

# The Toxicity Of Metallic Nanoparticles On Liver: The Subcellular Damages, Mechanisms, And Outcomes

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**Abstract:** Metallic nanoparticles (MNPs) are new engineering materials with broad prospects for biomedical applications; thus, their biosafety has drawn great concern. The liver is the main detoxification organ of vertebrates. However, many issues concerning the interactions between MNPs and biological systems (cells and tissues) are unclear, particularly the toxic effects of MNPs on hepatocytes and other liver cells. Numerous researchers have shown that some MNPs can induce decreased cell survival rate, production of reactive oxygen species (ROS), mitochondrial damage, DNA strand breaks, and even autophagy, pyroptosis, apoptosis, or other forms of cell death. Our review focuses on the recent researches on the liver toxicity of MNPs and its mechanisms at cellular and subcellular levels to provide a scientific basis for the subsequent hepatotoxicity studies of MNPs.

**Keywords:** metallic nanoparticles, hepatotoxicity, subcellular injury, dysfunctions, toxicity outcome

## Introduction

With the rapid development of nanotechnology, nanomaterials (NPs) are considered to have enormous application potential due to their unique properties over the past few decades.<sup>1</sup> Of all the NPs, metallic nanoparticles (MNPs) have generated considerable commercial interest owing to unique properties of NPs such as small size and the greater surface area to volume ratio as well as different electronic, magnetic, optical, and mechanical properties and also particle shape. MNPs mainly include metal nanoparticles and metal oxide nanoparticles, and MNPs have been widely included in a great diversity of products and the various fields, such as electronic devices, cosmetics, paints, additives in food, and biological and medical systems.<sup>2,3</sup> With the widespread application of MNPs, it is inevitable that MNPs will be released into the environment or contact with humans directly. Therefore, their potential risks to human health and the environment have gained even more attention.<sup>4</sup> MNPs can enter the body in various ways, for example, through the inhalation, gastrointestinal tract, or skin, and circulate via the blood or lymphatic system, eventually accumulating in various organs.<sup>5</sup> Previous studies for metal nanoparticles and metal oxides nanoparticles, including nano-Cu, nano-Ag, nano-Ni, nano-TiO<sub>2</sub>, and nano-ZnO, have shown that MNPs reached the lung and gastrointestinal tract through the respiratory and digestive tract, and further translocated to the systemic circulation, and then accumulate the potential target organs such as the liver and the mononuclear

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phagocytic system.<sup>6</sup> As a secondary exposure site, the liver is extremely important, as it has been shown to accumulate MNPs at much higher quantities compared with other organs.<sup>7</sup> Meanwhile, MNPs accumulate the liver typically results in interaction with hepatic cells and the possibility of changing the structure and function of hepatic cells. The liver is a complex network of inter-related cells, including about 60–80% of the hepatocytes, and the additional cells include Kupffer cells, liver sinusoidal endothelial cells, hepatic stellate cells, and so on. The interactions of MNPs with liver cells determine the fate of administered MNPs in vivo and the results of hepatotoxicity. In vivo studies are mostly focused on the accumulation of the MNPs at the organ level, while most in vitro studies are focused on hepatic cells and do not summarize changes in subcellular levels and their relationship with hepatotoxicity. It is important, therefore, to summarize hepatotoxicity studies of MNPs on animals, the cell level and subcellular level and its molecular mechanism and outcomes. Furthermore, the physicochemical characteristics of MNPs, such as size, surface properties, and chemical nature would change and influence their potential toxicity. From these facts, the aim of this review is to compile and discuss the hepatotoxicity effects of MNPs both in vitro and in vivo, particularly those involved in subcellular levels as well as to highlight its molecular mechanism of action of these MNPs.

## The Liver And Metallic Nanoparticles Toxicity

The liver is the primary organ for detoxification in human body. It possesses abilities of deoxidation, glycogen storage, and secreted protein synthesis. It acts as biological barriers by isolating and eliminating various exogenous compounds through phagocytosis. Previous in vivo studies have shown that different types of MNPs: nano-metal monomers and nano-metal oxides, tend to deposit in the liver with extensive toxic effects.<sup>8–10</sup> As shown in Table 1, MNPs entering the body cause changes in inflammatory cytokines. NiO NP increased the concentrations of pro-inflammatory cytokines (IL-1 $\beta$  and IL-6) but decreased the levels of anti-inflammatory cytokines (IL-4 and IL-10).<sup>11</sup> Liver dysfunction caused by MNPs leads to structural changes of liver. MNPs caused inflammation, which may lead to changes in liver coefficients.<sup>12–14</sup> By analyzing blood serum, significant decrease of total bilirubin and increase of alkaline phosphatase (ALP) with aspartate aminotransferase (AST) indicated liver injury.<sup>11,15,16</sup> The

damage mainly manifested in liver structural changes causing metabolic dysfunction. AgNP caused the increase of relative spleen weight and affected diffuse and severe hepatocyte necrosis and hemorrhage, as well as multifocal peribiliary microhemorrhages, occasional portal vein endothelial damage, which in turn affects the liver.<sup>17</sup> TiO<sub>2</sub> NP induced alterations in the liver structure including hepatic inflammatory cell infiltration, increased density of liver tissue collagen, initiation of fibrosis and Glisson capsule thickness increase.<sup>18</sup> AuNP was found to activate hepatic macrophages and then significantly aggravated the course of experimental immune hepatitis and liver injury.<sup>19</sup>

## The Hepatocytes And Metallic Nanoparticles Toxicity

Hepatocytes constitute the basic functional unit of the liver, the hepatic lobule, which contains 60% of the solid cells (hepatocytes) and 30% to 35% of the non-solid cells (hepatic stellate cells, Kupffer cells, and sinusoidal endothelial cells). NPs deposited in liver tissue may affect the normal physiological and biochemical functions of liver by affecting liver parenchymal cells and other cells along with important physiological functions in liver tissue. When studying the hepatotoxicity of MNPs, the effects on hepatocytes from different sources, including primary cells and cell lines, should be considered at the same time, so as to obtain more comprehensive information. It has been widely carried out in vitro toxicity research of MNPs, including the use of cell lines from different species and origins, as well as studies at the cellular, subcellular and molecular levels. MNPs cause liver cell toxicity mechanism including triggering inflammation, oxidative stress, and possibly eventually leading to different types of cell death outcomes. As shown in Table 2, due to the role of MNPs, the decrease in survival rate of hepatocytes is common, accompanied by a time- and dose-dependent relationship. The sensitivity of hepatocytes from different sources to MNPs was different. Compared with normal cell lines, MNPs seem to have more obvious toxic effects on cancer cells. In the study of Mei-Lang et al,<sup>22</sup> the IC<sub>50</sub> for SK-Hep-1 and HepG2 cells were 25 and 85  $\mu\text{g/mL}$ , respectively. Ali et al<sup>23</sup> also found that HepG2 cells were more sensitive to rGO-Ag than human CHANG liver cells. The activity of lipid peroxide, superoxide dismutase, and catalase increased and glutathione decreased. Previous studies have shown

Table 1 In Vivo Studies On The Liver Toxicity Of Metallic Nanoparticles (MNPs)

