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REVIEW

In vitro and in vivo toxicity assessment of nanoparticles

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Abstract

Nanotechnology has revolutionized gene therapy, diagnostics and environmental remediation. Their bulk production, uses and disposal have posed threat to the environment. With the appearance of these nanoparticles in the environment, their toxicity assessment is an immediate concern. This review is an attempt to summarize the major techniques used in cytotoxity determination. The review also presents a detailed and elaborative discussion on the toxicity imposed by different types of nanoparticles including carbon nanotubes, gold nanoparticles, silver nanoparticles, quantum dots, fullerenes, aluminium nanoparticles, zinc nanoparticles, iron nanoparticles, titanium nanoparticles and silica nanoparticles. It discusses the in vitro and in vivo toxological effects of nanoparticles on bacteria, microalgae, zebrafish, crustacean, fish, rat, mouse, pig, guinea pig, human cell lines and human. It also discusses toxological effects on organs such as liver, kidney, spleen, sperm, neural tissues, liver lysosomes, spleen macrophages, glioblastoma cells, hematoma cells and various mammalian cell lines. It provides information about the effects of nanoparticles on the gene-expression, growth and reproduction of the organisms.

Keywords Nanoparticles · Toxicity assessment · In-vivo toxicity · In-vitro toxicity · Diagnostics

Abbreviations

CdSe/ZnS	Cadmium selenide/zinc sulphide
CdTe	Cadmium telluride
CNTs	Carbon nanotubes
DCFDA	2′,7′-Dichlorofluorescein diacetate
DTNB	5,5′-Dithiobis-(2-nitrobenzoic acid)
EPR	Electron paramagnetic resonance
HBMVECs	Human brain microvascular endothelial cells
HEK	Human epidermal keratinocyte
HMSC	Human bone marrow derived mesenchymal stem cells
LD50	Lethal dose 50
LDH	Lactate dehydrogenase
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MW-CNTs	Multi-walled carbon nanotubes
PI	Propidium iodide
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SCGE	Single cell gel electrophoresis assay
SOD	Superoxide dismutase
SW-CNTs	Single-walled carbon nanotubes
TEMP	2,2,6,6-Tetramethylpiperidine
TUNEL	TDT-mediated dUTP-biotin nick-end labelling
WTS-1	Water soluble tetrazolium salts
XTT	2,3-Bis-(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide

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Introduction

Nanoparticles are produced in the size range 1–100 nm [1]. With the advancement in the technology, there has been a tremendous growth in their applications [2, 3]. Nanoparticles are used in additives for paints, ceramics, foods, paper, packaging, drug delivery, biosensor and cancer therapy [4]. They are also used as tumour detector [5], paclitaxel [6] and radiotherapy dose enhancer [7, 8]. They are in demand due to their small size and greater surface area to volume ratio [9]. These properties result in higher chemical reactivity and increased reactive oxygen (ROS) production [10, 11]. Nanoparticles have received much attention due to their toxicity imposed on the environment during production and disposal of consumer products [12] (Fig. 1). They impose toxicity by various processes. Nanoparticles can easily cross the cell

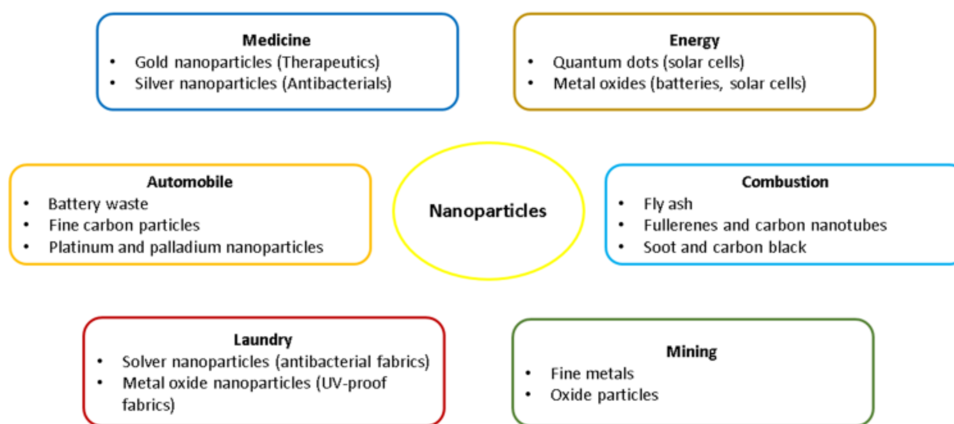

Fig. 1 Applications of nanoparticles in different occupation

membranes and interact with intracellular metabolism [13]. ROS formation is one of the mechanisms for nanoparticle toxicity [14, 15]. Interaction of nanoparticles with cells induces pro-oxidant effects leading to ROS generation, mitochondrial respiration and NADPH-dependent enzyme systems [16–18]. Upon internalization of nanoparticles, phagocytosis induces the production of reactive oxygen species ROS [17–19].

