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In–Vivo Models for Toxicity Screening of Nanohazards: Opportunities and Challenges

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Abstract: Advancement in nanotechnology has introduced several nanomaterials in the market. As these nanomaterials are of very small size and have high penetrative potency in the biological systems, the issues related to nanotoxicity are inevitable. The evaluation of nanotoxicity is thus a major concern for the safety of human health and the environment. There are currently only a few reported studies based on human nanomaterial toxicity data, mainly due to difficulties quantifying human exposure, specifically nanomaterials. Additionally, it involves various ethical constraints to collect data concerning human subjects in their particular environment. It is practically impossible to expose humans to specific nanomaterials under controlled laboratory conditions. Hence, *in-vitro* cellular systems and *in-vivo* animal models come into play. Although testing *in vitro* is a faster, easier, and comparatively low cost, it has its challenges, viz., translating *in vitro* results to *in vivo* situations. On the other hand, the model organisms and experimental animals provide *in vivo* results that are more realistic and exhibit systemic interaction to evaluate the toxic potential of these materials. The present review presents the pros and cons of using various organisms, namely *Saccharomyces cerevisiae*, *Caenorrhabditis elegans*, *Drosophila melanogaster*, *Daphnia*, *Danio rerio*, and rodents, for toxicity screening of nano hazards.

Keywords: nanomaterials; animal model; toxicity; nanoparticles.

1. Introduction

The nanomaterials (NM) industry is expanding at a constant rate, with thousands of nanoparticles being introduced into the market daily, and their increased production and use have revolutionized diverse areas of our lives. Tremendous research in developing nanomedicines, nanochips, nano gadgets, nanosensors, and nanopolymers leads to a new domain in science and technology [1,2]. These advancements are attributed to several unique features of NM, such as flexibility in chemical, physical, and biological properties, mainly due to nanoscale size and quasiparticle nature. NMs are mainly of four major types based on their composition, including carbon-based (Carbon nanotubes), inorganic-based (contain metal and metal oxides), organic-based (excluding carbon and inorganic NM), and composite-based (multi-phased combination of NM and nanoparticles) [3]. The formation of NMs usually may occur viz. incidental, engineered, and natural processes. The incidental NM are produced incidentally as a forest fire, vehicle exhaust smoke, etc.. In contrast, engineered NM is produced anthropogenically, deliberately for specific purposes, whereas natural NM are naturally formed in living organisms [4]. However, the distinction between incidental and

naturally occurring NM is unclear in the literature and sometimes is used as overlaps. All types of NM share a common feature of nanoscale dimension, which increases their application in the diverse fields and penetration within the biological systems. Due to such wide applications of nanoparticles viz. nanobatteries, surface coating of electrical goods, fuel cells, cosmetics, drug delivery, communication devices, high-frequency amplifiers, gas turbines, etc., deep exposure to human tissues is thus expected, and issues related to nanotoxicity becomes inevitable [5].

Assessment of toxicity is important to forecast NM's behavior and understand the way they interact with biological systems under various conditions. With the increasing use of NMs, the possibility of their exposure and toxic effects on consumers and the environment has also been highlighted [6]. The prime reasons for nanotoxicity comprise aggravated and faulty production, improper unethical bulk usage, and improper waste disposal and treatment. Improper disposal leads to their entry into the environment exposing humans and other organisms. NMs can easily enter into the human body through various routes, viz. oral, respiratory, gastrointestinal, and skin; they can interact with various tissues like the liver, kidney, heart, lungs, spleen, and brain. Interaction of nanoparticles may cause the generation of reactive free radicals and illicit the deleterious process of oxidative stress [7]. The free radicals are very reactive chemical species, which can react with proteins, lipids, nucleic acids, and other macromolecules to cause great damage and develop diseases like asthma, diabetes, emphysema, and cancer [8,9]. Further, the interaction of these materials with cellular components leads to a chronic defect in cellular uptake, phagocytosis, reticuloendothelial defects, cellular injury, and translocation defects. Besides this, interactions of nanoparticles with cellular forces such as van der wall steric, interfacial, and electrostatic interactions cause long-term damage, inflammation, and cell death [10,11]. The extent of damage depends on the tissue's origin, size, type, and a load of nanoparticles.

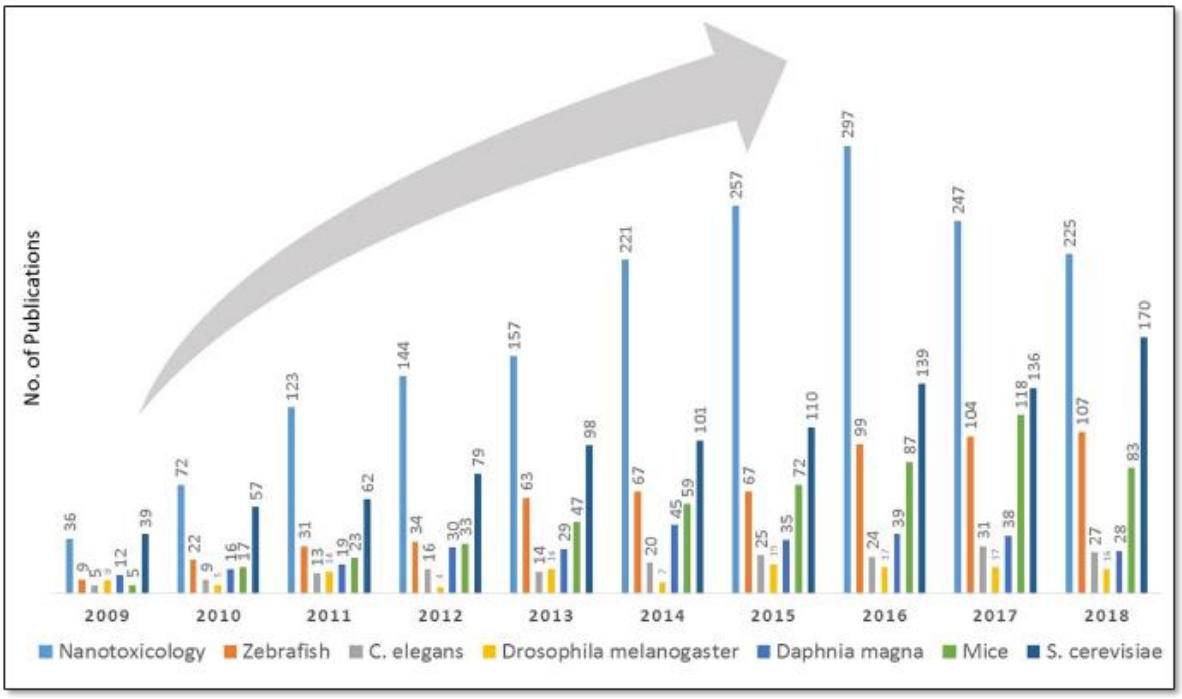

Figure 1. Increasing Trends in the research related to the toxicological assessment of the nanoparticles on different model organisms during the last decade (Source: PubMed, NCBI database. The search was conducted in March 2019. Note that the data was collected using the keywords “Nanoparticle toxicity” and “organism name”).

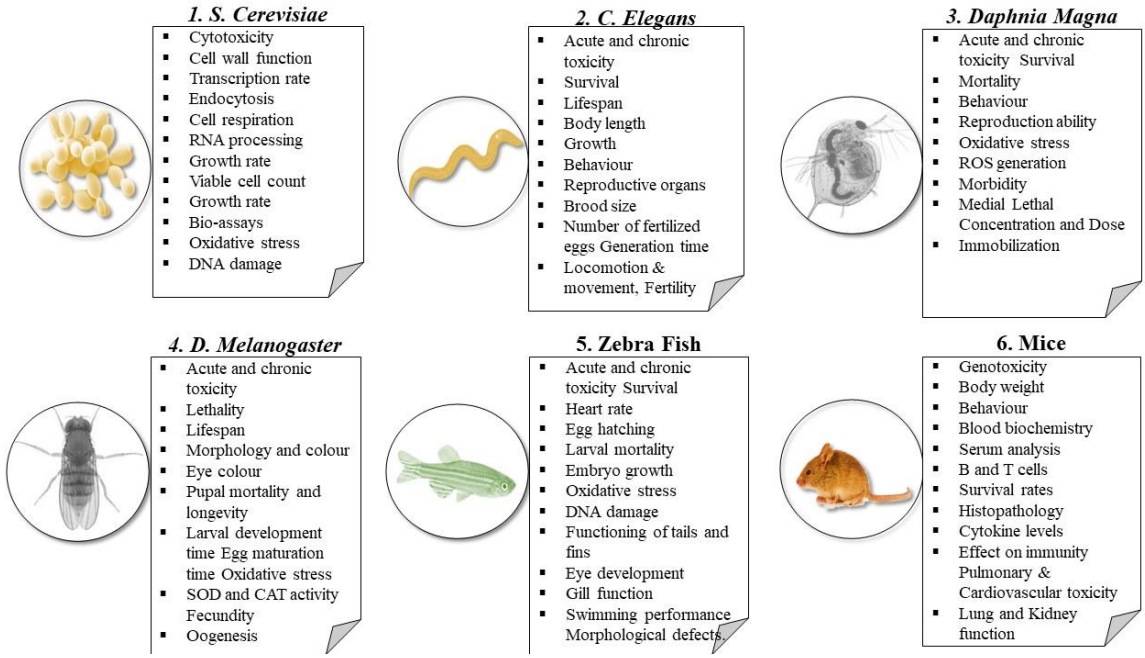

Figure 2. Toxicological endpoints commonly studied for different model organisms in nanotoxicology research.

As the exposure of these nano-based materials increases, the information on toxicological properties remains minuscule, which precludes the reliable assessment of their impact on human health [6]. To assess the toxicity of nanomaterials, several cellular assays and model organisms, for instance, *Sachharomyces cerevisiae*, *C. elegans*, *Daphnia*, *Drosophila*, Zebrafish, mouse, and many more, have been used [12]. Recent research based on a nanotoxicological assessment of engineered nanoparticles on model organisms is shown in Fig. 1. The information on human studies is still missing due to the difficulty in quantifying NM exposure in natural settings and the ethical concerns associated with human exposure. Additionally, several factors, such as tissue complexity, present many challenges in understanding cellular and tissue behavior. Thus, most laboratories across the globe sought cellular systems because *in vitro* testing provides information on various quantitative measurements and cellular assays, including DNA damage, apoptosis, necrosis, and oxidative stress biomarkers estimations. Several studies have been performed for toxicity testing of nanomaterials generated through photocopiers on various cell lines, which has given an insight into the toxicity of various nanomaterials [9]. Although testing the toxicity levels of nanoparticles *in vitro* is a faster, easier, and comparatively low cost, the translation of *in vitro* results to *in vivo* situations always stands highlighted as a major issue [13]. The living system functions as an interconnected unit of many systems and a self-responding immune system, hence the variation between the *in vivo* and *in vitro* results. Therefore, a living system such as animal models presents more realistic and proper insight into NM human interaction [13]. Such model-based studies also demonstrate the biodistribution, localized route, metabolism, and bio clearance of NM in different organs with various doses.

