuetic genetic material and DNA vaccines.8 The nanosilica also served as an effective targeting nanocarrier for anti-tumor drug delivery.9 Functional nanosilica was applied for magnetic resonance (MR) imaging to increase imaging sensitivity.10 Moreover, bioactive nanosilica with excellent surface properties played significant roles in the field of tissue regeneration.5 Recently, the 7 nm Cy5 encapsulated nanosilica was approved by the US Food and Drug Administration (FDA) for molecular imaging.11 The widespread exposure to biomedical products raised great concerns on the safety of nanosilica to human health.

The Organization for Economic Cooperation and Development (OECD) has proposed an urgent need for safety evaluation of nanosilica.12 Both in vivo and in vitro studies have been conducted to investigate the potential toxicity of nanosilica.13,14 Nanosilica was reported to pass through various biological barriers and distribute in nearly all organs through the blood circulation,13,15 exhibiting pulmonary toxicity, cardiovascular toxicity, genotoxicity and hepatotoxicity.16,17 The liver was considered as the main target organ of nanosilica toxicity.18 After intravenous injection in mice, nanosilica accumulated mainly in the liver and was retained for over 30 days.19 Moreover, a fatty liver pattern with a higher value of ALT (Alanine Aminotransferase) also occurred in nanosilica fed mice.20 Previously, we have demonstrated that nanosilica could cause acute toxicity of lymphocytic infiltration,
granuloma formation, and hydropic degeneration in liver hepatocytes. Furthermore, numerous in vitro studies have reported that nanosilica could penetrate the cellular membrane, deposit in organelles, alter protein expression and cause cell cycle arrest, DNA damage and cell death.

Due to the diversity and complexity of cell death mechanisms, the Nomenclature Committee on Cell Death (NCCD) has proposed recommendations of cell death classification respectively in 2005, 2009, 2012 and 2015. According to the NCCD, cell death at least can be classified into 4 subroutines: necrosis, apoptosis, autophagy and mitotic catastrophe. Different kinds of cell death like necrosis and apoptosis induced by nanosilica have been confirmed in various cell lines, such as A431, A549 and GES-1 cells. Our previous studies also demonstrated these cell death patterns in both HUVECs and HepG2 cells. For autophagy, it was originally considered as a degradation process to maintain cellular homeostasis, however, both autophagy and apoptosis dysfunction recently emerged as underlying mechanisms of nanoparticle toxicity. Although autophagy was reported as a potential toxic mechanism of nanomaterials, conclusions were still contradictory. The copper oxide nanoparticles and cat-ionic polystyrene nanospheres were reported to induce autophagic cell death, while cadmium-based quantum dots and ferroferric oxide nanoparticles induced pro-survival autophagy. Reactive oxygen species (ROS) were widely accepted as the main cause of both apoptosis and autophagy induced by nanoparticles and verifications have been performed in our previous studies. However, the relationship between apoptosis and autophagy was still not clear and it remains unknown whether autophagy contributes to the nanosilica-induced adverse effects and cell death. As a self-protection mechanism, cellular autophagy might serve as a susceptive response to defend against stimulation triggered by nanoparticles. All these continued debates and scientific issues suggest that further studies are needed to investigate the biological function of autophagy and cell death in the toxic effects of nanoparticles.

In the present study, we evaluated cell death subroutines of autophagy and apoptosis in ICR mice after nanosilica intra-venous injection and further explored the relationship between these two cell death pathways in vitro in Human hepatocellular carcinoma cells (HepG2). Based on our series of investigations, we further clarified the underlying molecular mechanisms of nanosilica-induced cell death. These biological effects and mechanistic insight might provide more persuasive evidence for safety evaluation and risk management of nanomaterials.

Materials and methods

Characterization of nanosilica

Amorphous nanosilica (silicon dioxide [SiO2]) was synthesized by the Stöber method. After the suspension was properly dried, the average particle size and morphology were examined using transmission electron microscopy (TEM) (JEOL JEM2100, Japan). The size distribution of nanosilica was analyzed using ImageJ software. To explore the hydrodynamic sizes and zeta potential, nanosilica in distilled water and serum-free cell culture medium were examined using a Zetasizer (Malvern Nano-ZS90, Britain). The nanosilica suspension was autoclaved for sterilization and sonicated to minimize aggregation (160 W, 20 KHz, 5 min) before being used for experiments.

