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Toxicity of copper oxide nanoparticles: a review study

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Abstract: Copper oxide nanoparticles (CuO NPs) use has exponentially increased in various applications (such as industrial catalyst, gas sensors, electronic materials, biomedicines, environmental remediation) due to their flexible properties, i.e. large surface area to volume ratio. These broad applications, however, have increased human exposure and thus the potential risk related to their short- and long-term toxicity. Their release in environment has drawn considerable attention which has become an eminent area of research and development. To understand the toxicological impact of CuO NPs, this review summarises the in-vitro and in-vivo toxicity of CuO NPs subjected to species (bacterial, algae, fish, rats, human cell lines) used for toxicological hazard assessment. The key factors that influence the toxicity of CuO NPs such as particle shape, size, surface functionalisation, time–dose interaction and animal and cell models are elaborated. The literature evidences that the CuO NPs exposure to the living systems results in reactive oxygen species generation, oxidative stress, inflammation, cytotoxicity, genotoxicity and immunotoxicity. However, the physio-chemical characteristics of CuO NPs, concentration, mode of exposure, animal model and assessment characteristics are the main perspectives that define toxicology of CuO NPs.

1 Introduction

Nanotechnology is a revolutionary field that deals with the synthesis, characterisation and applications of nanomaterials in the fields of material science, biology, chemistry and physics. The remarkable surface chemistry and chemical reactivity of nanomaterials distinct them from bulk counterparts. The increase in surface-to-volume ratio of nanomaterials leads to rise in reactivity or toxicity due to the availability of large number of reactive sites. Toxicology deals with the study of all those noxious elements that have adverse effects on living system and environment. In the last few decades, development in the field of nanotechnology and their subsequent applications has increased the exposure of nanoparticles (NPs) to the environment and human beings. The occurrence of NPs in the environment is of great significance regarding their impact on human health [1], hence nanotechnology coined with toxicology give rise to new term 'nanotoxicology' [2]. The mechanisms involved behind the interaction of NPs with the living systems that result in nanotoxicity are not well known yet.

This review focuses on the toxicity of CuO NPs because of their widely usage in various commercial applications such as catalysts, photovoltaic cells, gas sensors, heat transfer nanofluids and antimicrobial agent [3, 4]. CuO NPs have been reported effective against broad range of microorganisms [5, 6]. Additionally, these are also used as an anti-fungal agent when incorporated in plastics, textiles, coatings and so on [7, 8]. The electrochemical properties of CuO NPs make them appropriate to be used in graphite surface coating to improve its capacitance properties [9]. Besides these, copper (Cu) plays significant role in normal functioning of human body by maintaining its homeostasis while high Cu intake leads to jaundice, haemolysis and ultimately death. It also results in toxicity of respiratory system, gastro intestinal tract (GIT) and skin diseases when exceeds certain limits. Due to high impact usage of CuO NPs, there is an urgent need to critically assess the toxicity associated with CuO NPs. However at present, there is limited knowledge into the potential detrimental outcomes of human exposure to CuO NPs.

Throughout this review, emphasis has been done on the information relating to synthesis methodologies of copper oxide nanoparticles (CuO NPs), characterisation, advantages and disadvantages, experimental designs related to concentration of NPs, models used, duration of the CuO NPs exposure and assessment of endpoints. The information is used to understand the influence of experiments on toxicological examinations and their relevancy. In the light of above discussions, we tried to cover toxicological consequences of CuO NPs. In order to attain this objective, we targeted in-vitro and in-vivo toxicity of CuO NPs which include oxidative stress, immunotoxicity, genotoxicity, serum chemistry, haematology, urine chemistry, histology and mechanism involved behind toxicity. The present level of understanding the toxicity of CuO NPs has been achieved, and the gaps are covered, in order to highlight the research areas that should be addressed in the future.

2 Toxicity of metal oxide NPs

The genotoxicity, cytotoxicity and immunotoxicity of many metal oxide NPs have been reported. Due to several contributing factors, the mechanism of metal oxide NPs is not yet fully understood despite of extensive work. Irrefutably, the surface properties greatly affect the toxicity and interaction with the living systems. Before evaluating the toxicity (genotoxicity/cytotoxicity/immunotoxicity) of nanomaterial parameters like morphology, size, crystallographic appearance, chemical composition, surface properties (chemistry, charge) and aggregative behaviour should be characterised [10]. The release of metal ions from NPs is one of the major factors in the toxic potential. In inducing toxicity, the dissolution of metal ions from metal oxide NPs and the environment in which the NPs expose, plays a vital role [11]. Among different NPs, we hereby discuss toxicity of CuO NPs in detail due to its wide spread use in almost all fields.

3 Toxicity of CuO NPs

The complications that result in misunderstanding the toxicity rely on the fact of CuO NPs binding, their interaction to the living cells and subsequent change in surface chemistry. To elucidate the toxicity of CuO NPs, there is a need of understanding the characterisation and surface modification of CuO NPs, routes of exposure and mechanism or pathways that are involved in toxicity. Table 1 demonstrates the correlation between the synthetic methods of CuO NPs, characterisation and their toxic effects. The

complications that result in misunderstanding the toxicity rely on the fact of NPs binding, their interaction to the living cells and subsequent change in surface chemistry. To elucidate the toxicity of nanomaterials there is a need of understanding the characterisation and surface modification of NPs, routes of exposure and mechanism or pathways that are involved in toxicity [24–26].