The reviews published so far on nanoparticle toxicology provide information about toxicity of engineered nanoparticles to environmental microorganisms [20], titanium oxide nanoparticle toxicity [21], gold nanoparticle toxicity [21], risk management of inhaled nanoparticles [22], induced mitochondrial toxicity of silver nanoparticles [23] and single-wall carbon nanotube toxicity [24] and multi-wall carbon nanotube toxicity [25]. The published reviews are either too general or very specific (focusing on a single nanoparticle or toxicological effects on a particular organ) to explain the toxicity. Second, these reviews are unable to provide a comprehensive and detailed information on the toxicity assessment on the higher organisms and cell lines.

This review presents a detailed and elaborative discussion on the toxicity imposed by the nanoparticles on rat, mouse, pig, guinea pig, human cell lines and human. The review also focuses on (1) summarizing the techniques useful in determining the toxicity of nanoparticles. (2) Determining the toxic effects of nanoparticles (carbon nanotubes, gold nanoparticles, silver nanoparticles, aluminium nanoparticles and quantum dots, etc.) both in vitro and in vivo and (3) evaluating the effect of nanoparticles on the gene-expression, growth, behaviour and reproduction of organisms.

Assessment of nanoparticle toxicity

Various methods are available for the toxicity assessment imposed by nanoparticles on the organisms. Figure 2 presents the types of nanoparticles, experimental models and toxic effects imposed by nanoparticles. The methods for toxicity assessment can be categorized as in vitro and in vivo.

In vitro assessment methods

In vitro nanoparticle toxicity assessment is one of the important methods. The advantages include lower cost, faster and minimum ethical concerns. Assessment can be subdivided into proliferation assay, apoptosis assay, necrosis assay, oxidative stress assay and DNA damage assays.

Proliferation assays

This assay is used to measure the cellular metabolism by assessment of metabolically active cells. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) is the most commonly used tetrazolium salt for in vitro toxicity assessment of nanoparticles [26]. The technique is advantageous due to quick yields, reproducible results and minimum manipulation of the model cells [27]. The assay is based on the measurement of tetrazolium salt and it can sometimes lead to altered measurement due to changes in the culture media additives [28], media pH [29], ascorbate [30] and cholesterol [31]. The MTT assay also produces formazan; therefore, the assays such as such XTT or WST-1 which produce soluble dyes are preferred. [^{3}H] thymidine incorporation is a method used for assessment of cellular proliferation, but this method is avoided due to toxicity and relatively high cost [32]. Alamar Blue is used to measure the cellular redox potential and advantageous as compared to MTT assay due to simpler sample preparation [33]. But the success of the Alamar Blue is hindered due to unavailability of the biochemical mechanisms of the assay and reaction of non-porous silicon with Alamar Blue in the absence of the cells [34]. Another assay is known as cologenic assay where the proliferating cells are counted by visual inspection after nanoparticle exposure is also used [35].

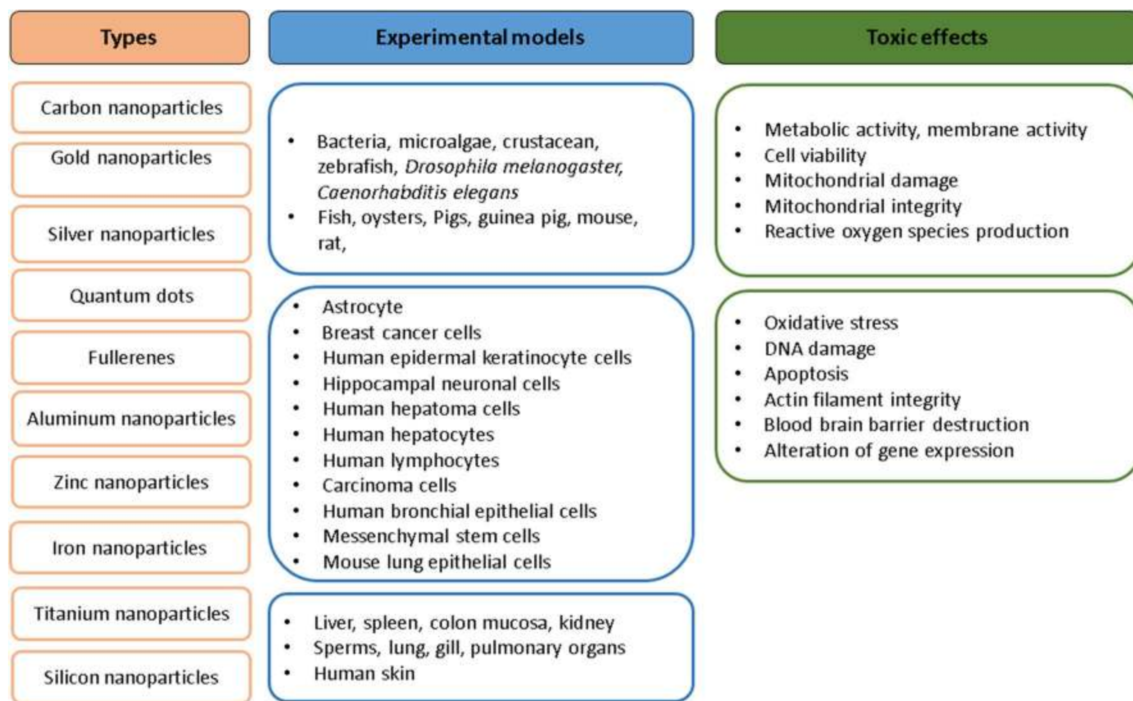

Fig. 2 Nanoparticles types, experimental models used for the studies and toxic effects ofnanoparticles