Moreover, biochemical, morphological, and physiological changes can also be studied at various time intervals in various tissues using blood, serum, metabolic excretions, and histopathological examination. These endpoints are represented in Fig. 2. Additionally, biodegradable and non-biodegradable NM assessment and their long-term and short-term toxic effects are also recorded with high precession.

Thus, *in vivo* and *in vitro* analysis both offer significance in assessing NM toxicity; *in vivo* studies indeed have an edge of their homology with the human system. Keeping this view, the present review presents elaborative and detailed information on models organisms and animal models with their advantages and disadvantages in nanotoxicological studies.

2. *Saccharomyces cerevisiae* (Yeast)

Saccharomyces cerevisiae (*S. cerevisiae*), commonly known as yeast, is considered a successful model organism as it is being used constantly to unravel the biological processes of higher eukaryotes. This ability owes specific features of its easy growth and genetic manipulation. Moreover, a short generation time (90 minutes) makes it possible to grow and analyze a large population of individuals in a single experiment. Its genome size comprises 6000 genes, out of which around 60% have an assigned function, and 40% genes have conserved amino acid sequences [14]. Considerable gene homology and conservation of fundamental biochemical pathways establish a prospective model for understanding fundamental cellular processes, cell cycle, and DNA repair mechanisms [15]. It is being used as an efficient model organism to evaluate cellular viability because of easy and inexpensive culture techniques.

Additionally, its small size, simplified morphology, and large surface area provide more sensitivity and reproducibility than other complex organisms [16]. Because it can be easily cultured on agar plates, its colony growth can thus be monitored after the administration of nanoparticles. It shares many similarities in the cell structure and other functional organizations with cells and plant cells, so it is more often used in cell biology and molecular studies. Thus, it can provide insight into nanotoxicity, too [17].

Yeast serves as the best model to replace animals in toxicity testing because various mechanisms associated with cytotoxicity in higher eukaryotes appear to be conserved in yeast [18]. A systematic collection of knockout mutants is available, allowing the recognition of every non-essential gene [19].

S. cerevisiae also allows assessing the toxicological impacts at genetic levels. Toxic compounds lead to the formation of bulky lesions that block DNA replication and lead to DNA damage. Therefore, cell cycle arrest occurs that leads to the inability of cells to form colonies [20]. The effect of silver nanoparticles was evident on the genome, and altered behavior of yeast cells was observed when it was fed on different concentrations. It was found that silver nanoparticles lead to differential expression of at least 17 genes in response to stress and chemical stimuli [21].

Nanotoxicity of zinc oxide nanoparticles was checked in *S. cerevisiae*, and no specific toxicity was found in wild-type yeast. In contrast, mutants with a deficiency in defense mechanisms showed significant toxicity [21]. Studies also indicated that manganese oxide was toxic to yeast cells mainly because it caused cell membrane damage (at around 170 mg/L conc.) and oxygen consumption, whereas iron oxide nanoparticles did not show any effect on cell membrane integrity [22].

Since *S. cerevicae* is unicellular, organism-level phenotypes that emerge from intercellular interactions are not captured. Extrapolation of toxicological assay results in humans is difficult because of the absence of organ systems and cellular pathways as present in humans. Moreover, different routes of administration cannot be explored in this organism as the only way of intake of the nanoparticle is through the medium. In addition, slight changes

in media composition can lead to unavoidable changes in nanoparticle uptake efficiency [19]. The studies on the toxic effects of NPs on *S. cerevisiae* are summarized in Table 1.

Table 1. Details of various studies on the toxicological effects of nanoparticles on *Saccharomyces cerevisiae.*

S.No.	Species	Nanoparticles studied	NP exposure conc.	Duration of exposure	NP size	Exposure/Routes	Toxicological endpoints/studies	Effects/Results	References
1.	*S. cerevisiae* S288C	ZnO NPs CuO NPs TiO_2 NPs	31.25–1000 mg/L 10–160 mg/L 250–4000 mg/L	24 h	ZnO: 50–70 nm CuO: 30 nm TiO2: 25–70 nm	Exposed in malt extract medium with 2-fold dilutions of NPs	Viable cell count (CFU/ml) Inhibition of growth rate	EC50 = 131–158 mg/L (24 h) EC50 = 20.7 mg/L (8 h) EC50 = 1297 mg CuO/L(24 h) No toxicity by nano- TiO2 Lysosomal and mitochondrial damage Cell death	[19]
2.	*S. cerevisiae* BY4741	CuO NPs	0.48−160 mg/L	24 h	30 nm	Cultivation/Incubation of yeast cells with varying NP concentration in 96-well microplates	Oxidative stress Toxicity Cell viability Growth Inhibition Spot Assay	IC50 = 4.8 mg/L Sorption on the yeast surface DNA damage	[19]
3.	*S. cerevisiae* S288C	ZnO NPs Ag NPs	1 mg/mL 0.095 mg/mL	48 h	50-70 nm 1-10 nm	*S. cerevisiae* exposed to a sub-inhibitory concentration of ZnONPs and AgNPs	Cytotoxicity Cell wall function Transcription rate Endocytosis Electron transport system RNA processing Cell respiration	1.3<MIC100<1.5 mg/mL (ZnO) 0.10<MIC100<0.12 mg/mL (Ag NPs) Cell membrane disruption Effects on liposomes cell membrane depolarization	[21]
4.	*S. cerevisiae*	TiO_2, ZrO_2, Fe^o, Fe_2O_3, and Mn_2O_3 NPs	1000 mg/L	10 h	ZrO2 (20–30 nm) Fe0 (40–60 nm) Fe2O3 (20–40 nm) Mn2O3 (30–60 nm) TiO2 (21 nm)	Yeast cells exposed to NPs amended with dispersant (100 mg/L) in YEPD medium	Cell membrane Cytotoxicity Bioassays	Inhibition of O_2 uptake Highest oxygen inhibition Mn_2O_3 by 50% at 170 mg/L Membrane damage Low toxicity of Fe^o NPs No toxicity of TiO_2, ZrO_2, Fe_2O_3 Nano-Mn2O3 most toxic NP	[21]
5.	*S. cerevisiae* InvSc1	Mn_3O_4 nanoparticles	10, 000 ppm.	12 h	10–25 nm	Co-culturing of yeast cells with Mn_3O_4 NPs	Toxicity studies Growth Cell count ER damage detection ROS generation	Dose-dependent toxicity IC50 = 340 ppm intracellular ROS accumulation ER dysfunction growth inhibition and apoptosis	[22]

Abbreviations used: NP: Nanoparticles; MIC: Minimum inhibitory Concentrations; IC: Inhibitory Concentration; YEPD: Yeast extract-peptone-dextrose *SOD:* Superoxide dismutase; GSH: Glutathione; CAT: catalase; ALP: Alkaline phosphatase; AST: Aspartate transaminase.

3. *Caenorhabditis elegans*

C. elegans is a nematode used to elucidate various basic aspects of biology such as RNA interference, miRNA function, and apoptosis. It offers several advantages such as small size so animals can be maintained in multi-well plates containing nutrient media. As a result, several substances and combinations can be evaluated in a short space at a wide range of concentrations. They have a three-day life cycle and a reproduction capacity of one with approximately 300 progeny per organism, resulting in millions of creatures. The majority of the tests can be done in a high-throughput manner, and they can be created quickly. Another advantage of using *C. elegans* as a model organism is its tough but transparent cuticle, through which internal structures can be visualized without dissection and specific gene expressed can be easily tracked. *C. elegans* can be easily maintained at 15-25ºC using simple techniques, and carbon dioxide incubators are not required for their growth [23].

In *C. elegans*, mapping somatic cell locations and lineages has already been studied [24], facilitating the morphological assessment of toxin-induced abnormalities. The signaling pathways and genes are found to be well conserved between *C. elegans* and humans [25,26]. It also stands out as one of the potential models for studying the assessment of human-relevant toxicity pathways due to the availability of mutant and transgenic strains for many genes. Moreover, two-thirds of human proteins tend to have *C. elegans* homologs, and also 80% of genes for a human inborn error of metabolism have analogs in *C. elegans* [27]. Researchers have used *C. elegans* in ecological risk assessment for metals, pesticides, and nanomaterials, suggesting it is a bioindicator of ecological health [28].

The life cycle and development of *C. elegans* can be controlled by varying temperature and time; therefore, allowing the use of worms at specific developmental stages. Such controls make it possible to study chronic or acute effects of nanoparticles on *C. elegans*. Transgenic strains of nematodes can be easily generated by microinjection or gene bombardment [29]. Mutants with expected sensitivity to specific toxicity mechanisms can be used to elucidate nanotechnology-related molecular pathways and mechanisms [29]. Mutations in enzymes that are involved in oxidant defense can be used to study the oxidative stress caused by nanoparticles. The morphological and physiological attributes that can be considered for toxicity testing are; survival rate, lethality, changes in growth pattern or length of the worm, the effect on reproduction and life span of subsequent generations, evaluating the fertility of treated worms, and the effects on offspring [30]. Various effects of nanomaterial toxicity on *C. elegans* have been shown in Fig. 3.