4 Key factors

4.1 Characterisation

Characterisation of CuO NPs involves the size, shape and charge of the particles. There is a correlation between size of the NPs and surface-to-volume ratio, smaller the size greater will be the surface-to-volume ratio and vice versa. The penetration and reactivity of particles is entirely dependent upon its size. However, it has been postulated that NPs having size >100 nm can penetrate the cells by crossing the cell membrane whilst size <40 nm can enter into blood to nuclei of cells. The mechanisms that are influenced by NPs size are cellular uptake, interaction mechanism

Table 1 Synthesis techniques, characterisation and applications of CuO NPs

S.no	Method	Technique	Chemicals	Characterisation	Results	Advantages	Disadvantages	Applications	References
1	physical	laser ablation	99.99% copper target, deionised water, d/f laser focusing conditions	ellipsoidal shaped NPs, monoclinic CuO and cubic Cu_2O 25–200 nm	laser ablation, pressure, temperature ultimately induced structural modifications in NPs	no capping, stabilising agents required	less concentration of NPs produced, high energy is required	nanolubricant, heterogeneous catalyst, sensors and so on	[12]
2	chemical	reduction method	copper (II) succinate as precursor	crystalline 45 nm average diameter	*controlled particle size*	simple, economical, efficient, surfactants prevents agglomeration of NPs, controlled size and shape of NPs	instability of NPs in solution	antimicrobial	[13]
		sol–gel method	sodium dodecyl sulphate as a surfactant	monoclinic, crystalline NPs	particle size increase with calcination b/c of agglomeration	controlled size and shape NPs	expensive and less eco-friendly, costly	applications in gas sensing	[14]
		chemical vapour deposition	copper nitrate tri-hydrate 0.02M, distilled water, 0.5 g sodium hydroxide at 5C	rod like structures, monoclinic 33 nm	widely dispersed	synthesis of controlled size and shaped NPs	needs high temperature	antimicrobial	[15]
		precipitation method	copper (II) acetate and sodium hydroxide	crystalline NPs 23 nm	reproducable procedure	simple and effective	inappropriate for the synthesis of pure, price stoichiometric phase	biocidal	[16]
		electro-chemical deposition	supporting electrolytes, NaOH, $NaCO_3$, sodium nitrate in water, water:acetonitrile (12:1), water:methanol (12:1), copper plate	well dispersed, granular spherical, round shaped, 20–25 nm, peaks at 2θ values	small sized particles	cost-effective, less laborious, pure and controlled size and shaped NPs are synthesised	lack of reproducibility, needs high energy, pressure and temperature	photocatalytic and antibacterial	[14]
		hydrothermal	copper sulphate and NaOH	crystalline monoclinic, 27 nm average size	large optical band energy observed	variable NPs produced	high pressure and temperature is required	—	[17]
		ultrasonication synthesis of CuO/ Cu_2O/Cu-NPs	2.0 g copper acetate monohydrate, ammonia solution (25% w/w)	monoclinic crystalline,bandgap 1.42 eV, major peaks at 2θ values	simple but specific instrument required	effective, simple, cheap, non-toxic	scale up problems	could be used as industrial catalysts for photocatalytic degradation of dyes	[18]

S.no	Method	Technique	Chemicals	Characterisation	Results	Advantages	Disadvantages	Applications	References
3	biological	*E. coli* mediated	citrate minimal medium, $CuSO_4$, *E. coli* mass, SDS-PAGE	micrometre dimensions and variable morphologies	use of SDS-PAGE resulted in reduction of Cu II to CuO NPs and their stabilisation	high yield, low cost, fast. Clean, reproducibility	tedious purification steps, difficult to understand the mechanism and to achieve control size and shape of NPs	antimicrobial applications	[19]
		fungi mediated (*Trichoderma asperellum*)	5 mM copper nitrate, TA-CFE (mycelial free water extract)	crystalline and cubic-faced structure, 110 nm average size	ROS production and cellular observation in A549 cancer cells and the activity increased with TA-CuO NPs	high yields secretory proteins related to increase NPs, scalability, easy downstream processing	risk of pathogenicity	TA-CuO NPs could be used as therapeutics in modern medicines	[20, 21]
		plant mediated Hibiscus rosa-sinensis (flower extracts)	1 M aqueous copper acetate solution, deionised water	crystalline, spherical, 45–80 nm	antimicrobial	cost- effective, eco-friendly, easy availability, high quality NPs, controlled size and shape	difficulty and time consuming	antimicrobial applications	[22]
		algae mediated (*Bifurcaria bifurcate*)	1 mM copper(II) sulphate, 2 ml algal extract	crystalline NPs, 5–45 nm average size	antimicrobial	eco-friendly, convenient, no chemicals involved	laborious and slow process	pharmaceutical	[23]

and intercellular stability. By making it abridged in comparison to large size NPs, the small NPs show more toxicity and are more suspected to cellular internalisation [24, 25]. The translocation process of NPs through the cell membrane is affected by shape and charge up to 60 orders of magnitude [25, 26]. Due to small size of Cu NPs, they can easily translocate between cells, cross cell membrane and causes cell disruptions (Lee *et al.*, 2016). Few studies have been conducted to demonstrate the shape and particle form of NPs affecting their toxicity and bioaccumulation. The reports elucidate that at the nanoscale, CuO NPs spherical in shape showed increased reactivity (antibacterial activity) than bulk CuO. Nano-cupric oxide (n-CuO) CuO nanosheets showed highest surface reactivity, electrochemical properties and antimicrobial activity than CuO.

There are three synthetic methods which influence the characterisation and properties of CuO NPs viz., physical, chemical and biological (Table 1). Laser ablation method has been used for synthesis of CuO NPs that produces 25–200 nm sized NPs with ellipsoidal shape and no capping or stabilising agents are required [12]. The chemical methods involved for CuO NPs synthesis are chemical reduction, sol–gel, chemical vapour deposition, precipitation electrochemical, hydrothermal and ultrasonication. One of the easiest and simple methods for CuO NPs synthesis is the chemical reduction method [13]. The CuO NPs produced are crystalline in nature and having average size of 45 nm which is enough to penetrate the bacterial cell membranes and cause cell disruptions at different levels. The CuO NPs synthesised by chemical vapour deposition and precipitation method produces monoclinic rod like structures, 33 nm in size.

The CuO NPs synthesised by sol–gel method, electrochemical and ultra-sonication showed high gas sensing and strong photocatalytic properties against safronine O and methylene blue dyes [14, 18, 27]. The advantages of these techniques are cost-effectiveness, non-toxic, controlled size and shape and efficient one. The disadvantages are lack of reproducibility, scalability, difficulty in understanding the mechanisms.

