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Current Updates On the *In vivo* Assessment of Zinc Oxide Nanoparticles Toxicity Using Animal Models

Ce Lynn Chong[1] · Chee Mun Fang[1] · Swee Yong Pung[2] · Chin Eng Ong[3] · Yuh Fen Pung[1] · Cin Kong[1] · Yan Pan[1]

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Abstract

Zinc oxide nanoparticles (ZnO NPs) is one of the most exploited nanoparticles in the biomedical field due to its enormous potential in disease diagnostics and monitoring. The upsurge in demand for ZnO NPs due to its peculiar properties and vast applications has led to an increase of inadvertent exposure of ZnO NPs to humans, resulting in higher risk for nanotoxicity. Previous literature is strongly suggestive of the notion that ZnO NPs may induce adverse health effects in humans and organisms in the environment. Hence, knowledge of the physiological interactions of ZnO NPs with the biological system as well as understanding the interplay between the physicochemical properties of ZnO NPs and its toxic effects are key factors in developing ZnO NPs that can be safely applied in the biomedical field. At present, many experiment models have been employed to unveil the possible unforeseen effect of ZnO NPs on the human, animals, and environment. Among these experiment models, animal models are of paramount importance as it allows the prediction of the possible toxic effects of ZnO NPs in individual organs or the body as a whole. The current review systemizes and summarizes the in vivo assessments of organ-specific nanotoxicity of ZnO NPs using animal models published between 2015 and 2020. Besides, this review attempts to outline the possible mechanisms of ZnO NPs–induced toxicity and factors influencing these toxic effects of ZnO NPs.

Keywords Zinc oxide · Nanoparticles · Nanotoxicity · In vivo · Animal models

1 Introduction

In the past few decades, nanotechnology has achieved tremendous progress in various fields. The growth and development of nanotechnology have led to an increase of inadvertent exposure of nanoparticles (NPs) to humans and the environment. Nanotoxicology is an arising discipline that deals with the potential toxicity of NPs to better understand and evaluate the health risks associated with the application of them [1]. Today, many analytical approaches and devices have been introduced to unveil the possible unpredictable negative impact of NPs on human, animals and environment. The toxicity of substances can be evaluated by in vitro studies using cells or cell lines, in vivo exposure on experimental animals as well as in silico computational models [2]. These in vitro, in vivo, and in silico studies would each provide pieces of research puzzle that contribute to vital toxicological information. The in vitro study is usually the first stage of the nanotoxicity assessment. However, the complexity of the biological events that take place in an organism cannot be fully duplicated in cell culture or in non-living systems [3]. The use of animal models in the study of nanotoxicity are crucial as they can capture events that are hardly mimicked in the in vitro studies such as the interaction of the NPs with different cell types, molecules and biological fluids in the tissues, as well as evaluate the kinetics of such interactions and the biodistribution of the NPs within the body [4].

The global consumption of ZnO NPs has been increasing throughout recent history due to its application in vast areas such as cosmetics, paints and coatings, rubber, agriculture, textiles, electronics, and food industries. In recent years, the applications of ZnO NPs have been extended to the

✉ Yan Pan
panyan1980@hotmail.com

Chee Mun Fang
https://orcid.org/

[1] School of Pharmacy, University of Nottingham, Semenyih, Selangor, Malaysia

[2] School Materials and Mineral Resources Engineering, Universiti Sains Malaysia Engineering Campus, Parit Buntar, Penang, Malaysia

[3] School of Pharmacy, International Medical University, Kuala Lumpur, Federal Territory of Kuala Lumpur, Malaysia

biomedical field. Multiple studies have shown promising results of ZnO NPs in cancer treatments [5, 6]. ZnO NPs have been explored for use in drug delivery, gene delivery, and tumor imaging in cancer treatment as a result of their ability to target specific cells or tissues [7]. A recent study on the anticancer effects of ZnO NPs suggested that the exposure of low-dose ZnO NPs effectively suppressed the proliferation and migration of bladder cancer (T24) cells through the alteration of histone methylation [8]. On top of that, ZnO NPs were demonstrated to enhance the efficacy of chemotherapy drugs, for instance, cisplatin, gemcitabine, and doxorubicin in treating non-small cell lung cancer cells and MCF-7 breast cancer cells [9, 10]. Lately, multiples studies have also found evidence showing the effectiveness of ZnO NPs against a broad spectrum of diseases other than cancer. It was found that ZnO tetrapod NPs showed strong microbivac efficacy against herpes simplex virus type-2 infection in murine models [11, 12]. Herein, ZnO tetrapod NPs trap the HSV-2 virus and inhibit the viral entry while allowing the activation of an immune response. This study had definitely offered a promising start point for the application of ZnO NPs as a live virus vaccine platform that could provoke a protective or therapeutic immune response without causing infection [12]. Moreover, in an interesting study, ZnO NPs had also been shown to be effective against diabetes. The hypoglycaemic study showed that ZnO NPs at the concentration of 8 and 14 mg/kg reduced approximately 25.13 and 29.15% of blood glucose levels in rats [13]. Although the current state of knowledge concerning the application of ZnO NPs in the biomedical field is still in the preliminary stage, however, the great potential of ZnO NPs by virtue of their peculiar physicochemical properties had driven an increasing number of publications and patents over the past few years. It is important to mentioned that ZnO NPs have entered phase III clinical trials for use in the treatment of denture stomatitis and pulpitis [14, 15]. The nano zinc oxide eugenol sealer was shown to lower severity of pain of patients with irreversible pulpitis. Hence, it is regarded as a promising substitute to the routine sealers [15, 16]. Table 1 enlisted some patents associated with the application of ZnO NPs in the biomedical field issued between 2015 and 2020.

According to a report published by Allied Market research, the global nano zinc oxide market is anticipated to reach $7677 million by 2022 [41]. ZnO has been graded by the US Food and Drug Administration (FDA) as a "GRAS" (generally recognized as safe) substance [42]. However, existing research data suggest that ZnO NPs may induce adverse health effects in humans and organisms in the environment. The toxicological profiles of these NPs are distinct from their larger counterparts due to their unique physicochemical properties [43]. Pieces of evidence showed that ZnO NPs are more toxic as compared to ZnO. A comparative study by Hao et al. on the toxicity of ZnO NPs and bulk ZnO showed that ZnO NPs caused more severe histopathology changes in the liver of juvenile carp than its bulk particles. This result is in accordance with the ZnO NPs induction of higher levels of intracellular oxidative stress in the liver cells [44]. Srivastav et al. supported this, showing higher upregulation of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphate (ALP), and gamma-glutamyl transferase (GGT) expression in ZnO NPs–treated rats. This indicates that ZnO NPs caused greater liver damages in oral-treated Wistar rats compared to their larger counterparts [45]. Therefore, there is a need to collect and disseminate accurate, reliable, and unbiased information on the risks and benefits of ZnO NPs, to evaluate if the potential advantages outweigh the risk associated.

In this review, the literature published between 2015 and 2020 which covers the in vivo assessments of organ-specific nanotoxicity of ZnO NPs using animal models were collected, analyzed, and discussed. Besides, information on the possible mechanisms of ZnO NPs–induced toxicity were outlined together with the factors influencing these toxic effects of ZnO NPs. To our best knowledge, this is the first review that discussed the current updates on the organ-specific toxicity caused by ZnO NPs in animal models.

2 Organs Specific Toxicity Caused by ZnO NPs in *In vivo* Studies Using Animal Models

2.1 Pulmonary Toxicity

ZnO NPs–induced pulmonary toxicity has received increasing concerns, given inhalation is the main route of exposure to ZnO NPs in the occupational environment. As shown in Table 2 and Fig. 1, the ZnO NPs–induced toxicity endpoints reported in previous in vivo studies included acute pulmonary inflammation, chronic inflammation, altered metabolisms, histopathological alterations in lungs, and airway irritation. A recent study reported ZnO NPs were the most inflammogenic NPs among the tested metal oxides (ZnO, TiO_2, Al_2O_3, and CeO_2) using mice models [51]. A proteomic analysis conducted by Pan et al. supported this by showing the upregulation the S100-A9 protein in mice 24 h after administration via intratracheal instillation. S100-A9 is a zinc-binding protein that is crucial in the regulation of the inflammatory processes and immune response. The upregulation of S100-A9 protein may result in the promotion of pro-inflammatory cytokines [48]. In rats and mice models, administration of ZnO NPs by inhalation or intratracheal instillation resulted in an elevation of the inflammatory cells in the bronchoalveolar lavage fluid (BALF) as well as activation of a series of cytokine cascade including the upregulation of interleukins (IL), tumor necrosis factor (TNF), and interferon [46, 49, 54]. Qiao et al. showed that the oropharyngeal aspiration of

Table 1 Patents of ZnO NPs and its biomedical applications published between 2015 and 2020

ID	Title	Possible application	Year	References
US10576100B2/US10675301B2	Chitosan zinc oxide nanoparticle formulation for treating drug resistant bacteria	Mitigate against multi-drug resistant bacteria from nosocomial infections	2020	[17]
CN111658816A	A drug-loaded metal nanoparticle/chitosan controlled-release hydrogel, and a preparation method and application thereof.	Drug delivery	2020	[18]
CN110721318A	Disulfiram nanoparticle, and preparation method and application thereof	Drug delivery	2020	[19]
CN110755607A	Zinc oxide and antigen co-loading medicine nanometer vaccine, and preparation method and application thereof	Drug delivery (vaccine) impairs	2020	[20]
US10593902B2	Quantum dot light emitting devices (QLEDs) and method of manufacture	Optical sensor, phototherapy, photomedicine and photobiomedicine.	2020	[21]
CN109512800A	Preparation method of drug-loaded zinc oxide and silicon dioxide composite nanoparticles	Drug delivery	2019	[22]
KR20190027346A	Delivery of biomacromolecules employing clusters of nanowires	Gene delivery	2019	[23]
US10398732B2	Compositions and methods for treating striated muscle injury, treating striated muscle atrophy and/or for promoting striated muscle growth	Treatment for striated muscle injury and/or atrophy and for promoting striated muscle growth	2019	[24]
US10478402B2	Solid nanoparticle with inorganic coating	Drug delivery	2019	[25]
CN109939267A	High molecular composite material for antibacterial medical catheter and preparation method of material	Antibacterial medical catheter	2019	[26]
EP3341034A1	Antimicrobial and enzyme inhibitory ZnO NPs	In-dwelling medical device comprising the antimicrobial material	2018	[27]
CN108653241A	Preparation method for anticancer drug nanoparticles	Drug delivery	2018	[28]
KR20180101767A	The composition for catheter the central venous catheter and the intravascular tube catheter prepared by using the same	Provide a composition for a catheter having better hemostatic power and antibacterial activity,	2018	[29]
CN107981441A	Medical high-efficient sterilizing mask	Biomedical materials	2018	[30]
CN107157951A	Self-assembled nano zinc oxide drug-loading capsule, and preparation method and application thereof	Drug delivery	2017	[31]
CN106823017A	Preparation method for hybridized biological functional coating based on halogen-ammonia compound and zinc oxide nanoparticles	Antibacterial medical implants	2017	[32]
CN106384737A	Flexible electroluminescent X-ray image memory display panel	Bioimaging	2017	[33]
CN106492266A	Method for preparing nano-silver/zinc oxide composite hydrogel dressing with surface antibacterial properties	Wound dressing	2017	[34]
KR20170021351A	Antitumor composition based on hyaluronic acid and inorganic nanoparticles method of preparation thereof and use thereof	Selective antitumor agents	2017	[35]
CN105853373A	Zinc oxide–based nano-drug composition, and preparation method and application thereof	Drug delivery	2016	[36]
CN105497087A	Slow-release gel loaded with sanqi and nanometer zinc oxide, preparing method thereof and applications of the slow-release gel	Treating oral diseases	2016	[37]
US2016220606A1	Silver-copper-zinc oxide wound care system	Antimicrobial wound care dressings	2016	[38]
CN105926042A	Three-dimension gene-tree biological chip and manufacturing method thereof	Biochip for disease diagnosis	2016	[39]
CN105599329A	Preparing method of antibiosis anti-ultraviolet contact lenses	Antibacterial anti-ultraviolet contact lens	2015	[40]

Table 2 The animal studies of ZnO NPs–induced pulmonary toxicity conducted between 2015 and 2020