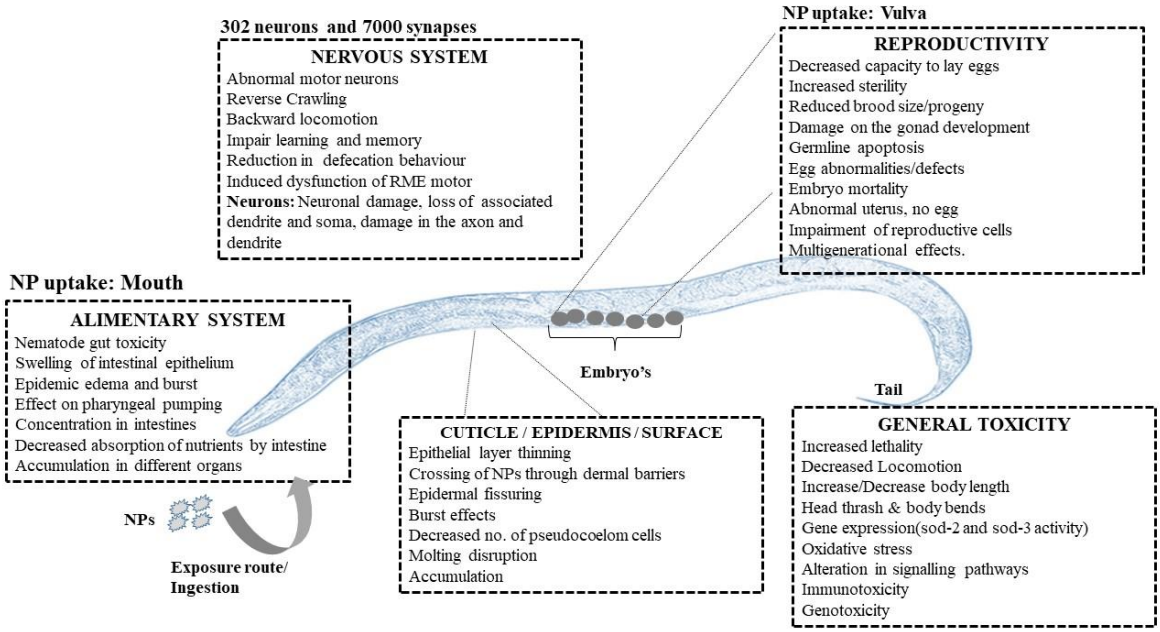

Figure 3. The potential detrimental effects of nanoparticles exposed to *C. elegans* through distinct uptake routes.

Gonzalez *et al*. reported that exposure to 17 pg/ml of Cerium oxide nanoparticles concentration was associated with a dose-dependent increase in the mortality in *C. elegans* [31]. It was also reported that gold nanoparticles lead to reproductive failure in *C. elegans* due to their persistence and accumulation in the reproductive system. Another study reported that chronic aluminum oxide nanoparticles caused a neurotoxic effect on locomotors behavior of

C. elegans by inducing severe ROS production and ROS defense mechanism disruption [32]. Other studies are discussed in Table 2.

Table 2. Details of various studies on the toxicological effects of some nanoparticles on *C. elegans.*

S.No.	Species	Nanoparticles studied	NP exposure concentration	Duration of exposure	NP size	Exposure test	Toxicological endpoints	Effects/Results	References
1.	*C. elegans*	Ag NPs (citrate capped)	0, 10, 50, 100, 500, and 1000 mg/L for survival. 0, 1, 5, 10, 50, and 10 mg/L for reproduction	24 and 48 h	50.6 nm	Transfer of 1-10 organisms to wells containing NGM agar plate mixtures with NPs suspensions	Survival Reproduction Interaction with biological surfaces Toxicity	Epidemic edema and bursting at 100 mg/L Epidermal fissuring and damage Increased toxicity NPs observed in digestive organs LC_{50} at 55 mg/L and NOEC at 10 mg/L (survival) EC_{50} at 1 mg/L and NOEC at 4100 mg/L (reproduction)	[33]
2.	*C. elegans*	Au NPs (citrate capped)	100 µg/ml	24 h	11 nm and 150 nm	Transfer of NGM grown nematodes with each plate containing 1000-2000 animals	Survival Brood size Reproductive effects Gene expression Cellular localization of NPs SEM-EDX studies Bio distribution assay	Decrease in reproductive output Decrease in brood size Ingestion of 11 nm Au NPs more than 150 nm Entry of NPs through the alimentary tract	[34]
3.	*C. elegans*	CuO NPs	3.8, 7.9, and 15.9 mg/L	4-96 h	28.4 nm	*C. elegans* has grown on NGM and exposed to CuO NPs	Average body length Feeding Brood size Neurodegeneration Stress Induction	Decrease average in body size Impacts on feeding Variable reduction in brood size Decrease in progeny Absence/Blebbed neurons Increased GFP expression of the *hsp-16.2* reporter strain Inhibits food uptake	[35]
4.	*C. elegans*	ZnO NPs	0.4, 0.8, 1.6, 4.1, 6.1 and 8.1 mgL^{-1}	8 h	20 nm	Transfer of 30 L1 juveniles by micropipette to the Petri plates containing different concentrations of NPs	Growth Reproductive capability LC_{50} (24 h) of NPs Lethality Worm length No. of eggs inside worm body Metal analysis	LC_{50} at 2.3 mgL^{-1} Acute toxicity Decrease in body length, no. of eggs per worm	[36]
		Al_2O_3 NPs	10.2, 30.6, 51.0,102.0, 203.9 and 407.8 mgL^{-1}		60 nm			LC_{50} at 82 mgL^{-1} Inhibition of growth Decrease in body length and offspring per worm	
		TiO_2 NPs	24.0, 47.9, 95.9, 167.8, 239.6 mgL^{-1}		50 nm			LC_{50} at 80 mgL^{-1} Reduction in body length Affects fertility	
5.	*C. elegans*	Ag NPs MWCNT	10^{10} particles/mL.	24, 48, 72 h	22.8-123.3 nm 1.9- 39 nm	Supplementation of NGM with required conc. of NPs and animal cultivation for 48 h.	Lifespan Movement Neuro ability Population studies Body length Gene expression analysis by oligonucleotide microarray Transcriptome analysis	Reduction in body length No effects of MWCNTs on survival 2-5 % dead animals Altered expression of multiple genes 34 over expressed and 9 suppressed genes in Ag NPs	[37]
6.	*C. elegans*	ZnO NPs	325–1,625 mg Zn/L	24 h at 20 °C	1.5 nm	10 worms per well (30 worms for each NP concentration) in 24-well plates	Lethality tests Behavior Reproduction ability Transgene expression in an *mtl-2 :GFP*	Increased mortality (100%) at high conc. Affected movement LC_{50} = 789 mg/L EC_{50} = 635 mg Zn/L EC_{50} (reproduction) = 46 mg Zn/L 3-fold increase in GFP expression	[38]
7.	*C. elegans*	CeO_2	1 mg/L for both NPs	24 h	15,45nm	Exposure to NPs on nematodes grown in K-medium	Growth Fertility Survival cyp35a2 gene expression by Semi-quantitative RT-PCR Stress-response gene expression profiling analysis	11% decrease in the number of eggs per worm No effects on growth 20 % decrease in survival rates Increased expression of the gene Reduced fertility capacity	[39]
		TiO_2			7, 20nm			Decrease in growth (9%) Reduced fertility potential 30% survival rate decrease	

Abbreviations used: NP: Nanoparticles**;** NGM: Nematode Growth Medium; NOEC: No Observed Effect Concentration; EC_{50}: Median Effective Concentrations; LC_{50}: Median Lethal concentration; *SOD:*Superoxide dismutase; GSH: Glutathione; CAT: catalase.

The disadvantage of using *C elegans* as one of the model organisms for nanotoxicity testing is that it lacks mammalian organs such as the kidney, lungs, liver, kidney, and eye.

Therefore, the direct effect of nanoparticles on these organisms cannot be elucidated. It lacks adaptive immunity and is not a good adsorption model compared to higher organisms, for *e.g*, zebrafish. Moreover, the assay results can also get altered significantly by small changes in nutrient, temperature or salt concentration. Extrapolation of these results to humans is questionable due to the above factors. Again routes of administration can also not be explored to their full extent in these organisms, which also adds to its disadvantages as a model organism.

4. *Daphnia magna*

Daphnia magna is a small planktonic crustacean belonging to the subclass *Phyllopoda*. It can be found in various freshwater environments, ranging from acidic swamps to snow runoff containing rivers, throughout South Africa and the Northern Hemisphere. Various features that make *Daphnia magna* one of the suitable model organisms for different studies include the ease by which they can be manipulated and maintained in the laboratory and their less generation time of approximately one week in culture (20ºC) which makes it possible for the researcher to track responses of various tests throughout their ontogeny [40]. Moreover, simple defined media is required for their growth, including algae and bacteria, in controlled conditions. These are also important models for experimental genetic studies. It is generally seen that *Daphnia* species are cyclical parthenogens, i.e., they have the capability of both clonal and sexual reproduction. It is seen in previous studies that clonal reproduction can serve as a medium for comparisons of various treatments and dosages under a defined genetic background [41]. The bioassays using *Daphnia magna* are recommended over other models to assess the toxicity of various materials, mainly due to filter-feeding, which leads to a substantial increase in oral exposure [42]. It was seen that bioassays with *D. magna* are viable and of great advantage in studies related to pharmacology. One of the other advantages of *D. magna* use in research is that it is possible to obtain insights into the physiological effects of the materials [43]. Several optical methods can be used to measure various parameters on animal physiology with noninvasive techniques. Various studies have shown that heavy metals, i.e., cadmium at very low concentrations, increased mortality and decreased the feeding rate and offspring production. These can be considered a hallmark of toxicity testing in *D. magna* [44]. This was also observed in checking the toxicity of various metals such as copper, lead, and zinc on these organisms that showed detrimental effects [45]. A correlation between *D. magna* immobilization and toxicity was observed when the level of insecticide were found to be increased in the stream and river waters [46]. The toxic effects of nanomaterials on *D. magna* are shown in Fig. 4. An overview of studies on toxicity and accumulation of engineered nanomaterials in *D. magna* is given in Table 3.

Inspite of the fact that *D. magna* has been extensively used in nanomaterial toxicity screening, it has various disadvantages, such as the lack of certain organs and their related metabolism compared with a superior mammalian model, making it difficult to extrapolate the results to the higher animals. Additionally, the low sample yield per individual makes it difficult to perform various assays from a small sample size [47]. It is somewhat difficult to manipulate this organism experimentally, and it does not offer the advantage of different routes of administration to the full extent. Genomic level homology is also less in the case of *D. magna* than other organisms.