The biological methods have also been used, i.e. bacteria, fungi, algae and plant for CuO NPs [19–22]. The NPs synthesised are of various sizes and shapes and shows strong antimicrobial, photocatalytic activities. The NPs synthesised show strong abilities to be used in therapeutics, biotechnological, environmental, pharmaceutical and industrial applications. The advantages of biological methods are cost-effectiveness, eco-friendliness, easy handling and availability. The drawbacks of using green techniques are tedious down streaming procedures, laborious, difficult in case of using bacteria, risk of pathanogenictiy in fungi mediated, time consuming and slow in case of algae and plant mediated synthesis of CuO NPs.

4.2 Surface modification

The potentiality of CuO NPs in wide range of applications is because of its high surface reactivity, chemical stability and thermoelectric properties [28]. Greater the surface reactivity, greater will be the reactive oxygen species (ROS) production and vice versa. The relationship between surface chemistry (capping agents) of Cu NPs and generation of ROS has been well documented [29]. Three surface modifiers named 8-mercaptooctanoic acid (MOA), 12-mercaptododecanoic acid (MDA) and 16-mercaptohexadecanoic acid were used and followed by dispersion in water, demonstrating the strong affinity of Cu NPs to thiolated functional groups as compared to carboxylic acids. The 8-mercaptooctanoic treated Cu-NPs showed a significant level of ROS activity. The findings showed that ROS generation abilities profoundly decreased with increasing long chains.

4.3 Dissolution

The dissolution of metal ions is another factor affecting the toxicity of NPs. The solubility parameter is one of the meaningful parameter which exploits the reasons of NPs toxicity to many organisms. The NPs exhibit faster dissolution because nanosized particles have larger surface area as compared to bulk ones to interact with solvent molecules. Thus, the nanosized particles exhibit higher toxicity. From literature it has been evidenced that dissolution plays significant role in oxidation of Cu NPs. The Cu-

based NPs (CuO NPs) release sufficed amount of Cu ions in relevant media to affect biological systems [11]. The toxicity of CuO NPs increases by 40-fold to bulk CuO and evident 40–50 fold increase in toxicity [30]. The toxicity of Cu ions from CuO NPs is significant in the growth medium but lower than the effects of particles themselves [31]. Other possibility is the release of Cu ions after penetration and accumulation in lysosomes, where the solubility parameter increases due to acidic pH leading to highly cytotoxic Cu2 + ions into the cytoplasm [32].

4.4 NP dose

In toxicology, NP dose is one of the key factors contributing towards toxicity. The NP dose applies to the initial concentration to the cells but also to the real number of NPs taken up by a one single cell. For public health risk assessment, it is important to elucidate real and relevant dose regimes for in-vitro and in-vivo experimental models. By all means to achieve a biological response, nanotechnologist should examine NPs toxicity based on real-world doses instead of unrealistic one. In nanomedicines, NP dose is considered critical phenomenon [2]. The human pulmonary epithelial cells (A549) when exposed to CuO NPs concentration in dose-dependent manner (10, 25, 50 μg/ml) indicated by depletion of glutathione and induction of lipid peroxidation, catalase and superoxide dismutase. The MIT assay indicated that the CuO NPs decreased the cell viability to 75, 66 and 48%, respectively [16].

4.5 Environment

The chemical transformation of nanomaterials in environment or living body significantly affects their toxicity risks. In the environment, NPs interact with air, soil and water. As discussed above, the interaction of NPs with substrates changes the surface properties of NPs or charge and results in aggregation [33]. The aggregation of NPs affects the cellular uptake and ultimately toxicity [34]. The release of Cu NPs in environment via Cu-based materials used in agriculture (pesticides) or effluent of wastewater treatment undergoes chemical transformations such as sedimentation, aggregation, dissolution, natural organic matter interactions and redox reactions [35].

4.6 Exposure route of Cu NPs

The primary exposure of NPs is by improper handling of raw materials and equipments by the workers during lab-scale production. The commercial synthesis of nanomaterials is another primary exposure route of NPs. The other forms of exposure route of NPs might be the errors that occur during the categorisation, packing and transportation of nano materials. On contrary, the applications and consumption of nanomaterials based products also lead to lethal effects. The main entry routes for NPs into the body are inhalation, ingestion or through skin and blood stream [36–38]. After entrance into the body, the NPs are transported to other regions of body through blood circulatory system [39, 40] and cause toxic effects at the different sites.

The Cu NPs exposure route is the main indicator which affects their toxicological outcomes. Due to small size of Cu NPs, they can easily translocate via tissue interstitially (between cells), pass through cell membranes and eventually enter into blood circulatory system. A very significant role is played by circulatory and lymphatic systems in the translocation of NPs from exposure site to downstream effects, ending in accumulation in the body organs.

4.7 Inhalation

Inhalation is the chief route of entrance of NPs into the body. During inhalation NPs penetrate into lungs and interact with the epithelium that leads to inflammation. The olfactory bulb is considered one of the hazardous routes for the inhaled NPs to get access to other organs of the body like CNS [41, 42]. The mechanism that lies behind the translocation of NPs seems to be through endocytosis of alveolar epithelial cells [43]. Amount of NPs, disposition in lungs, dimensional properties of NPs, persistent of NPs, and defence mechanism are the factors that affect the living system on inhalation of NPs. As the size of NPs decrease, the disposition rate of NPs significantly increases in lungs. The occupational workers are the cohort population exposed to Cu NPs and CuO NPs. Industrial emission of Cu NPs takes place in the production of asphalt and rubber [35]. The capability of Cu NPs to remain in lung tissues for indefinite time is accredited to its small size, ends in ROS production followed by oxidative stress and inflammatory responses because of sensitisation and irritation.