Animals	Type of exposure	Size and Concentration	Method of detection	Parameters	Toxicities	Reference
Rats	Oropharyngeal aspiration	41.7 nm; 2, 4, and 8 mg/kg	• ELISA • LDH assay • Bicinchoninic acid assay • H&E staining • TEM • Western bolt • miRNA microarray assay and bioinformatics analysis	• Serum levels of IL-8, IL-1β, and TNF-α, LDH, protein levels and activity in bronchoalveolar lavage fluid (BALF) • Lung histology • Morphology of exosomes miRNA profiling of serum exosomes	ZnO NPs–induced pulmonary neutrophilic inflammation in mice through modulation of miRNA expression • IL-8, IL-1β, and TNF-α ↑ • Number of cells and the percentage of neutrophils in the blood ↑ • LDH activity and total protein in BALF ↑ • Altered morphology of exosomes • 16 DEmiRNA ↑; 7 DEmiRNA ↓	[46]
	Intratracheal instillation	NA, 1, 5, and 10 mg/kg	• Acute pulmonary analysis: BALF cytology, BCA protein assay, LDH assay, ELISA • ICP-MS • H&E straining	• Body and lung weight • Cell morphology • Total protein in BALF and activity of LDH • BALF level of IL-6 and TNF-α • Zn level in lungs • Lung histology	ZnO NPs–induced multifocal acute inflammation which then progressed to chronic inflammation in the lungs of Sprague Dawley rats after 14 days of exposure • Macrophages, total cell count, and total protein concentrations in BALF ↑ • Histopathological alterations in lungs at day 1 and day 14 of exposure	[47]
Mice	Inhalation	13.2 + 5.4 nm; 212 mg/m3	• Macrophage depletion characterization • Inhalation bioassay and aerosol characterization	• Tidal volume	• Tidal volume ↓	
	Intratracheal instillation	20 nm; 100 μg/lung	• Protein digestion and iTRAQ labeling • LC-MS/MS • Protein functional analyses: protein analysis through Evolutionary Relationships (PANTHER) classification system • Protein network analysis	• Protein expression, function and network	Alterations of the protein levels in the lung tissues in the 24-hour follow-up and 28-day follow-up groups which then lead to the impairment of significant pathways • S100-A9 protein ↑	[48]
	Intratracheal instillation	Functionalized: 70–100 nm Non-functionalized: 58–93 nm 0, 1, 4, 8, 16, 32, 64, and 128 μg/mouse	• Protein analysis • H&E staining	• Cytokines levels in BALF • Lung histology	ZnO NPs–induced acute pulmonary inflammation with cell damage in bronchoalveolar lavage fluid • BAL cells, number macrophages and neutrophils ↑ • Mediators of acute inflammation and the acute phase response levels IL-6 and granulocyte-colony stimulating factor (GCSF)) ↑ • BALF protein content and LDH level ↑ • Histopathological alterations in lungs	[49]
	Intratracheal instillation	NA; 200, 400, 800 μg/kg	• Blood biochemical analysis	• Total protein, AKP, hydroxyproline, and LDH in BALF	ZnO NPs–induced acute toxicological effects in mice	[50]

Table 2 (continued)

Animals	Type of exposure	Size and Concentration	Method of detection	Parameters	Toxicities	Reference
			• Biochemical and cytological evaluation of BALF • Measurement of NO and MDA in lung homogenates • H&E staining	• NO and MDA levels in the lung homogenates Lung histology	• Total protein level, AKP and hydroxyproline ↑, no change in LDH level • Oxidative stress • Histopathological alterations in lungs	
	Inhalation	ZnO NPs 1: 13.2 ± 5.4 nm; 5, 58, 203 mg/m^3 ZnO NPs 2: 36.1 ± 18.1 nm; 4, 26, 53 mg/m3	• Estimation of deposited doses • Comet assay • BCA protein assay • Periodic acid-Schiff (PAS) hematoxylin staining	• Inhalable fraction (IF) • BAL cell composition • Total protein in BALF • Lung histology	ZnO NPs exposure–induced acute airway irritation and pulmonary inflammation. • Tidal volume ↓ • Neutrophils and lymphocytes in BAL ↑ • Total protein ↑ • Histopathological alterations in lungs	[51]
	Intratracheal instillation	9.1 ± 1.9 nm; 0.21, 0.43, and 0.86 mg/m3	• Bradford assay • CytoTox96 non-Radioactive cytotoxicity assay • H&E staining • ELISA • ICP-MS	• Total protein and polymorphonuclear neutrophil (PMN) • LDH activity • GOT, GPT and creatine phosphokinase (CPK) activities • Lung histology • monocyte chemotactic protein-1 (MCP-1) and chemokine (C-X-C motif) ligand 1 (CXCL1)	Inhaled aerosolized ZnO NP–induced acute pulmonary inflammation in mice. • PMN and total protein in BALF ↑; macrophages • MT expression in lungs ↑ • GOT, GPT and CPK ↑ • Histopathological alterations in lungs • MCP-1 and CXCL1 ↑	[52]
	Intratracheal instillation	30 nm; 5 mg/kg	• RNA extraction and qPCR • H&E staining	• Serum levels of eotaxin, TNF-α, and MCP-1 and other pro-inflammatory cytokines • Lung histology	ZnO NPs provoke acute pulmonary inflammatory reactions in mice • Histopathological alterations in lungs (inflammation) • Pro-inflammatory cytokines ↑	[53]
	Oropharyngeal aspiration	50 nm; 0.1 mg/kg, 0.5 mg/kg	• ELISA • ASK Liu's staining • Cytometric bead array mouse enhanced sensitivity flex set system • Total protein assay and LDH assay • H&E staining	• Total serum IgE • Inflammatory cell profiles in BALF • Inflammatory cytokines in BALF: s IL-4, IL-5, IL-6, IL-10, IL-13, IFN, and TNF-α • Total protein assay and LDH levels in BALF • Lung histology	ZnO NPs–induced eosinophilic airway inflammation in Balb/c mice. • Total cell count, neutrophils, lymphocytes and eosinophil ↑ • Cytokine in BALF ↑ • Total BALF protein ↑ • Histopathological alterations in lung (inflammation)	[54]

ZnO NPs resulted in the upregulation of IL-8, IL-1β, and TNF-α in treated rats [46]. It was postulated that these pro-inflammatory cytokines caused neutrophils to adhere to pulmonary capillaries and extravasate into the alveolar spaces, where they undergo activation. Activated neutrophils were shown to release a variety of factors, such as leukotrienes, oxidants, proteases, and platelet activating factor (PAF), which contribute to local tissue damage, accumulation of edema fluid in the airspaces, surfactant inactivation, and hyaline membrane formation [55]. Moreover, the in vivo exposure of ZnO NPs in mice increased the neutrophils and macrophages in the BALF [49]. These neutrophils and macrophages were believed to be responsible for the fibroblast growth observed in pulmonary toxicity. Fibrosis is caused by the abnormal accumulation of collagen and its presence can be evaluated by investigating the hydroxyproline in the BALF[55]. An experimentation showed that the hydroxyproline in the 400 μg/kg and the 200 μg/kg mice were significantly higher than those in the control mice, suggesting the presence of early lung fibrosis. The observations mentioned were in line with

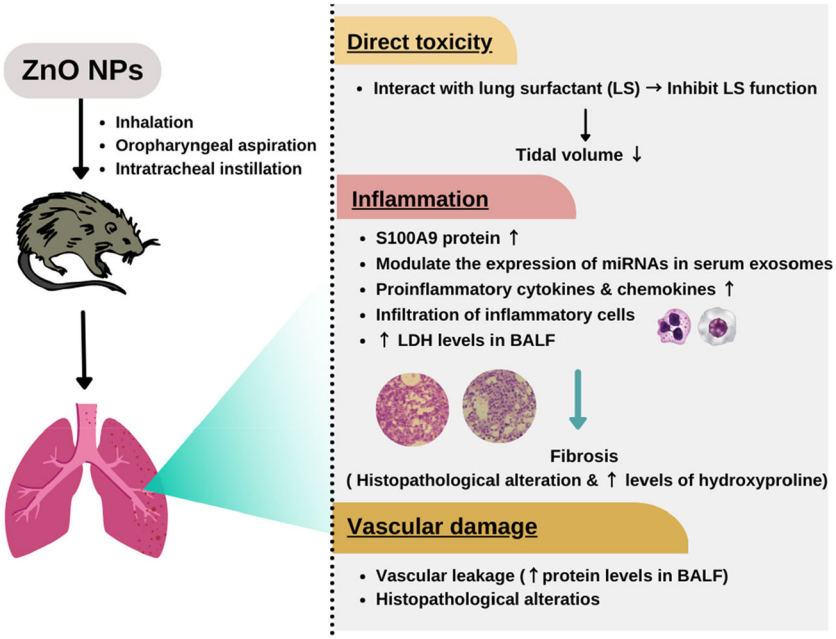

Fig. 1 Summary of ZnO NPs–induced pulmonary toxicity

the histological findings in the ZnO NPs–induced pulmonary toxicity [50]. The histopathological alterations reported included inflammation cells infiltration, thicken bronchial and vascular walls, internal hemorrhage, exudate and edema formation, alveolar interstitial fibrosis, as well as desquamation of bronchiolar epithelial cells [46, 47, 49–51, 53, 54]. Moreover, increment in the total protein levels, together with the elevation of lactate dehydrogenase (LDH) in the BALF were also observed in ZnO NPs–treated mice and rats [46, 49, 50, 54]. In healthy animals, the protein levels in the alveolar lining are considerably low; however, injury to the airways and alveolar structures may lead to the leakage of vascular protein into the alveolar space. Hence, the BALF analysis of total protein is regarded as the simplest and most accurate measure of acute lung injury and vascular leakage [56]. On the other hand, LDH is a cytoplasmic enzyme that has been used as indicators of pathological conditions in the lungs, such as cell damage or inflammation [57]. In addition to the inflammatory effects of ZnO NPs in lungs, inhalation of ZnO NPs were also shown to induce an acute decrease in the tidal volume in mice. Larsen et al. observed shallow breathing (reduced tidal volume) in mice shortly after the onset of exposure to ZnO NPs [58]. Taken together, the data from these studies implied lung injury in treated animals, in response to ZnO NPs exposure.

2.2 Nephrotoxicity or Renal Toxicity

Lin et al. injected the mice with 10 mg/kg of ZnO NPs either intravenously or intraperitoneally and to monitor their biodistribution and accumulation in various organs. Findings revealed that ZnO NPs accumulated in the tested organs including kidneys, suggesting that kidney could be one of the major target organs for the ZnO NPs–induced toxicity [59]. The recent findings of ZnO NPs–induced nephrotoxicity with various animal models were summarized in Table 3 and Fig. 2. The two main approaches used in these studies to evaluate the ZnO NPs–induced nephrotoxicity included histological examination of the kidney and serum biochemical analysis. Abdel-aziz et al. reported that the exposure of ZnO NPs to rabbits caused histological changes in the kidney, including loss of brush bored, vacuolation of cytoplasm, destruction of distal convoluted tubule, increase in Bowman's space, intra tubular protein deposition, infiltrations of inflammatory cells, and capillaries congestion in between tubules [65]. Similar findings were reported by Khorsandi et al. using rats. The kidney weight of rats treated with the ZnO NPs increased, indicating congestion of red blood cells, infiltration of leukocytes, and accumulation of ZnO NPs [61]. Chien et al. reported that the inhaled ZnO NPs lead to sustained inflammation of the kidney. The study observed an increment in the microscopic inflammatory foci with pronounced periglomerular inflammation and interstitial lymphocytic infiltration as well as tubulitis with lymphocytic infiltrate in the tubular epithelium [60]. These histopathological observations suggested that the kidney inflammation as a result of oxidative stress contribute to progressive renal injury in animals treated with ZnO NPs. The study of nephrotoxicity using blood test and biochemical analysis includes the measurement of blood urea nitrogen (BUN), uric acid, bilirubin, creatinine concentration, creatinine clearance, glutamate oxaloacetate transaminase (GOT) activity, and glutamate pyruvate transaminase (GPT) activity [60, 61, 66]. Elevated BUN, creatinine concentrations, glutamate oxaloacetate transaminase (GOT), and

Table 3 The animal studies of ZnO NPs–induced nephrotoxicity conducted between 2015 and 2020

