

## Review Article

# The Exposure and Hazards of Zinc Oxide Nanoparticles: *In Vitro* and *In Vivo* Studies



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## ABSTRACT

**Background:** Extensive application of zinc oxide nanoparticles has increased the likelihood of its release into the environment and subsequent human exposure and toxicity. The toxicity is thought to be a combined effect of intracellular particles and the release of dissolved zinc ions.

**Objectives:** This review outlines the possible mechanisms of zinc oxide toxicity in biological organs through *in vitro* and *in vivo* experiments.

**Methods:** We reviewed articles published between 2001 and 2021. In this way, we did a manual search of Google Scholar and scientific databases, including PubMed, Web of Science, Scopus, and Embase, with keywords such as “zinc oxide nanoparticles”, “toxicity mechanism”, and “*in vivo* and *in vitro* studies”. The other qualified papers contained the history of identifying zinc oxide nanoparticles, the toxicity of metallic nanoparticles, and physical, chemical, and biological side effects with topical and systematic approaches.

**Results:** The main mechanism suggested for zinc-based nanoparticles-induced cell damage is via the induction of increased levels of reactive oxygen species, which are oxidative stress markers. This mechanism has also been found to be a key mechanism for the cytotoxicity of other metal nanomaterials. Zinc-based nanoparticles were found to induce oxidative DNA damage, inflammation, progressive degenerative cell changes, cell cycle arrest, cytogenetic alterations, and ROS-triggered mitochondria-mediated apoptosis in human organs.

**Conclusion:** This review sheds light on the full understanding of *in vitro* and *in vivo* toxicity assessment of zinc oxide nanoparticles, highlighting the health concerns from the perspective of ZnO nanoparticles release to the ecosystem after their increasing application.

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## Introduction

**T**oday, with the increasing development of nanotechnology, the environmental accumulation of nanomaterials and the exposure of humans to these nanoparticles are unavoidable [1]. Safety evaluation or toxicity assessment of various synthesized nanoparticles have been reported in different studies [2-6]. With the gradual increase in the use of zinc oxide nanoparticles in biomedical fields, its subsequent release in the environment and direct exposure to the human body is inevitable. Despite the widespread use of zinc oxide nanoparticles, the safety of this nanomaterial for humans is still unclear. The reported side effects of zinc-based nanoparticles have cast doubts about their use [7]. The current state of knowledge concerning the application of zinc oxide nanoparticles in the biomedical field is still in the preliminary stage. Despite numerous studies on zinc toxicity, there is a lack of knowledge on the characteristics and effects of zinc-based nanoparticles [1, 7]. Due to their small size and larger surface area, zinc oxide nanoparticles have a higher penetration capacity into the human body than metallic zinc [7]. These compounds can have desirable biological activities, such as carrying capacity for biomedical treatment, penetrating cellular barriers to drug delivery, and undesirable side effects, such as cytotoxicity, induction of oxidative stress, cell dysfunction, or a combination of both [8].

Numerous studies have reported the toxicity, free radical production, and induction of programmed cell death due to the high amount of zinc nanoparticles in environments and living organisms [8, 9]. The two main ways of adsorption of the nanoparticles in living cells are active adsorption by endocytosis and passive adsorption through diffusion. The process of cell uptake, the site of accumulation within the cell, and the ability of nanoparticles to produce toxic effects depend on their size, chemical properties, electric charge, crystallization, shape, and solubility [10].

The toxicity of these nanoparticles can be evaluated through *in vitro* studies using cells or cell lines or *in vivo* exposure to experimental animal models [11, 12]. Laboratory studies are usually the first step in measuring the toxicity of nanoparticles. However, cell culture cannot fully assess the complexity and relationships between components of biological matrices in an organism since this category can record events difficult to investigate in laboratory research scales. Therefore, more *in vivo* studies are needed.

This review aims to provide a critical summary of recent scientific literature on the potentially hazardous effects of zinc oxide nanoparticles and information on the possible mechanisms of these nanoparticle-induced toxicity in *in vitro* and *in vivo* models.