Apoptosis assay

Apoptosis is one of the major markers observed in the in vitro assessment of nanoparticle toxicity. Generation of excessive free radical is considered the cause of apoptosis and DNA damage [36, 37]. Evidence suggested that apoptosis and DNA damage can be caused by oxidative stress in cell culture systems [36]. Many studies have reported the apoptosis induced by nanoparticles. In vitro studies indicated that silver nanoparticles caused apoptosis in mouse embryonic stem cells [38]. In another investigation, the release of apoptosis markers viz. caspase-3 and caspase-9 were examined on the treatment of larval tissues of *Drosophila melanogaster* with silver nanoparticles at concentrations of 50 and 100 µg/ml for 24 and 48 h. The results suggested the involvement of silver nanoparticles in the apoptotic pathway of *D. melanogaster* [39]. Up-regulation of p38 protein expression was also demonstrated in the exposure of silver nanoparticles in *D. melanogaster* in time- and dose-dependent manner. Up-regulation of genes and extensive DNA insult is responsible for inducing cell death and a cascade of apoptosis pathway [40, 41].

There are a number of methods for assessment of apoptosis. These include Annexin-V assay [42], Comet assay [43], TdT-mediated dUTP-biotin nick end labelling (TUNEL) assay [44] and inspection of morphological changes [45]. The DNA laddering technique is a technique which is used to visualize the endonuclease cleavage products of apoptosis [46]. An irregular reduction in the size of cells and by DNA fragmentation confirms the induction of apoptosis. Agarose gel electrophoresis can easily discriminate between apoptotic and necrotic modes of cell death [47, 48]. Genomic fragments obtained during electrophoresis of irregular sizes are typical of necrotic cells and a ladder-like electrophoretic pattern indicates apoptotic internucleosomal DNA fragmentation [49]. Annexin-V and propidium iodide (PI) are typical cell death markers used in toxicity assessment. The assay works on the principle that when Annexin-V bounds to phosphatidylserine, it shows increased fluorescence and hence indicates the externalization of the plasma membrane. This externalization of the plasma membrane is induced by activation of the caspase-dependent pathway. PI is an impermeable dye which stains the nucleus only when the integrity of the cell membrane is lost, which can be related to the late stage of apoptosis [21, 50]. When human HepG2 hepatoma cells were treated with silica nanoparticles, morphological changes in the nucleus and induction of apoptosis was observed [51]. The investigation demonstrated the induction of apoptosis measured by Annexin V/PI in HeLa cell lines treated with gold nanoparticle [52]. Single cell gel electrophoresis assay (SCGE), or comet assay, is a sensitive tool for the detection of the mutagenic potential of a test material [53]. It is used to detect single- and double-stranded DNA breaks in individual cells, both in vitro and in vivo [54, 55]. It is also used to quantify oxidative DNA damage, alkali-labile sites, DNA–DNA or DNA–protein cross-links

and abasic sites [56–58]. The assay is based on the principle that the damaged DNA fragments will migrate out of the cell when an electric current is applied, whereas the undamaged DNA will remain in the cell nucleus. In assay, damaged DNA resemble the tail and the intact DNA resemble the head. The extent of DNA damage is correlated with the size and shape of the tail and the distribution of DNA within the comet [59–61]. In the procedure, the cells are lysed to remove cellular protein and the damaged DNA is allowed to migrate away from the nucleus by undergoing electrophoresis. DNA-specific fluorescent dye is used to stain the samples. The gel is then analysed for the amount of fluorescence in the head and tail and the tail length [62–64]. Comet assay was used to assess the toxicity of zinc oxide nanoparticles at 25 mg Zn/L on *D. tertiolecta* which resulted in 55% nuclei damage [65]. In another investigation, comet assay was used to measure the toxicity imposed by SiO_2 nanoparticles on *D. tertiolecta* at 125 mg/L, which resulted in increasing genotoxic effects after 72 h [66]. In the similar study on *D. tertiolecta,* using TiO_2 nanoparticles for 24 h resulted in more than 70% damage to the nuclei after 72 h [66].

TUNEL assay is one of the most widely used methods for detecting DNA damage in situ TUNEL staining [67]. IT was initially described as a method for staining cells that have undergone apoptosis or programmed cell death and internucleosomal DNA fragmentation [47, 68–70]. TUNEL assay is based on the ability of the enzyme terminal deoxynucleotidyl transferase to incorporate labelled dUTP into free 3′-hydroxyl termini generated by the fragmentation of DNA [67]. IT is not limited to the detection of apoptotic cells only. It can be used to detect DNA damage associated with non-apoptotic events including necrotic cell death which is induced by exposure to toxic compounds [71]. TUNEL assay was reported to stain cells undergoing active DNA repair [72]. The pancreatic islet function of Goto Kakizaki rats was estimated by TUNEL assay of pancreatic β-cells after treatment with insulin-loaded selenium nanoparticles (25 IU/kg) daily for 2 weeks. In TUNEL assay, apoptotic cells declined to 2.3 % from initial 17.6 % in the treatment [73].