Animals	Type of exposure	Size and concentration	Method of detection	Parameters	Toxicities	Reference
Rats	Inhalation	NA, 1.1, 4.9 mg/m^3	• H&E and periodic acid (PAS) staining	• Kidney histology	Inhaled ZnO NPs result in sustained inflammation of the kidney. • Histopathological alterations in kidney	[60]
	Oral	NA, 5, 50, and 300 mg/kg	• Zn content examination (nuclear ingestion spectrophotometer) • Biochemical experiments • Assessment of lipid peroxidation • SOD activity (Ransod method) and GPx activity (Randox method) • H&E staining • TUNEL assay	• Organ weight • Zn concentration in blood and renal • Biochemical: uric acid, creatinine, bilirubin • MDA level, SOD, and GPx activities • Kidney histology • Percentage of apoptosis	ZnO NPs exhibited nephrotoxicity and induced apoptosis in proximal cells of rats at low dose. • Zn concentration in blood and renal ↑ • Uric acid, creatinine, bilirubin ↑ • Oxidative stress • Apoptosis ↑ • Histopathological alterations in kidney	[61]
	Intraperitoneal	NA, 10, 20, 30 mg/kg	• H&E staining	• Kidney histology	The ZnO NPs–induced nephrotoxicity is dose-dependent manner. • Histopathological alterations in kidney	[62]
	Oral	20-30 nm; 100, 200, 500 mg/kg	• H&E & PAS staining • Mercuric bromophenol blue staining	• Kidney histology	ZnO NPs have potential renal toxicity that may affect the function of the kidney • Histopathological alterations in kidney	[63]
Mice	Oral	NA, 150, 350 mg/kg	• H&E staining	• Kidney histology	ZnO NPs affect the kidney by causing histopathological changes.	[64]
	Intravenous or intraperitoneal	NA, 10 mg/kg	• ICP-AES spectrometry • Biochemical test • H&E staining	• Zn content in lung, liver, kidney, spleen, heart • Serum biochemical: GPT, GOT, BUN, creatinine. • Creatinine clearance • Kidney histology	ZnO NPs cause nephrotoxicity in rats • Zn concentration in organs ↑ • BUN, creatinine, GPT, GOT ↑ • Histopathological lesions in kidney	[59]
Rabbits	Intraperitoneal	NA, 100 ml/kg, 250 ml/kg	• H&E staining	• Kidney histology	ZnO NPs caused histopathological alterations of kidney through oxidative stress	[65]

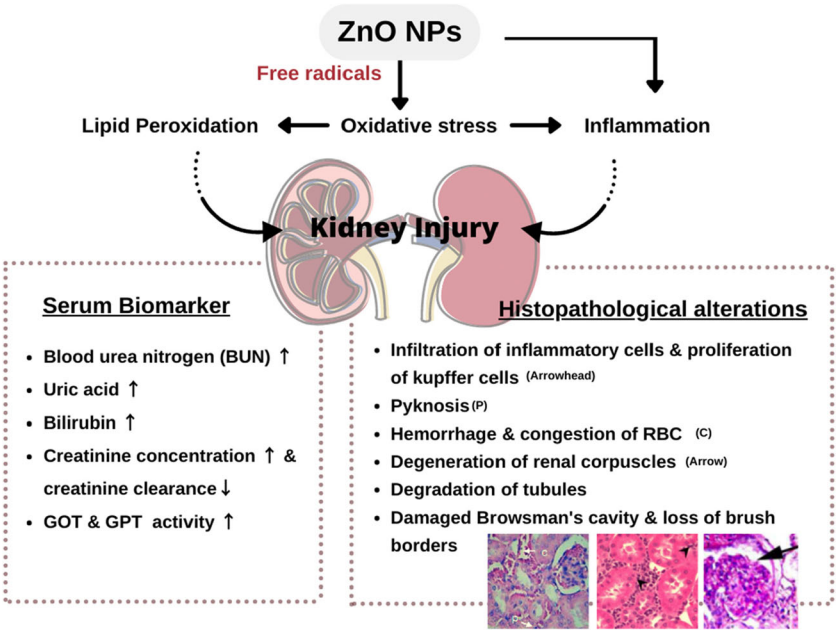

Fig. 2 Summary of ZnO NPs–induced nephrotoxicity

glutamate pyruvate transaminase (GPT) were reported in rats treated with 10 mg/kg ZnO NPs, indicating the dysfunction of the kidney[59].

2.3 Hepatotoxicity

Table 4 listed liver injury caused by ZnO NPs in numerous recent animal studies while Fig. 3 summarized the ZnO NPs–induced hepatotoxicity. The exposure of ZnO NPs was shown to induce hepatotoxicity, as revealed by the histological and histochemical examinations of treated experimental animals. The histopathological manifestations of ZnO NPs–induced hepatotoxicity include bile duct and Kupffer cells hyperplasia, steatosis, anisokaryosis, karyolysis, inflammatory cells infiltration, hepatocytes vacuolization, necrosis and apoptosis, hepatocytes glycogen depletion as well as congestion in dilated portal veins, sinusoids, and hepatic artery [67–70, 72, 73]. These alterations in the histological structures of the hepatic tissues, in turn, lead to impairment of the liver function. The degree of hepatic injuries induced by ZnO NPs can also be reflected using biochemical tests. The elevation of AST, ALT, GGT) and ALP as a result of ZnO NPs exposure were recorded, indicating the presence of liver injuries in the tested animals [67, 68, 71, 72]. Hegazy et al. found that the oral administration of ZnO NPs led to the increment of AST and ALT in rats even at a low concentration (1 mg/kg)[67]. It is noteworthy that the elevation of serum ALT is associated to cholestatic injury in the liver, while the increment in AST implied hepatocyte damage [74]. Moreover, another study demonstrated increased levels of ALP and GGT in the liver of rats orally treated with ZnO NPs [71]. Elevated ALP signified biliary injury in the ZnO NPs treated rats [75]. GGT, on the other hand, is an enzyme that plays a crucial role in the antioxidant system and its upregulation has been associated to oxidative stress. Indeed, the ZnO NPs oxidative stress and its mediated inflammation have been believed to be responsible for the ZnO NPs–induced toxic effect in the liver. The administration of ZnO NPs has been found to cause an elevation of cytokines (IFN-γ, TNF-α, and IL-12), which are responsible for the inflammatory responses and immune-cell differentiation process [71]. The release of these cytokines by Kupffer cells or hepatocytes mediate many of the pathological effects seen with hepatotoxicity including inflammatory cell infiltration, lipogenesis, fibrogenesis, and cholestasis [76]. Apart from inducing liver injury, the inflammation in the liver is also known to inhibit the expression and activity of drug metabolizing enzymes. (e.g., cytochrome P450 (CYP)). Tang et al. reported that exposure of ZnO NPs downregulated the mRNA expression of CYP1A2 and upregulated the CYP 2C11 and CYP 3A2 expression in orally treated rats. Additionally, a significant decrement in the activity of liver CYP2C11 and CYP3A2 was observed. The authors proposed that the significant decrease in the activity of CYP2C11 and CYP3A2 may be due to the protein structural or conformational alterations by the ZnO NPs–induced reactive oxygen species or inflammatory cytokines [71].

2.4 Neurotoxicity

In a recent study, De Souza et al. observed an increment in Zn accumulation in the brain tissues of mice intraperitoneally treated with ZnO NPs at environmentally relevant (5.625×10^{-5} mg/kg) and high concentrations (300 mg/kg) [77]. This finding suggested that ZnO NPs might overcome the blood–

Table 4 The animal studies of ZnO NPs–induced hepatotoxicity conducted between 2015 and 2020

Animals	Type of exposure	Size and concentration	Method of detection	Parameters	Toxicities	Reference
Rats	Oral	NA, 1 mg/kg	• Histological and immunohistochemical examination: light microscopic examination, H&E, Masson's trichrome, Anti-caspase 3 staining • Biochemical assay • Morphometric study	• Liver histology • Biochemical: aspartate aminotransferase (AST), alanine aminotransferase (ALT) • Caspase 3	ZnO NPs induce histological and biochemical adverse effects on liver of adult male albino • Histopathological alterations in liver • AST and ALT ↑ • Caspase 3 ↑	[67]
	Oral	NA, 5, 50, and 300 mg/kg ZnO NPs	• Zn content analysis • Biochemical tests • Estimation of lipid peroxidation and ROS • Histological examination: H&E assay • TUNEL assay	• Organ weight • ZnO NPs concentration in liver • Biochemical: ALT, AST, alkaline phosphate (ALP) • MDA level and SOD, GPx activities • Liver histology	Lower doses of ZNP has more hepatotoxic effects on rats • Liver weight ↑ • ALT, AST, ALP ↑ • Oxidative stress • Histopathological alterations in liver: inflammation	[68]
	Intraperitoneal	35 nm; 2 mg/kg	• Histological and histochemical analysis: H&E, PAS, Mallory trichrome, reticulin stain, Prussian blue reaction	• Liver histology	Histological alterations in the liver of male Wistar albino rats • Inflammation	[69]
	Oral	10–30 nm; 100, 250, 500 mg/kg	• Histological and histochemical analysis: H&E, PAS, Mercuric • bromophenol blue staining	• Liver histology	Histological alterations in the liver of male Wistar albino rats • Inflammation	[70]
	Oral	NA, 100, 300, 600 mg/kg	• Hematologic and biochemical analyses • Measurements of cytokines and antioxidant ability • RNA extraction and RT-PCR • CYP450 assay • Histological examination: H&E	• Blood chemistry • Inflammatory cytokines (IFN-γ, TNF-α, and IL-12) • ALP and GGT • Gene expression: constitutive androstane receptor (CAR), pregnane X receptor (PXR), CYP450 1A2, CYP450 2C11, and CYP450 3A2 • CYP 1A2, 2C11, and 3A2 activity • Liver histology	ZnO nanoparticles showed adverse effects on rat liver • Alterations in the blood chemistry • ALP and GGT ↑ • Expression: CYP450 2C11 and 2A4 ↑ ; CYP450 3A2 ↓ • Activities : CYP450 2C11 and 2A4 ↓; Cyp450 3A2 activity remain unchanged • Histopathological alterations of liver	[71]
Fish	Suspension in water	100 nm; 0.5, 1.0, 1.5 mg/L	• ICP-MS • Biochemical assay • Histological examination • Comet assay	• LPO, SOD, CAT, GSH • Gills and liver histology	ZnO NPs induce toxic effect in the liver of tilapia. • Zn in liver ↑ • Histopathological alterations in liver • Oxidative stress	[72]
	Suspension in water	NA, 30, 50, and 70 ppm	• Morphology examination • Histological examination: H&E staining	• hepato somatic index (HIS) • Liver histology	ZnO NP caused histological abnormalities in liver tissues of tilapia • Morphological alteration in adult fish • HIS ↓ • Histopathological alterations	[73]

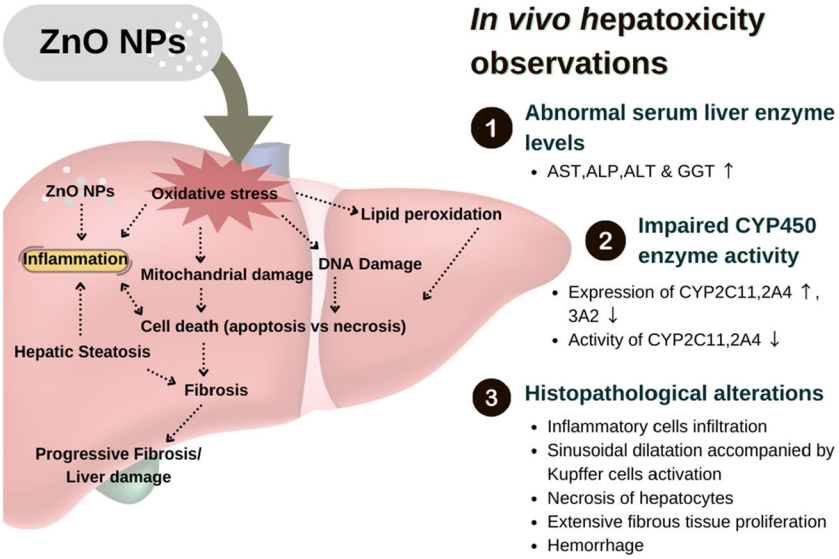

Fig. 3 Summary of ZnO NPs–induced hepatotoxicity

brain barrier (BBB) and translocate into the central nervous system (CNS) via the blood circulatory system. Prior studies have also shown that ZnO NPs could translocate into the CNS via the olfactory or taste nerve translocation pathway. Significant upregulation of Zn content in the nerves and brain tissues were reported in rats and mice exposed to ZnO NPs through tongue and intranasal instillation [78–80]. These accumulations of ZnO NPs in the brain subsequently caused neurotoxicity by inducing excessive production of oxidative stress, inflammatory response, as well as ultrastructure and histopathological alterations which potentially then lead to neurodegenerations and functional impairments of the brain. Increasing evidences suggested that the CNS was more vulnerable to the ZnO NPs–induced adverse effect owing to its high oxygen consumptions, low concentration of antioxidant enzymes, high content of unsaturated lipids, and restricted cellular regeneration of the neural tissue[81–83]. Previous studies found that the oral administration of ZnO NPs in rats resulted in enhanced oxidative and nitrosative stresses which are known to induce inflammation in the CNS by the activation of glial cells (astrocytes and microglial) and the release of pro-inflammatory mediators [78, 80]. Increased levels of TNF-α, IL-1β, and the NOS2 have been reported in animals treated with ZnO NPs [80, 84]. The increment of IL-1β in the brain was found to be involved in the development of acute neuronal lesions and neuronal cell death [83, 85]. These findings were consistent with a study performed by Aijie et al., who investigated the oxidative stress-related gene expression and immunological biomarkers of ZnO NPs–treated rats [79]. In this study, the upregulation of glial fibrillary acidic protein (GFAP) was reported in the ZnO NPs–treated rats. GFAP is an intermediate filament protein of the astrocytes and its upregulation is associated with astrocytosis and astrogliosis [86]. It's believed that this activation and subsequent accumulation of astrocytes in the CNS may impede the regeneration and growth of axons which undoubtedly lead to neurodegeneration in treated animal models [79, 87]. Moreover, high concentration of ZnO NPs was also shown to inactivate autophagy, leading to the formation of tau aggregates in the brain [88]. This elevation of tau in the brain is assumed to reflect axonal damage in the brain tissue [89]. To evaluate the neurotoxicity of ZnO NPs, the behavioral changes and alterations in cognitive functions have also been investigated using various animal models. Mice exposed to 250 and 500 mg/kg of ZnO NPs showed a deficit in normal motor functions. The motor behavioral tests used in this study included the beam balance, pole, and footprint tests [90]. Similar findings were observed in *Drosophila melanogaster*. It showed that the oral administration of ZnO NPs affected the neuromuscular coordination of the flies and caused impaired crawling ability [91]. Learning and memory impairment were also recorded in rats treated with 50 mg/kg of 50 nm sized ZnO NPs. Aijie et al. then further confirmed these behavioral performance results by examining the expression of learning and memory-related genes (*Sgk1*, *Drd2*, *Trappc4*, and, *Gnaq*) using qRT-PCR. However, contradicted results were also reported by Chuang et al., who found that there are no major alterations in the spatial cognition or learning ability of rats treated with ZnO NPs [88]. The neurotoxicity induced by ZnO NPs is summarized in Table 5 and Fig. 4.