## Evidence Acquisition

To gather relevant information, a review of published studies reporting mechanisms of action of zinc oxide nanoparticles-induced toxicity in *in vitro* and *in vivo* models in Scopus, PubMed, and Google Scholar databases was undertaken. Keywords like “Zinc oxide nanoparticles”, “toxicity”, and “*in vitro* and *in vivo* experiments” were used. In this review, the most important published literature of the last 10 years (2011 to 2021), related to *in vivo* and *in vitro* evaluations of organ toxicity to zinc oxide nanoparticles using animal models, were collected, analyzed, and discussed. In addition, the information related to the possible mechanisms of toxicity caused by zinc oxide nanoparticles, along with the influencing variables in this process, was summarized.

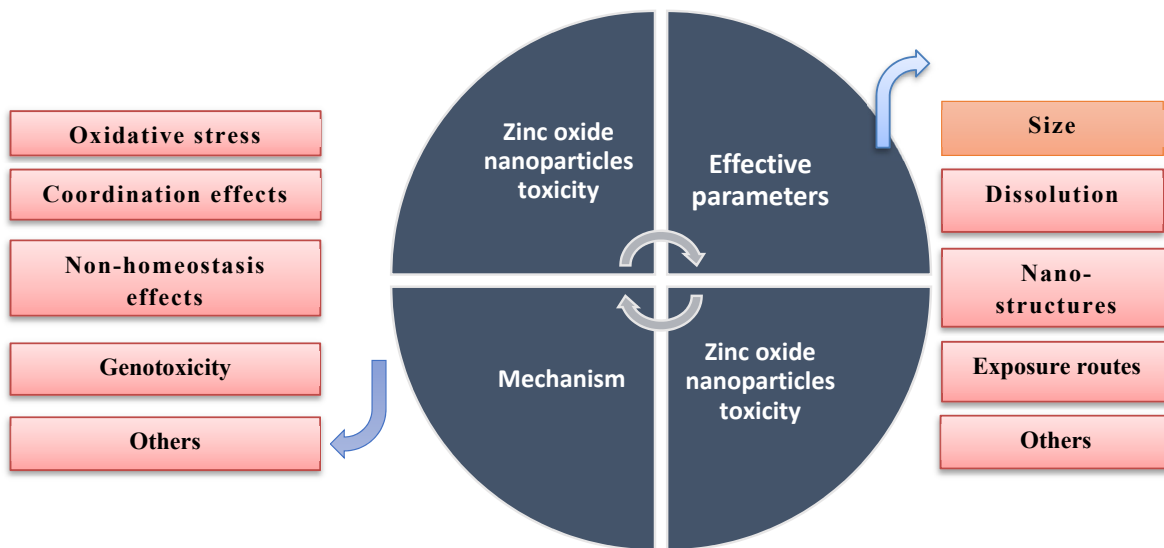
## Zinc Oxide Nanoparticles

Among the metal oxide nanoparticles, zinc oxide nanoparticles have received considerable attention largely due to their various uses in the biomedical field—their enormous potential in disease diagnostics and monitoring. However, the increasing use of zinc nanoparticles has led to their release into the environment, and the toxicity of these compounds on organisms has become a concern in the biomedical research community [13]. The research conducted in this field shows that releasing zinc oxide nanoparticles may negatively affect organisms and the environment [13] (Figure 1).

## Toxic Mechanisms of Zinc Oxide Nanoparticles

Zinc oxide nanoparticles are among the most important and multifunctional compounds. The ability of zinc oxide nanoparticles to release zinc is an important parameter from a risk assessment perspective [14]. Recent studies have shown that zinc oxide nanoparticles can be toxic to humans due to oxidative stress [14, 15], but the details and the full picture of their interplay have remained obscure. Figure 2 shows the possible role of zinc oxide nanoparticles in causing toxicity through reactive oxygen species (ROS) in biological organs.