Types	NP	Size (nm)	Subjects	Routes	Dose (mg·kg <sup>-1</sup> ·BW)	Time	Results	References
Monomeric MNPs	Cu	80	Male SD rats	Oral	100; 200;400	7 days	Cu NPs induced liver injury by increased levels of IL-2, IL-6, IFN- $\gamma$ , MIP-1, T-AOC, MDA through inflammation and oxidative stress; Cu NPs affected CYP450 activity and suppressed some nuclear receptors through the NF- $\kappa$ B signaling pathway.	12
	Ni	50	SD rats	Dorsal penile vein injection	1; 10; 20	Once at day 1 and once at day 14	Ni NPs increased the liver coefficient in a dose-dependent manner. Both 1 mg/kg and 20 mg/kg doses of Ni NPs significantly increased serum albumin levels. Serum ALT levels decreased significantly in the 1 mg/kg group as well as total bilirubin levels with 10 mg/kg. Both total and direct bilirubin levels were reduced due to exposure to 20 mg/kg.	16
	Ag	10	Male CD-1 (ICR) mice	Intravenous injection	10	24 hrs	Ag NPs damaged the liver through extensive hepatocyte necrosis. It caused multiple bleeding around the biliary tract, including gallbladder wall and wall hemorrhage, accompanied by portal vein endothelial injury.	17
	PVP-Ag	30	Male C57BL/6 mice (NAFLD)	Oral	100;300	2 weeks	PVP-Ag NPs damaged the liver by inflammation and inhibiting fatty acid oxidation, and promoted the transformation of NAFLD to steatohepatitis.	13
	PVP-Ag	20-30	Male SD rats	Oral	50; 100;200	90 days	PVP-Ag NPs increased ROS production in a dose-dependent manner as a protective mechanism for cell survival and DNA fidelity. The activities of SOD and CAT were stimulated, as well as IRS-1, PKB, mTOR, p53, p21, and caspase-3. However, excessive ROS production might lead to the exhaustion of survival mechanism in rat liver, thereby enhancing autophagy, especially insulin resistance.	73
	Au	-	C57BL/6 mice	Vein injection	12;120; 1200	8 weeks	Preactivation of hepatic macrophages induced by gold nanorods (NR) significantly aggravated liver injury and disease activity in mice with experimental immune-mediated hepatitis.	19
	Ti + Ag	Ti2I Ag<100	Male Wistar rats	Gavage	100	21 days	Ti NPs + Ag NPs reduced mitochondrial respiratory control ratio and had uncouple effects on the oxidative phosphorylation system. Ag NPs and Ti NPs have synergistic effects. When both NPs were exposed at the same time, the interference effect on mitochondrial function was more significant.	72

(Continued)

Table 1 (Continued).

Types	NP	Size (nm)	Subjects	Routes	Dose (mg·kg <sup>-1</sup> ·BW)	Time	Results	References
Metal oxide NPs	TiO <sub>2</sub>	21	Male albino mice	Oral	150	2 weeks	TiO <sub>2</sub> NPs caused oxidative stress, inflammation, DNA damage, and potential apoptotic mechanisms.	14
	TiO <sub>2</sub>	12–18 (diameter) × 40–80 (length)	SD rats	Intraperitoneal injection	0.5; 5; 50	24 hrs	TiO <sub>2</sub> NPs induced cell infiltration and hepatocyte necrosis in a dose-dependent manner. The activity of AST, ALT, LDH, and ALP was significantly higher than that of normal rats.	15
	TiO <sub>2</sub>	21	Male rats	Intratracheal instillation	0.5; 5; 50; 150	4 days, a month, 3 months	After 4 days of intratracheal TiO <sub>2</sub> NPs infusion, some pathological changes of liver tissue were observed, including necrosis of hepatocytes, increase of fibrosis, proliferation of histiocytes, and vasodilation. Injury effects were more obvious after 1 month and 3 months of infusion.	18
	TiO <sub>2</sub>	21	C57/BL6 mice	Oral	250; 500	14 days	Central venous congestion and hepatic sinus dilatation were observed in the liver tissue of rats poisoned by titanium dioxide NPs. Focal hemorrhage and coagulative necrosis of hepatocytes occur in the liver parenchyma. The proliferation of Kupffer cells was also observed.	36
	TiO <sub>2</sub>	5; 10; 60; 90	ICR mice	Intraperitoneal injection	5; 10; 50; 100; 150; 200	60 days	Mitochondria of hepatocytes were slightly swollen after 60 days of exposure by TiO <sub>2</sub> . Concentrated chromatin and apoptotic cells (5 mg/kg), swollen mitochondria and vacuoles (10 mg/kg), collapsed nucleolus, dispersed chromatin and obvious apoptotic cells (50 mg/kg) appeared in the liver of mice.	75
	ZnO	40	CD-1CR male mice	Oral	250	7 weeks	ZnO NPs reduced the body weight, promoted the activity of serum glutamic-pyruvic transaminase, and increased neutrophil count and HGB in blood.	20
	NiO	20	Male Wistar rats	Intratracheal instillation	0.015; 0.06; 0.24	Twice a week for 6 weeks	The wet weight of liver and liver coefficient increased in NiO exposed group. The pathological changes of liver were cellular edema, disappearance of hepatic sinuses, and binuclear hepatocytes. Total nitric oxide synthase (NOS) and inducible NOS activity increased, as well as NO content. The expression level of MT-1 was down-regulated while that of HO-1 was up-regulated.	11
	NiO	20	Male Wistar rats	Intratracheal instillation	0.015; 0.06; 0.24	Twice a week for 6 weeks	NiO NPs increased the activity of liver-related enzymes, including ALT, GGT, AST, and ALP. The liver showed cellular edema, sinusoidal disappearance, infiltration by neutrophils and lymphocytes. NiO NPs increased the concentration of pro-inflammatory cytokines IL-1 $\beta$ and IL-6 and inhibited IL-4 and IL-10.	21

**Abbreviations:** IL-2, interleukin-2; IL-6, interleukin-6; IFN- $\gamma$ , interferon- $\gamma$ ; MIP-1, macrophage inflammatory protein-1; TAOC, total-anti-oxidizing-capability; MDA, malonaldehyde; CYP450, P-450 cytochrome; NF- $\kappa$ B, nuclear factor kappa-B; DNA, deoxyribonucleic acid; SOD, superoxide Dismutase; CAT, catalase; IRS-1, insulin receptor substrate-1; PKB, protein kinase B; mTOR, mammalian target of rapamycin; p53, protein 53; p21, cyclin-dependent kinase inhibitor 1A; NAFLD, nonalcoholic fatty liver disease; ALT, alanine aminotransferase; LDH, lactate dehydrogenase; ALP, alkaline phosphatase; HGB, hemoglobin; MT-1, metallothionein-1; HO-1, heme oxygenase-1; GGT,  $\gamma$ -glutamyl transpeptidase; IL-1 $\beta$ , interleukin-1 $\beta$ ; IL-4, interleukin-4; IL-10, interleukin-10.