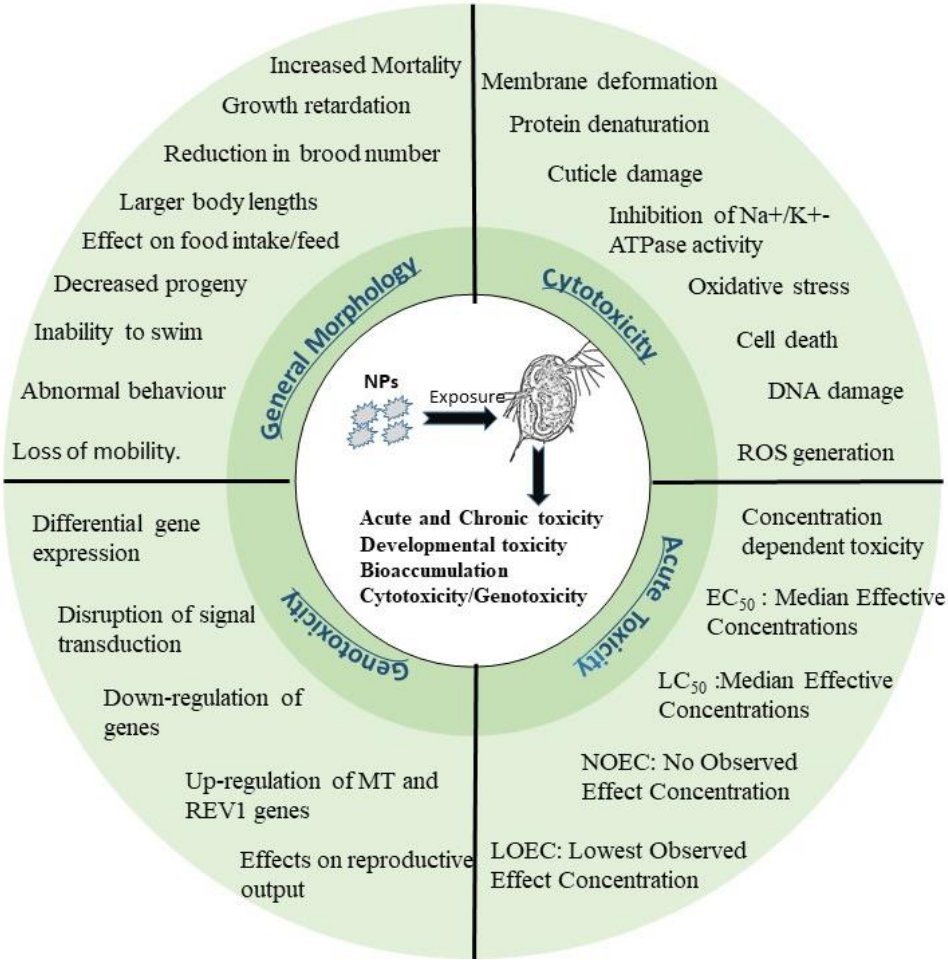

Figure 4. The acute and chronic toxic effects of nanomaterials in *Daphnia magna.*

Table 3. Details of various studies on the toxicological effects of engineered nanoparticles on *Daphnia Magna.*

S.No.	Species	Nanoparticles studied	NP exposure conc.	Duration of exposure	NP size	Exposure test	Toxicological endpoints	Effects/Results	References
1.	*Daphnia Magna*	Ag NPs (citric acid stabilized)	25, 50, 100, 150, 200, 250 and 300 µg Ag/L	24-48 h	30 nm	24 h old *D. magna* exposed to Nanoparticles in food under different feeding conditions	Survival Number of live neonates Reproduction Body length Acute and chronic toxicity tests	Larger body length EC50 value of 0.75_g Ag/L (Acute) Adhesion of algal cells to the exoskeleton Immobility 50% mortality at 1.6_g/L.	[48]
2.	*Daphnia Magna*	Ag NPs (citrate and detergent stabilized)	5, 10, 30, and 60 µg Ag/L	24 h	20-30 nm	10 neonates per 100 mL exposed to 30 µg Ag/L for 24 h	Chronic toxicity Behavior Mortality reproductive effects	LOEC = 19.2 µg Ag/L (citrate) LOEC = 27.5 µg Ag/L (detergent) Increased mortality at high conc. Decreased number of broods Higher body burdens for Cit-Ag NPs than det-Ag NPs Reduced reproduction	[49]
3.	*Daphnia Magna*	Nano-scale TiO_2 and ZnO	0.1–100 mg/L	48 h	20-30 nm (TiO_2) 200 nm (ZnO)	10 daphnids per concentration level Exposed according to OECD guidelines	Mortality Acute toxicity Chronic toxicity Reproduction ability	EC_{50} = 100 mg/L (TiO_2) EC_{50} = 1 mg (ZnO) NOEC (mortality) = 30 mg/L NOEC (offspring production) = 3 mg/L EC_{50} (reproduction) = 26.6 mg/L Acute toxicity observed	[50]
4.	*Daphnia Magna*	TiO_2 NPs	0.1, 0.5, 1, 5, 10, 50 and 100 mg/L	72 h -21 d	21 nm	6–24 h old neonates kept in 50 mL glass beaker with 30 ml NP solution	Body length Acute and chronic toxicity Mortality	Growth retardation Reproductive defects Bioaccumulation of TiO_2 20% immobilization at 100 mg/L EC50 and LC50 > 100 mg/L	[51]

S.No.	Species	Nanoparticles studied	NP exposure conc.	Duration of exposure	NP size	Exposure test	Toxicological endpoints	Effects/Results	References
								13% mortality at 0.1 mg/L LC50 was 2.62 mg/L (Reproduction) Effect on food intake Decreased body length	
5.	*Daphnia Magna*	Au NPs coated with MPA, citrate, PAH and Gold Nanorod coated with CTAB	1, 10, and 50 µg/L	24 h	4 nm NPs 12×50 nm AuNR	3 weeks old Daphnids exposed to different NP suspensions	Oxidative stress Cellular stress ROS production Expression of gst,CAT, heat shock protein 70 (hsp70), and metallothionein 1 (mt1)	ROS induction by all NPs Upregulation of gst expression upregulation in cat expression (0.8-fold)by MPA-AuNP downregulation of all genes by Citrate Au NPs 1.7-fold hsp70 at 50 µg/L) of PAH-AuNP Chronic toxicity	[52]
6.	*Daphnia Magna*	Cu NPs	36 to 216 µg/L	48 h	50 nm	Acute chronic toxicity tests according to OECD guidelines	Mortality Effect of aging Effect of pH Effect of DOC	LC_{50} decreased by 30% (7 day aging) LC_{50} increased 12 fold (DOC to 10 mg/L) 3-fold LC_{50} at pH 8.5 More contribution of Cu NPs towards toxicity than Cu ions	[53]
7.	*Daphnia Magna*	CuO NPs	0.1, 0.5, 1, 5, and 10 mg/L Cu	48 h	6 nm, 100 nm	Organisms exposed to NP acc to ISO standard testing conditions	Effect of NPs under different test conditions(acidity, in the presence of citric acid and humic acid)	Concentration dependent toxicity Immobilization increased at lower pH No immobilization at 5 mg/L (Humic acid) EC_{50} = 0.08 mg/L (6 nm) EC_{50} = 1.55 mg/L (100 nm) ROS generation Cell death DNA damage	[54]
8.	*Daphnia Magna*	CuO NPs	0.037, 0.092, 0.230, 0.575, 1.438mg Cu/l	48 h-21 d	50 nm	Chronic exposure to daphnids according to OECD guideline 211	Chronic effects Reproduction (no of offspring) Body length Survival	EC_{50} = 1.041mgCu/l NOEC= 0.604mg Cu/l LOEC = 1.413mg Cu/l Decreased populations Affected body length Ingestion of NPs by daphnids	[55]
9.	*Daphnia Magna*	TiO_2 NPs C_{60} fullerenes	0.2, 1, 2, 5, 6, 8, and 10 ppm	48 h	TiO_2 NPs : 10-30 nm	U.S. EPA standard operating procedure 2024 (48 h acute toxicity tests)	Acute toxicity Mortality	EC50: 5.5 mgl/L (TiO_2) LC_{50} = 5.5 ppm NOEC = 1 ppm LOEC = 1 ppm Dose-dependent toxicity	[56]
			C_{60}: 40, 180, 260, 350, 440, 510, 700, and 880 ppb		C_{60} : 0.72 nm			EC50: 460 µg/L (C_{60}) LC_{50} = 7.9 ppm NOEC: 0.2 ppm LOEC: 0.5 ppm Inability to swim Immobilization	
10.	*Daphnia Magna*	Colloidal silica NPs	4.0 mg/mL	24 - 96 h	30 nm	5 neonates in 10-mL well of a 6-well culture plate exposed to NP slurry	Acute and chronic toxicity Morbidity Body size Reproductive output	Slower swimming rates No morbidity observed Body sizes increase by 9% 1.86-fold increase in reproductive output	[57]

Abbreviations used: NP: Nanoparticles; NOEC: No Observed Effect Concentration; LOEC: Lowest observed Effective concentration; EC_{50}: Median Effective Concentrations; LC_{50}: Median Lethal concentration; MPA: Mercapto-propionic acid; PAH: Polyallylamine hydrochloride; CAT: catalase; ROS: Reactive Oxygen Species; CTAB: Cetyl Trimethyl-Ammonium Bromide; DOC: Dissolved Organic Carbon.

5. *Drosophila melanogaster* (Fruit fly)

Drosophila melanogaster offers several advantages, such as a short life cycle, ease of rearing and reproduction, simple genome structure, fast offspring turnover, and low maintenance cost compared to other models. One of the major advantages of using it as an animal model is that it can be manipulated experimentally much more easily than vertebrate models due to technical and ethical issues. *D. melanogaster* tends to have a simpler structure

than the mammalian genome; therefore, it is more prone to complex reverse genetics due to the availability of genetic analysis tools [58]. Many human diseases (around 75 %) genes share homology with *D. melanogaster*, and these genes are found to have more than 90 percent nucleotide sequence identity [59]. Due to these advantages, *D. melanogaster* has been used in several scientific research fields such as cellular patterning, cell death, and survival to understand animal growth and development [60,61].

The four discrete stages of the fly's life cycle, i.e., embryonic, larval, pupa, and adult, pose unique opportunities to access the toxicity of various nanoparticles at various stages. For instance, developmental studies like neuronal development and organogenesis upon nanoparticle exposure can be carried out at the embryonic stage. The wandering instar larvae can be used for physiological and behavioral studies. The adverse effect of nanoparticles on endoreplication and change in morphology can be observed during the late larval stage [62]. The main attraction for using this fly as a nanotoxicity testing model lies in the similarities in response to nanoparticles in behavior and gene regulation in *D. melanogaster* and the mammalian system.

The most common nanoparticle delivery method to the fly is through the mode of ingestion followed by injection and inhalation. The inhalation mode has great potential to study the effect of nanoparticles on the respiratory system [63]. This is of utmost importance as many similarities are found in the fly airway system of flies and mammals.

Systemic and reproductive evaluation of nanoparticle toxicity has been proposed by some authors using the *Drosophila* model [64,65]. In these studies, injection or direct micro-transfer were preferred over ingestion because of the variability in the mount and stability of nanoparticles in food. The microinjection form is more uniform and allows the accurate assessment of the nanogram range of nanoparticles, thereby permitting a large number of replicates and leading to the identification of the specific stage in which mortality occurs [66]. Other endpoints of the nanomaterials toxicity on different phases of the life cycle of *D. melanogaster* have been presented in Fig. 5.