Mitochondria produce ROS as a by-product of metabolism, and they react as transport molecules in many important cellular activities such as gene transcription,

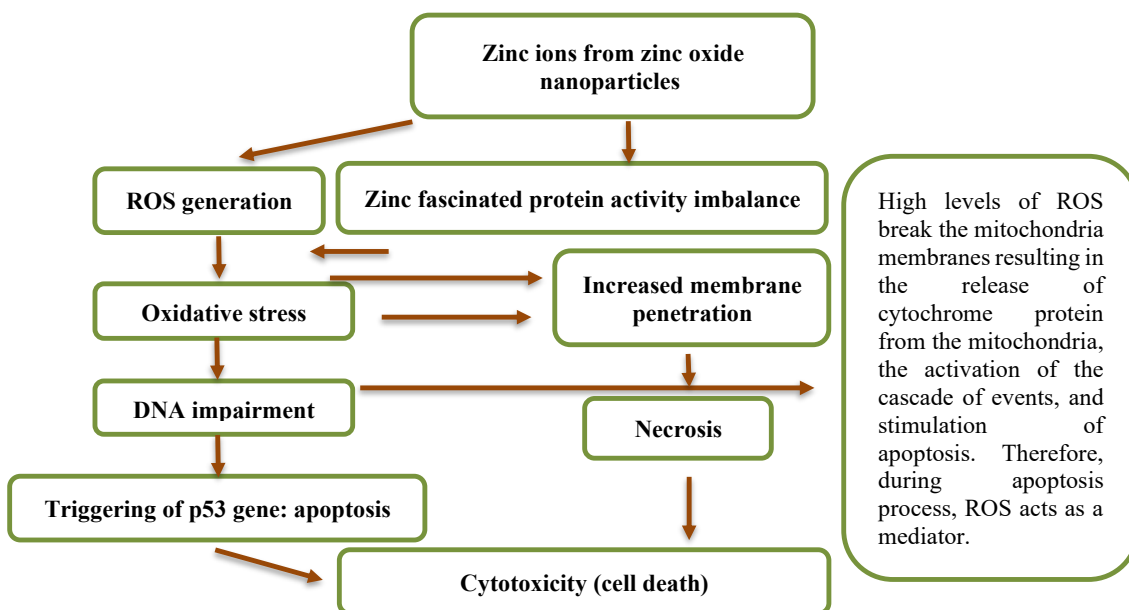


**Figure 1.** Schematic overview summarizing the toxic effects of zinc oxide nanoparticles

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signal transduction, and the immune response [11]. Furthermore, ROS has been suggested as an underlying reason for proliferative effects observed in human leukemia cells at low zinc concentrations [10]. Oxidative stress is a disorder of the oxidant-antioxidant balance, which disrupts normal cellular functions and leads to cytotoxicity or DNA damage [7]. Oxidant-antioxidant imbalance can result from a lack of antioxidant capacity due to impaired production and distribution of reactive oxygen species or their excess in relation to other factors. These events may cause oxidative damage to DNA, increasing the chromosomal aberrations associated with cell de-

formation [7]. Oxidative stress and its damage indicate an association between different forms of chronic liver damage [8]. In a study, the toxic effects of zinc-based nanoparticles in adult mice showed that high concentrations of these nanoparticles cause the cells to use different enzymes such as superoxide dismutase, catalase, glutathione peroxidase, and glutathione S-transferase to remove ROS [9]. Superoxide dismutase can convert internal oxygen radicals into  $H_2O_2$ . In addition, catalase and glutathione peroxidase enzymes can reduce oxygenated water to water and oxygen. Therefore, superoxide dismutase, catalase, and glutathione peroxidase can keep



**Figure 2.** Cytotoxicity mechanism of zinc oxide nanoparticles

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oxygen levels low and prevent cell poisoning [9]. The excessive generation of various deleterious ROS like hydrogen peroxide, hydroxyl radical species, nitric oxide or superoxide anion as a result of zinc nanoparticles exposure may result in oxidative damages on DNA, RNA, proteins, and so on.

In general, zinc-based nanoparticles were found to induce oxidative DNA damage, inflammation, cell degeneration, cell cycle arrest, cytogenetic alterations, and apoptosis. Cytotoxicity studies have shown that zinc-based nanoparticles have dose- and time-dependent cytotoxic potential, genotoxicity, and carcinogenicity [9, 10].