**Table 2** In Vitro Studies On The Liver Toxicity Of Metallic Nanoparticles (MNPs)

Types	NPs	Size (nm)	Surface Modification	Cell	Dose	Time	Results	References
Monomeric MNPs	Au	10; 30; 60	-	HepG2	10ppb/10ppm	16, 32 hrs	The excessive production of free radicals and ROS under the action of Au NPs lead to carbonylation of cellular proteins, lipid peroxidation, and DNA damage. The smaller the size of nanoparticles, the greater the effect	50
	Au	40±5	SiO <sub>2</sub> & folate group	HepG2	2.5; 5; 10; 20; 40ppm	48 hrs	GNRS@SiO <sub>2</sub> -FA enters cells through endocytosis, connects with tumor cells expressing high folic acid, and accumulates in cytoplasm. The cell viability decreased with the increase of nanomaterial concentration.	51
	Ag	10, 30-50	PVP	HSCs	20; 100; 250 µg/mL	96 hrs	Reduction of cell survival rate exerted by AgNPs on HSCs were size- and dose-dependent, which was associated with mitochondrial damage and apoptosis. MMP-2 and MMP-9 were inhibited by AgNPs.	32
	Ag	16.3±1.8 (68.5%), 58.1±2.6 (31.5%)	Citrate	HepG2	0-100 µg/mL	24 hrs	The IC50 value of citrate-coated AgNPs was 50 mg/L. Treatment with AgNPs resulted in cell membrane damage, decreased cell function and disordered antioxidant status. AgNPs exposure affected the respiratory chain of HepG2 cells. ROS production, GSH consumption, and SOD activity increased slightly but in a dose-independent manner.	24
	Ag	6.3±0.1 (93.3%), 28.58±0.4 (6.7%)	PVA					
	Ag	20	-	HepG2	50 µg/mL	2; 24 hrs	NPs lead to the ROS generation and oxidative stress due to mitochondrial damage and malfunction of respiratory chain.	25
	Ag	10; 50; 100	PVP	HepG2	≤10 µg/mL	6; 12; 24hrs	The IC50 values of 10-, 50- and 100-nm AgNPs at 24 hrs post exposure were 5.1, 7.6, and 6.4 µg/mL, respectively. AgNP-induced hepatotoxicity is mediated by AgNP-induced LMP and inflammation-dependent caspase-1 activation. AgNPs induce autophagy and lysosomal membrane permeation, leading to inflammation-dependent caspase-1 activation of NLRP3.	26
	Ag	20	-	C3A	1-4 µg/cm <sup>2</sup>	24 hrs	LC50 lactate dehydrogenase: 2.5 µg/cm <sup>2</sup> . AgNPs affect the homeostasis of hepatocytes by reducing albumin release. At sublethal concentration, AgNPs were distributed in the cytoplasm and nucleus of hepatocytes. AgNPs induced changes in inflammatory mediators, accompanied by increased expression of IL-8/macrophage inflammatory protein 2, IL-1β and tumor necrosis factor-α, and increased release of IL-8 protein.	80
	Ag	2	-	HepG2	0-20 µg/mL	72 hrs	AgNPs inhibited the proliferation of HepG2 cells through induction of apoptosis with caspase-3 activation and PARP cleavage. AgNPs with dose-dependent manner significantly increased the apoptotic cell population (sub-G1). AgNP-induced apoptosis was found dependent on ROS and affecting of MAPKs and AKT signaling and DNA damage-mediated p53 phosphorylation to advance HepG2 cells apoptosis.	82
	Ag	28-35	-	CHANG	0-10 µg/mL	24 hrs	The IC50 value was 4 µg/mL. AgNPs induced ROS generation and suppression of reduced GSH in human Chang liver cells. ROS generated by AgNPs resulted in DNA breaks, lipid membrane peroxidation, and protein carbonylation. Cell viability decreased due to apoptosis. AgNPs induced a mitochondria-dependent apoptotic pathway via modulation of Bax and Bcl-2 expressions, resulting in the disruption of mitochondrial membrane potential (Δψ/m).	27

(Continued)

Table 2 (Continued).

Types	NPs	Size (nm)	Surface Modification	Cell	Dose	Time	Results	References
	Ag	5-10	Cs/grape leaves aqueous extract (Cs/GLE)	HepG2	0.39-50%	-	Cs-Ag NPs induced mitochondrial intrinsic apoptotic pathway by upregulation of the expression of p53 and downregulation of the expression on Bcl-2 gene.	95
	Ag	16±2	Reduced graphene oxide	CHANG;HepG2	5-50 µg/mL	24 hrs	The rGO-Ag nanocomposite reduced cell viability and impaired cell membrane integrity of CHANG and HepG2 cells in a dose-dependent manner. It increased ROS and reduced mitochondrial membrane potential in both cells in a dose-dependent manner. The activity of lipid peroxide, superoxide dismutase, and catalase was increased and glutathione was reduced. The maximum DNA damage occurred at rGO-Ag nanocomposite (25 µg/mL) for 24 hrs. HepG2 cells are more sensitive to the effects of NPs.	23
	Fe	2-5	Tannic complexes	HepG2	0-30 µM	24 hrs	Fe-TA NPs can be taken up by HepG2.2.15 cells in concentration and in a time-dependent manner. A high uptake of Fe-TA NPs resulted in autophagic cell death.	103
	Cu	100±35	-	Primary hepatocytes of E. colioides	0; 2.4 mg Cu/L	24 hrs	Cu NPs impaired structure of membrane and anti-oxidant defense system by increased ROS and lipid peroxidation. Increased ROS may impair mitochondrial bioenergetics and physiological functions. The release of mitochondrial cytochrome c into the cytosol activated caspases, triggering apoptosis. Oxidative stress activated apoptosis-related genes (p53, p38), and TNF-α.	28

(Continued)

Table 2 (Continued).

Types	NPs	Size (nm)	Surface Modification	Cell	Dose	Time	Results	References
Metal oxide NPs	NiO	24.05±2.9	-	HepG2	25; 50; 200µg/mL	24 hrs	NiO NPs induced oxidative stress, DNA damage, apoptosis, and transcriptome alterations.	31
	CuO	50-70 (in length)	-	HepG2;SK-HEP-I	0; 10; 25; 50; 75; 100 µg/mL	24 hrs	The IC50 for SK-Hep-I and HepG2 cells was 25 µg/mL and 85 µg/mL, respectively. CuO NPs accumulated in cells, causing oxidative stress. SK-Hep-I has a lower degree of differentiation and is more sensitive to toxicity. CuO-NP causes severe DNA strand breakage (70%) in SK-HEP-I cells and causes DNA damage by increasing the level of gamma-h2ax.	22
	ZnO	71	-	Catfish primary hepatocytes, HepG2	0-200 mg/L	48 hrs	The IC50 values of nano-CuO and ZnO were 181.8 and 275.6 mg/L, respectively. Co3O4 had a stimulatory effect at 25 and 50 mg/L and inhibitory effect at 100 and 200 mg/L, respectively. This toxicity is caused not only by cell death caused by reactive oxygen species but also by damage to cell and mitochondrial membrane.	29
	TiO2	42.3	-					
	CuO	28	-					
	Co3O4	78.3	-					
	TiO2 Degussa P25a 3:1 mixture of anatase and rutile	21	-	Primary rat hepatocytes	0-1000 ppm	72 hrs	The LC50 values of P25, anatase and rutile TiO2 nanoparticles were 74.13 ±9.72 ppm, 58.35±4.76 ppm, and 106.81±11.24ppm, respectively. Prolonged exposure of hepatocytes to TiO2 NPs decreased two main specific functions of hepatocyte- urea synthesis and albumin synthesis. The exposure of hepatocytes to 50 ppm of P25 and anatase resulted in relatively highest ROS production while exposure to the same concentration of rutile demonstrated lesser ROS production.	30
	TiO2 Pure rutile	50	-					
	TiO2 Pure anatase	50	-					
	SPION	10	-	Primary rat hepatocytes	0-400 µg/mL	48 hrs	The LD50 values were calculated as 328.51 ± 18.25 and 319.79 ± 25.73 for one-shot and cumulative treatment styles, respectively. The time-dependent ROS production data from the current study illustrates that ROS can be used as an early and potent diagnostic marker for SPION-induced toxicity as well as other nanotoxicological inquiries in the liver.	76
Fe3O4-TiO2	20-25	-	HL-7702	Ti: 0; 6.25; 15.625 25 µg/cm <sup>2</sup>	12 hrs	Fe3O4-TiO2 NPs induced cellular ROS generation, reduced cell viability, and induced apoptosis in a dose-dependent manner in HL7702 cells. Mitochondrial membrane was damaged and cytochrome c was released, followed by regulation of apoptosis-related proteins.	96	