4.8 Ingestion

Gastrointestinal tract is also one of the potential exposure routes for NPs. In different food items and drugs, NPs are added directly or indirectly that are taken orally and absorbed through mechanism of GIT from where they enter in lymphatic cells [44]. The factors that influence the absorbance of NPs by GIT are surface chemistry, geometry, charge, shape, size, substrate and attachment potential to substrates [45]. The unstable NPs are excreted by body whereas the NPs that become agglomerated or form aggregates block the GIT and leads to death. It has been observed that accumulation varies in key organs like brain, spleen, heart and liver. The extent ability of body to excrete NPs in urine is not yet studied well or their bioaccumulation in distinguished organs (liver, spleen, heart and brain) and their possibility to block the excretory pathways. When the NPs enter hepatic circulation, they become hepatotoxic and cause gradual fibrosis. However, when NPs enter the GIT, they result in ulcers by altering the lining permeability, cause dysplasia/metaplasia by weakening the epithelium, malabsorption of the nutrients and in severe case may results in chronic bleeding. In viewing such aspects of toxicity of nanomaterials, greater attention ought to be paid in evaluating harmful effects before endorsing any parenteral administration [46, 47].The Cu NPs present in the food/water entered through direct ingestion or inhalation, exposed to GIT. According to the reports of Chen *et al.*, toxicity of Cu NPs after oral exposure in mouse was evaluated. The fate of the Cu NPs in the GIT followed by reaction with the stomach acid, diffusion into the microvilli, or entered into the intestines ending in systematic transport of Cu NPs by intestinal epithelium to organs [48].

4.9 Skin

Human skin acts as a first barrier towards the NPs and other harmful chemical substances. However, the presence of hair follicles and sweat glands makes this barrier susceptible by allowing the entrance of NPs [44]. There are more chances of penetration of NPs when the protective layer of skin is removed or wounded [49]. The penetration of NPs into the skin decides their fate and shows their diverse toxic forms. They may cause irritation, allergy or can damage the cellular or subcellular parts of body, which may initiate chemical reaction results in production of ROS [50].

Cohen *et al.* (2012) described the toxicity of CuO micro and NPs in human skin organ cultures using electron microscopy and biochemical markers. The particles adhere to the skin surface, react with the acidic environment leading towards generation of soluble Cu ions that make their way to inner sites. Particles suspension were made and topically applied onto intact/stripped epidermis for 24 and 72 h treatment. The reports evidenced that NPs showed more toxicity as compared to micro-sized particles and the effects were much stronger when the particles supplied in growth medium. In epidermis, the CuO NPs induced inflammatory responses followed by cytokine secretions and necrosis.

5 In-vitro toxicity of CuONPs

5.1 ROS and oxidative stress

ROS constitute oxygen containing molecules like O^{2-}, H_2O_2 and HO• that exhibit greater reactivity than molecular oxygen. Among antioxidants, GSH is the most copious compound comprising sulfhydryl groups and performs a substantial role in various life processes. It also establishes first line of defence against oxidative damages, and acts as a main redox buffer intracellularly [51] and

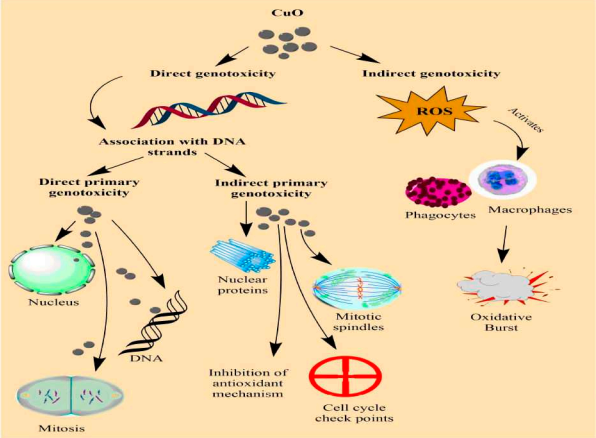

Fig. 1 *Pathways of genotoxicity of NPs*

co-substrate in the GSH peroxidase catalysed reaction of H_2O_2 or lipid peroxidation leading to its depletion. Superoxides, hydroxyl, hydrogen peroxide and other oxygen radicals among ROS are quite important as they are directly involved in oxidation of biological molecules like DNA, proteins and lipids [52]. Under normal physiological conditions, damages induced by ROS are regulated via antioxidative enzymes and non-enzymatic molecules like catalases, superoxide dimutases, glutathione peroxidase, tocopherol, glutathione and ascorbic acid present in the cell [53]. However, when generation of oxygen containing radicals increase, they generate toxic intermediates that are usually responsible for oxidative stress due to imbalance between the level of ROS and antioxidants that detoxify toxic intermediates and restore damages [54]. Body's defence systems collapse to counterbalance oxidative stress and lead to the malfunctioning of biomolecules [55, 56], change in the level of antioxidant enzymes, depletion of glutathione and perturbation of mitochondria. Damages also induce in DNA and consequently cell death occurs [57]. The report addressed by Mortimer *et al.* exhibits the effect of CuO NPs (80 mg/l) on *T. thermophila* cell membrane [31]. *T. thermophile* regulates its membrane's lipid composition to more rigid configuration by lowering its unsaturated fatty acids and increasing saturated fatty acids. Studies also evidence the lipid peroxidation in rainbow and *C. reinhardtii* when exposed to CuO NPs [58, 59]. The studies also report cytotoxicity of CuO NPs in primary liver cells of cat fish and *HepG2* due to generation of ROS [60, 61].

The cytotoxicity of CuO NPs is also observed in human cell lines like human lung epithelial A549, human cardiac microvascular endothelial, kidney and neuronal cells [16, 62–66]. CuO NPs cytotoxicity and oxidative stress have been examined in *A549* cells that leads to high level of lipid peroxidation and ROS production while lower level of antioxidant (GSH) in *HepG2* (human hepatocellular carcinoma) cells. They observed that MDA, a lipid peroxidation marker and antioxidant enzymes like SOD and CAT significantly increased followed by reduction in GSH level. These outcomes suggest that oxidative strain might be the key mechanism behind the CuO NPs [16, 67]. The reports also show that oxidative stress is the main reason for cytotoxicity of CuO NPs [63, 68].

Lanone *et al.* stated that engineered NPs including CuO NPs alter level of antioxidant enzymes like SOD and CAT along with glutathione [69]. Lower level of GSH and inhibition of SOD and CAT activities by CuO NPs contributing to oxidative damages was also observed in embryo and while change in zebrafish physiology like failure in hatching, small body length and reduction in reproductive potential were also due to CuO NPs affect [70]. CuO NPs produce ROS along with blockage of antioxidant defence system. Rat administered with CuO NPs displayed lower CAT, TAC and GSH activities while higher level of nitric oxide (NO) was observed [71].