### In vitro toxicity studies

The in vitro studies on the toxicity of zinc oxide nanoparticles confirmed the induction of diverse adverse effects in pulmonary-blood pathways and central nervous system-derived cell toxicities [14]. This process can be related to excessive dissolved ionic  $Zn^{2+}$  in the culture medium or inside cells, leading to oxidative stress, cytotoxicity, and mitochondrial dysfunction [15, 16]. In this line, Kao et al. investigated an olfactory bulb-brain translocation pathway for zinc oxide nanoparticles in rodent cells in vitro and in vivo conditions. They concluded that an olfactory bulb-brain translocation pathway for airborne zinc oxide nanoparticles exists in rats and that endocytosis is required for the interneuron translocation of these particles [15]. Coating zinc oxide nanoparticles with polymeric compounds may reduce cytotoxicity effects and ROS generation in WIL2-NS cell lines in humans [15]. Further studies on in vitro exposure to zinc oxide nanoparticles are summarized in Table 1. In many references, zinc oxide nanoparticles are also considered the most toxic nanoparticles with the lowest  $LD_{50}$  value among the other metal oxide nanoparticles. In this line, Cao et al. evaluated zinc oxide nanomaterials' developmental and neurotoxic effects with different shapes/sizes compared to zebrafish and SH-SY5Y cells. They found the  $LD_{50}$  of treated zebrafish larvae with zinc oxide nanoparticles was 11  $\mu\text{g}/\text{mL}$  for 144 h [10]. The  $LD_{50}$  values were calculated to compare the toxicity of three zinc oxide nanomaterials. These values for short and long zinc oxide nanorods were estimated at 15.4  $\mu\text{g}/\text{mL}$  and 17  $\mu\text{g}/\text{mL}$ , respectively. In another study, Ng et al. investigated the acute toxicological effects of zinc oxide nanoparticles in mice after intratracheal instillation. Their results also showed that the  $LD_{50}$  in the intratracheal instillation of zinc oxide nanoparticles (48 nm) was 493.85  $\mu\text{g}/\text{kg}$  body weight [11].