**Abbreviations:** HSCs, hepatic stellate cells; MMP, matrix metalloprotein; IC50, half-maximal inhibitory concentration; PVA, Polyvinyl alcohol; GSH, glutathione; NLRP3/NLRs, pyrin domain containing 3; LC50, median lethal concentration; MAPK, mitogen-activated protein kinase; AKT, protein kinase B; CHANG, human Chang liver cells; LD50, median lethal dose; SPION, superparamagnetic iron oxide nanoparticles.



that MNPs can destroy the function of mitochondria and cell respiratory transmission.<sup>24,25</sup> MNPs induced decrease of ATP levels, activated the signaling pathway of inflammation, apoptosis, and autophagy.<sup>26–28</sup> The changes in oxidative stress and inflammatory factors suggest the mechanism of cell fate induced by MNPs.<sup>24,29,30</sup> In addition, MNPs can also damage DNA, which may explain the cause and mechanism of liver damage caused by MNPs at the organelle and molecular level.<sup>31</sup>

## Effects Of MNPs On Organelles

A small organ-like structure present inside the cell is called a cell organelle, which is the basic structural, functional, and biological unit of all known living organisms. The integrity of organelle determines the fate of the cell. Liver cell membrane is sensitive to free radical and lipid peroxidation injury. Liver cell membrane injury was characterized by decreased fluidity and increased permeability. Wang et al<sup>29</sup> found that ZnO NPs were distributed in the nucleus or concentrated on the surface of primary hepatocytes membrane microvilli and other organelles in catfish. Under light micrographs, numerous MNPs caused changes in cell membrane permeability, as well as distinct damages under TEM. According to Vrček et al,<sup>24</sup> treatment of Ag NPs and Ag ion on human hepatocytes both led to cell membrane damage, which was manifested as LDH leakage and decreased albumin synthesis with ALT activity inhibited. Changes in liver membrane fluidity can damage the enzyme activity, receptor and transportation function, and inhibit the function of liver cells. The internal structure of the membrane may be disturbed by MNPs, as they can cause changes in membrane permeability by causing the plasma membrane to partially dissolve and form pore structures. The exposure of MoS<sub>2</sub> NPs made reduction of the phospholipid bilayer domain of the liver cancer cells and an increase in membrane fluidity.<sup>33</sup>

The nucleus controls the cellular genetic material and plays an important role in cell growth, metabolism, proliferation, and differentiation. MNPs reached the nucleus and affected genetic materials, thus destroying nuclear morphology, damaging DNA, and affecting gene expression concretely.<sup>34</sup> The mouse hepatocyte exposed to ZnO NPs exhibited karyopyknosis, nuclear membrane irregularity with indentation, and chromatin fragmentation. Shrunken micronuclei of hepatocytes with reticular-pattern chromatin condensation and apoptotic activity were further observed.<sup>35</sup> TiO<sub>2</sub> NPs orally administered into C57/BL6 mice caused liver metabolic genes (Oatp1, Mrp3, Cyp2b10, Cyp2c37) to increase under

high dose treatment.<sup>36</sup> TiO<sub>2</sub> NPs can also regulate the expression of mRNA p53 and the downstream genes regulating DNA damage response (p21 mdm2, gadd45) temporarily. Also, exposure of HepG2 cells to TiO<sub>2</sub> NPs resulted in DNA strand breakage and sustained growth of purine oxide.<sup>37</sup> Overall, MNPs could cause intracellular DNA damage, which induced different cell outcomes, for example, activating caspase-3 and caspase-7 mediated apoptosis,<sup>25,38,39</sup> regulating relevant genes (Bax, Puma, Noxa),<sup>39</sup> and promoting caspase-1 induced pyroptosis.<sup>38</sup>

Mitochondrial dysfunction enticed by MNPs included morphological changes, increased production of ROS, changes in calcium content, descending mitochondrial membrane potential, inhibition of various enzyme activities, inhibition of electron transport chains, inhibition of cellular respiration, decline of ATP synthesis, etc., which could further lead to insufficient energy supply and affect cell viability such as apoptosis and necrosis.<sup>40,41</sup> ZnO NPs caused a series of morphological changes in mouse hepatocyte mitochondria, such as enlargement, elongation, angulations, swelling, cristolysis, lacked cristae, and ruptured membranes.<sup>35</sup> Apart from morphological changes, three types of TiO<sub>2</sub> NPs (commercially available rutile, anatase, P25) induced oxidative stress in primary rat hepatocyte, downregulated mitochondrial dynamin OPA-1 and mitochondrial fusion protein MFN-1 gene expression, significant loss of mitochondrial membrane potential (MMP), and decreased activity of mitochondrial Mn-SOD enzyme.<sup>30</sup>

Endoplasmic reticulum (ER) changes caused by MNPs include endoplasmic reticulum swelling, endoplasmic reticulum stress, misfolding of proteins, and increasing or decreasing protein synthesis.<sup>42,43</sup> In liver, ER plays an important role in the synthesis of protein and steroid hormones, as well as promoting lipid metabolism and calcium storage. ER damage is related to the loss of protein synthesis initiation and liver detoxification function. The ER of mouse hepatocytes treated with ZnO NPs demonstrated ER pleomorphism in the form of dilatation, loss of parallel arrays, stacks shortening, vesiculation, upregulated transcription of genes encoding ER-resident molecular chaperones such as Grp78, Grp94, pdi-3 and xbp-1, and accelerated the process of protein kinase R-like reticulum kinase (PERK) and eukaryotic initiation factor 2 $\alpha$  (eIF2 $\alpha$ ) phosphorylation. ER stress is considered to be one of the early sensitive indicators of cytotoxicity caused by MNPs.<sup>35,44</sup> Chen et al showed that the level of xbp-1s and Chop mRNA elevated with mice exposed to Ag NPs.



In addition, the upregulation of ER stress marker proteins (hsp70, bip, p-ire1, p-perk, and chop) was dose-dependent for Ag NPs exposure.<sup>34</sup> Ultra-small superparamagnetic iron oxide nanoparticles (USPIO-NP) act on L02 cells, causing the expansion and vacuolation of ER, and increasing the level of calcium ions in ER cavity. ER stress and unfolded protein response to PERK/ATF4 signaling pathway were finally activated.<sup>45</sup>

MNPs enter lysosomes mainly through passive diffusion or endocytosis, causing changes in lysosomal structure.<sup>46</sup> The destruction of lysosomal cristae in Kupffer cells was obviously observed in rats injected intraperitoneally with an interval of 48 hrs.<sup>47</sup> ZnO NPs entered the lysosome mainly through endocytosis, leading to damage to lysosomal morphology during the interaction with the acidic environment, releasing a large amount of Zn<sup>2+</sup> to the cytoplasm. And Zn<sup>2+</sup> captured partially by mitochondria triggered the generation of ROS, causing mitochondrial dysfunction and apoptosis of cells.<sup>46</sup> MNPs released ions under the lysosomal acidic environment, and then the lysosomal membranes were ruptured by MNPs or ions and the contents entering the cell lead to damage.<sup>38,48</sup> Low-dose Ag-NPs (10 µg/mL) activated autophagic lysosome pathway in HepG2 cells and found increased level of lysosome activity, LC3-II protein expression, caspase-1, and IL-1 beta levels. Using ATG5 siRNA or chloroquine to destroy the autophagic pathway, Ag NPs induced increased caspase-1 activation and LDH release, suggesting that Ag NPs induced-cytotoxicity is associated with lysosomes damage and inflammatory bodies.<sup>26</sup>

## Properties Of Metal Nanomaterials Affect Cell Absorption And Distribution Of MNPs

Toxicity of MNPs is largely dependent on cellular uptake and subcellular distribution. The size and surface properties of MNPs and the types of liver cells play a critical role in determining the outcome of interaction with the cells and other biological entities.<sup>49</sup>