Fahmy and Cormier [63] observed no difference in case of SOD activity while 25 and 29% inhibition in catalase and GR activities was observed. They found 100 and 150% increase in 8-isoprostanes and GPx activity upon exposure of *HEp-2* cells to 80 lg/cm^2 of CuO NPs. Increase (150%) in oxidised to total glutathione ratio shows that oxidised GSH directs the failure of epithelial cells to inhibit ROS produced by CuO NPs. This leads to the generation of oxidative stress that was responsible for oxidative damage and cell death. Besides this, CuO NPs was more effective at blocking the antioxidant defence of the cell [63, 72].

Higher level of 8-isoprostane in the supernatant of *HEp-2* cells upon treatment of CuO NPs demonstrate oxidative damages to the cells [73]. CuO NPs also significantly increase SOD, and TBARS while reduce CAT activity [74]. Jing *et al.* conducted an experiment on *HBEC* and *A549* cells exposed to CuO NPs. They observed that upon 4 h exposure, significant higher level of ROS was found in *HBEC* followed by *A549* cells [75].

Furthermore, scientists have found that cytotoxicity links with CuO NPs due to the elevated ROS production in several cell lines like *HL60* cells, human laryngeal and alveolar type-I epithelial cells [63, 76–79]. Results show downregulation of catalase around 3.18 and CA3 to 4.05 fold in the liver while 2.19 fold of glutathione peroxidase 3 in the kidney via using 2D-DIGE technology in rats intoxicated with CuO NPs (200 mg/kg). Decline in the levels of NPSH (non-protein thiol groups) and PSH (protein thiol groups) were also observed in the liver and kidney. These outcomes demonstrate that CuO NPs weaken antioxidant system of the liver and kidney. Besides this, increment in the level of MDA suggests that lipid peroxidation also establishes in liver and kidney when exposed to CuO NPs [80]. Studies support that CuO NPs cause cytotoxicity through primarily generating oxidative stress followed by genotoxicity.

6 Genotoxicity and nanomaterials

Genotoxicity is chromosomal aberrations, oxidative DNA damage and DNA strand breaks caused by different mutagens that lead to mutations. There is paradigmatic relationship between the syntheses of ROS and DNA damage [81]. The proposed mechanism of toxicity studied by Thit *et al.* demonstrates the uptake of NPs by cell via endocytosis that results in synthesis of ROS [82]. ROS causes DNA damage that triggers a signalling pathway which ends in apoptosis or cell death. Genotoxicity of nanomaterials is of particular concern since an alteration of the genetic material may favour cancer development or fertility impairment.

6.1 How nanomaterials induce genotoxicity?

Nanomaterials induce DNA alterations by two pathways: via direct association with DNA strands or through indirect route by induction of oxidative stress upon exposure to NPs (Fig. 1). In direct genotoxicity, NPs penetrate either through nuclear pores or during mitosis directly interact with DNA molecules or disrupt their replication and transcription. During indirect genotoxicity, NPs induce toxicity by interaction with the nuclear proteins, mitotic spindles, checkpoints and inhibition of antioxidant defence mechanism. The secondary genotoxicity of NPs is mediated by ROS production in inflammatory cells (phagocytes, macrophages). The oxidative burst caused by activated phagocytes might be a potential explanation for the genotoxicity of NPs [83].

There are many factors that are involved in genotoxicity induced by nanomaterials. Magdolenova *et al.* and Sun *et al.* elucidated the factors that influence the genotoxic potential of NPs [83, 84]. These include size of NPs, shape, surface properties, composition, solubility, aggregation/agglomeration, particle uptake, presence of mutagens and transition metals affiliated with the particles also influence the mechanisms of genotoxicity. CuO NPs carry out higher proportion of DNA damages and cell deaths as compared to other metallic NPs [16, 85]. The genotoxic potential of CuO NPs has been observed by Wang *et al.* in the nucleus by hypothetically disrupting the nuclear membrane, permitting the NPs to pass through the cell nucleus and directly interact with DNA [11].

6.2 CuO NPs and genotoxicity

Plenty of reports are there that verify the genotoxic potential of CuO NPs among different organisms. Table 2 presents data on the test organisms that have used for the determination of genotoxicity exhibited by CuO NPs. The genotoxicity is dependent upon size, shape, duration of exposure and concentration of NPs. Generation of free oxygen radical and oxidative stress evoke a cascade of cellular events including DNA damage and apoptosis.

The p53 protein is known as master guardian of the cell cycle check points, DNA repair and apoptosis. In case of genetic toxicity, the p53 protein is triggered to arrest the cell cycle to provide time for the DNA repair mechanism or ultimate apoptosis [94] The production of ROS induces the expression of p53 and p38 proteins that damage the DNA. The ROS production also leads to the synthesis of inflammatory cytokines. These findings support that the CuONPs causes cytotoxicity through mediation of oxidative stress followed by genotoxicity.

7 In-vivo toxicity of CuONPs

7.1 CuO NPs toxicity and behavioural analysis

The toxic effects of nano-sized CuO towards neurons results in hippocampal dysfunction (impairing the voltage gated sodium channels) which attributes to learning and memory. The nano-CuO caused spatial impairment and electrophysical alteration in rats [95]. In behavioural experiment, Morris water maze (MWM) showed significant decrease in memory and learning in rats treated with nano-CuO for 14-days. The accumulation of Cu in the hippocampus region remarkably evidenced the notions that NPs have ability to cross blood brain barrier and disrupt central nervous system physiological functions. The occurrence of oxidation–antioxidation imbalance, disruption of homeostasis, damaging of neurons in the hippocampus, oxidative damages induced by nano-CuO resulted in neurotoxicity. Apart from cognitive malfunctions and reduction in spontaneous activity, there were no evidences of anxiety/stress observed in the rats treated with nano-CuO.

To evaluate learning and memory behaviours, the MWM test was administrated with CuO NPs to Male Wister rats at 0.5 mg/kg/day for 2 weeks [96]. The test showed the toxic behaviours of CuO NPs on the rat's cognitive functions. After the MWM test, the long-term potentiation test was conducted to demonstrate the field excitatory postsynaptic potential. The adverse effects of CuO NPs on the cognitive behaviour of rats is because of the suppression of postsynaptic receptors and release of presynaptic glutamate ended in diminishing long-term potentiation and correspondingly other induced cognitive deficits.