In earlier studies, Ahmad and his associates fed the fly larvae with four carbon nanotubes and found no physical or reproductive effects [67]. However, impaired locomotors' function, grooming behavior, and mortality were observed when these were administered to adult flies. Thus, it was speculated that the adhesion of nanoparticles to the body surface of the fly influences the grooming behavior leading to the transfer of nanoparticles inside the fly's body [68]. In the case of flies treated with metal oxide nanoparticles, size, dose, and material-dependent toxicity were observed [69,70]. Silver nanoparticles were found to cause increased mortality, decreased fecundity, and developmental delay in flies. Similarly, Iron oxide nanoparticles modified with pristine citric acid (300-600 µg/g) showed a significant decrease in female fecundity, delay in the developmental stages from egg pupa to pupae adult transitions, ovarian defects, and delays in egg chamber development. Further, exposure of larvae to different concentrations of food-grade E171 titanium oxide has shown no change in survival rate and fecundity but significantly increased the pupation time.

Copper oxide nanoparticles were found to cause a significant dose-dependent increase in DNA damage in larval hemocytes and caused mutation indicating oxidative stress in these flies [71]. When copper-doped zinc oxide nanoparticles were administered, no loss in climbing, activity behavior and protein content levels was observed [72]. In addition to nanotoxicity testing, more sophisticated and creative research is emerging, Xia *et al*. (2006) evaluated the development of nanoparticle-based drug or gene delivery systems by using the fly as a model

organism. He showed that novel organically modified silica had no significant effect on their viability despite penetration into a fly's brain. Similarly, nano alumina was administered to the flies to evaluate their potential effects on CNS. It was observed that 15 minutes after the ingestion of aluminum nanoparticles, a significant decrease in the average neuronal activity was found, indicating the adverse effects of all nanoparticles on the CNS [73]. Studies are also available regarding the detrimental effects of green and chemically synthesized ZnO NPs and GO NPs on the growth and development in drosophila flies [74,75]. More *in vitro* toxicity studies of nanomaterials on *D. melanogaster* are discussed in Table 4.

Drosophila is extremely sensitive to different conditions they are exposed to, such as different raising and handling practices, humidity, day/ night cycle. This led to the variation in biological effects on flies and, therefore, confound the toxic effects due to nanomaterials [76]. Nanomaterials may have differential effects on drosophila's food intake and feeding patterns; these are not well established yet and, therefore, need proper investigation for accurate studies. Even the different routes of nanomaterials exposure to *drosophila* may affect their stability, which remains to be studied [76]. It can be assessed that despite high structural homology and genetic similarity between humans and *drosophila*, the translation of nanotoxicological findings to the impact of these nanoparticles on human health remains challenging.

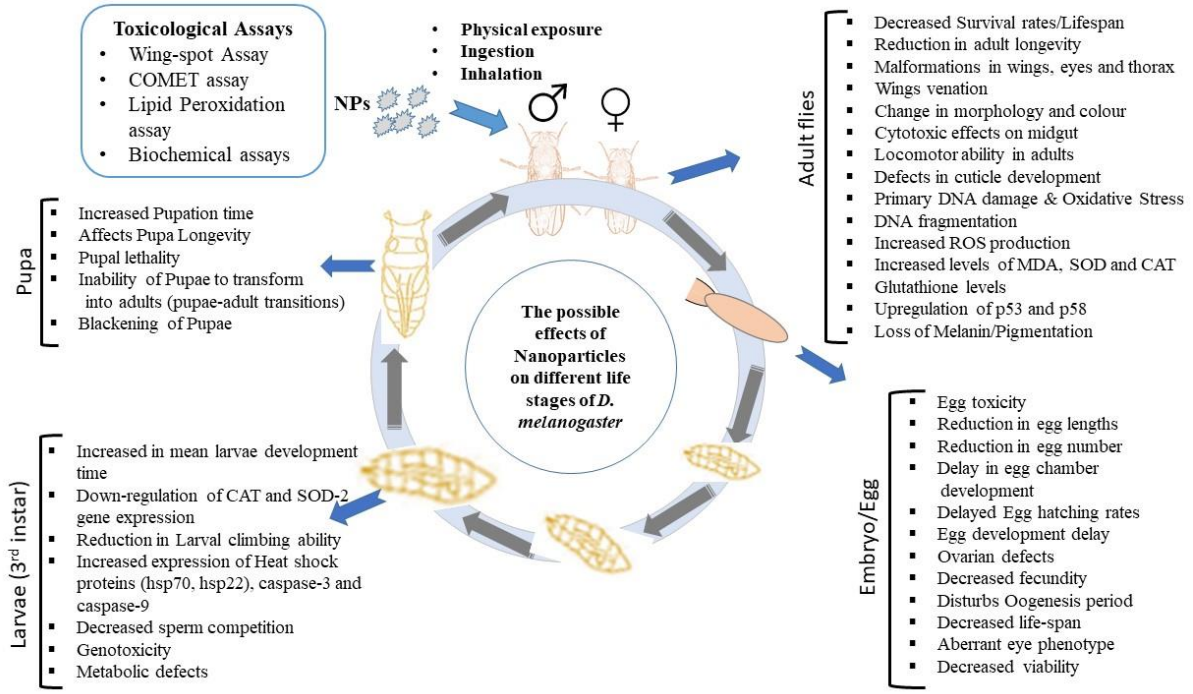

Figure 5. The possible toxicological effects of nanoparticles on various life stages of *Drosophila melanogaster.*

Table 4. Details of various studies on the potential toxicological effects of nanoparticles on *Drosophila Melanogaster.*

S.No.	Species	Nanoparticles studied	NP exposure conc.	Duration of exposure	NP size	Exposure test	Toxicological endpoints/studies	Effects/Results	References
1.	*D. melanogaster* Larvae, *pupae* and adults	Silver nanoparticles (Ag NPs)	10, 50, 100, 200 ppm	6-72 h	Ag NPs: 5-22 nm	Transfer of 10 *Drosophila* larvae transferred to Petri dish containing NP	Pupal mortality Toxicity Pupal longevity Larval development time	Increased mortality with increasing NP conc. 93.3 % mean mortality at 200 ppm	[77]

S.No.	Species	Nanoparticles studied	NP exposure conc.	Duration of exposure	NP size	Exposure test	Toxicological endpoints/studies	Effects/Results	References
		Sulfur nanoparticles (S NPs)			S NPs: 8 nm	(400 μL) impregnated in diet Release of groups of 3-days old females into Petri dishes containing media with NPs	Morphology & colour	No significant effect at 10 ppm No toxicity of sulfur NPs Increase in Mean larval development period Pupae longevity not affected	
2.	*D. melanogaster*	Ag NPs	10-100 ppm	--	20 nm, 100 nm, 500-1200 nm	Transfer of 50 fly eggs(12 hours old) and 30 mL food into secondary flask containing NPs Suspension	Egg pupation and maturation	Size dependent toxicity Reduction in ability of pupae to transform into adults Egg toxicity 100 nm: 59% egg pupation 500-1200 nm: 34% pupation Accumulation at 10 ppm	[78]
3.	*D. melanogaster* larvae	Ag NPs (Acacia gum coated)	50 µg/ml and 100 µg/ml	24 h and 48 h.	10 nm	Mixing of 2 conc. of NPs with food and feeding to third instar larvae	Heat shock stress DNA damage Apoptotic assays Oxidative stress SOD and CAT activities GSH levels	Induced stress High Malondialdehyde level High SOD and CAT content Upregulation of p53 protein and cell signaling protein p38 High caspase-3 and caspase-9 markers	[79]
4.	*D. melanogaster* w^{1118}	Magnetite NPs - Citric acid and APTS (3-aminopropyltriethoxylsilane) coated	300 and 600 µg/g	24(Fecundity) 3 d	15 nm	Rearing in glass vials containing different NP conc	Fecundity Oogenesis Ovarian effects Development	Reduced female fecundity Reduced egg lengths Ovarian defects Delays egg chamber development Development delay 10% decrease in the pupariation at 600 µg/g dose NPs observed in ooplasm and vitelline membrane of the eggs	[80]
5.	*D. melanogaster*	Gold nanoparticles (Citrate coating)	2.05×10^{-3} µg/µl	96 h	15 nm	Transfer of 10 flies in vial with addition of NPs to food, mixing and sonicating before feeding to flies	Phenotypic alterations Genotoxicity Reproductive performance p53 gene expression DNA damage (TUNEL assay)	Decreased fecundity Lower number of eggs laid Lethal genetic mutations Haemocyte DNA damage Production of ROS 3-fold over-expression of p53 gene Cellular defects Mutagenesis: Mutated flies Wings deformation Abnormal phenotypes Eyes defects: Malformations	[81]
6.	*D. melanogaster*	Titania nanoparticles	50, 100, 200, 250 mg/L	24 h	30 nm	Feeding of flies with different NP conc. and collection of eggs laid on food	Survival Life cycle Behaviour patterns Phenotype Body weight Mechanosensory organ structure	Delays life cycle and pupation Abnormal behaviour Wing venation Generation of ROS 63% hatching at 200 mg/L conc. 56% hatching at 250 mg/L conc Pupae blackening Damage of the gut inner layer Reduced body weight and crawling rates Affected bristles	[82]
7.	*D. melanogaster* larvae and adults	TiO_2 NPs	0.005%, 0.01%, 0.05%, 0.1%, 0.5% w/v	24 h	50 nm	Addition of different NP conc. to standard molasses culture medium for flies and larvae	Fecundity(No. of eggs) Egg and larval development Reproduction	Progeny loss Decline in female fecundity No morphological changes Tissue damage Developmental toxicity	[83]

S.No.	Species	Nanoparticles studied	NP exposure conc.	Duration of exposure	NP size	Exposure test	Toxicological endpoints/studies	Effects/Results	References
8.	*D. melanogaster* larvae	Silica NPs	0.1 to 10 mM	24 h	6, 15, 30 and 55nm	Transfer of 3-day-old larvae to plastic vials containing medium and 9mL NP suspension	Genotoxicity Oxidative stress Wing-spot assay Comet assay	DNA damage No toxicity at low conc. Increased ROS levels Indirect dose–response Less toxicity and no genotoxicity	[84]
9.	*D. melanogaster* w1118 strain	CuO NPs	0, 0.05, 0.1, 0.15 or 0.25 mg/mL	-	40-50 nm	Oral route of administration – NPs added to food	Egg-to-adult survivorship Viability Development Cellular ROS detection Assay gstD1 expression TUNEL Assay	Dose-Dependent Toxicity Accumulation of NPs Developmental delay Increase in apoptosis and ROS Apoptosis of the gut epithelial cells NPs observed in lumen Inhibition of the TF Nrf2 Induction of Cell Death and Oxidative Stress in the Gut Reduced viability increase in the expression of GstD1	[85]

Abbreviations used: NP: Nanoparticles; CAT: catalase; *SOD:* Superoxide dismutase; GSH: Glutathione; MDA: Malondialdehyde; gstD1: Glutathione S transferase; ROS: Reactive Oxygen Species;

6. *Danio rerio* (Zebrafish)

Zebra fish is accepted as a modern experimental animal model gradually. Its widespread use is due to its certain characteristics, such as small size, high reproducibility, transparent embryos, and quick development [86]. These advantages fit this model organism between traditional cell culture and mammalian models. Due to transparency, all cells are observable since the early larval stages, along with organs and tissues [87,88]. The cost of zebrafish husbandry is quite low compared to other vertebrate models. Many animals can be housed in a small space and, therefore, it becomes easy to handle and maintain. Zebrafish also possess high fecundity and produce many embryos in a single breeding experiment. Under preferable conditions, a female can spawn around 300 eggs per week [63]. Several signaling pathways also tend to be similar in both zebrafish and humans, with an increased level of homogeneity. Moreover, the development of transgenic fishes is easy; therefore, knockdown experiments can be easily performed [89].