2.5 Reproductive Toxicities

Although many studies focused on the effects of ZnO NPs on the primary organs that are exposed to ZnO NPs such as lungs, livers, and kidney, recently, the toxicity of ZnO NPs on the reproductive system has been increasingly reported in both in vitro and in vivo studies. The animal studies with regard to reproductive toxicity of ZnO NPs in recent years have been shown in Table 6. ZnO NPs may translocate and accumulate

Table 5 The animal studies of ZnO NPs–induced neurotoxicity conducted between 2015 and 2020

Animals	Type of exposure	Size and concentration	Method of detection	Parameters	Toxicities	Reference
Rats	Intraperitoneal	NA, 50, 200, 400 mg/kg	• H&E staining • Ultrastructure analysis: transmission electron microscope	• Brain and spinal cord histology	ZnO NPs exposure–induced ultrastructure and histopathological alterations in the brain and spinal cord in a dose depending manner.	[92]
	Oral	NA, 40 and 100 mg/kg	• Biochemical analysis • Cayman's superoxide dismutase assay • Measurement of nitrite • ELISA: competitive enzyme immunoassay and quantitative sandwich enzyme immunoassay • DNA fragmentation • Comet DNA assay	• SOD activity • Oxidative stress markers: MDA, GSH, CAT • Nitric oxide production • Brain levels of heat shock protein-70 (HSP-70) • Inflammatory markers: TNF-α and IL-1β • Apoptotic markers: Fas and caspase-3 • DNA damage markers	Recurrent oral exposure of ZnO NPs–induced neurotoxicity in Sixty Wistar albino rats. • Oxidative stress • Brain concentration of nitrites increased • HSP-70 ↑ • Inflammatory cytokines and apoptotic markers ↑ • DNA oxidation	[78]
	Tongue-instilled	50 nm; 50 mg/kg	• ICP-MS • TEM • Colorimetric assay • qRT-PCR • H&E staining • Immunohistochemistry assay • Morris water maze (MWM)	• [Zn] • Oxidative stress markers (SOD, MDA, GSH, GSH-Px, and GSH/GSSG) • Oxidative stress–related genes • Brain histology • Spatial learning and memory	ZnO NPs translocated into the brain via the taste nerve pathway and induced neurotoxicity. • [Zn] in taste nerves and brain tissues ↑ • Oxidative stress • GFAP expression ↑ • Histopathological alterations in brain • Significant deficits in behavioral performance	[79]
	Intranasal instillation	10-30 nm; 20 μg/g	• ICP-MS • Oxidative stress–related biomarker assay • ELISA • Transmission electron microscopy • H&E staining	• [Zn] in cerebral cortex, striatum, hippocampus, and olfactory bulb. • MDA and GSH levels • IL-1β and TNF-α levels • Ultrastructure of olfactory bulbs, hippocampus, striatum, and cerebral cortex • Hippocampus, striatum and cerebral cortex histology	ZnO NPs–induced neuronal toxicity in Wistar rats. • [Zn] in striatum, olfactory bulbs, hippocampus ,and cerebral cortex ↑ • Oxidative stress • IL-1β and TNF-α ↑ • Ultrastructural changes and histopathological alterations in olfactory bulbs, hippocampus, striatum, and cerebral cortex	[84]
	Tongue instillation and oral gavage	42.31 ± 17.9 4 nm; 50 mg/kg	• ICP-MS • qRT-PCR • ELISA • H&E staining • Immunohistochemical analysis	• [Zn] in brain and nerve tissue • Gene expression of TNF-α, IL-1β, IL-6, IL-10, IFNG, and NOS2 • Tongue and brain histology • Astrocyte and microglial cell activation	ZnO NPs–induced neuroinflammation via taste nerve translocation pathway • [Zn] in tongue, nerve, cerebellum, brain stem, cerebral cortex, hippocampus ↑; no significant changes in blood • TNF-α, IL-1β, and NOS2 ↑ • Histopathological and immunohistochemical alterations in the brain	[80]
	Intratracheal instillation	50 nm; 5 and 10 mg/mL	• Morris water maze • Elevated plus maze • Spectrophotometer and HPLC • Cytokine assay using Luminex • H&E staining • Immunohistochemistry assay	• [Zn] • DNA concentration and purity • DNA markers (8-NO_2Gua, 8-OHdG, 15N5-8-OHdG, and 13C3-8-NG)	Neurotoxicity was caused by acute pulmonary exposure to 380 ZnONP in rats. neurotoxicity was caused by acute pulmonary exposure to 380 ZnONP in rats.	[88]

Table 5 (continued)

Animals	Type of exposure	Size and concentration	Method of detection	Parameters	Toxicities	Reference
				• Inflammation markers (IL-1α, IL-1β, IL-6, and TNF-α) • Brain histology • Spatial cognition or learning abilities	neurotoxicity was caused by acute pulmonary exposure to 380 ZnONP in rats. acute exposure of 382 ZnONP could induce neurotoxicity Acute exposure of ZnONPs could induce neurotoxicity. • 8-OHdG/dG ↑ • No alterations in spatial cognition or learning abilities • IL-1β, IL-6 ↑ • Histopathological and immunohistochemical alterations in the brain	
–	Oral	30–40 nm; 250, 500, mg/kg	• Beam balance walking • Footprint test • Pole test • H&E staining	• Body weight • Behavioral assessment • Brain histology	ZnO NPs–induced neurotoxicity and leads to motor function impairments in Swiss albino mice. • Deficits in normal motor functions • Nuclear area of motor cortex area neurons enlarged	[90]
	Intraperitoneal	NA, 5.625×10^{-5}, 300 mg/kg	• ICP-MS • Open-field test • Elevated plus maze test • Forced swim test	• Brain mass and body weight • [Zn] in brain tissue • Behavioral assessment	ZnO NPs at environmentally relevant concentrations induced behavioral changes in Swiss mice. • Anxiogenic behavior of treated mice in open field test	[77]
Bees	Oral	NA, 0.8, mg/ml	• Feeding rate and survival assessment • Enzyme activity assay • BCA protein assay	• Stress level • Acetylcholinesterase (AchE) and glutathione S-transferase (GST) activity • Total protein content in brain	ZnO NPs are potentially neurotoxic to bees. • Total brain protein content ↓ • Survival of bees ↓ • AChE and GST activities increased	[93]
Flies	Oral	NA, 1, 10, 50, 100, 200, and 300 μg/ml	• Crawling assay	• Larval crawling behavior	ZnO NPs synthesized by both green and chemical methods induced behavioral changes in *Drosophila melanogaster*.	[91]

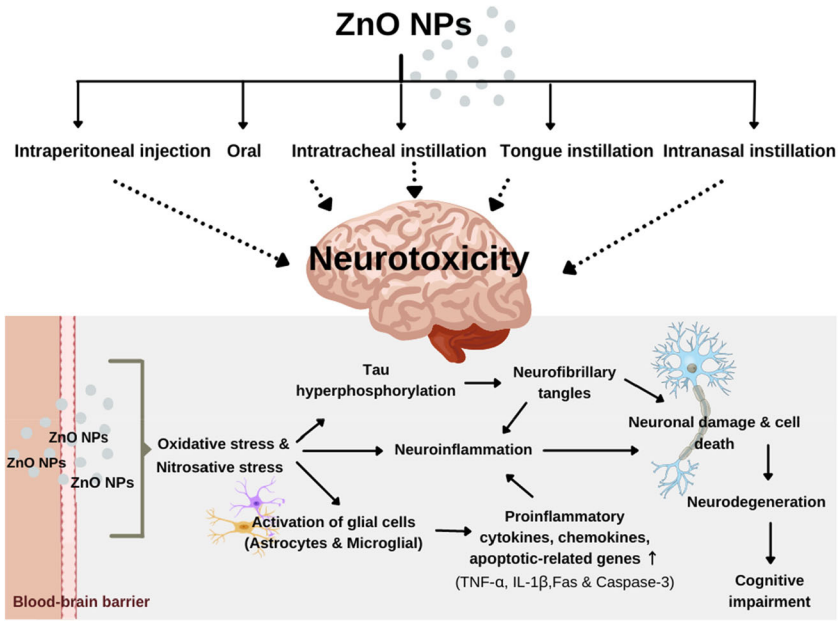

Fig. 4 Summary of ZnO NPs–induced neurotoxicity

in the reproductive organs such as ovary and testis, making them potential reprotoxicant. Pieces of evidences showed that ZnO NPs were able to bypass the blood testes barrier and accumulate in the epididymis and testis [97, 101]. This accumulation of ZnO NPs leads to testicular damages, spermatogenesis abnormalities, and impaired hormonal regulation. Histological examination is the most commonly used method to access the reproductive toxicities in animal studies. Mice and rats exposed to ZnO NPs exhibited histopathological alterations in the testis, in which germ cell loss and the reduction in the thickness and diameter of seminiferous tubules were observed [96, 97, 102, 103]. This histopathological observation in the testis agreed with the testicular weight change shown in Radhi et al. study. The decrement in the testis's weight illustrated the changes in seminiferous tubules [100]. Tang et al. demonstrated that an oral administration of 50 mg/kg ZnO NPs lead to the segregation of the seminiferous tubules, low sperm number in the lumen, and a random germ cell layer. All this suggested the presence of testicular atrophy. In mice treated with 150 mg/kg ZnO NPs, the seminiferous tubules were degraded and vacuolization of the Sertoli cells was observed; 450 mg/kg treatment of ZnO NPs caused necrosis of the seminiferous tubules with a low number of germ cells, and degeneration of spermatogenic cells and necrotic signs of shedding, inter-tubular edema and Leydig cells vacuolization[97]. Target cells of ZnO NPs toxicity namely Sertoli cells and Leydig cells play crucial roles in spermatogenesis and steroidogenesis respectively. Other than the vacuolization of the Sertoli cells, multinucleated spermatid cells were also spotted in Mozaffari et al. study [103]. Thus, ZnO NP–induced Sertoli cell damage eventually lead to decreased production of inhibin and spermatogenesis abnormalities, including the reduction of sperm number and quality. The damage of Leydig cells gives rise to the impairment of hormone production and regulation. However, these changes may not be identified using light microscopy in histological examination; hence, ELISA was carried out to quantify the serum hormone levels. Yousef et al. revealed that the treatment of ZnO NPs in rats could decrease levels of testosterone and thyroid-stimulating hormone (TSH), and increase the follicle stimulating hormone (FSH), luteinizing hormone (LH), tri-Iodothyronine (T3), and thyroxin (T4) levels [96] (Fig. 5).