Sun *et al.* examined dose-dependent toxic effects of CuO NPs on the behaviour (locomotion) of zebra fish larvae [97]. The locomotion behaviour was estimated by total movement distance, angular velocity, velocity, fast movement time, medium movement time and slow movement time. Three groups were used in the trial, control, exposed to 12.5 and 50 mg/l CuO NPs. The behavioural tests were performed three times after exposing the larvae groups to CuO NPs for six days. The larvae groups exposed to CuO NPs showed reduction in movement distance, velocity and angular velocity and no significant difference was examined in fast movement time. Moreover, the exposed groups medium movement time and slow movement time were longer than the control group. The study also showed that the CuO NPs exposure to zebra fish larvae resulted in reduced locomotion capacity. The delayed development of the central nervous system or the effective neuromuscular system ended in the changed larval behaviours.

A study conducted by Mashock *et al.* quantified the *Caenorhabditis elegans* (nematode) behaviour (feeding and average body length) and reproduction in response to CuO NPs and copper sulphate exposure [98]. Three strains of wild nematode and wild type strain were examined to explore the influence of Cu on their physiological responses. As compared to CuSO4, CuO NPs showed significantly effects on the average body length of the wild strains at 7.9 mg/l concentration. Whilst at 15.9 mg/l copper sulphate demonstrated no impact on the average body length. In terms of feeding behaviour, CuO NPs showed greater toxicity trend than $CuSO_4$ in *Caenorhabditis elegans* strains. Reproduction was considerably reduced only at the utmost Cu dose, though still more noticeable with CuO NPs than copper sulphate treatment.

8 Biochemical alterations induced by CuO NPs

Many reports verify substantial toxicity of CuO NPs, induced damages in nephrotic and hepatic tissues of rats that affect physiology and biochemical functioning of kidney and liver [11]. Mohammadyari *et al.* [99] found rise in ALT and ALP upon treatment of CuO NPs demonstrating disturbance in liver's membrane that lead to cellular release of these enzymes. Lee *et al.* [100] observed dose-dependent increase in AST, ALP, ALT, TBIL, CRE, BUN and LDH while the level of TP (total protein) and TG (triglyceride) decreased significantly. Moreover, rats displayed dysregulation in the electrolytes balance. Manna *et al.* [101] also observed elevation in AST, ALT, TB, BUN and CRE. Arafa *et al.* [71] conducted an experiment on rats treated with 3 and 50 mg/kg of CuO NPs. They found significant increase in liver enzymes (ALT, AST and ALP) in dose-dependent manner as compared to the control. Lei *et al.* reported significant biochemical alterations in rats administered with 200 mg/kg as compared to the control group [102]. They noticed five-fold increase in AST, TB, CRE and BUN while about two-fold increase were observed in ALT, TG and total bile. Furthermore, level of ALP and TCHO (total cholesterol) reduced while TCHO and TG were slightly increased in mid- and low-dose treated groups. Comparative intoxication of male and female rats with CuO NPs was done by Wang *et al.* [103]. Rats treated with 1250 mg/kg CuO NPs showed reduction in TG, Na and Cl and elevation in ALT, AST, BUN, LDH and TCHO in male while increased level of CPK and LDH were found in female rats. Additionally, significant increase in the level of ALT, AST, TCHO, CPK, LDH and total proteins and decrease in TG, Cl and K were reported in female rats exposed with 2500 mg/kg CuO NPs. Kim *et al.* also observed strong inflammation in mice upon exposure of CuO NPs that led to higher neutrophils and total cell recruitment in lungs while LDH activity was enhanced in the bronchus [104]. Doudi and Setorki studied the effect of 10, 100 and 300 mg/kg of CuO NPs on hepatic enzymes, i.e. SGOT (serum glutamate oxaloacetate transaminase) and SGPT (serum glutamate pyruvate transaminase). Their results showed that after two weeks, noteworthy difference was observed in SGOT in the rats treated with 10 and 300 mg/kg of CuO NPs while the level of SGPT elevated significantly in rats intoxicated with low does (10 mg/kg) [105]. Meng *et al.* [106] compared effect of micro, nano and ionic Cu in mice and found that nano-Cu induced higher toxicity as compared to others due to higher SC level and lower elimination rate. On the other hand, Chen *et al.* found that nano-Cu was more toxic as compared to micro and ionic Cu due to higher level of TBA, ALP, Cr and BUN [48].

8.1 Immunotoxicity induced by NPs

The function of immune system is to preserve the integrity of body by preventing it from environmental agents like chemicals, microbes or any entity that could disturb the homeostasis [107]. The interaction of NPs with the immune system has been an active research area in the field of biotechnology and nanotechnology. Tremendous efforts have been made in providing novel tactics to the existing therapies and diagnostics in health sector. The applications of nanotechnology in medical are trying to proffer many therapeutic solutions in the form of desirable physico chemical characteristic of NPs to enhance bioavailabilty, biodistribution and to reduce toxicity [108].

There are four possible mechanisms of NPs interactions with immune cells that are phagocytosis, endocytosis, passive uptake and receptor interaction based uptake [108]. The phagocytosis mechanism is well known for removing biological agents like microbes. The capture and internalisation of NPs take place in the phagosomes following by lysosomal degradation. The pH level of phagolysosomes results in the release of metallic ions that ends in disruption of mitochondrial processes leading to ROS production via Fenton type reactions. Similarly, in clathrin-independent and