The main reason for using zebrafish as a model organism for nanotoxicity testing is, firstly, it offers biological complexity of multi-organ events, which are difficult to monitor in the case of cell lines. Secondly, due to its high fecundity, a wide dose range of a large number of nanomaterials can be assessed in a single assay. Likewise, well-characterized developmental stages allow easy targeting of specific organs to see the effect of nanoparticles on the zebrafish embryos.

Previously, several studies have been done to assess the toxicity of nanoparticles on zebrafish embryos [90]. Developmental malformations can be used as a parameter for toxicity testing. The malformations include incomplete organ development or deformities of body parts such as the bent notochord, fin malformations, *etc.*, which are also shown in Fig. 6. It was found that when zebrafish embryos were treated with carbon fullerenes, caudal malformations were observed at a concentration of 200 ppb, whereas pericardial edema, yolk sac edema, and pectoral fin malformations were observed at a concentration of 2500 ppb [91]. Similarly, delay in hatching can also be considered one of the criteria for nanotoxicity testing. Usually, the zebrafish embryos hatch within the first three days after fertilization. Recently research was done using titanium oxide nanoparticles to understand the hatching events being undertaken by

evaluating the relationship between the success rate of hatching and hours post-fertilization. Premature hatching was observed in embryos treated with titanium oxide nanoparticles [90].

Zebrafish behavioral response can also be used as an indicator of nanotoxicity. It was found that titanium oxide nanoparticles affect larval swimming parameters like the velocity and subsequent activity level [91]. Kokel *et al*. (2011) generated a behavioral "barcode" to understand the toxicity caused by various nanoparticles. Genotoxicity is also considered the main risk factor for the effects of nanoparticles. Studies on the effect of cadmium nanoparticles on zebrafish are available using RAPD and RT PCR [92]. Reproductive toxicity can also be used as one of the measures to assess the toxicity primarily due to the high reproduction rates of zebrafish. Chronic exposure to titanium oxide nanoparticles leads to disturbance in zebrafish reproduction. A 9.5% decrease in the collective number of zebrafish eggs was observed after 13 weeks of titanium oxide nanoparticles exposure [93].

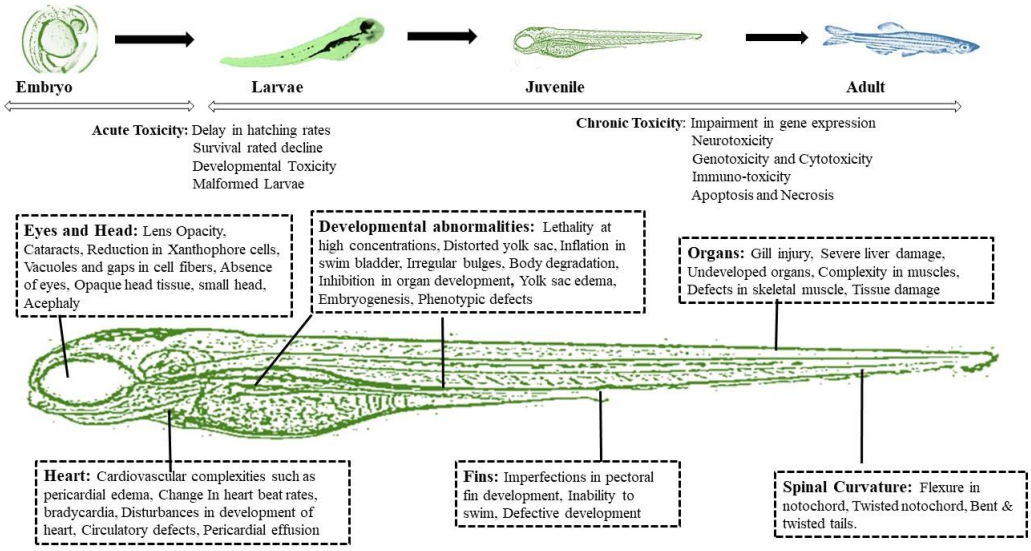

Figure 6. The developmental stages of Zebra fish and various toxicological responses produced by nanoparticle exposure.

Table 5. Summary of various studies on the toxicological effects of nanoparticles on Zebra Fish *(Danio rerio)*.

S. No	Species	Nanoparticles studied	NP exposure concentration	Duration of exposure	NP size	Exposure test	Toxicological Endpoints	Effects/Results	References
1.	Zebra Fish embryos	ZnO NPs	1,5,10,20,50 and 100 mg/L	144 hpf	50-100 nm	Exposed to a solutionm ZnO in a24-well multi-plate.	Developmental Toxicity DNA damage Oxidative stress mRNA expression Measurement in SOD, CAT, GPx and MDA content.	Reduction in hatching rates Induction of morphological malformations Generation of ROS at high concentrations Hyperaemia Pericardial enema Tail deformities Spinal curvature abnormalities Lower SOD activity Inhibition of CAT, MDA activity	[97]
2.	Zebra fish embryos	Ag NPs	5, 10, 25, 50 and 100 mg/L at 28°C	4, 24, 48 hpf	10-20 nm	Incubation at different nano Ag conc. in 24-well plate	Mortality Deformity rates Heartbeat Physical studies Expression of relevant genes (*sox17, gsc, ntl, otx2*)	100% lethality at high concentrations No change in heartbeat rates Irregular bulge in fertilized eggs Distorted yolk sac Phenotypical imperfections (twisted notochord) Pericardial enema Effects on the translation activity of genes at high conc.	[98]
3.	Zebrafish embryos	(a) CuO NPs	0.01, 0.1, 1, 5, 10 mg/L	120 hpf	197 nm	8 newly	Survival	Reduced survival	[99]

S. No	Species	Nanoparticles studied	NP exposure concentration	Duration of exposure	NP size	Exposure test	Toxicological Endpoints	Effects/Results	References
		(b) ZnO NPs	0.01, 0.1, 1, 5, 10 mg/L		100 nm	fertilized embryos exposed for 120 h in 24-well plates	Malformations Hatching rates Autometallography	increased malformation prevalence Hatching impairment Sub lethal toxicity Malformed larvae	
		(c) TiO_2 NPs	0.1, 1, 10, 50, 100 mg Ti/L		10-400 nm				
4.	Adult zebra fish	TiO_2 NPs	0, 10, 50, 100, 150, 200 and 300 mg/L	96 hours	20-70 nm	In a 2-L glass beaker containing 1.5 L of the test solution	Acute toxicity Oxidative stress and damage	No mortality at 50 mg/L conc. 100% death at 300 mg/L LC_{50} 124.5 mg/L Affected activity of SOD, CAT and MDA Reduced glutathione and protein carbonyl Cell membrane damage High stress Generation of OH radicals	[100]
5.	Zebra fish embryos	$CoFe_2O_4$ nanoparticles	10–500 µM for 96 hours	96 hpf	40 nm	Exposed to different nanoparticle concentration in tank containing E3 medium	Survival rate Malformations Heart and tail functioning Hatching	Acute toxicity with increasing concentration Apoptosis of cells in head and tail region Flexure of spinal cord/tail Finfold malformations Yolk sac edema Delay in hatching Decrease in metabolism Oxidative stress	[67]
6.	Zebrafish embryos	Silica NPs	25, 50, 100 and 200 µg/mL	4-96 hpf	62 nm	10 embryos in 2 mL solution/well in 24-well plate at 28^0 C	Cardiovascular Toxicity Larval Mortality Embryonic malformation Heart rate Whole-embryo death	Pericardia toxicity Bradycardia Inhibition of angiogenesis Disturbs formation of heart Development toxicity Head malformation and bent tails Pericardial edema Yolk sac edema Necrosis and Apoptosis	[101]
7.	Zebrafish embryos	Ag NPs	0.4 mg/L	4 hpf	40 nm	50 embryos in 8 mL medium in a 6-mm dish	Eye(Lens) development Phenotypic studies Apoptosis Histopathological study	Lens opacity(Cataracts) Formation of vacuoles and gaps in lens fibers Down regulation of structural crystalline genes Do not affect Retina Decline in xanthophore cells	[102]
8.	Zebrafish embryos	(a) Ag NPs	10, 25, 50, 75, and 100 µg/ml for all three NPs	72 hpf	15-35 nm	40 embryos in each well of a 6-well plate	Mortality rates Hatching Metal accumulations Physical defects	Drop in hatching and heartbeat rate Growth retardation Twisted tails Inability to swim Pericardial effusion Defective development of pectoral fin Abnormal cardiac morphology and circulatory defects Defects of the eyes	[103]
		(b) Au NPs			5-35 nm				
		(c) Pt NPs			3-10 nm				
9.	Zebrafish embryos	ZnO NPs	1, 5, 10, 25, 50, and 100 mg/L withdilutions in E3 medium	96 hpf	30 nm	1 embryo per well in 24 well plate	Toxicity studies Hatching Developmental effects	Dose dependent toxicity Decreased hatching rates at 10 mg/L No hatching at 50 and 100 mg/L Tail defectsShortening of larvae body length Inhibition of embryonic development	[104]
10.	Zebrafish embryos	colloidal Au and Ag NPs	0.25, 2.5. 25, 250 µmol diluted in egg-water suspension	120 hpf	3, 10, 50 and 100 nm	cAu and cAg egg-water solutions at 28.5^o C for 5 days	Morphological defects Survival Toxic adverse effects.	Toxicity of cAg more than cAu Mortality with increasing conc	[105]