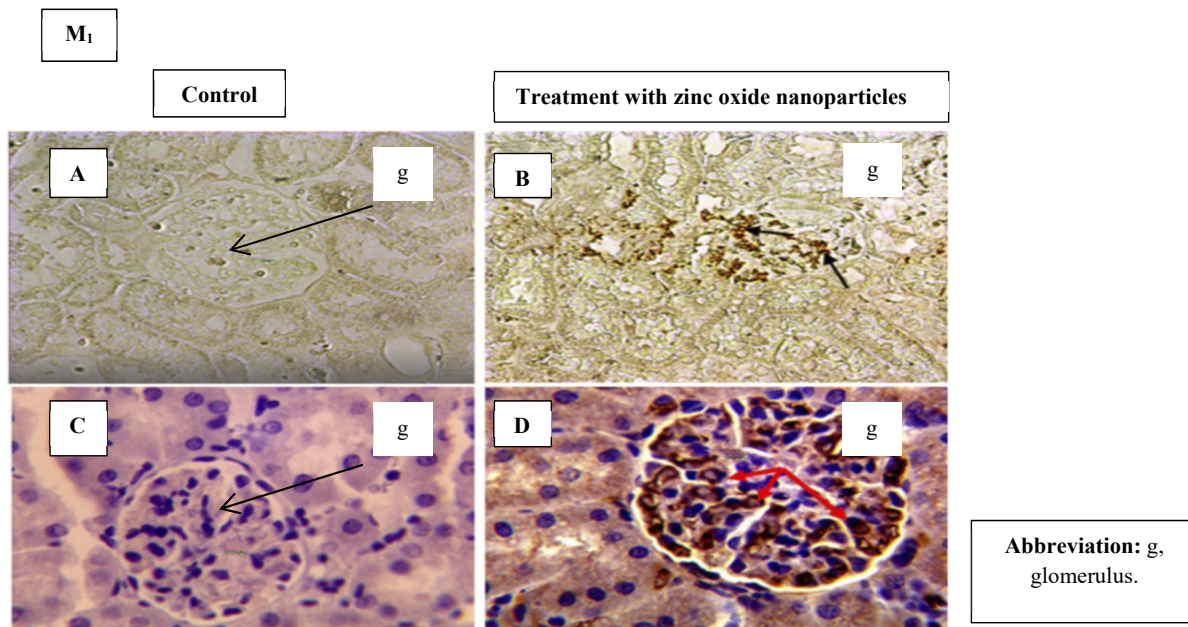
### In vivo toxicity studies

Toxicology results after exposure to zinc oxide nanoparticles are different and depend on various parameters [17, 18]. Particle penetration is mainly determined by their physicochemical properties, especially particle size. The zinc oxide nanoparticles toxicity increases malondialdehyde and glutathione S-transferase activity and decreases total antioxidant levels in the blood serum and liver of treated rats. Finally, this process leads to the formation of free radicals and an increase in the rate of oxidative stress [10]. Therefore, the induction of enzymatic antioxidant defense after zinc oxide nanoparticles exposure can be a successful adaptive response. It is a compensatory mechanism that enables the cell to overcome the damage. Other inflammatory responses and immune system activation are also commonly suggested in biochemical observations after exposure to zinc oxide nanoparticles. Detrimental health effects were also observed through intraperitoneally administrated zinc oxide nanoparticles in rats. Most symptoms were non-specific toxicity and caused more nerve damage in the caudate nucleus and hippocampus [11]. The treated rats with zinc oxide nanoparticles also showed different tissue changes, including vacuolation and pyknosis in renal tubular epithelial cells and glomerular damage. Zinc oxide nanoparticles caused ultrastructural changes in the proximal convoluted tubule of renal tubules and certain glomerular cells. These particles significantly increased cell apoptosis in rat kidneys (Figure 3) [12].

In this study, the size of zinc oxide nanoparticles was about 20 nm in diameter, which could facilitate their entrance into cells. Zinc oxide nanoparticles with a size less than 20 nm have been shown to accumulate in the liver and spleen of rats, while zinc oxide nanoparticles of larger size accumulate in the kidney [12]. In a study, Amara et al. investigated behavioral performances and the brain contents of some monoamine neurotransmitters with zinc oxide nanoparticles exposure. The results revealed that acute intravenous injection of zinc oxide nanoparticles does not affect neurotransmitter contents, locomotor activity, or spatial working memory in adult rats [17].

Pasupuleti et al. evaluated the toxicity effects of zinc oxide nanoparticles through the oral route [18]. Their experiments aimed to determine the importance of the affected dose (5, 50, 300, 1000, and 2000 mg/kg body weight) by comparing zinc oxide's acute oral toxicological potential in dimensions (20 nm) to its micro size. In the nano-dimensions of zinc oxide, microscopic lesions in the liver, pancreas, heart, and stomach were more





**Figure 3.** M<sub>1</sub>: Light micrographs of apoptotic cells in the glomerulus of zinc oxide nanoparticles-treated rat kidney; A) Control group, negative tunnel reaction; B) Zinc oxide nanoparticles group, positive tunnel reaction (Arrows); C) Control group, no positive P<sub>33</sub> reaction; D) Zinc oxide nanoparticles group, positive P<sub>33</sub> reaction (Arrows). The image was taken from Yousef MI et al. study with permission from Elsevier [12].

frequent in lower and higher doses. However, the incidence of the above lesions was higher in mice treated with higher doses of zinc oxide in micro dimensions. In this study, nano-sized zinc oxide showed more toxicity in lower doses. Therefore, they concluded that future nano-scale toxicological research should focus on the importance of dose exposure criteria [18]. The other effects of in vivo exposure to zinc oxide nanoparticles are summarized in Table 1.

## Discussion

One of the main risks of application and exposure to zinc oxide nanoparticles is the dissolution and release of Zn<sup>2+</sup> ions that are toxic to cells [18]. Among the effective cytotoxic effects of zinc oxide nanoparticles, we can mention the increase in ROS production along with lipid peroxidation, genotoxicity, and induction of inflammatory pathways [11]. The review of the published articles in this field shows that increased oxidative stress is associated with decreased antioxidant activity in the studied models. This problem is justified by lower levels of glutathione and total antioxidant capacity and inhibiting the activity of antioxidant enzymes such as superoxide dismutase, catalase, glutathione peroxidase, and glutathione-S-transferase [12, 19, 20]. Zinc oxide nanoparticles have been reported to interact with mammalian intracellular enzymes. These nanoparticles may disrupt these

antioxidants' defense mechanisms through ROS production. The development of this process leads to inflammatory response, disruption, and destruction of mitochondria. Finally, we will see apoptosis in human liver cells, lung epithelial cells, and even some cancer cells [22, 23, 27]. In a controlled environment (in vitro conditions), inflammatory status has been confirmed in many studies by higher circulatory levels of tumor necrosis factor-alpha (TNF-α) and interleukin (IL)-6 in the treated rats with zinc oxide nanoparticles at both mRNA and protein levels. Identifying zinc oxide nanoparticles as external particles by immune cells may produce ROS, reactive nitrogen species (RNS), signaling pathway disturbance, and altered cytokine levels [23, 27, 28].