### Size

Size is a key factor determining the subcellular distribution. Numerous researches have indicated that MNPs, which mainly distributed in lysosome, cytoplasm, and nucleus, enter cells through endocytosis.<sup>50-52</sup> When comparing three different sizes (8.9 nm, 27.6 nm, 56 nm) of gold NPs exposed HepG2 cells, the size of MNPs between 3 and 10 nm entered the nucleus, while the particles of 25 to 60 nm

accumulated in the cytoplasm, which indicated the size is a key factor to determine the subcellular distribution.<sup>50</sup> Another study showed that the shape of Au NPs affected the ratio of endocytosis. The highest cell uptake was triangular, followed by rod-shaped and star-shaped.<sup>53</sup> It is worth noting that the phenotype, internalization, and dissociation kinetics of each type of cells in liver have impacts on the quantity and absorption rate to hepatocytes,<sup>38,54,55</sup> which will ultimately determine the liver toxicity caused by MNPs. Previous studies have shown that the liver preferentially cleans larger nanomaterials.<sup>56,57</sup> Because of their higher surface ligand density, they were more likely to be absorbed by primary rat Kupffer cells as well as immortalized mouse macrophages.<sup>55</sup>

## Surface Modification

The interaction between nanomaterials and cells begins with the recognition of surface ligands and biofilm receptors. Current research is devoted to the surface modification of innovative materials to improve the specificity of cell recognition. Sykes et al<sup>56</sup> studied the binding of nanoparticles to MDA-MB-435 cancer cells. It was found that within 60 nanometers, transferrin-modified ANP could be absorbed by cancer cells more quickly, while PEG-coated materials could penetrate into cancer cells more deeply, but the absorption rate was slow. Surface modification reduces the toxicity of some metal nanomaterials. Gao et al<sup>8,51,58</sup> synthesized a spherical silicon-coated gold nanomaterial (GNRS@SiO<sub>2</sub>), which was conjugated with amino terminus by folic acid as receptor, and finally produced GNRS@SiO<sub>2</sub>-FA. In the concentration range of 0–40 ppm, the composite has almost no toxic effect. Compared with unmodified GNRS@SiO<sub>2</sub>, the material can enter HepG2 cells quickly and distribute in cytoplasm and nucleus, while the internalization of unmodified nanomaterials is not obvious. Surface modification with enhanced biocompatibility can be used as an ideal material for targeted cancer therapy.<sup>59</sup> Magnetic nanoparticle-aptamer probe demonstrates efficient *in vitro* MR imaging of the cancer cells and enhanced delivery of an anticancer drug into the cancer cell.<sup>60</sup> Christopher et al<sup>61</sup> modified NPs with bilayer nano-chitosan mercaptan and phosphatidylcholine and found that HepG2 cells consumed more new materials than gold polyvinyl glycol nanoparticles. Further studies showed that the structure of phosphatidylcholine-modified nanoparticles was similar to that of liposomes in hepatocytes, which enhanced the transport of gold nanoparticles. *In vivo*, the biodegradation and removal rate of PEG nanoparticles in liver and spleen is faster because PEG nanoparticles are more specific to tumors.<sup>62</sup> Surface

modification of metallic nanomaterials can enhance electrocatalytic activity.  $\text{TiO}_2$ <sup>63</sup> and  $\text{CeO}_2$ <sup>64</sup> nanocomposites modified by platinum nanoparticles enhanced the electrocatalytic activity of the materials for redox reaction. This may be due to the increase of oxygen capacity caused by strong electron coupling between composite structures.

## Ion Release And Solubility

MNPs have the character of ion release. The toxicity of nano metallic monomer and metallic oxide not only comes from the NPs themselves but also from the release of metal ions or their interaction. Biological effects of MNPs in cells are shown to be mainly caused by the exposure to solubilized metallic ions. Han et al<sup>65</sup> found that some MNPs decreased the activity of LDH. The similar deactivation mode of  $\text{Cu}^{2+}$  indicates that the decrease of LDH activity is mainly due to the dissolution of Cu NPs. Kinetic analysis showed that the Cu content in blood of Cu NPs exposed rats was 15–25% lower than that exposed by  $\text{Cu}^{2+}$ . The Cu level in the organs (especially in the liver, kidney, and spleen) of the treated rats significantly increased. In the blood and organs of rats treated with  $\text{Cu}^{2+}$  and Cu, respectively, Cu reached the highest level later and lasted for a shorter time.<sup>66</sup>  $\text{Zn}^{2+}$  and ZnO NPs can increase the Zn content of liver metallothioneins (MTs) and vitellogenin-like protein in plasma. It is noteworthy that MTs were upregulated by  $\text{Zn}^{2+}$  and ZnO NPs exposure, and the combination of Zn and Cu with MTs increased.<sup>67</sup>

The different results of subcellular distributions revealed that liver has different detoxification pathways for ZnO NPs and  $\text{Zn}^{2+}$ . Metallothionein-like protein was the main effector of  $\text{Zn}^{2+}$ , and ZnO NPs were mainly related to metal-rich granule.<sup>68</sup> IvanaVinkovi Vrček et al<sup>24</sup> compared the toxic effects of silver NPs and silver ions on HepG2 cells and found that the absorption of silver in the two forms was almost the same; the half-maximal inhibitory concentration (IC50) value of Ag NPs (50mg/L) was about 100 times higher than the corresponding value of  $\text{Ag}^+$  ( $0.5 \text{ mg}\cdot\text{L}^{-1}$ ). The possible reason was that  $\text{Ag}^+$  directly combined with SOD and GSH-Px and inhibited the enzyme activity.

## Mechanisms of hepatotoxicity induced by metallic nanoparticles

### Oxidative Stress

Reactive oxygen species (ROS) are active molecules produced during cell metabolism. In biology, ROS refers to superoxide anion radicals, hydroxyl radicals, and hydrogen peroxide.<sup>69</sup> ROS are produced in the process of

mitochondrial and cytoplasmic oxidation and help maintain cell function in the process of cell physiology. Excessive production of ROS can break the redox balance, resulting in oxidative stress, which leads to cell damage and cell death. Previous studies have shown that oxidative stress leads to lipid peroxidation and hepatocyte apoptosis, which is related to the occurrence and development of hepatitis, liver failure, ischemia-reperfusion injury, alcoholic liver disease, and other diseases.<sup>70</sup>

MNPs accumulated in the liver cause oxidative stress by altering the content and activity of antioxidant enzymes. The process of oxidative stress is accompanied by increased activity of antioxidant enzymes such as SOD, CAT, and glutathione peroxidase (GSH-px), as well as activity of non-enzymatic antioxidants such as ascorbic acid (ASA) and GSH.<sup>71</sup> Due to the combined action of Ag NPs and Ti NPs, oral exposure in rats caused a strong level of oxidative stress in the liver. The endogenous antioxidant system showed decreased GSH/GSSG ratio and increased formation of reactive substances.<sup>72</sup> After administration of polyvinylpyrrolidone-coated AgNPs (PVP-AgNPs) in male Sprague Dawley rats, the activities of SOD, CAT and TBARS increased and showed a dose-dependent effect.<sup>73</sup> In addition,  $\text{Fe}_3\text{O}_4$  NPs treatment caused significant increase in enzyme activities of GSH-Px, GR, and glutathione s-transferase (GST) with decrease in GSH content in Wistar rat organs.<sup>74</sup>