S. No	Species	Nanoparticles studied	NP exposure concentration	Duration of exposure	NP size	Exposure test	Toxicological Endpoints	Effects/Results	References
								80% mortality at 250 µmol Small head and opaque head tissue Retarded growth (Tail defects and body degradation) Bent spines	
11.	Zebrafish	Copper(Cu) NPs	0.25 mg/L, 0.25 mg/L, 1.5 mg/L, 100 μg/L	48 hpf	80 nm	Exposure in 10 L tanks containing 10 female zebrafish per tank	Histopathological analysis of organs(liver and kidney toxicity) Gill toxicity BUN and PALT levels Microarray analysis	Acute toxicity at LC_{50} of 1.5 mg/L Gill damage and impairing of function Damage to gill lamellae Edema of primary and secondary gill filaments Hypertrophy of epithelial cells Increased BUN levels No effect on PALT Differential expression of 82 genes	[106]
12.	Zebrafish	Nickel NPs	10 to 1000mg/L	24 hpf	30, 60, 100 nm, aggregated 60 nm entities	25 embryos in six well plates	Histological studies Study of developmental defects Immunohistochemistry Embryo Visualization by staining	Dose-dependent toxicity Skeletal muscle fiber separation Defects in skeletal muscle Inflation of swim bladder Accumulation of NPs in digestive tracts Intestinal defects Defects in jaw cartilage at higher conc.	[107]
13.	Zebrafish embryos	Selenium NPs	0.05, 0.1, 0.2, 0.5, 1, 2 and 5 mg/L	24-120 hpf	25-250 nm	1 egg per well in 96-well plates at 28° C (egg-water medium)	Mortality Hatching rates Calculation of LC_{50} at different NPs conc	LC50 value at 1.77 mg/L Delayed hatching(75%) at 72 hpf [0.2 mg/L] Higher mortality (56.25%) at 72hpf even at 1 mg/L	[108]
14.	Zebrafish	Au NPs	0, 0.26, 0.53, 1.1, 2.1, 4.2, 6.3 µg/ml	120 hpf	11.6 nm	2 embryos/ well in 24 well plates exposed to 2 mL suspension	Survival rates Hatching Effects on embryonic development EDS of the embedded nanoparticles in tissues and cells.	Concentration-dependent toxicity Stochastic effects Finfold abnormality and acephaly Cardiac malformation Yolk sac edema	[109]
15.	Zebrafish	ZnO NPs	50, 10, 5, 1, 0.5, 0.1, 0 mg/L	96 hpf	50 – 360 nm	Eggs in an aqueous suspension containing NPs	Dose-dependent Toxicity studies Embryos or larvae survival Hatching rate Malformation	Tissue damage and ulcerations Decreased survival and hatching rates LC_{50} at 1.793 mg/L Zero survival at 50 mg/L Pericardial edema Body arcuation	[51]
		Nano TiO_2	500, 100, 50, 10, 1,0 mg/L		100 – 550 nm			No effects on hatching rates and survival No significant toxicity	
		Nano Al_2O_3	1,000, 100, 10, 1, 0 mg/L		285 – 2,450 nm			Non-toxic to embryos	

Abbreviations used: NP: Nanoparticles CAT: catalase; GPx: Glutathione peroxidase; *SOD:* Superoxide dismutase; MDA: Malondialdehyde; LPO: Lipid Peroxidation; AP: Acid Phosphatase; BUN: Blood Urea Nitrogen; PALT: Plasma alanine aminotransferase; LC_{50}: Median Lethal concentration; hpf: hours post-fertilization

Other studies were also done to assess the overall toxicity of different nanoparticles. Silver nanoparticles were found to cause oxidative stress and further apoptosis in zebrafish liver, confirming the hepatotoxic behavior of silver nanoparticles [94]. Toxicity of the mixture of copper oxide and cerium oxide was tested on zebrafish embryos, and concentration as less than 0.01 µg/ml was toxic to those embryos [95]. Similarly, carbon nanotubes were also found to affect zebrafish lung and dermal cellular behaviors [96]. Further studies on zebrafish are also discussed in Table 5.

In zebrafish, rapid developmental stages make performing a systematic embryo-based nanotoxicity assay challenging. In addition, the lack of an established protocol limits its comparison to other model organisms [89]. It is poikilothermic, and the placenta is absent in the developing embryos, which shows that nanoparticles may be metabolized differently from mammals. As seen in other organisms, the environment influences the results obtained through the toxicity testing assays. Again extrapolation of the results obtained from zebrafish studies to humans is challenging.

7. *Rodents* (mice and rats)

In various studies, rodents (especially Mus musculus and Rattus norvegicus) are extensively used tomodel organisms. It has developed into a premier model system for genetic research owing to its close genetic and physiological similarities to humans. About 99% of rodents' genes are equivalent in humans. Therefore, they are considered an ideal animal model for studying various diseases such as cancer, cardiovascular diseases, and diabetes. Although many species (such as dogs, pigs, and non-human primates) are more closely related to humans than rodents, working with these species tends to be more expensive and is full of ethical concerns [110]. The studies about genetic homology between humans and rodents have been a benchmark for creating transgenic, knockout out, and knock-in mice.

Due to their small size, the housing of rodents is more convenient and easy to maintain. Rodents are far better than flies or worms for studying complex biological systems such as the immune system, nervous system, endocrine system, etc. Even genetic manipulation at the embryo level is also possible. As these animals have been used in research over decades, researchers have developed an understanding of mouse biology and genetics and developed large numbers of tools and techniques to study them, which are not yet available for larger mammals [110]. In recent years, a shift in rodent-based research has taken place in favor of rats rather than mice as the major model of choice in biological research. It is easier to monitor the physiology of the rat, and it has been observed that a volume of data has developed in rats that will take years to be replicated in the mouse. In several studies, the physiology of rats is more like the corresponding human condition, which gives an in-depth knowledge of the effects of various chemicals or diseases [111]. In nanoparticle toxicity testing, the animal's size enhances its use as a model because the size of important substructures in organs is proportional, giving the distance effects of nanoparticle administration to specific anatomical areas.

Moreover, serial blood drawing is easier in rats to see its effects on the circulatory system. The advantages of the mice model are that due to their small size, they are easier to handle, and genetic modulation (especially transgenic mice) is more convenient [112]. Nanomaterials toxicity has various endpoints on mice, as discussed in Fig. 7.

Research studies have shown that 1mg/kg of the bodyweight of Ag nanoparticles increases levels of IL- 1,6,4,10,12 and TGF-B; eventually, these cause organ toxicity and inflammation in mice [113]. Similar effects were seen when purified single-walled carbon nanotubes were instilled by pharyngeal installation leading to inflammation, fibrosis, and granulomatous pneumonia in mice [114]. Similarly, intragastric administration of titanium oxide nanoparticles in a size range of 5-6 nm leads to ovarian aberrancy and alteration in anatomic gene announcement levels, thereby proving its toxic nature to the reproductive system of mice [115]. It was also seen that single-walled and multi-walled carbon nanotubes were found to promote an inflammatory response in the production of TNFα and MCP-1 (Monocyte

Chemoattractant Protein-1) [116]. It was seen that administration of quantum dots in mice led to lung inflammation, injury, and DNA damage. It was also found that surface area is correlated well with inflammatory response [117]. Nano-copper has also been shown to cause severe impairment in the liver, kidney, and spleen in mice models, thereby showing the toxic effects of copper oxide nanoparticles [118].

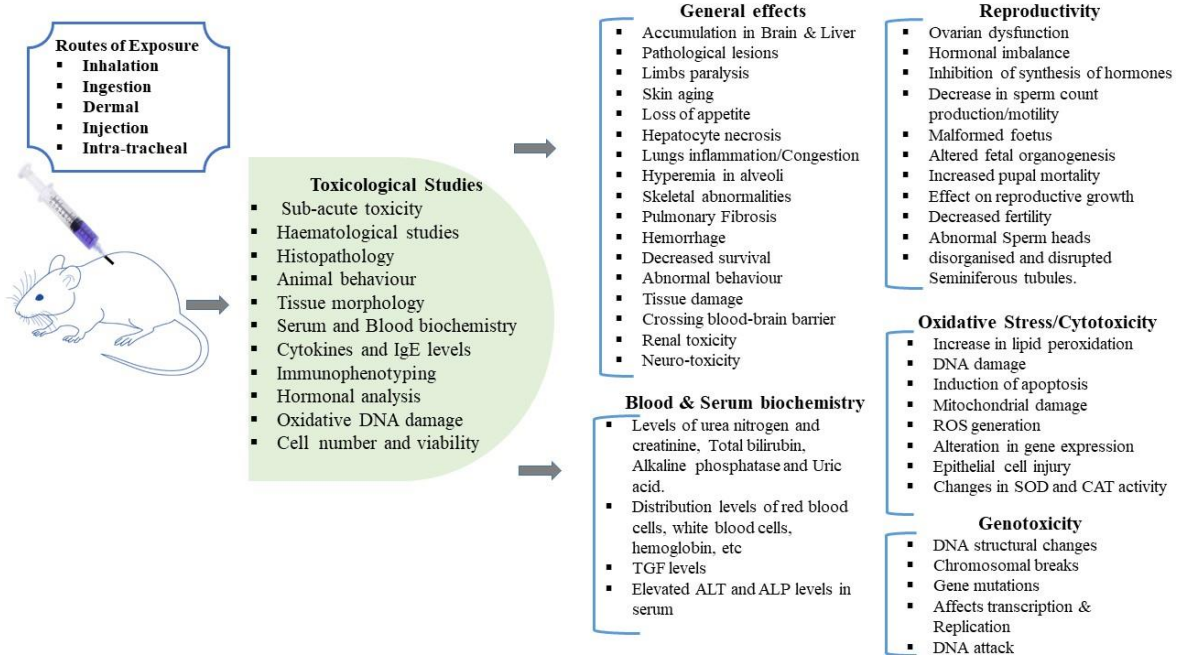

Figure 7. An overview of nano-toxicity produced by nanomaterials in rodents (*Mus musculus and Rattus norvegicus).*

Similarly, several studies have also been performed on rat models. Abdelhalim *et al*. showed that gold nanoparticles cause nephrotoxic effects demonstrated histochemical and histological alterations in the renal tissues of rats [119]. Similarly, Gui *et al.* showed the toxicity caused by titanium dioxide nanoparticles, assessed by physiological and gene expression modifications in rats [120]. Silver nanoparticles were also found to accumulate= in the immune system organs of rats, leading to mild irritation in the spleen and thymus[121]. In other studies using rats, it was also observed that synthesized HANPs were nontoxic to rats at a cellular level when they were exposed to them dermally [122]. The toxicity studies of NiO NPs on rats showed that they induce genotoxicity in rats when administered through the oral route. But extensive mechanistic investigations are needed before drawing any conclusion on their toxicology [123]. Other studies are summarized in Table 6.