Necrosis assay

Necrosis is measured by the integrity of the membrane and it is commonly used to determine the viability of the cells. Membrane integrity is measured by uptake of the dye such as Neutral Red [74] and Trypan Blue [75]. The need for a reliable, rapid, inexpensive and reproducible quantitative in vitro assay for screening of nanoparticles is generally acknowledged. Neutral red (2-amino-3 methyl-7-dimethyl-aminophenazoniumchloride) is a weakly cationic supravital dye which at slightly acid pH yields a deep red colour. The Neutral red readily diffuses through the plasma membrane. It concentrates in the lysosomes and binds by electrostatic hydrophobic bonds with anionic sites in the lysosomal matrix [76, 77]. Alterations of the cell surface lead to lysosomal fragility [76]. Such changes brought about by the action of xenobiotics or nanoparticles [77, 78] can result in a decreased uptake and binding of Neutral red. It is thus possible to distinguish between viable and dead cells [79, 80]. In a study, endosome–lysosome system stability which was measured by neutral red assay decreased after exposure to the silver nanoparticle (30%) [81]. Another method is called Trypan blue exclusion test. The dye trypan blue enters dead cells and is excluded from living cells [82]. A trypan blue exclusion assay was performed for the evaluation of cell membrane stability. The medium was then replaced with zinc nanoparticles at 12, 61, 123, 184, 369 and 737 µM. The results demonstrated that Zn compounds exerted considerable cytotoxicity at 369 µM and higher [83].

Oxidative stress assay

Exposure of nanoparticles leads to the production of reactive ROS and reactive nitrogen species (RNS) [84]. The method for detection of ROS and RNS involves the reaction of 2,2,6,6-tetramethylpiperidine (TEMP) with O_2^- stable radical which can be detected using X-band electron paramagnetic resonance (EPR) [85]. The application of these methods is hindered due to their high cost. Fluorescent probe molecules have emerged as an alternative and cost-effective approach [86]. But there are limitations with fluorescent probes as they are inefficient due to their ability to react with a variety of reactive species. This property leads to misleading results sometimes [87]. The above problems can be solved by the use of 2′,7′-dichlorofluorescein diacetate (DCFDA), a non-fluorescent probe. DCFDA is reactive to HO·, RO·, ROO· and H_2O_2 in the presence of cellular peroxidases [88]. Oxidative stress can also be assessed by measuring lipid peroxidation C11-BIODIPY assay and TBA assay for malondialdehyde [89]. Availability of numerous other assays makes the assessment much convenient. These assays include lipid hydro peroxide's measurement using Amplex Red assay, antioxidant depletion measurement by 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) and superoxide dismutase (SOD) activity by Nitro blue tetrazolium assay [90].

In vivo toxicity assessment methods

The in vivo toxicity assessment is normally performed on animal models such as mice and rat. The assessment methods for in vivo toxicity include bio distribution, clearance, haematology, serum chemistry and histopathology. Biodistribution studies examine the localization route of nanoparticles to the tissue or organ. Nanoparticles are detected in the killed or live animals through radiolabels [91]. Clearance of

nanoparticles is performed by the examination of excretion and metabolism of nanoparticles at various time points after exposure [92]. Another method for in vivo toxicity assessment is the examination of changes in the serum chemistry and cell type after exposure of nanoparticles [93]. Histopathology of the cell, tissue or organ after exposure is used to determine the toxicity level caused by a nanoparticle [94]. Histopathology examination has been used to nanoparticles' exposed tissues such as lung, eyes, brain, liver, kidneys, heart and spleen [93, 95]. The advancement of toxicity assessment includes use of micro-electrochemistry and microfluidics [96].

In-vitro toxicity of nanoparticles

Cell viability and lethality

Cell viability and lethality are the two parameters which are used to measure the toxicity caused by the nanoparticles. Among the various nanoparticles, carbon nanoparticles (CNTs) are used most frequently for assessment of viability and lethality of cells. They are widely used in chemical, industrial and biomedical applications due to their unique properties [97, 98]. They are synthesized as single-walled carbon nanotubes (SW-CNTs) and multi-walled carbon nanotubes (MW-CNTs) [99]. The anti-microbial properties of CNTs have been observed by studies in various bacteria due to the mechanical damage caused by the nanotubes [100–102]. A recent study has indicated that functionalized CNTs affect soil bacterial diversity [25]. The toxicity studies on a micro crustacean (*Daphnia magna*), freshwater microalgae (*Raphidocelis subcapitata* and *Chlorella vulgaris*) and a fish (*Oryzias latipes*) revealed inhibited the growth of the algae *C. vulgaris* and *R. subcapitata* with effective SW-CNT concentration 30.96 mg/mL and 29.99 m/L, respectively [103]. The nanoparticles synthesized in the form of iron oxide were also reported toxic in murine macrophage cells, human macrophages, human hepatocellular carcinoma cells and rat mesenchymal stem cells. Iron oxide nanoparticles reported toxicity at 25–200 µg/mL for 2 h exposure on murine macrophage cells. The study observed effects include the decrease in cell viability [104]. Another study reported a reduction in the cell viability when murine macrophage cells were treated with 0.1 mg/mL iron oxide nanoparticles for 7 days [105]. Another toxicity study which was performed on rat mesenchymal stem cells at 0.1 mg/mL for 2 days reported a decrease in cell viability [106]. Silica nanoparticles were also reported for toxicity to human keratinocytes. In a study, the toxicity of the silica nanoparticles at 30–300 µg/mL was evaluated using CHK (human keratinocytes). The results suggested a decrease in cell viability [107].