Zinc oxide nanoparticle toxicity is affected by different factors, including the routes of exposure, physio-chemical properties, and environmental conditions, making comparing the data of various studies difficult. Environmental conditions in any organ may affect the toxicity potential of nanoparticles. For instance, the acidic environment in lung lining fluid may dissolve zinc oxide (1 and 5 mg/kg of body weight), leading to the transient increase in the concentration of Zn<sup>2+</sup> ions and local toxicity. Their solubility determines zinc oxide nanoparticles' toxicity. In lung injury, the main cause of zinc oxide nanoparticle-induced toxicity is pH-dependent dissolution inside phagosomes [12, 22].

**Table 1.** In vitro/in vivo toxicity effects of zinc oxide nanoparticles

Size(nm) Model	Concentrations	Exposure Time	Outcomes, Reference
<50 Rat PC-12 and human SH-SY5Y neuronal cells	(10, 100, 1000, 10000 $\mu$ M)	24 h	Newport-Green DCF-2 K <sup>+</sup> -conjugated with zinc oxide nanoparticles and the membrane probe FM1-43 demonstrated endocytosis of zinc oxide nanoparticles by PC12 cells; FluoZin-3 measurement showed elevation of cytosolic Zn <sup>2+</sup> concentration in cells [15].
<100 In vitro: MRC5 human lung fibroblasts In vivo: the fruit fly <i>Drosophila melanogaster</i>	(0, 1, 10, 25, 50, 75, and 100 $\mu$ g/mL)	24, 48, and 72 h	The occurrence of cytotoxicity and genotoxicity in human lung fibroblasts under zinc oxide nanoparticles exposure in vitro and in <i>D. melanogaster</i> in vivo conditions [11].
~50 Rat retinal ganglion cells	(5, 10, and 20 $\mu$ g/mL)	24, 48, and 72 h	Zinc oxide nanoparticles could decrease the mitochondrial membrane potential, increase the production of reactive oxygen species (ROS) and lead to the overexpression of caspase-12 in RGC-5 cells, suggesting that zinc oxide nanoparticles induced toxicity by ROS overproduction [19].
80.145 In vitro: mouse ovarian germ cells	(10 and 30 $\mu$ g/mL)	1 d	Zinc oxide nanoparticles have cytotoxic effects in a concentration- and time-dependent manner in mouse ovarian germ cells [20].
~ 100 nm hepatic and renal toxicity	(100 mg/kg BW)	75 d	Hepato- and nephro-toxicity through epigenetic changes in the gene expressions of mtTFA and PGC-1 $\alpha$ may subsequently cause mitochondrial dysfunction, instigating the generation of ROS and oxidative stress [12].
229.6 human EA.hy926 cells coated with 4 mM aqueous solution of Pluronic F-127	(8, 16, 24, 32, 40, 48, and 56 $\mu$ g/mL)	48 h	In vitro cytotoxicity evaluation: Pluronic F-127 modification significantly improved the biocompatibility and reduced the cytotoxicity of zinc oxide nanoparticles toward the normal endothelial human EA.hy926 cells [16].
41.7 MicroRNAs (miRNAs) enclosed in exosomes	(2, 4, and 8 mg/kg)	24 h	Zinc oxide nanoparticles induced significant increases in the serum levels of interleukin 8 (IL-8), interleukin-1 beta (IL-1 $\beta$ ), and tumor necrosis factor $\alpha$ (TNF- $\alpha$ ) and elevated the number of cells and the percentage of neutrophils in the blood [21].
100-500 rat lung	(1, 5, and 10 mg/kg)	14 d	Histological examination indicated that multifocal acute inflammation occurred in the zinc oxide nanoparticles-treated samples and progressed to chronic inflammation by day 14. A partial recovery of lung injury was observed within two weeks after exposure [22].
30, 80, and 200 nm human lung epithelial (A549) cells for the in vitro exposure study	(0.1, 0.5 mg/kg)	24 h	Zinc oxide nanoparticles can stimulate a strong inflammatory and antioxidant response in A549 cells. Exposure to a single but relatively high dose of zinc oxide nanoparticles by intranasal instillation may provoke acute pulmonary inflammatory reactions in vivo [12].
30-40 In vivo: Male Wistar rats, intravenous injections	(25 mg/kg BW)	14 days	Zinc oxide nanoparticles caused no changes in neurotransmitter contents, no deterioration in locomotor activity, and spatial working memory was observed [17].
30-40 Male Wistar rat	(25 mg/kg BW)	Single dose, intravenous injection	Zinc oxide nanoparticles did not affect neurotransmitter contents, locomotor activity, or spatial working memory in adult rats [23].
68.96 $\pm$ 33.71 Adult male Swiss mice	(5.625 $\times$ 105 mg/kg BW)	Aqueous exposure	Even briefly, the environmentally relevant concentration of zinc oxide nanoparticles induced behavioral changes related to anxiety in mice [24].
30 Carp ( <i>Cyprinus carpio</i> )	300 mg/kg BW)	14 days, aqueous exposure	A significant decrease in several enzymatic activities was observed under zinc oxide nanoparticles (50 mg/L) treatment. The most sensitive organs were the gill, liver, and brain [25].
10-30 Zebrafish ( <i>Danio rerio</i> ) embryos	(0.5, 5, 50 mg/L) (1, 5, 10, 20, 50, and 100 ppm)	96 h	Retarded developments of the nervous and vascular systems were observed under treatment with zinc oxide nanoparticles [26].