Table 2 Genotoxic potential of CuO NPs in different organism

Organism	Part used	Shape	Sizes	NPs mode of concentration	Effects	References
frog	kidney epithelial cells	polydispersed	6 nm, <100 nm, <6nm	Size-dependent NPs, <100 nm NPs more toxic than 6 nm	<100 nm NPs more toxic than 6 nm DNA damage, decreased cell viability, reduced levels of GSH, cell death	[82]
Danio rerio (fish)	not specified	polydispersed	<50 nm	dose-dependent manner	small size, cross-cell membrane, release Cu^{2+}, ROS production, DNA damage, cell death	[86]
mice	lung tissues	nearly spherical	<30–>80 nm	dose-dependent manner	cell apoptosis	[87]
mouse	neuroblastoma cell line	spherical	30–40 nm	dose-dependent manner	DNA fragmentation, DNA methylation, chromosomal damage, lipid peroxidation, micronucleus formation	[65]
rat	lung tissues	spherical	15–20 nm	time- and dose-dependent manner	cell proliferation, inflammation, upregulation of proinflammatory cytokines	[88]
human	A549 cells	spherical	<100 nm	time- and dose-dependent manner	DNA damage, DNA lesions, neurotoxicity, cytotoxicity	[32]
human	pulmonary epithelial cells	spherical	50 nm	dose-dependent manner	DNA damage mediated through lipid peroxidation and oxidative stress, apoptosis	[16]
human	cell line A549	spherical	50 nm, 3 μm	size-dependent manner	Cu NPs more toxic than micro-NPs, DNA damage	[89]
human	HepG2 cells	spherical	22 nm	dose-dependent manner	induce cytotoxicity, ROS production, p53 and apoptotic gene caspase-3 upregulation, apoptosis in HepG2 cells via mitochondrial pathway	[77]
human	bronchial epithelial cells BEAS-2B	approx. spherical	20–200 nm	size-dependent manner	CuO NPs more toxic than CuO MPs, induction of oxidative stress, cell cycle arrest, apoptosis	[90]
human	skin keratinocytes cells	spherical	50 nm	time- and dose-dependent manner	apoptosis, necrosis, DNA damage via oxidative stress	[91]
human	airway epithelial cells	spherical	—	dose-dependent manner	increased inflammation, collagen deposition in lungs	[92]
human	cancer cell lines HT-29 and SW620	polydispersed	20 nm	dose-dependent manner	apoptosis, inhibition of proteins expression	[93]

dependent endocytosis, lysosomal fusion with the endosome takes place [109, 110].

The passive uptake involves the internalisation of CuO NPs which passively enter the cell or escape the endocytic/phagocytic vesicles, directly interacts with normal autophagic process and modulate the NLRP3 inflammasome [111–113]. The interaction of CuO NPs with cell surface receptors results in intracellular cascades activation such as the scavenger receptor pathway, TLR4 cascade, MAPK pathway and the lectin pathway. The extracellular consequences include exocytosis, cytokine secretion and ended in complement activation [108].

8.2 Immunotoxicity induced by CuO NPs

Immunotoxic effects of dose-dependent CuO NPs on macrophages and signalling pathways of peritonitis model (mouse) showed that after 1 h of exposure, the injection site had noticeably macrophages recruitment (Arancibia *et al.*, 2015). The reports showed that the CuO NPs recruited the first cells of the innate immune system. In comparison, no significant changes were observed in the control group, T and B cells, natural killer cells and mast cells. The inflammatory mediators and free radicals (NO) are required by macrophages for the removal of pathogens and other harmful substances. For evaluation, peritoneal macrophages were modified with lipopolysaccharide (LPS) and treated with different NPs exposure to demonstrate their effect on NO production. The in-vitro results elucidated that the LPS-mediated NO production was not affected by SiNP, TiNP and AlNP while stimulation of primary macrophages treated with LPS, co-treatment with CuO NPs prevented the production of NO. The production of intracellular free radicals by CuO NPs leads to activation of genes and different signalling pathways and ends in upregulation of arginase activity that indirectly enhances protein expressions. The arginase activity regulation results in modulation of CuO NPs induced proinflammatory cytokines secretion (TNFα, MIP-1β) and induction of COX-2 (cyclooxygenases derived prostaglandin) and prostaglandin E_2. These results clearly evidence that CuO NPs suppresses macrophaged innate immune response by upregulating the arginase activity.

The induction of ROS generation by CuO NPs exposure caused death in human lymphocytes through the generation of oxidative damage [114]. From healthy male individuals, lymphocytes were isolated via Ficoll polysaccharide followed by gradient centrifugation. The lymphocytes were exposed to varying concentrations of CuO NPs and incubated for 6 h. CuO NPs resulted in decreased cell viability, ROS formation, lipid peroxidation, alteration in glutathione levels followed by mitochondria and lysosomal damage. Another findings by Yaqub *et al.* elucidated the effect of sub-lethal concentrations of CuO NPs on the haematological parameters (white blood cells, red blood cells, haemoglobin and platelets count) of Mus musculus (Home mouse) [115]. The intravenous injection of sub-lethal concentration of CuO NPs into the Mus resulted in elevated level of WBCs, evidential decrease in RBCs, Hgb and platelets. The findings also evidenced the toxic effects of CuO NPs on haematological and metabolic enzymes of caspian trout (Juvenile) [116]. The experiment was run in triplicate in which the Caspian trout was exposed to lethal concentration of CuO NPs (LC50) for 28 days. Blood samples were taken to examine the short- and long-term effects of CuO NPs. The biochemical tests revealed an elevation in WBCs, neutrophils, monocytes, RBCs, Hgb and Hct after acute exposure of CuO NPs as compared to control group ($p < 0.05$). Moreover, after CuO NPs induction, there was a significant

elevation in the alkaline phosphatase, lactate dehydrogenase and aspartate aminotransferase levels.

The functional analysis of RAW264.7 cell line macrophages revealed a decrease in glutathione levels, phagocytosis inhibition, LPS-mediated NO production [117]. The findings evidenced that the Cu -based NPs profoundly affect the macrophages functions. The proteomic analysis also showed alterations in oxidative stress responses (superoxidases and peroxidases), mitochondrial proteins, glutathione biosynthesis, actomyosin.

The immunotoxic effect of CuO NPs and $CuSO_4$ on *Metaphore Posthuma* (Indian earthworm) immune associated parameters [phagocytic response, total count, cytotoxic molecules (superoxide anion, NO)], enzymes activities (alkaline phosphatase, catalase, superoxide dismutase, phenoloxidase, acid phosphatase) and coelomocytes total protein has shown that the exposure of 1000 mg/kg of CuO NPs and $CuSO_4$ for 14 days resulted in meaningful decrease in total coelomocyte followed by NO production, lower phenoloxidase and cytotoxic activity in 7–14 days, respectively [118]. Immune compromisation and decrease in earthworm's population density is observed among the species habituating in unrestricted contaminated soil by CuO and $CuSO_4$ NPs.