The reproductive toxicity of ZnO NPs in the female reproductive system remains scarce. A study on the toxicity of ZnO NPs in female rats showed an increment in the ovary and uterus weight [109]. These weight changes correlated well with the histopathological observation in Hosseini et al. study [94]. In the histopathological evaluation of ZnO NPs–treated rats, atrophic ovary was observed with hyperemia, increased corpus luteum, follicular cysts, inflammatory cells infiltration, and fibrosis. The histological changes in the uterus included epithelial destruction and hyperplasia of endometrial glands. This morphological changes in the ovary were possibly due to the elevation of the circulating endogenous sex hormones namely estrogen and progesterone [94]. These sex hormones act directly on the uterus causing endometrial hyperplasia. The increase in the circulating endogenous sex hormones induces negative feedback control on the production and release of FSH and LH leading to ovary atrophy. A study on the hormonal alteration in ZnO NPs–treated fish have shown significant decrement in the serum levels of FSH and LH which disrupted the gonadal function of the fish. The impaired

Table 6 The animal studies of ZnO NPs–induced reproductive toxicity conducted between 2015 and 2020

Animals	Type of exposure	Size and concentration	Method of detection	Parameters	Toxicities	Reference
Rats	Intraperitoneal	10–30 nm; 4, 8, 25, 50, 100, and 200 mg/kg	• ELISA • H&E staining • MDA assay	• Serum hormones: estrogen and progesterone hormones • Ovaries and uterus histology • Ovary biochemistry: MDA	ZnO NPs caused adverse effect in the uterus and ovary of rats • Estrogen and progesterone ↑ in low doses and ↓ in a dose-dependent manner at high doses. • Histopathological alterations in ovary and uterus	[94]
	Oral	NA, 5, 10, 20, and 40 mg/kg	• ELISA	• Serum hormones: testosterone, LH, FSH, and inhibin B • Testes and pituitary gland histology	Altered in function of pituitary-testes axis • Testosterone and FSH hormones level ↑; LH and inhibin B level ↓ • Histopathological alterations in pituitary and testes	[95]
	Oral	100 nm;100 mg/kg	• RNA extraction and RT-PCR • DNA extraction and fragmentation • ELISA • TBARS assay • Griess test • Total antioxidant capacity colorimetric kit • Semen characterization • H&E staining	• Testicular gene expression: mtTFA and UCP2 • TNF-α, IL-6 • Semen characteristics • Plasma hormones: Testosterone, FSH, LH, TSH, T3, T4 • Plasma antioxidant enzymes and free radicals: GPX, GST, CAT, SOD, GSH, TBARS, NO, and TAC • Steroidogenic enzymes: 17β-hydroxysteroid dehydrogenase (17β-HSD) and 17-ketosteroid reductase (17-KSR) • Testes histology	Altered testicular architecture and caused fertility • Changing gene expression of proteins involved in the mitochondrial biogenesis and function (mtTFA and UCP2) • Induction of oxidative stress, lipid peroxidation, nitric oxide production, apoptotic, and necrotic cell death pathways in the testicular tissues	[96]
Mice	Oral	30 nm; 50, 150, and 450 mg/kg	• ELISA • RNA extraction and RT-PCR • Immunofluorescence assay • H&E staining	• Sperm count • Testes histology • Serum hormones: Testosterone • Total zinc in testis and epididymis • Gene expression in testes: BIP, XB1s, IRE1-α, JNK, C/EBP, CHOP, Bax, Bcl-2, caspase 3, caspase 12, StAR, and CYP450scc	Testicular damages and spermatogenesis abnormalities via activation of apoptosis and ER stress • Number of sperms in the epididymis and testosterone level in serum ↓ • Histopathological alterations in testes • ER stress associated gene ↑ • Caspase 3 and caspase 12 ↑ • StAR ↓	[97]
	Oral	50 nm; 100 and 200 mg/kg	• Hancock staining method [98] • Krzanowska method [99]	• Weight and of testicles, epididymis (head and tail), seminal vesicles and prostates • Sperm quality	Increasing concentrations of ZnO-NPs and durations of exposure leads to negative effects on the male reproductive efficiency of albino mice • Body weight ↓ • Weight of testes and epididymis (head and tail) ↓ • Weight of seminal vesicle and prostate ↑ • Sperm abnormalities	[100]
	Intragastric	50 nm; 40, 80, and 160 mg/kg	• Graphite furnace atom absorption spectrophotometry • ELISA • Eosin-nigrosin staining • Bicinchoninic acid assay • Benzoic acid colorimetric assay • Ammonium molybdate assay • Thiobarbituric acid (TBA) colorimetric assay • H&E staining	• Zn contents in the livers, testes, and serum • Blood glucose and blood lipid: TG, TC, HDL, LDL • Serum hormones: FSH, LH, testosterone, estradiol • Semen quality • T-SOD, GSH-Px, CAT, H_2O_2, MDA • Testes histology	ZnO NPs affect antioxidant system in the liver and testes and induce lipid metabolism disorder, hyperlipidemia, and reproductive toxicity to male mice. • Semen quality ↓ • FSH, LH, testosterone, estradiol ↓ • Oxidative stress	[101]
	Intravenous	70 nm; 1.0 and 5.0 mg/kg	• Eosin staining	• Testes histology	Structural alterations in the seminiferous epithelium and sperm abnormalities	[102]

Table 6 (continued)

Animals	Type of exposure	Size and concentration	Method of detection	Parameters	Toxicities	Reference
	Intraperitoneal	NA, 250, 500, and 700 mg/kg	• H&E staining	• Testes histology	Histopathological alterations in testes	[103]
Hen	Oral	NA, 25, 50, 100 mg/kg	• Transmission electron microscopy (TEM) and energy disperse spectroscopy (EDS) • Inductively coupled plasma atomic emission spectroscopy (ICP-OES) • ELISA • RNA-seq transcript profiling • mRNA qRT-PCR • Immunofluorescent staining	• Zn contents in ovary • Tissue mineral element: Zn, Cu, Mn, K, Fe, Mg, Ca, P • AST/GOT and ALT/GPT • Plasma hormones: estrogen and progesterone • Gene expression of neuron factors in the pubertal hen ovary tissue • Protein expression of neuron factors: NCAM1, DXC, ROBO1, SEMA3O • Population of neuroendocrine cells	ZnO NPs negatively affect ovarian developmental at puberty. • Ovary organ index ↓ • Concentration of essential elements Zn, Fe, K, Ca, and Mn in ovaries ↑ • Altered gene and protein expression of neuron factors in the pubertal hen ovary tissue • Altered the population of neuroendocrine cells	[104]
Fish	Suspension in water	Zero to 9.5 μ/l	• Sub-lethal exposure experiment • ELISA • RNA extraction and cDNA synthesis • RT-PCR	• Lethal concentration (LC50) • Serum hormone: fish GH, TSH, ACTH, FSH, LH, T3, T4, estradiol, testosterone, insulin and glucagon • Gene expression: GH, IGF-1, insulin and insulin receptor A	Hormonal and molecular alterations in fish • Serum GH, TSH, T3, T4, FSH, LH, E2, testosterone and insulin hormones ↓; ACTH and cortisol levels ↑; no change in glucagone levels. • GH, IGF-1, insulin and insulin receptor A gene ↓	[105]
	Suspension in water	50 nm, 0, 50, and 100 μg/L	• qPCR • Bradford assay • Enzyme immunoassay • ICP-OES spectrometry	• Gene expression of transcription factor: dmrt1, activin B, dax1, foxl2, ad4bp/sf-1, wnt5, and wnt4; steroidogenic enzyme: 20b-hsd and cyp19a1 • antioxidant enzymes: CAT, SOD, and GST • Serum hormones: Testosterone and 11-ketotestosterone • testis histology	Adverse effect of Zn on the testis function in common carp • Gene expression of transcription factor and steroidogenic enzyme ↓ • Serum hormones ↓ • Antioxidant enzymes ↑ • Histopathological alterations in testes	[106]
C. elegans	Suspension in medium	0.4, 4, and 40 mg Zn/L	• Total RNA extraction and RT-PCR • Apoptosis assay and SYTO 12 staining	• Germ cell apoptosis • Gene expression: ced-13, ced-3, ced-4, ced-9, cep-1, dpl-1, efl-1, efl2, egl-1, egl-38, lin-35, pax-2, and sir-2.1	ZnO NP exposure promotes apoptosis in germ cells • Number of apoptotic germ cell ↑ • cep-1, efl-2, egl-1, lin-35, and sir-2.1 ↑	[107]
Water flea	Suspension in water	0.04, 0.08, 0.16, 0.32, 0.64, 1.29, 2.57 mg Zn/l	• RNA extraction • Microarray • Hierarchical clustering analysis	• Gene expression: *p*-element somatic isoform c	Downregulation of a gene coding for *p*-element somatic isoform c	[108]

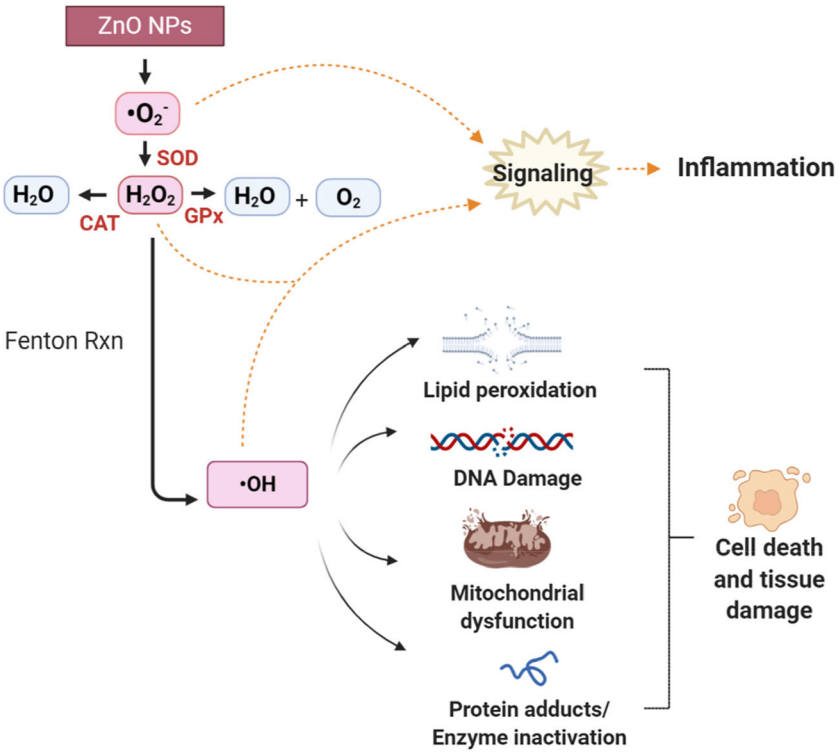

Fig. 5 The mechanism of ZnO NPs–induced toxicity

function of gonad was portrayed by the downregulation of the estradiol and testosterone levels [105].

2.6 Developmental Toxicities

Developmental toxicity is the adverse effect that interfere the development of an organism as a result of parental exposure to xenobiotics [110, 111]. It can be then further divide into teratogenicity and embryotoxicity [112].

During the process of pregnancy, the placenta serves as a protective barrier that protects the fetus from potentially invasive and harmful agents. However, Feng et al. found that the repeated exposure of ZnO NPs in mice dams lead to a significant increase in the accumulation of Zn in various organs of their offspring. This indicated the presence of maternal absorption and placental transfer of Zn. The placental transfer of the ZnO NPs has been suggested to be size-dependent. Teng et al. revealed that upon the exposure of ZnO NPs, the accumulation in the placenta, fetal accumulation was only observed when exposed to 13 nm but not 54 nm ZnO NPs. In another study, the exposure of 540 mg/kg ZnO NPs caused an increment in the placental and fetal Zn content, whereas no significant difference was observed in the 60 mg/kg, 20 mg/kg treatment, and control group [113] Additionally, ZnO NPs were also known to cause placenta dysfunction which is highly correlated with the fetal growth restriction and miscarriage. Chen et al. has shown that the oral administration of ZnO NPs resulted in damage of placental structure, decrease in spongiotrophoblast layer and impairment of placental function. This consequently lead to the fetal growth restriction, and a decrement in the fetal number [114]. As shown in Table 6, evidences had shown that the maternal exposure of ZnO NPs gave rise to the neurotoxicity, behavioral abnormalities, reproductive toxicity, and liver dysfunction of the offspring. Apart from the maternal exposure, Manzo et al. demonstrated that paternal exposure of ZnO NPs in sea urchin, induced DNA damage in the sperm cell, affecting the development of the deriving offspring even at lowest concentration (1 mg/kg food)[115].