Oxidative stress injury is closely related to mitochondrial changes.  $\text{TiO}_2$  NPs can produce excessive ROS and reduce the antioxidant capacity of cells by destroying mitochondria. Further observations showed that  $\text{TiO}_2$  NPs could significantly reduce the mRNA levels of various detoxifying enzymes in the liver of mice, including SOD, CAT, GSH-px, and MT. Cytochrome P450, family 1, subfamily A, polypeptide 1 (CYP1A1), and heat shock protein 70 (HSP70) also came down by NPs and were involved, respectively, in toxic metabolism and DNA repair of hepatocyte damage.<sup>75</sup>

In vitro studies,  $\text{Fe}_3\text{O}_4$  NPs exposure to primary rat hepatocytes showed that the excessive production of ROS was mainly due to the damage of mitochondria by MNPs.<sup>76</sup> The possible reason for excessive ROS production through mitochondria by MNPs was the accumulation of calcium ions, which interferes with the electron transport chain of mitochondria and makes mitochondria produce more oxygen-free radicals.<sup>77</sup> Another study also confirmed that the effect of  $\text{TiO}_2$  NPs on HepG2 cells could activate NF-E2-related factor 2 (Nrf2) signals.<sup>78</sup> In

addition, the damage of ROS induced by MNPs to the production of endoplasmic reticulum cannot be ignored. ZnO NPs consumed antioxidants in the liver and induced ROS to affect the structure and function of the endoplasmic reticulum of mouse hepatocytes, which is believed to be related to apoptosis and autophagy.<sup>44</sup>

The results of ROS and antioxidant enzymes induced by MNPs are closely related to cell differentiation. Mei-Lang et al<sup>22</sup> compared the effects of CuO NPs on different cells of cancer cell lines, showing that excessive CuO NPs can induce alter membrane permeability, damage the mitochondrial respiratory chain, and break DNA strands. Cells eventually died. SK-Hep-1 cells could not effectively remove the accumulated hydrogen peroxide due to low differentiation level and inadequate activity of CAT and GRx. SK-Hep-1 cells are more sensitive to oxidative stress induced by CuO NPs than HepG2 cells, and the cell damage is more serious.

## Inflammation

Inflammation promotes the necrosis of parenchymal cells in organs and increases the accumulation of extracellular matrix in tissues. Mild damage leads to fibrosis while severe damage can lead to changes in the structure of organs and tissues. MNPs entering the body or liver cells induce inflammation. Liu et al showed that the liver of male Wistar rats was infiltrated by inflammatory cells because of exposure to ZnO NPs, TiO<sub>2</sub> NPs, and Ag NPs. Steatosis of hepatocytes and necrosis of the central part of hepatic lobules were also observed. Serum IL-1 $\beta$  level increased significantly in MNP-exposed group, serum IFN- $\gamma$  and TNF- $\alpha$  level decreased in ZnONP and TiO<sub>2</sub> NP groups as well as the concentrations of TNF- $\alpha$  increased significantly in Ag NP groups.<sup>79</sup> Kupffer cells, a kind of phenotype, are the resident macrophages in liver. MNPs accumulated in the liver are mainly ingested by Kupffer cells, with little uptake for hepatocytes, inflammation of the liver, Kupffer cells proliferation, and increased IL-1 $\beta$  release.<sup>38,55</sup> Similar to the effect of Ag NPs on the expression of inflammation in vivo, the exposure of human hepatocyte line C3a to Ag NPs increased the expression of IL-8, macrophage inflammatory protein 2, IL-1RI, and tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ).<sup>80</sup> Ag NPs or AgNO<sub>3</sub> contributed to the transition from hepatic steatosis to steatohepatitis. Ag NPs or AgNO<sub>3</sub> acted on HFD mice caused the increase of serum total cholesterol, HDL, and LDL levels. More importantly, elevated levels of IL-6 and TNF in mouse liver suggested inflammation.<sup>13</sup> The production

of inflammation corpuscle NLRP3 is the core of inflammation induced by MNPs. The generation and activation of NLRP3 involve MAPK, NF- $\kappa$ B, and ROS signals. Manna et al<sup>81</sup> showed that exposure to Cu NPs reduced liver index in a dose-dependent manner, resulting in oxidative stress and liver dysfunction. Cu NPs also increased the transcriptional activity of NF- $\kappa$ B. Ag NPs activated MAPK and PKB signaling pathways and induced ROS-mediated DNA damage in HepG2 cells.<sup>82</sup> These signals are not only related to inflammation but also induce ROS to promote apoptosis. Cu NPs affect CYP450 activity and suppress some nuclear receptors through the NF- $\kappa$ B signaling pathway. In fact, the regulation of P450 is also related to ROS.<sup>12</sup>

## The Outcomes Of Liver Cell Caused By MNPs

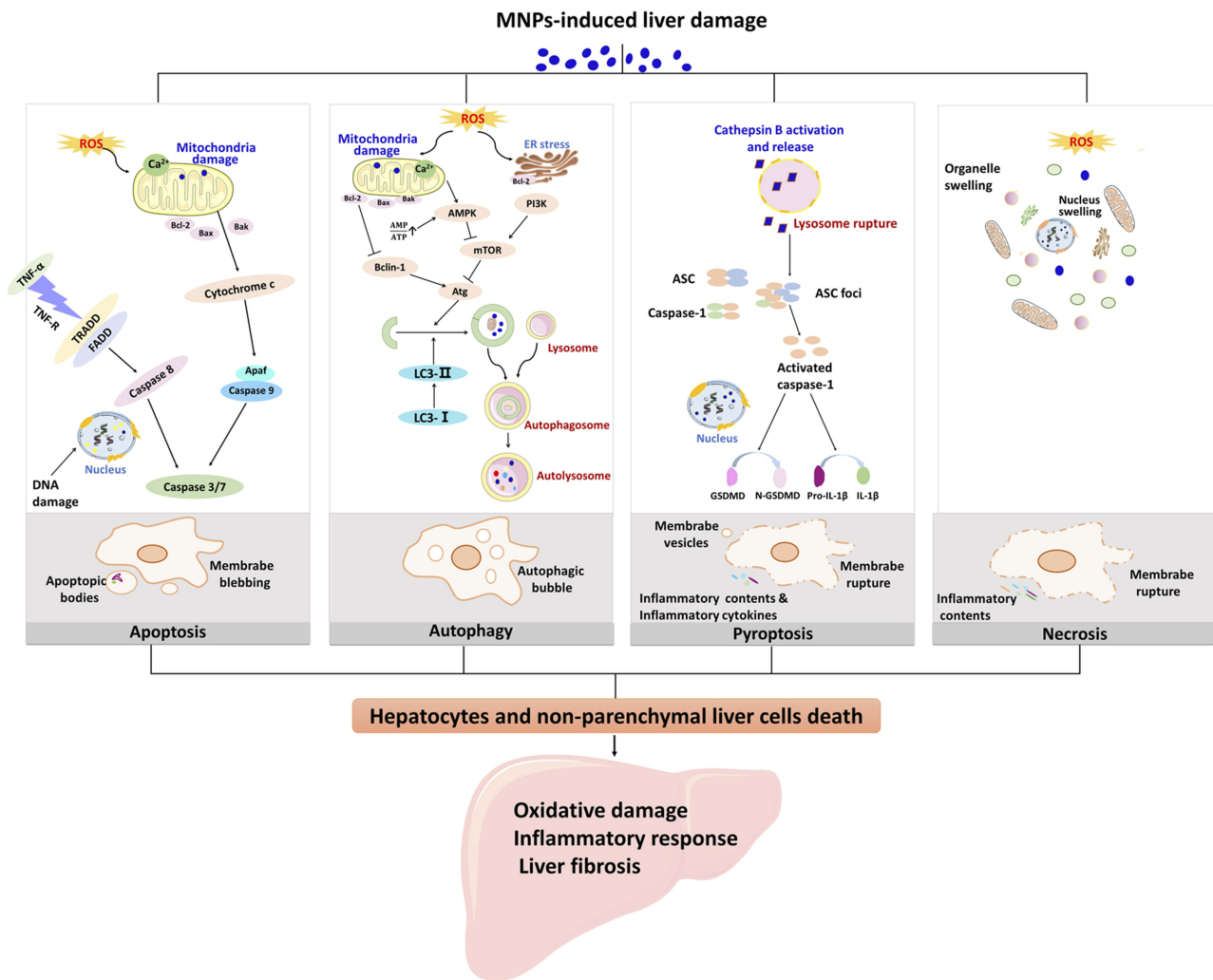
MNPs which reach the liver enter the cells and cause damage to the liver cells. As the basic unit of liver, different forms of cell death cause a series of damage, leading to liver dysfunction and pathological changes. Recent studies have highlighted the role of different death pathways in the pathogenesis of liver injury induced by MNPs as described in Figure 1.