Animals and treatment

The ICR mice (8 weeks old and 20–22 g in weight) were purchased from Weitong-Lihua Experimental Animal Center (Beijing, China). They were housed in a ventilated animal room maintained at 20 ± 2 °C and 60 ± 10% relative humidity with a 12 h light-dark cycle. The mice were given water and sterilized food. Prior to treatment, the mice were not fed overnight. A series of doses were set based on our previous LD50 estimating study. Mice were intravenously injected with nanosilica at 29.5, 103.5 and 177.5 mg per kg body weight through the mouse tail vein. Injections of sterile physiological saline were also given to the mice as a control. After intravenous injection, all animals were sacrificed at 14 d for subsequent experiments. All animal care and experimentation were approved by the Animal Ethics Committee at Capital Medical University (approval number 2011-X-072).

Cell culture and nanosilica exposure

Human hepatocellular carcinoma HepG2 cells were purchased from Cell Resource Center, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. The cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) (Gibco, USA) supplemented with 10% fetal bovine serum, 100 U per mL penicillin and 100 µg per mL streptomycin at 37 °C in a humidified incubator with 5% CO2. Cells were seeded in culture plates followed by exposure to nanosilica for 24 h, which was suspended in DMEM of certain concentrations (25, 50, 75 and 100 µg mL−1). Serum-free DMEM without nanosilica were used as the control group.

Histological analysis

The livers were removed and fixed in 10% formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E) for histological examination using standard techniques. The sections were examined under a light microscope (Olympus IX81, Japan).

TUNEL assay

Apoptosis induced by nanosilica in murine liver tissue was analyzed by the terminal deoxynucleotide-transferase (TdT)-mediated dUTP nick end labelling (TUNEL) assay. Ultrathin frozen sections were fixed and permeabilized, and then subjected to TUNEL staining based on the manufacturer’s instructions (Keygen, China). The nuclei were stained with 4’,6-diamidino-2-phenylindole (DAPI, Sigma) after the TUNEL assay. Afterwards, the apoptotic cells were visualized using a light microscope (Olympus IX81, Japan).
Immunohistochemistry

The murine liver tissue sections were formalin-fixed and paraffin-embedded. After deparaffinization and rehydration, the sections were placed in a 10 mM citrate buffer solution (pH 6.0) for antigen retrieval. Endogenous peroxidase was blocked with 3% H2O2 in PBS for 5 min and washed in PBS. After blocking with 10% normal goat serum for 10 min at 37 °C, the sections were incubated overnight with monoclonal LC3 antibody (CST, USA) at 4 °C or an equivalent amount of normal goat IgG as a negative control. The sections were then treated with an avidin–biotin affinity system for 30 min at room temperature, stained with 3,3′-diaminobenzidine (DAB) and hematoxylin. The examination was finally performed under a light microscope (Olympus IX81, Japan).

Cell viability assay

Cells were seeded and grown in 96-well plates at 10^3 per well overnight. The cytotoxicity of nanosilica was determined using the MTT assay. After pretreatment with or without 5 mM autophagy inhibitor 3-methyladenine (3-MA) or 20 μM apoptosis inhibitor z-vad-fmk for 1 h, cells were incubated with various concentrations of nanosilica (25, 50, 75, and 100 μg mL⁻¹) for 24 h at 37 °C. 10 μL of MTT was then added to each well followed by a 4 h incubation. Dimethyl sulfoxide (DMSO, 150 μL) was added to dissolve the resulting formazan crystals. The absorbance at 492 nm was then measured using a microplate reader (Thermo Multiscan MK3, USA).

Lactate dehydrogenase assay

Lactate dehydrogenase (LDH) released from damaged cells was measured using a LDH Detection Kit (Jiancheng, China) according to the manufacturer’s instructions. After the cells were treated with nanosilica for 24 h, the supernatants (100 μL) were collected for LDH measurement to reflect the cell membrane integrity. The absorbance of the reaction product was measured at 440 nm by a UV-visible spectrophotometer (Beckman DU-640B, USA).