The embryotoxicity of NPs in vivo are usually studied in zebrafish. Zebrafish embryos are optically transparent and developed externally; hence, the development statuses can be observed throughout the experiment using simple microscopic techniques. Additionally, the development process of vertebrates is highly similar, making the zebrafish development largely comparable with that of the mammalians. Previous studies widely used classification of morphological alterations as well as genetic and molecular technologies to evaluate the toxicity of ZnO NPs in zebrafish embryos. A study on the toxicity of ZnO NPs on zebrafish embryos showed that ZnO NPs caused mortality, impaired cardiovascular activity, retardation of hatching embryos, and morphological alterations in the zebrafish embryo. This morphological alterations involved pericardial edema and notochord bending [116]. Other morphological changes that have been observed in zebrafish embryo due to ZnO NPs exposure included shortened body length, hyperaemia, yolk sac edema, tail deformity and spinal curvature [117–119]. Similar results were also reported when

Table 7 The animal studies of ZnO NPs–induced developmental toxicity conducted between 2015 and 2020

Animals	Type of exposure	Size and Concentration	Method of detection	Parameters	Toxicities	Reference
Teratogenicity						
Rats	Intragastric	50 nm;500 mg/kg	• Histopathological examinations: H&E staining • ICP–MS elemental analysis • TEM analysis • Biochemical parameter assay • Total RNA extraction and RT-PCR • MWM test	• Body and organ weights • [Zn] in tissues of offspring • Brain histology • Immunohistochemistry: Ki-67, TUNEL, and 8-OhdG • Ultrastructure observation • Antioxidant in the brain: MDA, SOD, GSH-PX • Gene expression: SOD1, Fmo2, Txnip, Gsr, Keap1, Krit1, Gstt1, Alox12b • Spatial learning and memory	Prenatal exposure of ZnO NPs causes impaired learning and memory capabilities in rats. • [Zn] in offspring brain ↑ • Abnormal neuron ultrastructure • Histopathological alterations in brain of offspring • Oxidative stress • Alterations of gene expression of oxidative stress-related genes • Slowing of learning abilities and deteriorated memory in ZnO NP-exposed rats.	[122]
	Intravenous	NA, 2, 10, and 30 mg/kg	• Prenatal development toxicity study (OECD 414 protocol) • Plasma and serum isolation and analysis • Histopathological examination (H&E staining) • Zn analysis (ICP-MS analysis)	• Hematological parameters: WBC, RBC, HGB, HCT, MCV, MCH, MCHC, RET, NEU, LYM, MON, EOS, BAS, LUC, RDW, HDW, PLT • Serum parameters: GLU, BUN, CREA, TP, ALB, A/G, AST, ALT, TBIL, ALP, TCHO, TG, Ca, IP, K, CK, PL, Na, Cl • *Corpora lutea*, implantations, resorptions, dead fetuses, aborts, mortality, female-to-male ratio, pre and post implantation lost • Uterine and body weights of dam • Body weight of fetuses and placenta weight • Livers, kidneys, and lungs histology • Skeletal or visceral examination.	ZnO NPs caused adverse effect on the pregnant rats and embryo-fetal development • Alterations in the hematology and serum biochemistry in a dose-dependent manner • Histopathological abnormalities in the kidney, liver, and lung of dams. • Total post implantation lost ↑; ↓ body weight of fetus • Zinc levels in liver of fetuses ↑	[123]
Mice	Intravenous	30 nm; 16 mg/kg	• Isolation and culture ovary of mouse embryos • Isolation and culture germinal vesicle (GV)-intact oocyte • Immunofluorescent assay of fetal oocyte cytospreads • Ovary immunostaining • TEM and SEM • Western blot • qRT-PCR	• Number and body weight of fetuses • Morphologies and percentage of leptotene, zygotene, pachytene, and diplotene oocytes • Gene in ovarian tissue: Scp1, Scp3, Dazl, Rad51, Rec8 • Genes encoding proteins typical of meiotic oocytes: MVH, Dazl, Scp1, Rad51, Spo11 • Fetal oocyte cytospreads: chromosome (SCP3 stained) and DNA damage (expression of γH2AX) • BAX, BCL-2 • Number of oocytes and primordial follicles in the ovary and percentage of follicles • Mvh and Lhx8 mRNA levels • Percentage and rates of germinal vesicle breakdown (GVBD), PBE (polar body exclusion) and spinal defects • Number of fetuses and sex ratios	ZnO NPs affect affect pre- and post-natal oogenesis in vivo • Number and body weight of fetuses ↓ • DNA oxidation • Number of MVH positive oocytes ↓ • Oocyte specific proteins MVH, NOBOX, and LHX8 and the transcripts Mvh and Lhx8 in these ovaries ↓ • Number of oocyte and primordial follicles ↓ • Normal meiotic maturation capabilities of oocytes	[124]
	Oral	30 ± 10 nm; 20, 60, 180, and 540 mg/kg	• ICP-AES analysis • Histopathological examination (H&E staining) • Hematological analysis • Serum hormone analysis • RT-qPCR • IHC	• Hematological and organ coefficient; body weight growth of marternal mice • ZnO NPs distribution in the uterus, placenta, and fetus • Fetal body and tail length, fetal and placental weight, fetal number and malformation rate • Placental histology • Gene related to oxidative stress: Gclc and HO-1 • Gene related to *ER* stress*: eif2α, ATF4, Chop, JNK*	ZnO NPs could induce dam injury and fetal growth restriction through oxide stress and ER stress • Body weight ↓; the organ coefficient of thymus ↓ • White blood cells, mean corpuscular hemoglobin concentration, and platelet counts ↑; Red blood cell ↓ • Zn content in uterus, placenta and fetus of dam ↑ • Weight and number of fetuses ↓ • Histopathological change in placenta	[114]

Table 7 (continued)

Animals	Type of exposure	Size and Concentration	Method of detection	Parameters	Toxicities	Reference
				• Apoptotic related marker: *Caspase 3 and 12, Bcl-2* • Growth factors: *eNOS, VEGF, IGFR1* • Gene related to glucose transport	• Oxidative stress related gene ↓ • ER stress and apoptotic ↑ • Anti-apoptotic gene, growth factors (*eNOS and VEGF*) ↓; *IGFR1* ↑	
	Oral	13.2 ± 3.7 nm, 57.1 ± 4.1 nm; 200 mg/kg	• Maternal systemic toxicity analysis • Fetal developmental toxicity analysis • ICP-MS analysis • Histopathological examination (H&E) • ELISA	• Stability of ZnO particles in gastric fluid (dissolution rate) • Body weight, organ coefficient and biodistribution in maternal organ (Intestine, liver, lung, heart, kidney, spleen) • Zn content in placenta and fetus • Placenta coefficient and the levels of cytokines (VEGF, PGF, sFlt-1) • Morphological examination • Placenta histology	Small ZnO NPs can enter fetal circulation system by crossing both the intestinal barrier and placental barrier leading to fetal development toxicities • Zn content in maternal organ ↑ • Zn content in fetuses ↑ • Placenta dysfunction • VEGF and PGF ↓; sFlt-1 ↑ • Histopathological alterations in placenta	[113]
Chicken	Oral	30 nm; 200 mg/kg	• Transmission electron microscopy (TEM) and energy disperse spectroscopy analysis • Western blot • Immunofluorescent staining	• Hen body weight and egg production • Zn content in liver, ovary and yolk • Fertilization rate and embryonic developmental failure rate • De-phosphorylation enzymes: PP4C, PP4R3β, PP4R2, PP2ACα, and PP2A • NF-κB, tumor necrosis factor(A20) and γ-H2A • Protein levels of PCNA and apoptosis markers	ZnO NPs treatment inhibited embryo development following oocyte stage exposure, and the γ-H2AX and NF-κB pathways involve in the inhibition of embryo development. • No significant change in body weight and egg production of hens • Egg yolk lipid content ↓ • Embryonic developmental failure rate ↑ • De-phosphorylation enzymes ↑ • NF-κB and γ-H2AX ↓	[125]
Sea urchin	Oral	14 nm, 100 nm; 1 mg and 10 mg Zn/kg	• Cytotoxicity (neutral red assay) • Genotoxicity (comet assay) • Ecotoxicity analyses (assessment of fertilization and offspring quality)	• Evaluation of offspring quality: skeletal and/or gut malformations, pre-larval arrest and retarded larvae • DNA damage of sperm cells and fertilization process	ZnO NPs provoked adult echinoids damages to immune cells and transmissible effects to offspring • Malformation of larvae • DNA damage of sperms cells, but no significant effect on the fertilization process	[115]
Embryotoxicity						
Fish	Suspension in water	20–30 nm; 0.01, 0.1, 1, and 10 mg/L ZnO NPs	• Fish embryo acute toxicity test (FET) • Total RNA extraction • RNA labeling and microarray • qRT-PCR	• Embryonic malformations • LC25 • Gene expression: *aicda, cyb5d1, edar, intl2, ogfr12, tnfsf13b* and *beta actin*	ZnO NPs affect genes related to inflammation and the immune system, resulting in yolk sac edema and pericardia edema in embryonic/larval developmental stages. • Overexpression of *ogfrl2* and *cyb5d1* genes	[119]
	Suspension in medium	30znm, 10, 30, 60, 90, or 120 mg/L	• AO staining and flow-cytometric analysis • Biochemical analysis • Measurement of ROS and singlet oxygen • ELISA • Single-cell electrophoresis • RT-PCR • Protein extraction and western blotting analysis	• Hatching rate and embryonic heart rate • Biochemical analysis: SOD, MDA • Gene expression: genes encoding antioxidant proteins (*sod1, cat, gpx1a, and pparα*) genes and protein related to apoptosis (*bax, bcl-2, apaf1, puma, cytochrome c*) • Apoptotic cells • Caspase 3 and caspase 9 activities • Mitochondrial membrane potential • DNA damage (OTM)	ZnO NPs increase production of ROS which activate apoptosis pathway mediated by mitochondria and caspases. • Rate of embryo hatching ↓; heart rate ↑ • Genes encoding for antioxidant proteins ↑ • Apoptotic cells ↑ • Altered regulations of pro-apoptotic and anti-apoptotic genes • Cytochrome c release ↓ • Mitochondrial potential ↓ • Activation of caspase 3 and caspase 9	[126]
	Suspension in water	NA, 0.1, 1, and 10 mg/L	• Embryotoxicity experiment	• Embryo mortality • Heart rate, percent total hatching success (%), hatching time, malformation percentage (%) and characteristics of newly hatched larvae	ZnO NPs induce embryotoxicity to marine medaka • Mortality, heart rate and malformation percentage ↑ • Total hatching success ↓, hatching time ↑ • Deformities in larvae	[120]
	Suspension in water	NA, 0, 10, 25, 50, 100, 250 μg/ml	• Embryo and larvae cytotoxicity assays	• LC50 • Hatching rate and heart rate	ZnO NPs induce in vivo cytotoxicity to zebrafish • LC50, hatching rate and heart rate ↓	[116]

Table 7 (continued)

Animals	Type of exposure	Size and Concentration	Method of detection	Parameters	Toxicities	Reference
			• Examination of cellular ROS • Acridine orange staining for apoptosis analysis.	• Morphological deformities and organ malfunctions • ROS and apoptotic cells	• Deformities in exposed embryos • Apoptotic cells in trunk and tail region of larvae ↑	
	Suspension in water	NA, 1, 10, 25, or 50 mg/L	• Embryo toxicity test • H&E staining	• LC50 and EC50 • Hatching rate • Embryo histology	ZnO NPs caused malformations in marine fish by affecting embryonic growth and development. • Hatching inhibition and lethality ↑ • Histopathological alterations in embryos	[127]
	Suspension in water	20–30 nm;100 mg/L	• Danio rerio embryo toxicity test (DarT) • Early-life stage test (ELS) • Gene expression Danio rerio embryo test (Gene DarT): RNA extraction and RT-PCR	• LC50 and LC10 • ELS survival rates • Gene expression: *ogfrl2, cyb5d1, intl2*	Alteration in the expression of ZnO NP-specific gene • Mortality ↑ • Gene alterations	[128]
	Suspension in water	NA, 0.01, 0.1, 1.0, 5.0, 10.0, 20.0, 30.0, 40.0, and 50.0 mg/dm3	• OECD 236 method protocol	• Mortality: LC 10, 20, 50 • Number of hatched embryos and hatching rate • Heartbeat rate • Retardation of hatching the embryos: EC 10, 20, 50	ZnO NPs negatively affects Danio rerio embryogenesis • Heartbeat rate ↑ • Mortality of embryos is the highest at 10 mg/dm3 • Retardation of hatching the embryos at 0.012 ± 0.001 mg/dm3	[129]
Sea urchins	Suspension in water	14 nm,100 nm; 0.1, 0.5, 1, 3, 10, 30 μM	• Sea urchin bioassay • Cytogenetic endpoints • Embryological endpoints	• Mitotic index and presence of chromosomal aberrations • Developmental abnormalities • LC50 and EC50	ZnO NPs is cytotoxic to sea urchin embryos • ZnO NPs interfere with cell cycle • Percentage of normal larvae (N) ↓ • Morphological changes in larvae	[130]
Frog		63 ± 29 nm and 334 ± 208 nm; 1, 10, 50, and 100 mg/L	• FETAX assay • SOD assay • Light and electron microscopy analyses	• Malformation percentage and growth retardation • SOD activity • Small intestine histology	ZnONPs produced toxicity in *X. laevis* embryos • Malformations	[131]
Chicken		< 50 nm; 50 μg/ml	• Alizarin red staining • Whole-mount embryo and cell immunostaining • RNA sequencing (RNA-seq) transcript profiling • RNA isolation and qPCR • Dihydroethidium staining	• Craniofacial skeleton development • Inflammatory markers and oxidative stress markers (TLR4, MYD88, IL-2, Nrf2, HO-1, HQO1, Cat, GLXR, NOS, GAPDH)	ZnO NPs–induced oxidative stress restricts cranial neural crest development during chicken embryogenesis • suppressed production and migration of cranial neural crest cells (CNCCs) • Defects of craniofacial skeleton development • Activated TLR4/NF-κB signaling pathway in CNCCs • Apoptosis of CNCC • Oxidative stress ↑	[121]