Table 6. Details of various studies on the toxicological effects of nanoparticles on *Mus musculus.*

S.No.	Species	Nanoparticles studied	NP exposure conc.	Duration of exposure	NP size	Exposure/Routes	Toxicological endpoints/studies	Effects/Results	References
1.	ICR mice (6 weeks)	Ag NPs	0.25 mg/kg, 0.50 mg/kg, 1.00 mg/kg	14 d 28 d (repeated toxicity)	22 nm, 42 nm, and 71 nm	Oral administration of NPs for 5 mice per group and 6 mice per group (repeated toxicity)	Serum TGF-β level B cell distribution Body weight Histopathology blood chemistry Cytokines level Organ toxicity Inflammation	Accumulation of Ag in organs (brain, lung, liver, kidney, etc.) TGF-β level increased for all NPs Increased distribution of CD8+ T cells Increase in B cells Body weight not affected Increased ALP and AST levels Dose dependent increase in cytokine(IL-1,IL-6, TNF-α) Neuronal degeneration	[124]

S.No.	Species	Nanoparticles studied	NP exposure conc.	Duration of exposure	NP size	Exposure/Routes	Toxicological endpoints/studies	Effects/Results	References
2.	BALB/c line mice	Ag NPs	5; 3.3; 2.5;1.6; 1.0;0.7; 0.5;and 0.05 (10^{-3} Ag/L)	30 d	9 nm	Intraperitoneal injection of NPs (in distilled water, 0.2 ml)	Genotoxicity Survival rates Lethal dose	Abnormal sperm heads Paralysis of the hind limbs Decreased motor activity at high doses LC_{50} = (2.74 ± 0.67) × 10^{-3} Ag/l Decreased survival DNA damage Oxidative stress	[125]
3.	Male C57/BL 6 mice	Au NPs	40, 200, and 400 µg/kg/day	8 d	12.5 nm	Intraperitoneal injections of 100 µL of Au NPs solution	Survival and behavior Body weight Morphology Blood biochemistry Accumulation Serum analysis Histopathology	Increased Au levels in the blood Dose-dependent accumulation No mortality No inflammation No morphological changes	[126]
4.	CD1 mice	Cadmium oxide nanoparticles	240 mg CdO NPs/m^3	7 d	15 nm	Exposure for 3 hours a day through nose-only exposure tubes	Lung damage Inflammation Systemic immunity Pulmonary effects Differential blood counts LDH activity Cytokine levels	Cell injury Alteration in immune function Pulmonary inflammation 4.5-fold increase in protein conc. of BALT 6–8% decrease in body weight Elevation of LDH activity	[127]
5.	BALB/c hairless mice	TiO_2 NPs	5% TiO2	60 d	4- 90 nm	Dermal exposure of NPs in dorsal skin in the interscapular form	SOD, MDA, and HYP analysis Organ structure Histopathology	Pathological lesions Oxidative stress Skin aging Reduced body weight ROS generation Accumulation of NPs in spleen, heart, and liver Increase in MDA levels Decreased SOD activity Necrosis	[128]
6.	C57Bl Mice	TiO_2 NPs	50, 100, 250, and 500 mg/kg TiO2	5 d	21 nm	NPs were given with drinking water and calculation of water intake	Genotoxicity Oxidative DNA damage Inflammation	Dose-dependent response DNA deletions Increase in eyespots Increased DNA strand breaks 1.5-fold increase in 8-OHdG level Upregulation of TNF α, IFN-γ and IL-8	[129]
7.	Male Swiss albino mice	ZnO NPs	5, 50, 300 mg/kg	14 d	30 nm (TEM) 272 NM (DLS)	Oral routes of exposure	Body weight Histopathology Genotoxicity TUNEL assay Lipid peroxidation (LPO) assay Oxidative DNA Comet assay Apoptosis	Accumulation of NPs in liver No difference in body weight No mortality Cell injury Increased ALT and ALP levels Pathological lesions Oxidative stress No increase in lipid peroxidation	[130]
8.	Female mice	ZnO NPs	40 mg/ml	5 d	20-30 nm	Exposure through oral gavage.to mice	Effects on serum elements and sexual hormones Histopathology	Hepatic injury Necrosis & Liver damage Loss of appetite Inflammation in lungs Swelling in epithelial cells Hyperemia in alveoli and edema	[131]
9.	Adult female KunMin g mice	Iron Oxide Magnetic NPs	3 mg/mL	-	10,20, 30, and 40 nm	0.1 mL NPs (20 mg/kg) administered to mice via consecutive tail-vein injections	Blood biochemistry Mice weight Food intake Haematology Histological examination Toxico-kinetics Biodistribution Physiology	Accumulation in liver and spleen High concentration of iron in the liver Entry of NPs into brain and uterus No effect on body weight and food intake Increased levels of total bilirubin (TBIL), direct bilirubin (DBIL) Inflammation & tissue damage	[132]
10.	C57BL/ 6 mice	CuO NPs	2.5 mg/kg, 5mg/kg, 10 mg/kg	7-28 d	46.5 nm	Intranasal Delivery of different conc. of NPs	Genotoxicity and Cytotoxicity TUNEL staining Oxidative stress	Pulmonary inflammation Apoptosis of epithelial cells Epithelial cell injuryROS production Pulmonary Fibrosis Upregulation of TGF-β1	[133]

S.No.	Species	Nanoparticles studied	NP exposure conc.	Duration of exposure	NP size	Exposure/Routes	Toxicological endpoints/studies	Effects/Results	References
								Excessive production of ROS	
11.	Female F344 rats	Gold NPs	1,000 mg GNPs/kg body weight	1, 7, 14, 21, 28 d	15 nm	Intravenous exposure	Survival Histopathology Immunohistochemistry Biochemical profiles Oxidative stress Inflammation	Fatality within 24 hours Higher accumulation in spleen Greater fecal excretion NPs internalized by tissue macrophages and vascular endothelial cells	[134]
12.	Sprague Dawley rats	Silver nanoparticles	5.36 mg/kg, 13.4 mg/kg	1-6 months	8.93-33.4 nm	Oral exposure	Testicular toxicity Hormonal assay SOD and MDA levels Sperm morphology and viability	Decrease in testosterone at 6th month No significant changes in LH and FSH Abnormal sperms (double head or tailless) Decrease in DNA chromatin integrity	[135]
13.	Sprague – Dawley rats	Silver nanoparticles (citrate and PVP coated)	0.5-1 mg/kg	1, 7, 21 d	20-110 nm	Intratracheal instillation	Toxicity in lungs Macrophage quantification Silver deposition quantification	Persistence in lungs after 21 d Silver positive macrophages Ag deposition in subepithelial basement membrane No effects of particle size	[136]
14.	Male wistar albino rats	CuO NPs	5, 50 mg/kg	14 d	50 nm	Oral exposure	Glutathione levels Catalase, superoxide dismutase (SOD), Lipid peroxidation assay assay	No mortality Alterations in enzyme levels Decrease in GSH, CAT, and SOD Increased levels of MAD Dose-dependent toxicity	[137]
15.	Male Sprague – Dawley rats	ZnO NPs	Whole air inhalation	1 d	35 nm	Inhalation	Effect on the respiratory system 1H and J-resolved NMR analysis Metabolomics	Increased isoleucine and valine Decreased acetate, trimethylamine n-oxide, taurine, glycine, formate, ascorbate and glycerophosphocholine Membrane impairment in rat kidneys Inflammation and oxidative stress	[138]
16.	Male Wistar rats	NiO NPs	0.13–0.37 mg/m3	28 d	54 nm	Inhalation exposure by aerosols	BALF of rat lungs SPD levels (surfactant protein D) Phospholipid conc. Total protein conc. Histopathology of lungs	Inflammation highest in C_{60} Fullerenes and lowest in NiO NPs No change in phospholipid concentration in C_{60} SPD levels increased in NiO NPs Pulmonary surfactants	[139]
17.	Male Wistar rats	PEG-coated iron oxide nanoparticles	0.03 mg/kg body weight	2 h, 24 h, 7 d	35 nm	Intravenous	Effects on rat liver Body weight TXRF spectroscopy	Change in the chemical composition of liver tissue Elevation of Cu, Ca and Zn in serum Short term accumulation of NPs in liver Inflammatory response	[140]
18.	Male Wistar rats	Cadmium sulphide (CdS) NPs	10 mg/kg body weight	45 d	5-9 nm	Gavage feeding	Effects on kidney Cd conc. in kidney Creatinine and uric acid levels Metallothionein conc. Sulfhyfryl contents Kidney histopathology	No significant effect on body weight Cd- metallothionein induced Decrease in renosomatic index Higher creatine levels Glomerulonephritis Epithelial degeneration	[140]

Abbreviations used: NP: Nanoparticles; EC_{50}: Median Effective Concentrations; LC_{50}: Median Lethal concentration; CAT: catalase; GSH: Glutathione; *SOD:* Superoxide dismutase; ALP: Alkaline phosphatase; AST: Aspartate transaminase; BALT: Bronchus associated lymphoid tissue

The major drawback of using a rodent model is its cost. It is very costly compared to other model organisms and has many ethical concerns about experimentation. Moreover, high throughput testing cannot be done using a rodent model because testing a large number of NMs requires many animals in one go that is very difficult to obtain and take care of. The

development of rodent embryos occurs inside their body; therefore, it is difficult to observe the effect of NMs on different stages of development of mice embryos.

8. Conclusion

This review systematically provides general information on various model organisms used in nanotoxicology analysis, considering their pros and cons. *In vivo* testing is important for advancement in nanotechnology as these studies play a key role in making responsible regulatory decisions. However, most of the reported literature is based on *in vitro* studies, which do not correspond enough to the *in vivo* results. Several factors such as the absence of the evaluation of organ systems, the difference in dose administration, nanoparticles, and the problem of extrapolation of these results to humans are involved. Therefore, proper animal model studies are required wherein the systematic evaluation of different characteristics of nanostructures can be done, and their correlation to *in vivo* behavior can be easily established. Hence, the present review considers the risk of nanotoxicity and s on using appropriate model organisms for NM testing by supplying safe nanoparticles in society.

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Conflicts of Interest

The authors declare no conflict of interest.

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