Intracellular uptake of nanosilica

The cellular uptake of nanosilica and ultrastructural features in HepG2 cells were observed using transmission electron microscopy (TEM). The cells were seeded into a 6-well plate at a density of 3 × 10^5 per well. After incubation for 24 h with nanosilica (50 μg mL⁻¹), the cells were washed, collected and then centrifuged at 1500 rpm for 10 min. After the supernatants were removed, the cell pellets were fixed in 2.5% glutaraldehyde overnight. Ultrathin sections were sliced and imaged under a TEM device (JEOL JEM2100, Japan).

Flow cytometry analysis

Double staining with AnnexinV-FTTC and PI (KeyGen, China) was performed to detect apoptotic cells. About 3 × 10^5 cells were seeded into a 6-well plate for 24 h. After pretreatment with or without 3-MA, z-vad-fmk or N-acetyl-L-cysteine (NAC) for 1 h, the HepG2 cells were treated with nanosilica (50 μg mL⁻¹) for 24 h, then trypsinized and collected (centrifuged at 1500 rpm for 5 min). Cell pellets were resuspended in binding buffer, stained with AnnexinV-FTTC (5 μL) and PI (10 μL) and incubated for 15 min in the dark at room temperature. Apoptosis analysis of 10 000 cells was carried out at 488 nm excitation and 525 nm emission in a flow cytometer (Becton Dickinson, USA). The results were expressed as a dot plot with clearly distinguishable quadrants: viable (lower left, FITC−PI−), early apoptotic (lower right, FITC+PI−), late apoptotic and early necrotic (upper right, FITC+PI+), and late necrotic (upper left, FITC−PI+). The percentage of cells was determined by using the embedded FACS software.

Immunofluorescence

Cellular immunofluorescence was performed to detect the localization of microtubule-associated protein 1 light chain 3 (LC3) in the cytoplasm. The cells, cultured on glass coverslips, were treated with nanosilica for 24 h, fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton-X 100 in PBS for 10 min. The samples were blocked in 1% bovine serum albumin and incubated with LC3 antibody [1 : 200, rabbit anti-LC3 antibody, Cell Signaling Technology (CST), USA] overnight at 4 °C. Subsequently, glass slides were washed in PBS and incubated with the Alexa Fluor®-conjugated antibody (CST) for 1 h at room temperature. The nuclei were stained with 5 μg mL⁻¹ DAPI followed by LC3 protein localization detection using laser scanning confocal microscopy (LSCM) (Leica TCS SP5, Germany).

Western blot

To explore the nanosilica-induced autophagy and underlying mechanism in HepG2 cells, autophagy hallmarks of LC3, p62 and autophagy related proteins of Beclin-1, mammalian target of rapamycin (mTOR) and p-mTOR were examined by Western blot. After HepG2 cells were treated with nanosilica for 24 h, the cells were lysed in RIPA lysis buffer for 30 min. The total cellular protein in lysates was determined by the bicinchoninic acid (BCA) protein assay (Pierce, USA). Equal amounts of protein (40 μg) were loaded onto 12% and 15% SDS-PAGE and electrophoretically transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, USA). The membrane was then blocked with 5% nonfat milk, incubated with primary antibodies [1 : 1000, rabbit antibodies, Cell Signaling Technology (CST), USA] overnight at 4 °C and anti-rabbit Ig G secondary antibody (CST, USA) for 1 h at room temperature. The protein expression was finally detected using the ECL chemiluminescence reagent (Pierce, USA), while relative densitometric analysis was performed using Image Lab software (Bio-Rad, USA).

Statistical analysis

Data were expressed as means ± S.D. and significance was determined by using one-way analysis of variance (ANOVA). Student’s t-test was performed to draw statistical comparisons between two treatment groups. Differences were considered statistically significant at p < 0.05.
Results

Characterization of nanosilica

The characterization of spherical and well dispersed nanosilica is described in ESI Fig. 1A.† The average diameter was calculated to be about 62 nm. The hydrodynamic sizes and zeta potentials of nanosilica both in distilled water and in DMEM were measured. As depicted in ESI Fig. 1B and C,† the nanosilica showed a hydrodynamic size of 109 nm and zeta potential of −38 mV. These properties ensure the stability and dispersibility of nanosilica in both the stock solution and culture medium.