Effects on cell lines

The toxicity of nanoparticles was evaluated on various cell lines. The effect of SW-CNTs was observed by various researchers on human cell lines, including human HEL 293 cells, HEK cells, A549 cells, human macrophage cells and human epithelial-like Hela cells [108–110]. In a study lung fibroblast cells were treated with CNTs to evaluate the toxicity [53]. In another study when A549 cells were exposed to SW-CNTs at 250–500 µg/ml for 72 h, it resulted in oxidative response and membrane damage, induced by inflammatory response [111]. Another study reported suppression of inflammatory mediators including IL-6, IL-8 and MCP-1 in vitro [112]. The effects of multi-wall carbon nanotube were also evaluated on human epidermal keratinocytes [74]. It was suggested that toxicity induced by multi-wall carbon nanotubes is mediated by pro-inflammatory effects which are facilitated by NF-κB and ROS [113]. In vitro studies reported various toxicological effects of MW-CNTs including oxidative stress, DNA damage and apoptosis in mammalian cells lines. Other effects include VE-cadherin distribution and actin filament integrity in human aortic endothelial cells [114–118].

Gold nanoparticles (AuNPs) in MRC-5 human lung fibroblasts induced autophagy with oxidative stress [119]. In a study, the cellular motility was used to demonstrate the cytotoxicity of metal and semiconductor nanoparticles on animal cells. The study utilizes the potential of electrical cell-substrate impedance analysis as a highly suitable method to quantify the in vitro cytotoxicity of gold nanorods and quantum dots. The method was validated by fluorescence and dark field microscopy [120]. Toxicity of starch-coated silver nanoparticles was studied on human glioblastoma cells (U251) and human lung fibroblast cells (IMR-90). The study resulted in dose-dependent reduction in ATP content and DNA damage. The study demonstrated that the DNA damage is due to silver nanoparticle deposition and interaction with DNA followed by cell cycle arrest in G_2/M phase [121]. Cytotoxic study on fibroblast cells NIH3T3 showed that silver nanoparticles induced mitochondria-dependent apoptosis associated with JNK activation and reactive oxygen species [122]. Toxicity of silver nanoparticles was also evaluated on human hematoma cell line HepG2 using micronucleus test, viability assay and DNA microarray analysis [123]. A study reported the toxicity of silver nanoparticles in HeLa cells. Exposure resulted in upregulation of ho-1, mt-2A and oxidative stress genes [124]. The results of three different types of silver nanoparticle treatment on *E. coli* suggested compromised replication fidelity of the risk gene [125].

Long-term exposure of CdTe quantum dots was observed on the live cells. The study was focused on assessment of intracellular Cd^{2+} concentration in human breast cancer cells (MCF-7) which were treated with cadmium telluride

(CdTe), cadmium selenide/zinc sulphide (CdSe/ZnS) nanoparticles capped with cysteamine, mercaptopropionic acid and N-acetyl cysteine conjugated to cysteamine. The study demonstrated that CdTe quantum dots were cytotoxic with significant lysosomal damage and production of ROS [126]. The effects of different surface coatings on quantum dots were observed with relation to the toxicity on the human epidermal keratinocytes (HEKs). The results suggested that carboxylic acid-coated quantum dots enhanced the release of IL-1β, IL-6 and IL-8. It was determined that surface coating is the primary determinant of immunotoxicity and cytotoxicity in HEKs [127]. The cytotoxicity of thiols stabilized CdTe, CdTe/CdS/ZnS core–shell–shell structured quantum dots and CdTe/CdS core shell structure was observed on cell lines including HEK293T and K562. The results demonstrated that CdTe QDs was highly toxic for cells [128]. A hippocampal neuronal culture model was used for the neurotoxicity investigation of cadmium selenium quantum dots. The study focused on voltage-gated sodium channel and cytoplasmic calcium level. The results evidenced induced neuron death and elevated cytoplasmic calcium levels [129]. The exposure of CdSe quantum dot on enterocyte-like Caco-2 cells as a model for intestine epithelium was investigated. Results suggested that acid treatment of PEG-coated quantum dots increased the toxicity [130]. Another study investigated the in vitro and in vivo toxicity of CdTe nanoparticles on human hepatoma HepG2 cells [131].