marine fish and Medeka fish were used to evaluate the embryotoxicity of ZnO NPs. Cong et al. observed an increment in the mortality, heart rate, deformities, and hatching retardation of the Medeka fish embryos exposed to ZnO NPs [120]. In addition to the conventional embryo toxicity test, genetic technologies have also been employed to study the toxicities of ZnO NPs in the fish embryos. Choi et al. suggested that gene expression test was a better alternative to the early-life stage (ELS) test in evaluating the ZnO NPs–induced embryotoxicity [119]. The expressions of potential marker genes for ZnO NPs including opioid growth factor receptor-like 2 (*ogfrl2*), cytochrome b5 domain-containing 1 (*cyb5d1*), and intelectin 2 (*intl2*) were investigated. The ZnO NP *cyb5d1* lowest observed effect concentration (LOEC) was 0.001 mg/L while the LOEC of ELS was 0.016 mg/L. Gene expression *D. rerio* embryo test showed a 16 times lower LOEC than that of the ELS, suggesting that it could be a better alternative when the NPs concentration used is too low to show visible toxicity [119]. Other than zebrafishes, chicken embryo were also used to study the the toxic effects of ZnO NPs. It was observed that ZnO NPs significantly increased the incidence of shortened or curved coracoids as well as defect in the craniofacial skeleton and parietal bone in chicken embryos. These observations indicated that ZnO NPs treatment increased the incidence of craniofacial dysplasia in chicken embryos [121]. The embryotoxicity of ZnO NPs using animal models have been summarized in Table 7.

3 Oxidative Stress as an Underlying Mechanism for ZnO NPs–induced Toxicity

In the past decade, there has been a vast amount of literature designed to unveil the possible underlying mechanisms of ZnO NPs–induced toxicity, yet the exact details and the full picture of their interplay remain obscure. Among the several mechanisms proposed, ROS production and consequent oxidative stress has been the most documented underlying mechanism responsible for ZnO NPs toxicities, which is discussed in more detailed below. Saliani et al. ascribed this oxidative stress to the combination of the following events: (1) generation of ROS on the particle's surface, (2) dissolution and release of Zn^{2+} ions, and (3) the physical interaction of ZnO NPs with the biological molecules[132]. Figure 1 has summarized the how ZnO NPs contributed to organ toxicity via ROS.

ROS are oxygen-containing radicals generated by mitochondria as a metabolic byproduct, which serve as a signaling molecules in many crucial cellular activities such as gene transcription, signaling transduction, and immune response [133, 134]. However, in high concentrations, ROS was identified to be the main culprit for the occurrence of oxidative stress in the cells, tissues, and organs. In the animal studies, the levels of glutathione (GSH) as well as the expression and activity of the major antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPx), glucatalase (CAT) were quantified as oxidative stress biomarkers [135]. SOD is one of the antioxidant enzymes that catalyze the dismutation of superoxide into oxygen and to the less reactive peroxide (H_2O_2) which can be then neutralize by CAT and GPx [136–138]. These antioxidant enzymes regulate the concentration of the ROS in the cells and limit the overproduction of hydroxyl radicals and other ROS. The physiological imbalance between the antioxidants and ROS consequently leads to oxidative stress and damage of essential biomolecules such as RNA or DNA, protein, and lipids, which inevitably results in cellular damage and cell death[139, 140]. Aijie *et al.* identified that the tongue instillation of ZnO NPs in rats caused a significant reduction in the brain *SOD, CAT, and GPx* levels. In the same study, the authors revealed that ZnO NPs modulated the expression oxidative stress related markers (*Gsr, Nqo1, Fmo2*, and *SOD2*) in the brain of ZnO-NP-treated rats [79]. Similarly, depletion in SOD and CAT were observed in rats orally exposed to ZnO NPs for 7 consecutive days [78]. Quite a number of experimentations using animal models suggested that ZnO NPs might disrupt the redox homeostasis leading to oxidative stress and a cascade of ROS mediated toxicity [61, 68, 72, 78, 79, 84, 117, 122, 141, 142].

Lipid peroxidation is an oxidation process in which ROS attack the phospholipid or unsaturated fatty acids in the cellular membrane leading to the alteration in the membrane fluidity and permeability as well as impairment of membrane bound enzymes [2, 143, 144]. It has been demonstrated that the lipid peroxidation results in the formation of conjugated diene hydroperoxides and unstable substances that disintegrate into various aldehydes which may serve as biomarkers in quantifying the oxidative stress [145]. Malondialdehyde (MDA) is known as the most mutagenic lipid peroxidation end product, which could react with nucleosides to form adducts that may induce mutations, strand breaks, cell cycle arrest and eventually apoptosis, contributing to the development of inflammation and associated disease[146, 147]. Prior research showed ZnO NPs increased the MDA levels in treated animals[50, 61, 68, 78, 79, 84, 101, 122, 142]. This increase in the MDA levels of treated glass clover snail (*Monacha cartusiana*) did not return to normal levels even after recover period [142].

The excessive generation of ROS as a result of ZnO NPs exposure may end to oxidative damages on the DNA. It is known that the HO• radicals are highly reactive with the deoxyribose backbone and all bases, thereby triggering DNA strand breakage, nucleotide oxidation, and formation of DNA adduct, which all can ultimately lead to mutagenicity and carcinogenecity [148]. Elevation of the phosphorylated histone H2AX (γH2AX) has been documented, indicating the presence of DNA strand breaks in ZnO NPs–treated animals [125, 149, 150]. These observations were in agreement

with studies from other groups that highlighted the potential of ZnO NPs in inducing DNA damages. A study using rats model revealed that prenatal exposure of 500 mg/kg of ZnO NPs significantly increased the 8-hydroxy-2-deoxyguanosine (8-OHdG) levels in the brains of the offspring [122]. 8-OHdG has been long used as a biomarkers for oxidative DNA [151]. This production of 8-OHdG has shown correlation with the development of cancer and degenerative diseases due to its ability to induce GC-TA mispairing mutations [152]. Apart from this, high levels of micronuclei formation were observed in the peripheral blood and blood marrow cells of mice treated with ZnO NPs [150]. These DNA damages may trigger apoptosis via the activation of the p53 pathway and subsequently lead to tissue damages [96, 153, 154].

Early animal experimentation by Zhao et al. proved that the ZnO NPs profoundly depleted the mitochondrial membrane potential ($\Delta\Psi m$) of exposed zebrafishes [126]. This finding was consistent with research from Yousef et al., who demonstrated the impairment of mitochondrial biogenesis and function loss as a result of the enhancement of the mitochondrial uncoupling protein 2 (UCP2) expression and suppression of mitochondrial transcription factor A (mtTFA) in rats treated with 100 mg/kg ZnO NPs[96]. These ZnO NPs–induced mitochondrial dysfunctions did not only enhance the production of excessive ROS but also upregulated the Bax levels and activated the caspase 12, caspase 9, and caspase 3, while downregulating the Bcl-2 levels [67, 78, 117, 126, 155, 156]. The assessments of the Bcl-2/Bax and caspases expression in these animal investigations implied that ZnO NPs could provoke apoptotic cell death via the intrinsic mitochondrial mediated apoptotic signaling pathway in response to oxidative stress.

In addition to the oxidation of biomolecules, ZnO NPs could also cause damaging effect on organs by implication of oxidative stress mediated inflammation. In fact, the ZnO NPs–induced oxidative stress and inflammation acted concertedly in accentuating progressive damage in the tissues or organs. Biswas et al. lucidly depicted the interdependence between oxidative stress and inflammation in their review. The authors proposed that oxidative stress contributed to the development of inflammation whereas inflammation might lead to the production of ROS which would further exaggerate oxidative stress [157]. Thus, both oxidative stress and inflammation have been often simultaneously reported in experimentations pertaining the adverse effects of ZnO NPs in various organs [78, 84]. In animal models, ZnO NPs–induced inflammations in various organs by recruiting inflammatory cells and triggering the pro-inflammatory cytokines and mediators in resident tissue cells, via the mitogen-activated protein kinase (MAPK) and NF-κB signal transduction pathway [46, 53, 69, 71, 158]. It should be specially noted that these cytokines and chemokines generated largely depend on the tissue type due to the difference in the types and numbers of the resident immune cells in the tissues. Hence, the immunomodulatory changes which result in tissue damages may vary among different tissue types [159].

4 Factors Affecting the Toxicities of ZnO NPs in *In vivo* Studies

4.1 Physicochemical Properties

It is well documented fact that the physicochemical properties of ZnO NPs significantly influence their toxicity. Khare et al. showed that the NPs uptake, released of Zn ions, oxidative stress induction, as well as the growth, behavior, and reproduction of the *Caenorhabditis elegans* (*C. elegans*), were all highly influenced by the size of ZnO NPs. The study also demonstrated that the smaller sized ZnO NPs possessed higher surface area, hence faster release of Zn^{2+}. This, in turn, affected the NPs uptake by the cells, causing the modulation of the pro-apoptotic and anti-apoptotic gene expression following a size-dependent manner. The pro-apoptotic genes *cep-1, egl-1, ced-13, ced-4, ced-3* was significantly upregulated with decreasing particle size, while the anti-apoptotic gene *ced-9* was downregulated only in worms treated with 21 nm ZnO NPs. As a result, the smaller sized ZnO NPs showed higher toxicity towards *C.elegans* than those of bigger ones at the same concentration [160]. This was in line with Kaya et al. study, in which the oral administration of 10-30 nm ZnO NPs showed a threefold inhibition of gill Na^+, K^+-ATPase activity leading to higher osmoregulatory damages as compared to tilapia fish (*Oreochromis niloticus*) exposed to 100 nm ZnO NPs[161]. Sergey et al. assessed the toxicity of Sergey et al. ZnO NPs with size 10–30 nm and 80–200 nm using larvae of the *Artemia salina* (brine shrimp). The result obtained agrees with the studies mentioned above, in which smaller ZnO NPs possed higher toxicity in brine shrimp [162]. Moreover, the maternal-fetal transfer and fetal developmental toxicity of ZnO NPs were also proven to be size-dependent. ZnO NPs of 13 nm were reported to cause fetal developmental toxicity following oral administration in pregnant mice since they were small enough to bypass the intestinal and placental barrier, while these observations were not found in pregnant mice treated with 57 nm ZnO NPs or bulk ZnO [113]. However, contrary results were reported by Zhou et al., in which there was no significant difference in the mortality of zebrafish exposed to ZnO NPs with the size ranging from 4 to 70 nm [163].

The shape of the ZnO NPs is known to be another one of the major determinants of their toxicity. Different shapes of ZnO NPs have been reported including one-dimensional nanorods nanoring, nanohelix, nanocombs, nanowires, and nanodisk, two-dimensional nanosheets, nanopellets, and nanoplates, as well as three-dimensional nanoflowers,

nanomesosphere and nanourchins [164]. According to Hua et al., ZnO nanorods induced higher toxic effects in zebrafish embryos compared to the ZnO nanosphere and ZnO cuboidal submicron particles. ZnO nanosphere and ZnO cuboidal submicron particles were showed to form larger aggregates in water resulting in lower surface area of exposure and thus lower toxicity [165].

Besides, the surface charge also plays a vital role in determining ZnO NPs toxicity, as shown by Park et al. The histopathological examinations in their study revealed that both positively and negatively charged ZnO NPs resulted in gastric inflammatory and degenerative lesions, however, higher degree lesions were observed in rats orally treated with positively charged ZnOSM20(+) [166, 167]. Taken together, the physicochemical characterization of ZnO NPs is generally of high importance in, providing comparable data as well as better understanding of the correlation between the physicochemical properties and their induced toxicity.