In vivo study

Biodistribution of nanosilica. In order to confirm the subcellular distribution of nanosilica in liver tissue, transmission electron microscopy (TEM) observation was performed in 177.5 mg kg⁻¹ nanosilica treated mice. Particles or aggregates of nanosilica were found to be ingested into the cytoplasm of Kupffer cells (Fig. 1A). Moreover, autophagic ultrastructural features of multi-membrane vacuoles appeared near the particles (Fig. 1B). These representative images indicated nanosilica distribution and autophagy induction in the liver after nanosilica exposure.

Histological analyses. A histopathological liver examination was performed to investigate the liver injuries induced by nanosilica. In the control group, results showed that normal hepatic cells featured a complete cytoplasm as well as an intact nucleus. However, representative liver lesions of lymphocytic infiltration and granuloma formation were seen in 29.5, 103.5 and 177.5 mg kg⁻¹ nanosilica treated mice (Fig. 2). The numbers and sizes of granulomas in the liver increased in a dose-related manner in nanosilica treated groups, indicating that the nanosilica may be hepatotoxic.

Immunohistochemistry staining of LC3. LC3 was detected immunohistochemically in paraffin embedded liver sections as an autophagy marker. As shown in Fig. 3, LC3 positive cells were lower in the control group as well as 29.5 and 103.5 mg kg⁻¹ nanosilica treated groups, whereas strong positive signals of LC3 were found in the hepatocytes of mice treated with 177.5 mg kg⁻¹ of nanosilica (Fig. 3). The results suggest that an autophagic phenomenon induced by intravenous nanosilica occurs in murine liver tissue.

Fig. 1 TEM images of the liver in ICR mice after acute exposure to nanosilica. (A) Particles of nanosilica were deposited in the endosome, lysosome, mitochondria and autophagic vacuoles either freely or as aggregates in the cytoplasm in Kupffer cells. The mitochondrial cristae were fractured or disappeared and the mitochondria were swollen with vacuolation. (B) The multi-membrane autophagosome formation and nanosilica were internalized in autophagosomes. En, endosome; Ly, lysosome; Mi, mitochondria; Av, autophagic vacuoles; Au, autophagosome. (Scale bar: 0.2 μm, 0.5 μm and 2 μm).

Fig. 2 Histological analyses of the liver in the nanosilica treated mice. Representative liver sections taken from the control mice, 29.5 mg kg⁻¹, 103.5 mg kg⁻¹ and 177.5 mg kg⁻¹ administered mice. White arrows denote granuloma and lymphocytic infiltration (white arrows) in the liver. (Scale bar: 20 μm).

Fig. 3 Immunohistochemistry staining of LC3 in ICR mice liver tissue sections. The LC3 positive staining (white arrows) were mainly diffused in the cytoplasm in hepatocytes of the 177.5 mg kg⁻¹ treated group. (Scale bar: 20 μm).
**TUNEL assay.** TUNEL assay was used to detect apoptosis in the liver of nanosilica exposed mice. As shown in Fig. 4, the hepatic cells in the control group and 29.5 mg kg$^{-1}$ nanosilica treated group showed normal morphology. Few positive cells were found in the medium dose of the nanosilica (103.5 mg kg$^{-1}$) treated group. However, obvious TUNEL staining was visible in the liver sections of the high dose group (177.5 mg kg$^{-1}$), suggesting that intravenous nanosilica could induce apoptosis in the liver of mice at a high dose level.

**In vitro study**

**Cytotoxicity.** Cell viability was measured to investigate the potential cytotoxicity of nanosilica (25, 50, 75 and 100 μg mL$^{-1}$). Results showed that nanosilica induced a dose-dependent decrease of cell viability in HepG2 cells (Fig. 5A). In addition, pretreatment with the autophagy inhibitor 3-MA and apoptosis inhibitor z-vad-fmk significantly improved cell viability after nanosilica exposure (Fig. 5A). The suppression of decreasing cell viability suggested that both autophagy and apoptosis contribute to cell death induced by nanosilica.

**Lactate dehydrogenase leakage.** Cell membrane permeability was evaluated as indicated by lactate dehydrogenase (LDH) leaking from cells exposed to nanosilica. It showed that nanosilica exposure induced a dose-dependent increase of LDH release in HepG2 cells (Fig. 5B). The extracellular levels of LDH in the cell culture medium confirmed that nanosilica could result in cell membrane damage to HepG2 cells.