The aluminium nanoparticles in the size range 1–10 µM were used for 24 h on human brain microvascular endothelial cells (HBMVECs). The study demonstrated that treatment resulted in the decrease in mitochondrial function, cell viability and an increase in oxidative stress [132]. Mammalian cells were treated with 10–400 µg/mL aluminium nanoparticles to examine the toxic effects. The observed results suggested that no significant toxicity was observed on cell viability in the study [133]. In another study cell viability was determined with respect to the interaction of human bone marrow derived mesenchymal stem cells (HMSC) with aluminium nanoparticles in the range 25–40 µg/mL. The results from the study indicated a decrease in the cell viability [134]. Another study investigated the effect of increasing concentration of aluminium nanoparticles on rat blood cells at 500–2000 mg/kg for 72 h. It was observed from the study that the toxicity was dose dependent [135]. A study conducted on mammalian cell lines suggested that at 0–5000 µg/mL, aluminium nanoparticles were responsible for DNA damage after treatment for 2 h [136].

Mechanistic studies

Mechanistic studies were performed to assess the effects of nanoparticles in vitro. In vitro studies have revealed CNTs disrupt the membrane potential, membrane integrity, metabolic activity and cellular reproduction [137, 138]. Gold nanoparticles are responsible for mitochondrial damage, affecting cellular micro mobility, autophagy and oxidative stress [119, 139]. In vitro studies on silver nanoparticles' toxicity have suggested that interference with DNA replication, fidelity, apoptosis, oxidative stress, cytotoxicity, chromosome instability, intracellular calcium transients, JNK activation and cell cycle arrest in mammalian cells [121–125, 140, 141]. Fullerenes are responsible for DNA damage and oxidative stress in mammalian cells lines [142–144]. In a study on FE1-Muta™ mouse lung, epithelial cells were investigated to observe the effects of C_{60} fullerenes and SW-CNTs for cytotoxicity, genotoxicity and ROS production [142].

In-vivo toxicity of nanoparticles

Dose and LD50

The toxicity of nanoparticles is determined by the exposure conditions, exposure duration and dose. In a study, 3T3 cells were treated with 1–10 mm size SWCNTs with an exposure time of 1 h. The treatment resulted in 20% viability [145]. In another study immortalized epidermal keratinocytes with 80% confluency were treated with SWCNTs in concentration range 0.06–0.24 mg/mL for 2, 4, 6, and 8 h. The treatment resulted in decreased viability after 4 h and ~ 65% viability on exposure to nanoparticles at 0.24 mg/mL [146]. When human embryonic kidney cells were treated with SWCNTs at 0.7812–200 µg/mL for 24–120 h; then it was observed that cytotoxicity was time and dose-dependent with G1 cell cycle arrest in 43.5% cells after day 1 [147]. But when mouse peritoneal macrophage-like cells were treated with SWCNTs in the range 0–7.3 µg/mL for 4, 8, 12 and 18 h, the cells ingested the nanoparticles without toxic effects [148]. When guinea pig alveolar macrophages were treated with SWCNTs at 1.41–226 µg/cm^2 for 3 h, the study resulted in cytotoxicity at 0.38 µg/cm^2 and necrosis at 3.06 µg/cm^2 [149]. It was observed that the cytotoxicity of SWCNTs was dose dependent and 60–80% reduction in cell number was observed when rat alveolar epithelial cells, NR8383 and human alveolar epithelial cells, A549 were treated at 5–100 µg/mL for 24–96 h [150]. Strongest adverse effect of SWCNTs was observed when HEKs were treated at 0.8–100 µg/mL for 24–120 h. The study resulted in 79, 50 and 31% viability at 100 µg/mL when treated for 1, 3 and 5 days, respectively [151]. The toxicity of MWCNTs was also evaluated on different cell lines. In a study when HEK cells were treated with MWCNTs with 80% confluency at 0.1–0.4 mg/mL for 1, 4, 8, 12, 24 and 48 h, the study resulted in ~ 73% viability at 0.4 mg/mL. When purified

MWCNTs were used to examine the toxicity on human skin fibroblasts with 70% confluency at 0.06–0.6 mg/mL for 24 and 48 h, it was observed that the toxicity was dose dependent [152].

Gold nanoparticles' exposure at 150 pm for 3 h on HeLa and 3T3/NIH cells resulted in cell viability reduction by 20 and 5%, respectively [153]. In another study, gold nanoparticles were exposed to macrophage cells with particle size range 10–100 µm for 24–72 h. The study demonstrated that the 100 µm nanoparticles decreased the cell viability to 85% in 72 h [154]. In a study on human dermal fibroblasts, cells were used to assess the toxicity imposed by gold nanoparticles at 0–0.8 mg/mL for 2–6 days. The results from the study demonstrated dose-dependent decrease in the cell area and density [155]. Gold Nano shell was also used to study the cytotoxic effects. A study conducted on Vero cells at 0.001–200 µg/mL for 6 and 24 h resulted in decreased cell viability on exposure to gold Nano shells [156].

Fe_3O_4 nanoparticles' exposure to human fibroblast cells at 0–1000 µg/mL for 24 h resulted in 25–50% reduction in cell viability [157]. In another study conducted on mouse macrophages, Fe_3O_4 nanoparticles were exposed at 0.2 mg/mL for 1 and 4 days. The study demonstrated that cytotoxicity was dose dependent [158]. When human breast carcinoma SK-BR-3 cells were exposed to Fe_3O_4 nanoparticles at 10–400 nm for 1–48 h, the study resulted in 91% viability [159].