## Apoptosis

Apoptosis is a programmed process of cell death that is used to clear unwanted cells from the body and a safe and controllable process that does not affect surrounding cells.<sup>83</sup> Apoptosis of hepatocytes leads to dysfunction, proliferation inhibition, cycle arrest, and decreased viability, thus causing liver fibrosis,<sup>84</sup> nonalcoholic fatty liver diseases related to cirrhosis and hepatocellular carcinoma (HCC).<sup>85</sup> Apoptosis is also regarded as the basis for chronic inflammation.<sup>86</sup>

Apoptosis is a prominent feature of hepatic damage of MNPs. Hepatocyte apoptosis is characterized by nuclear chromatin condensation, nuclear rupture, cell contraction, plasma membrane vacuolation, DNA damage, lack of nutrition and cytokine release, which reflect the activation of cell surface death receptors and apoptotic factors.<sup>87-90</sup>

Apoptosis is classified into endogenous apoptosis and exogenous apoptosis. Endogenous apoptosis, also known as mitochondrial pathway apoptosis, is a core event in which mitochondrial membrane permeability increases and mainly induced by activated BH3-only protein, which increases Bcl-2. Two proapoptotic molecules of the lymphoma 2 family, BAX (Bcl-2 related X protein)



**Figure 1** Different death mechanisms of liver cells are involved in the pathogenesis of liver injury induced by MNPs. Liver damage caused by MNPs is associated with oxidative damage, inflammatory response, and liver fibrosis in the liver. Apoptosis, autophagy, pyroptosis, and necrosis are all pathways of hepatocyte death. ROS induced by MNPs is responsible for the lipid peroxidation injury of the hepatic subcellular organelles. Apoptosis is considered as type I programmed cell death and mainly mediated by endogenous mitochondrial pathway and exogenous death receptor pathway. Mitochondrial ROS inhibited Bcl-2, and Fas-related death domain proteins (FADD) were activated, all of which eventually activated caspase 3 or caspase 7. Autophagy cell death is a programmed cell death different from apoptosis with initiation, nucleation of autophagosomes, phagosome expansion and completion, and autolysosome docking. Mitochondria and endoplasmic reticulum oxidative stress cause changes in the upstream molecules of autophagy and regulate autophagy-related (Atg) molecules. Pyroptosis is a form of inflammatory cell death that characterized by caspase-1-dependent formation of plasma membrane pores, and mainly manifested by lysosome rupture, ROS production and the activation of inflammation, leading to the release of pro-inflammatory cytokines and cell lysis. Necrosis is due to the production of ROS or instability of lysosome, release of calpain, and decrease of ATP level. The characteristics of necrosis include plasma membrane rupture, mitochondrial swelling, lysosome rupture, and intracellular contents release. Cell necrosis leads to inflammation that is not related to caspase cascade.

and BAK (Bcl-2 antagonist or killer), form oligomers in the outer membrane of mitochondria, which constitute a supramolecular channel-mediated cytochrome c release, which causes other proteins to be released from the mitochondria into the cytoplasm, thereby activating Caspase 9 and the Caspase cascade, triggering endogenous apoptosis.<sup>91–93</sup>

For 40 adult male albino rats, histopathological examination of liver in the exposed group of TiO<sub>2</sub> NPs showed that oral administration of TiO<sub>2</sub> NPs caused obvious apoptotic damage, which is manifested in the increase of Bax

gene and the decrease of anti-apoptotic Bcl-2 gene level.<sup>94</sup> Ag NPs entering human liver cells induced ROS production, inhibited the production of reduced glutathione, caused DNA fragmentation, lipid membrane peroxidation, and protein carbonylation. In addition, the mechanism of cell damage caused by Ag NPs is mitochondrial-dependent endogenous apoptotic pathway. By regulating the expression of Bax and Bcl-2, Ag NPs destroyed mitochondrial membrane potential, induced cytochrome c release in cytoplasm, and activated caspase-9 and caspase-3.<sup>27</sup> Exposure



of the liver cells (HL7702 cells, CHANG cells, HepG2 cells) to MNPs (Cs-Ag NPs, rGO-Ag NPs, TiO<sub>2</sub> NPs, Fe<sub>3</sub>O<sub>4</sub>-TiO<sub>2</sub> NPs) has been proved to have the same damage effects.<sup>23,95,96</sup>

Xue et al<sup>97</sup> found that Ag NPs acting on HepG2 cells not only caused mitochondrial-dependent apoptosis induced by ROS but also activated the Fas death receptor pathway by downregulation of NF- $\kappa$ B and activation of caspase-8 and caspase-3. This process illustrates the death receptor-mediated exogenous apoptosis pathway.

Another crucial mechanism involved in apoptosis is mediated by JNK-activated ER stress. Yang et al<sup>44</sup> showed that ZnO NPs significantly reduced the expression of anti-apoptotic gene Bcl-2 in liver tissue of mice. The phosphorylation of JNK protein in mouse hepatocytes was activated, and the activities of caspase-3, caspase-9, and caspase-12 were observed.

## Autophagic Cell Death

Autophagy, an important process of self-regulation and homeostasis of cells, is involved in cell cycle, cell death, self-renewal of stem cells, establishment of pluripotent-induced stem cells, and resistance to foreign pathogenic microorganisms.<sup>98</sup> More and more studies have shown that autophagy, as a double-edged sword effect, plays a two-way regulating role in affecting cell survival and death.<sup>99</sup> In liver metabolic diseases, autophagy is closely related to the occurrence of NAFLD, viral hepatitis, and even cancer.<sup>100</sup>

Numerous studies have shown that MNPs activate autophagy after entering the liver cells by endocytosis. MNPs (CTAB-GNR NPs, Ag NPs) entered the L02 cells and HepG2 cells, activated low levels of autophagy, increased protein expression of LC3-II, and observed double-layer membrane-coated autophagosomes under TEM. At this concentration, no significant cytotoxicity and lysosomal damage were observed, and MNPs induced ROS-mediated protective autophagy.<sup>26,101</sup> Rare earth doped up conversion nanoparticles (UCNs) activated autophagy in Kupffer cell which caused a decrease of cell survival and an increase in liver damage. However, inhibiting the formation of autophagosomes with 3-MA increased the survival rate of Kupffer cells and further eliminated the hepatotoxicity induced by UCNs,<sup>102</sup> suggesting autophagy played a role in damaging cells.