**TEM observation.** The cellular uptake and ultrastructural features were estimated by TEM and the images are shown in Fig. 6. Nanosilica can deposit in mitochondria and endosomes in the cytoplasm (Fig. 6C). Particles were also dispersed either singly or in the form of agglomerates within cytoplasmic multi-membrane vesicles, which are typical features of autophagosomes and autolysosomes (Fig. 6C). Some of the vacuoles contained remnants of degraded cytoplasmic materials (Fig. 6D). The occurrence of particles in the cytoplasm indicated cellular internalization of nanosilica, while the appearance of autophagic characteristics suggested that nanosilica might cause autophagy in HepG2 cells.

**Detection of autophagy marker LC3.** Autophagy is characterized by the autophagosome, while the molecular marker of LC3 protein is localized in autophagosome membranes. Autophagy induction was verified by measuring the levels of LC3-I and LC3-II in nanosilica exposed HepG2 cells. Consistent with the autophagic ultrastructures of autophagosomes in TEM, western blot analysis demonstrated that the expression of LC3-II protein changed significantly with a dosage increase of nanosilica (Fig. 7A). Results from densitometric analysis showed that LC3-II was gradually increased in a dose-dependent manner (Fig. 7B).

**Cellular localization of LC3.** Immunofluorescence was performed to visualize the cellular localization of LC3 by confocal microscopy. The representative images showed that the LC3 puncta were diffuse and weak in control cells. By comparison, the number and fluorescence intensity of bright green LC3 dots were increased in cells exposed to nanosilica, suggesting autophagosome formation in nanosilica-treated cells (Fig. 8).
Evaluation of the autophagy process. The autophagosome scaffold p62 (also known as SQSTM1/sequestome 1) was further analyzed to evaluate the complete process of autophagy. As shown in Fig. 10A, the p62 protein expression was gradually enhanced. Densitometric analysis showed that nanosilica caused p62 elevation in a dose-dependent manner (Fig. 10D). The increase of p62 revealed dysfunction of autophagic degradation and autophagosome accumulation, indicating the blockage of the autophagy progress. These results showed valid evidence that nanosilica could induce autophagy, autophagosome accumulation and subsequent autophagy dysfunction in HepG2 cells.

Cellular apoptosis analysis. To further verify apoptosis induced by nanosilica, Annexin-V/PI double staining was carried out by Flow cytometry. Statistical data were extracted from the color dot plots (Fig. 9A and B) according to the cell percentage in different quadrants and are summarized in Fig. 9C and D. After the cells were incubated with different concentrations of nanosilica, both the late and total apoptotic rates were increased significantly compared with those in control cells (Fig. 9C). However, when the cells were pre-treated with 3-MA, z-vad-fmk or ROS scavenger NAC, the total apoptotic rate was significantly decreased (Fig. 9D). These results suggested that nanosilica caused apoptosis and autophagy or ROS might contribute to apoptosis in HepG2 cells.

The expression of proteins involved in autophagy. To further investigate the molecular mechanism of autophagy induced by nanosilica, autophagy-related proteins of Beclin-1 and mTOR were measured by Western blot. As shown in Fig. 10A, the expression of Beclin-1 was down-regulated by...
Fig. 8  The representative immunofluorescence images of cellular localization of LC3. The number and fluorescence intensity of bright green LC3 dots in cells exposed to nanosilica were obviously higher compared with diffuse and weak LC3 puncta in control cells, suggesting autophagosome induction by nanosilica. (Scale bar: 25 μm).

Fig. 9  Apoptosis of cells induced by nanosilica were shown by flow cytometry. (A) Effects of various concentrations of nanosilica on apoptosis in HepG2 cells. (B) Apoptosis of cells incubated with nanosilica followed pre-treatment with z-vad-fmk, 3-MA and NAC for 24 h. (C) The apoptotic rate was increased in a dose-dependent manner and (D) total apoptosis by 50 μg mL⁻¹ of nanosilica was significantly suppressed by z-vad-fmk, 3-MA and NAC when compared to corresponding nanosilica treatment alone. *p < 0.05 vs. control and #p < 0.05 vs. corresponding nanosilica treatment, Student’s t-test. Data are expressed as mean ± S.D. n = 3.
nanosilica exposure for 24 h. Total mTOR protein had no significant change, while the expression of phosphorylated mTOR was decreased after HepG2 cells were exposed to nanosilica. Further densitometric analysis showed that the relative ratio of both Beclin-1 (Fig. 10B) and p-mTOR/mTOR was decreased in a dose-dependent manner (Fig. 10C). These results suggested that nanosilica-induced autophagy was through negatively regulation of mTOR signaling but not the Beclin-1 associated pathway.