Treatment of Hela cells with CdSe quantum dots with sizes 1, 10 and 100 nm for 2 h resulted in survival of 90% cells [160]. In another experiment human lymphoblastoid cells were treated with CdSe quantum dots at 0.2 µm size for 12 h; the study resulted in decreased cell activity [161]. When three cell lines viz. primary hepatocytes, Hela cells and Vero cells, were treated with 0–4 mg/mL CdSe quantum dots for 24 h, the study resulted in low damage at 0.1 mg/mL, while increased damage was observed at 0.2 mg/mL. HepG2 cells' and Wister mice cells' treatment with CdSe quantum dots at 10–400 ppm for 12–72 h resulted in more than 80% cell viability at up to 400 ppm [162]. CdTe quantum dots were also examined to observe the cytotoxic effects on the cells. Treatment of rat pheochromocytoma cells with 0.01–100 µg/mL CdTe quantum dots for 24 h resulted in a decrease in metabolic activity by 50% [163]. Similarly, treatment of human hepatoma HepG2 cells with CdTe quantum dots at $0–10^{-5}$ M for 24 h resulted in ~ 50% reduced viability at 10^{-5} M [164].

Effects on organ systems

In in vivo models, the effect of CNTs appears to be related to their method of administration. Various research groups have found that exposure of nanoparticles to the respiratory system could result in asthma, bronchitis, emphysema and lung cancer. Entry of nanoparticles through the gastrointestinal tract could lead to Crohn's disease and colon cancer. Furthermore, it has been discovered that the nanoparticles' exposure to the circulatory system may result in blood clotting and heart disease (reviewed in [99, 165]). In vivo toxicity of carbon nanotubes toward animals was evaluated in a few studies which focused on organisms including guinea pig, mouse and rat. The effect of soot-containing carbon nanotubes containing Co/Ni was observed on guinea pig [166]. Effect of single-walled carbon nanotubes containing metals was evaluated in mouse and rat by various researchers [167–170]. In one of the above studies, acute lung toxicity was evaluated for intratracheally instilled SW-CNTs in rats [170]. In this study cell injury, multifocal granulomas and transient inflammatory reactions were observed. Another study reported lymphocyte and macrophage influx, early neutrophil accumulation, elevation of pro-inflammatory cytokines and fibrogenic transforming growth factor [169]. Effect of MW-CNTs was also evaluated in a study conducted on rat [171]. In the study, MW-CNTs were administrated intratracheally in rats. The study evaluated the inflammation, lung persistence and fibrotic reactions both histologically and biochemically. Pulmonary lesions were observed in the bronchial lumen which was characterized by collagen rich granulomas. Stimulation for the production of TNF-α was also observed. Toxicity of carbon nanotubes was also observed on an aquatic organism such as rainbow trout [172]. In the study, rainbow trout were treated with SW-CNTs at 0.1–0.5 mg/L for 10 days. The study recognized the SW-CNTs as a respiratory toxicant, responsible for neurotoxicity and cell cycle defects. MW-CNTs are also known for increased micronuclei frequency and chromosomal aberrations, promotion of allergic response in mice, activation of cyclooxygenase enzymes through suppression of systemic immune function in spleen and altered gene expression in the liver [173–177]. Other effects of MW-CNTs include apoptosis, phenotypic defects, toxicity in bacteria and formation of abnormal spinal cords in zebrafish embryo [178–180].

In vivo exposure of gold nanoparticles reported apoptosis and acute inflammation in the liver, bioaccumulation in organs and penetration ability in sperm head and tail regions [181–183]. In a study 13 nm sized PEG coated gold nanoparticles were used to observe the toxicity in the liver. It was observed that the particles accumulated in liver and spleen up to 7 days after injection. These nanoparticles induced apoptosis and inflammation in the liver. The study also demonstrated the presence of PEG-coated gold nanoparticles in spleen macrophages and lysosome of liver [181]. In vivo studies have reported the astrocyte swelling, blood–brain barrier destruction, oxidative stress induced by free radicals, alteration of gene expression and neuronal degeneration on exposure to silver nanoparticles [184–186]. The distribution

and accumulation of silver nanoparticles were investigated in rats by subcutaneous injection. The results indicated that particles were distributed in liver, spleen, kidney, brain and lung [186]. The ability to form oedema and permeability to blood–brain barrier was investigated on rats by intraperitoneal, intracerebral and intravenous administration. The results suggested that nanoparticles were able to induce oedema formation in the brain by influencing blood brain barrier in vivo [185]. A study utilized silver nanoparticles (25 nm) to evaluate its effects on the gene expression in the different regions of the mouse brain. The study suggested that the silver nanoparticles were able to produce neurotoxicity by the generation of free radicals [184]. The results demonstrated uptake of silver nanoparticles in liver, gills and kidney. The study also evidenced induced expression of cyp1a2 in the gills suggesting an increase in oxidative metabolism [187].