In vivo conditions, physicochemical properties, nano-fabrication, and biological factors (such as species, age, gender, and size of the tested animals) seem to be common factors that govern zinc oxide nanoparticles' toxicity [9, 11]. Regarding the complexity and diversity of reactions related to human biological systems, the generalization of animal model results to humans is often inconclusive. Concentrations used or determined in animal models do not necessarily imply the negative effects of exposure to zinc oxide nanoparticles in humans. Very low mass concentrations of these nanoparticles are needed in diagnostic interventions in humans. Therefore, future studies should rely on using real concentrations of these nanoparticles in the environment to evaluate their toxicity in living organism models. Parameters such as exposure routes, physicochemical properties, and environmental conditions should also be considered in these studies. The lack of sufficient understanding of the mechanism of toxicity caused by zinc oxide nanoparticles is one of the main obstacles to their use in various applications. More attention should be paid to the efforts made in understanding the mechanism of toxicity caused by zinc oxide nanoparticles and its control methods.

## Conclusion

It is necessary to continue monitoring the process of release and exposure of Zn-based nanoparticles and their harmful effects on living systems. Zinc oxide nanoparticles currently have a wide range of applications in cancer treatment, gene-drug delivery, and biological materials for tissue engineering due to wide band-gap semiconductors, which can readily absorb UV rays. With their unique properties, zinc oxide nanoparticles can damage cancer cells using the mechanism of selective cytotoxicity through oxidative stress via ROS generation. Oxidative damage caused by free radicals during the toxicity of zinc nanoparticles leads to a cascade effect. Ultimately, this effect will have adverse consequences in both in vitro and in vivo conditions (typically liver, lung, reproductive tract, kidney, central nervous system function, dysregulation, and reduced mitochondrial respiration). The main mechanisms during zinc-based nanoparticle toxicity are ultrastructural, ROS, oxidant, antioxidant enzymes, and cell apoptosis-related factors in different model systems (in vitro/in vivo). More attention should be paid to the penetration mechanism, the dose used, and the shape and dimensions of nanoparticles in the body's cell lines. Along with these cases, future studies should also focus on understanding the penetration mechanism of zinc oxide nanoparticles in combination with other materials and their penetration routes.

## Ethical Considerations

### Compliance with ethical guidelines

There were no ethical considerations to be considered in this review.

### Funding

The study was self-funded.

### Authors' contributions

All authors equally contributed to preparing this review.

### Conflict of interest

The authors declared no conflict of interest.

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