Discussion

While the potential adverse effects of nanosilica on human health is gaining widespread attention from environmentalists and toxicologists, there is still a lack of cell death-related studies in vivo and in vitro. The objective of this study was to investigate the effect of nanosilica on cell death by autophagy and apoptosis in vivo, and to illustrate the possible relationship between these two cell death modalities in HepG2 cells in vitro. The present study might provide a deeper insight into the toxicity mechanisms and toxicological consequences of nanosilica on human health and environmental safety.

Nanoparticles may enter organisms via various routes, including inhalation, ingestion, and absorption by skin. Once these nanoparticles enter the body, they can translocate to sites far away from their site of entry, causing toxic effects. The acute toxicity of intravenously administrated nanosilica in mice has been evaluated in our previous study. However, the mechanisms responsible for nanosilica toxicity remain unclear. In the present study, nanosilica induced liver injuries of lymphocytic infiltration and granuloma in mice (Fig. 2) and caused cytotoxicity in a dose-dependent manner in HepG2 cells in vitro (Fig. 5A). To investigate the origin of the hepatoxic effects, the biodistribution of nanosilica was performed by transmission electron microscopy (TEM) observation. Particle aggregates of nanosilica were found ingested in the cytoplasm of Kupffer cells (Fig. 1A). Translocation of nanoparticles first occurs across cell membranes and then transfers into certain organ cells and tissues. The lactate dehydrogenase leakage (LDH) release suggested the enhancement of cell membrane permeability (Fig. 5B), which facilitated the cellular entry of nanoparticles. Nanoparticles were allowed to enter cells through both phagocytosis and endocytosis. Receptor-mediated endocytosis is a two-step process: the first is binding to the cell membrane via cell surface receptors and the second is cellular internalizing. Scavenger receptors have been shown to mediate the uptake of silver nanoparticles by macrophages. The subcellular localization clarified that nanosilica particles deposit in mitochondria, endosomes and multi-membrane vesicles in HepG2 cells (Fig. 6C). Previously, we have reported that nanosilica are taken up by HepG2 cells via endocytosis. Recently, nanosilica uptake was further demonstrated through the cellular clathrin- and caveolin-dependent endocytosis. The endocytic nanoparticles tend to be stored in lysosomes for degradation and prolonged...

Fig. 10 The expression of Beclin-1, mTOR, p-mTOR and p62 was measured by Western blot assay and the relative densitometric analysis of the protein bands is further presented. *p < 0.05 vs. control. Data are expressed as mean ± S.D. \( n = 3 \).
accumulation might cause tissue lesions, cytotoxicity and cell death.

Apoptosis (type I programmed cell death) is the most comprehensively studied means of cell death resulting from extrinsic or intrinsic pathways.28 These pathways differ by the nature of their activation. Extrinsic apoptosis starts through cell surface death receptors, whereas intrinsic apoptosis originates from mitochondria and activates a cascade of proteolytic events.27 In this study, the TUNEL-positive cells in liver tissue indicate the appearance of apoptosis in vivo (Fig. 4); *in vitro* evaluation further confirmed nanosilica induced apoptosis in a dose-dependent manner (Fig. 9A and C). Extensive amounts of literature have shown that cytochrome C (Cyt C) release, Becl-2 inhibition, Bax, caspase-9 and caspase-3 activation are all involved in nanosilica-induced apoptosis in various human cell lines.30,48,49 Nanosilica was also reported to induce apoptosis in endothelial cells via JNK, p53 and NF-κB pathways.50 Previously, we have demonstrated that nanosilica caused apoptotic cell death respectively through a Chk1-dependent G2/M checkpoint in HUVECs24 and an intrinsic pathway in HepG2 cells accompanied with mitochondria damage, Cyt C release and caspase-3 activation.40 Herein, the inhibition of cell death (Fig. 5A) and apoptosis (Fig. 9B and D) by z-vad-fmk, a cell-permeable pan-caspase inhibitor, further confirmed that nanosilica-induced apoptosis was caspase-dependent. Although many efforts have been made to investigate the underlying mechanism of apoptosis induced by nanosilica, the complex molecular mechanism is still far from clear. The most obvious instance is that the autophagy inhibitor 3-MA exhibited similar suppression effects with that of apoptosis inhibitor z-vad-fmk on both cell death (Fig. 5A) and apoptosis (Fig. 9B and D), suggesting that autophagy might contribute to apoptosis in HepG2 cells after nanosilica exposure.