It was observed from the in vitro studies that quantum dots are able to cross the placental barrier and reached mice pups in pregnant mouse. They also affect mouse oocyte development and were able to penetrate the UV-radiation and compromise skin barrier [188–190]. Titanium oxide-containing sunscreen was used on the human skin to evaluate its toxicity [191]. The results revealed that nanoparticles penetrated the open part of the hair follicles. The penetration of the nanoparticles was reported in a study where the sunscreen was applied to relatively hairy skin [192]. Previous studies reported pulmonary toxicity where the silica nanoparticles' administration was intra-tracheal. The results from the study evidenced acute pulmonary inflammation and neutrophil infiltration to the lung tissues in a dose-dependent manner [193]. Similar studies using silica nanoparticles reported induction of anti-inflammatory mediators and reversibility of fibrotic changes [194, 195]. A few studies in lung tissues suspected translocation and diffusion of silica nanoparticles away from the lung tissue through systematic circulation and deposition in extra pulmonary organs [196–198].

Mechanistic studies

In vivo, mechanistic studies were performed to assess the toxicity caused by nanoparticles. Silver nanoparticles are also known for genotoxicity and cytotoxicity in fish including accumulation in gill tissues and lysosomal destabilization in adult oysters. They are also known for adverse effects on oyster embryonic development, oxidative stress and expression of p53 protein in zebrafish [187, 199–202]. In another study conducted on zebrafish, it was observed that silver nanoparticles caused induction of apoptosis and oxidative stress in the liver. After nanoparticles' treatment, there was upregulation of the p53-related pro-apoptotic genes Noxa, Bax and p21 [200]. Oyster embryo was used to examine the toxic effects of silver nanoparticles on embryonic development. The study evidenced a significant increase in metallothionein gene expression in embryos [201]. Another study demonstrated the genotoxicity and cytotoxicity of silver nanoparticles on fish cells. The results evidenced the induced aneuploidy and chromosomal aberrations after treatment. The study demonstrated that nanoparticles are genotoxic and cytotoxic to fish cells [202]. In *D. melanogaster*, they are known as oxidative stress, heat shock stress, upregulation of p53 proteins [39]. In *Caenorhabditis elegans*, they are known for oxidative stress and a decrease in reproduction potential [203].

Cytotoxicity of quantum dots was also determined in another study where cadmium sulphate CdS quantum dots were synthesized. The study revealed that CdS quantum dots are more toxic as compared to microsized CdS and they elevate ROS production by 20–30%. The study proposed that CdS quantum dots' cytotoxicity is mediated by intracellular ROS production, cadmium ions (Cd^{2+}) release and GSH depletion [204]. Quantum dots are responsible for phototoxicity in *Daphnia magna* under UV-B light [205]. In vivo studies suggested that fullerenes are responsible for elevated gene expression of MHC class II molecules, increased pro-inflammatory cytokines and increased T cell distribution in lungs [142, 206–210]. Oxidative damage in the liver, colon mucosa and lung was observed in the study conducted on rat by oral exposure of C_{60} fullerenes and SW-CNTs. Doses of SW-CNTs increased the levels of 8-oxodG in lung and liver. C_{60} fullerenes administration increased the hepatic 8-oxodG level and high dose generated 8-oxodG in the lung [206]. In oysters they are known for affecting oyster embryonic development, cellular damage in the alimentary canal in *Daphnia magna*, growth inhibition in freshwater fish *Carassius auratus*, increase in mortality rates in gestating daphnids, nitric oxide production in *Mytilus hemocytes [211–215]*.

The toxicity of zinc nanoparticles has been reported on human cervix carcinoma cell line (HEp-2), human hepatocyte HEK 293 cell line and human bronchial epithelial cells. Zinc nanoparticles in the range 10–100 µg/mL were used for 24–48 h on HEp-2 cells. The observed results from the study demonstrated that the zinc oxide nanoparticles were toxic for the cells causing DNA damage and reduction in cell viability [216]. Another study focused on the assessment of in vivo toxicity of zinc nanoparticles at 14–20 µg/mL for 12 h to determine the effects of treatment on cell viability, DNA damage, ROS production and apoptosis [217]. When HEK 293 cell line was treated with 0–100 µg/mL zinc oxide nanoparticles for 24 h, it was observed that there was a reduction in cell viability. Results also demonstrated DNA damage, oxidative stress and mitochondrial damage [218]. Similarly, when human bronchial epithelial cells were treated with zinc oxide at 100 µg/mL, the observed effects include the release of LDH, decrease in cell viability and oxidative

stress [219]. The toxicity of titanium nanoparticles affects differentiation, cell proliferation, apoptosis and mobility [220, 221]. The toxicity of the titanium oxide nanoparticle was observed when keratinocyte cell line (HaCaT), human dermal fibroblasts and human immortalized sebaceous gland cell lines (SZ95) were used. The cytotoxicity affected cellular functions including differentiation, cell proliferation and mobility which resulted in apoptosis [220].Upregulation of fibrogenic mediators including IL-4, IL-10 and IL-13 was also observed contributing to fibrotic changes [195].

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