Cells recognize MNPs as external stimuli, activate ROS, and then cause mitochondrial damage. In order to maintain cell stability, autophagy is used to remove dysfunctional

organelles. Autophagic damage occurs when autophagy fails to cope with environmental changes. Fusion of autophagosomes and lysosomes to form autophagic lysosomes is an important process of cell autophagy. Iron(III)-tannin complex (Fe-TA NPs) induced the endocytosis of HepG2 cells and initiated the formation of autophagosomes. The intracellular nuclear vesicles and multivesicular (MVBs) produced by Fe-TA NPs were fused with autophagosomes, which could be degraded by regulating lysosomal functions.<sup>103</sup> This could be considered as one of the mechanisms by which MNPs induce autophagic cell death from excessive self-digestion.

In addition, there is a close relationship between autophagy and apoptosis induced by MNPs in hepatocytes – apoptosis may be an autophagy-related death pathway. In adult male SD rats exposed to PVP-Ag NPs, the ratio of LC3-II/LC3-I increased together with increased caspase-3, p53, and p21.<sup>73</sup> Kermanizadeh et al<sup>104</sup> cultured HepG2 cells and A549 cells with Ag and ZnO NPs for 6 hrs, resulting in the expression of autophagy-related genes LC3B, Atg4b, p62 upregulated, Atg12 and Atg5 declined. However, in the latter stages autophagy was impaired by caspase-dependent apoptotic cell death.

## Pyroptosis

Pyroptosis, also referred to as cellular inflammatory necrosis, is one way of caspase-1-mediated programmed cell death.<sup>105</sup> Gasdermin D (GSDMD) is one of the downstream Gasdermin protein families. The basic mechanism of the cell pyroptosis is that the inflammatory complex of the upstream protein activates caspase-1, which cleaves the GSDMD, and then the GSDMD protein releases the N-terminal fragment to recognize the phospholipid molecules on the cell membrane. Further, a hole is formed on the cell membrane, resulting in changes in ion concentration and osmotic pressure inside and outside the cell. Finally, the cell membrane is broken and cell contents are released, accompanied by pyroptosis.<sup>106–108</sup>

MNPs activated hepatocyte pyroptosis after entering cells by endocytosis. Its main features are cell membrane rupture and proinflammatory cell content release, which will cause the pathogen released from the dead cells, phagocytized and degraded by other cells, thus reducing the burden of infection, activating a strong inflammatory response and releasing plenty of inflammatory factors.<sup>109</sup> In addition, MNPs could also cause liver nuclear condensation, DNA shearing, and fragmentation.<sup>110</sup> Mirshafiee et al<sup>38</sup> found that Gd<sub>2</sub>O<sub>3</sub> could cause Kupffer cell swelling,

giant blebbing, cell membrane pore, caspase-1 activation, and IL-1 $\beta$  creation. The formation of cell membrane pore depends on GSDMD, which activates caspase-1. It disturbs the ion flow inside and outside the cell membrane, causes cell swelling, forms membrane vesicles, and leads to the leakage and intracellular substances release. Therefore, the death pattern of macrophages and hepatic parenchymal cells caused by MNPs can be reversed by knocking out the Gastermin D protein.

The apoptosis induced by MNPs has been confirmed to be associated with autophagy. Ag NPs induce caspase-1 activation and autophagic flux in HepG2 cells. When the autophagy-lysosome system was blocked, NLRP3 inflammatory bodies activated caspase-1 to a higher degree.<sup>26</sup>

The release of IL-1 $\beta$  and the increase of N-GSDMD expression induced by cell death promote the occurrence and development of some liver diseases.<sup>111</sup> N-GSDMA activates NLRP3 inflammatory bodies and induces cell death through typical pathways.<sup>112</sup> The pathogenesis of hepatitis C has been confirmed to be closely related to caspase-1 and caspase-3 signal-mediated cell burnout.<sup>113</sup>

## Necrosis

Cell necrosis refers to the irreversible loss of metabolic function and structural integrity of the cell serosa, the loss of integrity of the serosa, and the activation of non-inflammatory bodies. It is characterized by mitochondrial impairment and ATP depletion. ROS generation induced by MNPs leads to cell death and damage via hepatocyte necrosis. Adult female rats were continually exposed to PbO NPs 24 hrs a day with an average concentration of 10<sup>6</sup> particles per cubic centimeter. Six weeks later, the liver changed, showing hepatocyte swelling and hydropic degeneration, lobular hypertrophy with nuclear size changes, hepatocyte necrosis, inflammation around the portal vein and accumulation of lipid droplets.<sup>114</sup> Wang et al<sup>28</sup> co-cultured primary hepatocytes with Cu NPs and CuSO<sub>4</sub> for 24 hrs and observed that the apoptosis and necrosis rate of primary hepatocytes were apparently higher than that of control group. Significantly increased intracellular ROS and MDA, multiplied cytochrome c release, downregulated anti-oxidation related genes [SOD, CAT, GSH-Px4] expression, upregulated apoptosis-related genes (p53, p38 and TNF- $\alpha$ ), and increased activities of caspase-3, caspase-8, and caspase-9 all indicated that ROS might be involved in the process of cell necrosis, and there could be a certain correlation between necrosis and apoptosis.

Necrosis is one of the prominent features in acute liver injury.<sup>115</sup> Death of necrotic cells is also a distinct feature of hepatic ischemia/reperfusion injury.<sup>86</sup> In chronic hepatitis B virus infection, from local inflammation led hepatocyte apoptosis and necrosis to liver regeneration, a vicious circle is formed, which may be the potential mechanism of hepatocellular carcinogenesis.<sup>116</sup>

## Outlook

With the advances in the fields of nanotechnology, the potential exposure of MNPs is likely to increase, so there is an urgent need to further study the possibility of any detrimental health effects, target organ damage, and its mechanism. The toxicity of MNPs to the liver is an important basis for the safety assessment of MNPs. At present, studies on the hepatotoxicity of MNPs are still in their infancy. The toxicity of MNPs is mainly due to the special physical and chemical properties, such as size, surface chemical modification and metal ion release. The liver is particularly susceptible to MNPs because the liver has a much higher accumulation of NM than other organs. For the evaluation of hepatotoxicity of MNPs, on the one hand, a full understanding of the distribution and metabolism of MNPs in the liver, and detecting the changes in liver function, degree of injury, and recovery of liver function in vivo which are prerequisites for evaluating their liver toxicity. On the other hand, it is important to better understand the mechanisms by focusing on the complex biological process between MNPs and cells. MNPs entering cells change the structure and functions of organelles, affect the normal biological functions of cells, and ultimately impact the amount of toxicity and threshold dose caused by MNPs. It is worth noting that once the MNPs accumulate in the liver, it may cause changes in liver function. When the MNPs enter the cell, it will damage it and produce a large amount of free oxidative free radicals, thereby destroying the oxidation/deoxidation balance. MNPs can also enter the nucleus and can directly or indirectly destroy DNA, leading to changes in gene expression and even apoptosis. If there is a long-term liver injury, the HSC will turn into an active state. Along with changes in the activity of several intracellular signaling pathways, extracellular components are involved in the extracellular matrix, which ultimately leads to fibrosis and may eventually progress to cirrhosis. However, the detailed mechanism of MNPs leading to liver fibrosis remains unclear.

To study the molecular mechanisms of liver injury caused by MNPs, it is necessary to perform experiments from in vivo to in vitro involving in molecular biology especially biomarker

screening, which is crucial for understanding the detailed mechanism of liver injury. Although immune inflammation, apoptosis, and oxidative stress related to liver injury have been investigated, the aspects of energy metabolism, protein metabolism, and lipid metabolism should be studied in detail. Current research into the toxicity of MNPs has been limited to animal experiments in vivo and in vitro; the relationship between subcellular damage and related mechanisms is still unknown. Therefore, the toxicology of MNPs must be studied in-depth to improve the quality and safety of those nanoparticles. Research in this area has a long way to go.

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## Disclosure

The authors report no conflicts of interest in this work.

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