To clarify this unsolved issue, the occurrence of autophagy induced by nanosilica was evaluated both *in vivo* and *in vitro*. The positive staining of the autophagy marker LC3-II (Fig. 3) and autophagosome formation (Fig. 1B) indicated detectable autophagy in the murine liver after intravenous nanosilica treatment. In addition, all the autophagic ultrastructural features (Fig. 6C and D), LC3-II expression (Fig. 7) and cellular LC3-II localization (Fig. 8) further confirmed autophagy initiation in nanosilica exposed HepG2 cells. Autophagy is generally considered as a conserved self-degradation response to maintain cellular homeostasis, which is activated by various chemical, physical or biological stresses.51 Recently, autophagy was also considered as the effector and player in the DNA damage response of cells to genotoxicants,52 including nanoparticles like Quantum Dots (QDs), gold nanoparticles, poly(amidoamine) (PAMAM) dendrimers and single-walled carbon nanotubes (SWCNTs).53–55 Moreover, PAMAM dendrimers and SWCNTs caused autophagic cell death through Akt-TSC2-mTOR signaling53,55 and cationic polystyrene nanospheres induced autophagic cell death through endoplasmic reticulum (ER) stress.14 However, QDs and ferroferric oxide nanoparticles were reported to induce autophagy as a cytoprotective mechanism.15,16 To investigate the possible mechanism responsible for nanosilica-induced autophagy, the protein expression of Beclin-1, mammalian target of rapamycin (mTOR) and p62 (SQSTM1/sequestome 1) was measured. The Beclin1/VPS34 complex and mTOR are two major regulators of the signaling network underlying the autophagy mechanism.51 Beclin-1 accounts for autophagy initiation, while mTOR is a well-known gate-keeper in autophagy and exerts an inhibitory effect on autophagy.56 Detailed mechanisms of nanoparticle-induced autophagy were dependent on the species of nanoparticles. For instance, single-walled carbon nanotubes were reported to induce autophagy via the Akt/mTOR signaling pathway,55 while Fe₃O₄ nanoparticle autophagy was revealed through enhancing Beclin 1 and VPS34 expression instead of mTOR phosphorylation.57 Herein, the decreased expression of Beclin-1 (Fig. 10A and B) and mTOR (Fig. 10A and C) suggested that nanosilica induced autophagy is via negative regulation of mTOR signaling but not the Beclin-1 associated pathway. Autophagic flux could be blocked by lysosomal dysfunction or cytoskeleton disruption,58 while nanosilica has been demonstrated to cause lysosome damage and affect the distribution of the cellular cytoskeleton,32,59 suggesting the potential of nanosilica to block autophagic flux. Moreover, the p62 protein is a scaffold of autophagosomes and is selected as a targeted substrate for lysosomal degradation in the final process of autophagy.59 Generally, cellular expression levels of p62 are decreased along with autophagosome degradation. However, an increase in p62 (Fig. 10A and D) indicates accumulation of autophagosomes due to a degradation failure, suggesting autophagy dysfunction induced by nanosilica in HepG2 cells.