4.2 Nanofabrication

Presently, the advancement in nanotechnology allows the precise synthesis of nanoparticles with adjustable properties for specific applications. A myriad of techniques and approaches have been employed for use in the fabrication of ZnO NPs, each with different obtainable physicochemical properties and subsequent applicability and toxicity. These techniques could be subdivided into bottom-up and top-down methods, which can be then further classified into physical methods, chemical methods, green synthesis or biological methods, and microfluidic reactor-based methods. A notable example of the effect of nanofabrication on the toxicity of ZnO NPs was demonstrated by Shubha et al. who compared the toxicity of green hydrothermally synthesized ZnO NPs (PeZnO NPs) with commercially available ZnO NPs (DZnO NPs). The effects of DZnO NPs and PeZnO NPs on silkworms were assessed, with the larvae death rate of 96.67 % and 26.27%, respectively at the highest tested concentration of 0.032 mg/ml. Meanwhile, DZnO NPs were also shown to cause 10.33% pupae growth inhibition, even at least concentration, indicating its higher toxic effect on the growth and development of the larvae. It is evident from this study that green synthesized nanoparticles possessed lower toxicity than those that are chemically synthesized [168]. Similarly, in work by Parvathi et al., differently synthesized ZnO NPs, (chemical precipitation, green chemistry approach, and commercially available) have been tested for their genotoxicity, immunotoxicity, and organ toxicity in zebrafish. Among all the ZnO NPs, the commercially available ZnO NPs incite the highest toxicity while the ZnO NPs synthesized using neem leaves (*Azadirachta indica*) as precursor material induced the lowest toxicity in zebrafish models on the comparison. The higher toxicity levels in the physically and chemically synthesized ZnO NPs can be ascribed to the toxic or hazardous chemicals used, as these chemicals may reside in the NPs.

On the other hand, it was hypothesized that surface modification during the nanofabrication of ZnO NPs can reduce their toxicity for use in biomedical applications. Dehkourdi et al. coated ZnO NPs with polyethylene glycol (PEG) (PEG-ZnO NPs) and tested them on 84 male rats. It was concluded that the ZnO NPs showed greater effects on the kidney function of the rats as compared to the PEG-ZnO NPs. Elevation of creatine, BUN, and uric acid was observed in rats intraperitoneally treated with ZnO NPs; however, there is no significant increase of these biochemical markers by the PEG-ZnO NPs [169]. Alternatively, in an intriguing study, Xia et al. showed how iron doping affects the toxicity of ZnO NPs. The authors tested the iron-doped ZnO NPs (Fe-doped ZnO NPs) on rodent lung and zebrafish embryos, which resulted in a reduction of pulmonary inflammation in rodents as well as improved hatching rates in zebrafish. These results imply that iron doping could reduce the degree of toxicity of ZnO NPs as it changes the particle-matrix and slows down the dissolution of Zn^{2+} [170].

4.3 Biological Factors

It should be noted that the in vivo toxicity of ZnO NPs does not only depend on its physicochemical properties but also on the organism related biological factors such as species, age, gender, and size of the tested animals. According to Brown et al., the animal's susceptibility to ZnO NPs toxicity varied among different species. It was demonstrated that *Daphnia magna* (water flea) were of higher sensitivity to ZnO NPs–induced toxicity than *Lytechinus variegatus* (sea urchins) [171]. This interspecies difference in response to ZnO NPs was then further confirmed by Vimercati et al. The authors investigated the toxicity of ZnO NPs in two marine crustacean species and revealed that the LC50 of ZnO NPs is considerably higher in amphipod *Corophium insidiosum* (1.75 ± 0.39 mg/L) as compared to copepod *Tigriopus fulvus* (0.60 ± 0.06 mg/L) [172]. These interspecies differences may be accredited to the variation in the anatomy and physiology among different species, which, greatly impact the toxicokinetics (absorption, distribution, metabolism, and excretion) as well as tissue injury and repair mechanism of the species tested [173].

Wei et al. highlighted the importance of the animal's life stage in evaluating the toxicity of ZnO NPs. They found out that the hepatotoxic effect of ZnO NPs was only observed in aged mice but not in young mice. Wei et al. attributed this to the age-related increase in oxidative stress and inflammation as well as the higher absorption of NPs in aged mice due to the age-related increase in intestinal permeability [174]. Since aging is regarded as the greatest factor regulating neuronal plasticity, it is critical to understand the possible age-related neurotoxicity of NPs. The developing and aging nervous system

may not be equally susceptible to NPs as the adults [175, 176]. A comparison of the adverse effects of ZnO NPs on adult (6 months old) and old (18 months old) mice has shown that old individuals are more susceptible to ZnO NPs–induced neurotoxicity. A more substantial memory loss was reported in the ZnO NPs–treated old mice. This was not only due to the greater nerve tissue injury in the hippocampus but also due to the suppression of cAMP/CREB signaling in an age-dependent manner, which was then further downregulated after ZnO NPs exposure[177]. Hence, the age or life stage of the animals should be taken into consideration in the evaluation and application of ZnO NPs, to prevent underlying risk.

5 An Alternative to In vivo Studies in ZnO NPs toxicity: *In silico* Computational Approaches for Predicting Nanotoxicity of ZnO NPs

In silico computational approaches have sparked considerable interest in the field of nanotoxicology lately. The recent advancement of these computational approaches makes it possible to predict the risk and safety of NPs while reducing the cost, time, and test animals needed in evaluating the toxicity profiles of these NPs. The in silico computational approaches used in the prediction of the toxicity of NPs could be classified classified as ligand-based (LB) and structure-based (SB).

One structure-based approach involves the successful use of molecular docking to elucidate the possible interaction of nanoparticles with biological macromolecules such as proteins. These interactions of NPs and proteins are known to cause physiological changes resulting in various pathological implications. To gain a better understanding on the mechanism of ROS alteration mediated by ZnO NPs, Verma et al. investigated the possible interaction of ZnO NPs with sod1 protein, a known regulator of oxidative stress using in silico molecular docking approach. The result obtained showed a good correlation with their in vivo experimental results, ZnO NPs were predicted to form hydrophobic and hydrogen bonds with the sod1 protein leading to alteration in the sod1 protein configuration which significantly downregulates its function [116]. ZnO NPs were also reported to interact strongly with the active sites of chemokine and cytological proteins such as MAPK, NF-kB, and matrix metallopeptidase-9. Singh et al. showed that ZnO NP has strong binding affinity with these proteins with docking energies − 8.81, − 7.64, and − 7.27 Kcal/mol, respectively, forming hydrogen, metal acceptor, and electrostatic bonds [178]. The strong interaction of ZnO NPs on the activation site of these proteins inexorably inhibits the chemokines and cytological proteins. Molecular docking could also be paired with molecular dynamics to study the time-dependent dynamic behavior of a molecular arrangements, providing imperative information on the internal motions and conformational changes of a protein [179]. The molecular dynamic stimulation for the interaction of ZnO NPs with insulin showed that ZnO NPs unfold insulin and change its secondary structure by converting its helix structure to random coil [180]. Hossienzadeh et al., in order to improve the predictive power of molecular dynamics, coupled replica–exchange method with molecular dynamics, to study the interaction of ZnO NPs with insulin [157]. Replica exchange molecular dynamics (REMD) is an approach that is designed to overcome the local minima problem found in the conventional molecular dynamic stimulation of insulin [158].

Alternatively, quantitative structure-activity relationship (QSAR) approach have been used to assess the safety and toxicity of NPs. QSAR is a ligand-based approach that correlates the chemical structure and physicochemical properties of a NP with their biological activity using mathematical statistics and machine learning methods [181, 182]. The basic workflow of the approach includes the construction NPs library as well as quantum chemistry calculations, followed by predictive modeling, in which the QSAR model is developed, mechanism interpretation and experimental validation [183]. Cao et al. successfully established nano-QSAR models to predict the acute cytotoxicity of 21 kinds of MeO NPs in human lung adenocarcinoma cells (A549 cells). All these models showed high prediction accuracy, in which the LC50 of ZnO NPs predicted using the these models (3.02 $molL^{-1}$ in model I, 3.27 $molL^{-1}$ in model II, 3.27 $molL^{-1}$ in model III, and 3.23 $molL^{-1}$ in model IV) were close to that of 3.21 $molL^{-1}$ obtained from the in vitro experimentation. Cao et al. characterized the MeONPs and build the nano-QSAR models by employing a total of six physicochemical parameters to the improved SMILES-based optimal descriptors. The study revealed that individual size and aggregation size were the key descriptors affecting the cytotoxicity of MeO NPs in the A549 cells, followed by cationic charge and zeta potential [184]. In the QSAR models by Zhou et al., the cytotoxic effects of 17 metal oxides (MeO) NPs including ZnO NPs in *Escherichia coli* were studied using six molecular descriptors. The authors adopted multiple linear regression (MLR) and support vector machine (SVM) methods, respectively, to build both linear and non-linear QSAR model that possessed great predictive performance and stability [185]. Most of the QSAR models published uses cell death as the toxicological end point. However, it is discernible from the above-mentioned studies that MeO NPs may cause implications other than cell death such as inducing inflammatory cells and triggering the pro-inflammatory cytokines and mediators. Hence, in a more recent study, Huang et al. developed a QSAR model for prediction of inflammatory potential of MeO NPs based on their physicochemical properties, with predictive accuracy exceeding 90%. The result of the QSAR model based on IL-1β demonstrated that the metal electronegativity, ζ-potential, and cation charge are the key intrinsic physicochemical properties affecting the inflammatory potential of MeO NPs [183].

Although the results of the aforementioned studies seem promising, yet, it is notably that each of these in silico approaches have its advantages and limitations, hence it should not be use as a standalone method for nanotoxicological prediction.

6 Limitations of Current Studies on ZnO NPs Toxicity And Future Directions

1. Stress were introduced to the animal models during nanotoxicity testing. Bailey et al. showed that the inherent, multi-facted stress introduced to the animal models may negatively impact the well-being of the animals, both psychologically and physiologically. Significant elevations of heart rate, blood pressure, and hormone levels were observed indicating fear, stress, and distress in terrestrial vertebrates in laboratories [186]. These might in turn lead to misleading indication of hazard and compromise the scientific quality of the data.
2. The dosage of ZnO NPs required to produce toxic effects in animals may never be reached in humans. It was estimated that the ZnO NP environmental concentrations range from 0.001 to 0.058 μg/L in surface waters and from 0.24 to 0.661 μg/kg in soil [187]. The ultrahigh ZnO NPs concentration used in these animal models may lead to unrealistic results that cannot be extrapolated in human scenario since usually only small amount of ZnO NPs is needed in the diagnostic and therapeutic interventions. Moreover, to date, the studies that evaluate the adverse effects of ZnO NPs at environmental concentration remain scarce. Hence, future studies in the ZnO NPs–induced nanotoxicity should rely more on the realistic NPs dosage.
3. Lack of uniformity in the current nano-toxicological studies. The nanotoxicity of the ZnO NPs is affected by various factors including the routes of exposure, physiochemical properties, and environment, making the comparison between the data of different studies difficult.
4. The transferability of data from these animal studies to human remains unclear. Primarily due to the complexity and the variability of the biological system. A study showed that the exposure of 540 mg/kg ZnO NPs caused an increment in the placental and fetal Zn content, whereas no significant difference was observed in the 60 mg/kg, 20 mg/kg, and control group [114]. However, it is difficult to relate these findings with the placenta toxicity of ZnO NPs in humans due to the difference in the placental physiology and anatomy. The rodent's placenta consists of three trophoblastic layers, while there is only one layer in human placenta [188]. Hence, NPs may bridge through the placenta even at low dose. Moreover, most of the animal models used in these studies constitute a homogenous (genetic) populations, the differences in the response to ZnO NPs due to the high genetic diversity in humans may not be predicted using these animal models.
5. Most of these studies only focus on the short-term effects of ZnO NPs. The long-term effects of ZnO NPs should be emphasized in future studies to better understand the toxicological profiles of ZnO NPs.
6. The lack of knowledge in the mechanism of the ZnO NPs–induced toxicity is the main impediment in the use of nanoparticles in the biomedical field. More attention should be paid to the efforts in investigating the actual mechanism of the ZnO NPs–induced toxicity and ways to control these adverse effects to allow the production of ZnO NPs that can be safely applied in food industry or biomedical field.

7 Conclusion

In conclusion, nanotechnology offers the potential of ZnO NPs for various biomedical applications such as cancer therapy, drug delivery, gene delivery, and tumor imaging. As a result, the safety and toxicity of ZnO NPs have become a matter of increased public attention. In this review, we showed that the ZnO NPs could exert adverse effects in various vital organs (lungs, kidney, liver, CNS, reproductive system, and fetal development) of animal models. However, the ZnO NPs–induced toxicity is multifactorial and their exact mechanism for toxic effect in these organs remains unclear. Taking everything into account, the upsurge in demand for ZnO NPs due to its expanding utilization in various field with the constrained information concerning their impacts on human wellbeing firmly proposed that extra studies are ought to be performed, including further investigations that correlates the in vivo, in vitro, and in silico data.

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Declarations

Ethics approval We furthermore declare that there is no ethical issue in our experiments.

Consent to participate This article does not contain any studies with human participants or animals performed by any of the authors.

Consent for publication This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest The authors declare that they have no conflict of interest.

Research Involving Humans and Animals Statement None.

Informed Consent None.

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