Although autophagy and apoptosis are independent and diverse cell death subroutines with different molecular mechanisms, there exists crosstalk between these two process modalities even though the details of molecular switching points are not still fully elucidated.61 Autophagy dysfunction that can lead to cell death and mechanisms have also been proposed recently to support the issue of autophagy activated apoptosis.62,63 Evidence has shown that LC3 and ATG5 may directly activate caspase-dependent apoptosis through interactions with Fas and Fas-associated protein with death domain (FADD).64,65 Moreover, truncated ATG5 has been reported to compete with the anti-apoptotic protein Bcl-XL, thereby inducing apoptosis.66 Reactive oxygen species (ROS) are widely accepted as triggers of both apoptosis and autophagy. In nanoparticle-exposed cells, ROS could result from nanoparticles themselves and mitochondria. Nanosilica was reported to induce stable surface radicals and sustained release of the hydroxyl radical (·OH), which is one of the most active ROS.14 Li et al. also demonstrated that nanosilica induced ·OH generation through an iron-dependent mechanism.67 Nanosilica has been demonstrated to cause both apoptosis and autophagy through reactive oxygen species (ROS) generation, which was verified herein and in our previous study,33,39 while limited studies were focused on the interaction between these two cell death pathways induced by nanosilica. Consistent with the inhibition effects of z-vad-fmk, autophagy inhibitor 3-MA suppressed cell death and apoptosis (Fig. 9B and D),...
suggesting that autophagy is involved in apoptosis after nanosilica exposure. Recently, cerium dioxide nanoparticles were reported to induce both autophagy and apoptosis, while magnetic iron oxide nanoparticles caused autophagy prior to apoptosis and autophagy enhancement further induced apoptosis by zinc oxide nanoparticles. However, it remains unclear how autophagy promotes cells to undergo apoptosis in cells exposed to nanosilica and even other nanoparticles. Previously we have demonstrated that nanosilica induced apoptosis through mitochondria damage via Cyt C and caspase-3 in HepG2 cells. By comparison, protein expression changes in autophagy marker LC3 was much more significant than that of apoptosis executor caspase-3, suggesting autophagy was more susceptible and occurred before apoptosis in HepG2 cells exposed to nanosilica. One possible mechanism underlying autophagy-mediated apoptosis might involve the degradation failure and thus accumulation of autophagosomes which contain damaged mitochondria, ultimately enhancing persistent Cyt C release and activating apoptotic executor caspase-3. Moreover, the p62 protein was already confirmed to regulate apoptosis and function as an important activator of caspase-8-dependent apoptosis. The up-regulation of p62 could accelerate caspase-8 activation and initiate apoptosis, showing a dependence of apoptosis on autophagy. As a result of autophagy dysfunction, the accumulated p62 (Fig. 10A and D) might account for the apoptosis induced by nanosilica in HepG2 cells in the present study. Furthermore, mTOR is not only a potent inhibitor of autophagy, but also plays crucial roles in cell proliferation, growth and survival to resist apoptosis. Reports showed that mTOR inhibitor Rapamycin can induce apoptosis and mTOR inhibition results in enhanced apoptosis. Accordingly, as a negative regulator of both autophagy and apoptosis, the suppressed p-mTOR (Fig. 10A and C) might accelerate the mechanistic response as well. The putative mechanistic molecular pathway underlying autophagy-mediated apoptosis induced by nanosilica in HepG2 cells is presented in a schematic diagram (Fig. 11).

This extensional study provides a potential relationship between autophagy and apoptosis in nanosilica-induced cell death. As an emerging new mechanism of nanosilica-induced cytotoxicity, autophagy might be a more susceptible indicator for toxicological consequence evaluation in nanoparticle toxicity.

Conclusions

In summary, we demonstrated that nanosilica induced autophagy and apoptosis both in mice liver and in HepG2 cells. An In vitro study further clarified that autophagy induction was mediated by negative regulation of mTOR cellular signaling but not the Beclin-1 related pathway. The blockage of autophagy degradation in the autophagy process led to autophagosome accumulation and autophagy dysfunction. The nanosilica activated autophagy preceding apoptosis and autophagy dysfunction might further result in apoptosis in HepG2 cells via cytochrome C (Cyt C) release, p62 accumulation and mTOR down-regulation. It is speculated that autophagy was involved in apoptosis and might be a more susceptible toxicological consequence which accounts for cell death induced by nanosilica. Our findings may enrich an in-depth insight about cell death and the toxic consequences induced by nanosilica, promoting a risk assessment of nanosilica on human health and environmental exposure.

Author contributions

Yongbo Yu and Zhiwei Sun designed all of the experiments and wrote the manuscript. Yongbo Yu and JunChao Duan contributed to all the experiments and characterization. YangLi, YangZou and QiuLingLi performed the in vitro study. YangYu, YangLi and LiZhen Jiang conducted the in vivo experiment. YongBo Yu and YuMei Yang analyzed the data. All authors have read and approved the final manuscript.

Conflict of interest

The authors declare they have no actual or potential competing financial interests.

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