The impact of Cu-based NPs (Cu and CuO) on adult mussel's immune system showed that the soluble Cu accumulated in gills and haemolymph whilst CuO NPs noticeably caused damages to gills and cumulated in the haemocytes. The increased ROS production, lower multi-drug resistance transporter activity and lysosome abundance in the haemocytes were results of the Cu and CuO NPs cellular toxicity. The exposure of Cu and CuO NPs to adult mussels resulted in lowering their haemocytes phagocytic activity and elaborating the risks in pathogenic bacterial proliferations.

8.3 Haematological alterations induced by CuO NPs

Significant changes have been observed in the haematological profile of rats upon administration of CuO NPs. Lee *et al.* observed that CuO NPs induced haemolysis of RBCs (red blood cells), decrease in level of RBCs, HB (haemoglobin), iron, HCT (haematocrit), MCV (mean corpuscular volume) while increased RET (reticulocytes) was observed [100]. They also found reduction in LYM (lymphocytes) percentage was due to the differential WBCs count that in turn effect defence system of the organisms. Meanwhile, increased percentages of MON and NEU (monocytes and neutrophils) indicate inflammation in the exposed organs. Other scientists also observed similar finding where they found destruction of RBCs in rodents that cause anaemia and reducing RBCs count, HCT, MCH, HB, MCV and WBCs (leukocytes) [119, 120]. Moreover, higher level of Cu prevents absorption and utilisation of iron hence level of iron reduces and eventually diminishes in the serum [121, 122]. These findings demonstrate that CuO NPs disturb RBCs count and immune system of the treated organism.

8.4 Urine chemistry alterations induced by CuO NPs

Administration of CuO NPs also effect urine parameters as reported by many researchers. Lei *et al.* observed higher level of glucose, citrate, amino acid, acetate, lactate, succinate and TAMO while creatinine level dropped in urine spectra of rats treated with Cu NPs [102]. Lee *et al.* [100] reported significant increase in protein, white blood cells, ketone bodies, specific gravity, nitrite and occult blood in urine. Meng *et al.* [106] compared biochemical analysis (serum Cu, SC; serum ceruloplasmin, CP; and urine Cu, UC) of mice after 24 and 72 h intoxication with micro, nano and ionic Cu. They found higher level of SC after 24 h and that remained higher after 72 h in case of nano-Cu as compared to micro-Cu while ion Cu reduced after 72 h. This was due to accumulation and transformation of nano-Cu into ions while macro-Cu cannot. The UC level increased significantly in mice treated with both ion- and nano-Cu as compared to control while no increase was found in micro-Cu. Moreover, higher CP level was found in ionic Cu than nano-Cu might be due to binding of serum Cu with CP, i.e. acute phase protein type reactant.

A study conducted by Lischkova *et al.* [123] erected the presence of NPs in the biological samples of exposed occupational individuals. During the study two groups were subjected to engineered NPs (Fe, Mn and C compounds) and CuO NPs. Biological samples of the post-shift were collected and analysis was performed using transmission electron microscopy (TEM) and energy-dispersive spectroscopy. The biogenic ones (K, C, Cl and O) were the most identified chemical elements whilst the metals containing NPs were found in urine, blood sample and exhaled breath condensate.

8.5 Histological alterations induced by CuO NPs

Administration of CuO NPs causes deleterious effects in the structure of various organs that can be traced via histopathological studies. Many reports verify toxic effects of CuO NPs in different organs (Table 3).

Doudi and Setorki [105] treated rats with different doses (10, 100 and 300 mg/kg) of CuO NPs and studied their effect on liver and lungs. They found that all doses of CuO NPs showed changes in the liver, i.e. appearance of central vein vasculature, portal triad vessels and loss of hexagonal lobules while thickening of air sacs and increase in fibrous tissues in the lungs. Lei *et al.* intoxicated rats with 100 and 200 mg/kg of Cu NPs and observed their effects on liver and kidney [80]. The Cu NPs caused necrosis in rat's liver intoxicated with 200 mg/kg while 100 mg/kg Cu NPs did not cause any alteration. Noteworthy changes were also observed in nephrotic tissues including extensive necrosis in proximal renal tubule along with presence of cellular fragments tubule lumen upon treatment with 200 mg/kg of Cu NPs. Rats treated with 100 mg/kg of Cu NPs showed swelling of proximal tubule. Wang *et al.* [103] studied gender-based effect of different doses of Cu NPs on rats. They noticed histological alterations in liver, spleen and kidney in male (1250 mg/kg), and female (2500 mg/kg) rats. Liver of male and female rats with above-mentioned doses also displayed slight inflammatory cell's infiltration, dilation in sinusoids and vacuolation. Same was observed when male and female rats treated with Cu ions (625 mg/kg). Inflammatory cell's infiltration, cellular fragments deposition in the tubule, hyaline cast, tubular dilation, atrophy of glomerulus were found in kidney treated with Cu NPs while mild cast and dilation was observed in the kidney's tubule when exposed to Cu ions. Multinucleated spleen along with decline in cell's white pulp was observed in both sexes administered with Cu NPs while no such alterations were found in Cu ion treated rats. Arafa *et al.* [71] conducted an experiment on the female rats treated with CuO NPs and CuO NPs conjugated with quercetin. They observed effects in liver. Rats administered with CuO NPs induced severe damages to liver like lobular liver structure, liver cells with ballooning and bi-nucleated cell infiltration, microsteatotic, dilated sinusoids and congested central vein as compared to control and CuO NPs conjugated with quercetin. Lee *et al.*[100] studied the comparative effect of nano- and micro-Cu on spleen, thymus, hepatic and nephrotic tissues of rat. Their results determined that nano-Cu is more toxic and causes major alterations in these organs while micro-Cu did not exhibit any change. Major alterations found in these organs when exposed to nano-Cu were appearance of atrophic white pulp, yellow coloration and decreased cellularity and follicular number observed in spleen, interrupted demarcation of cortex and medulla. Reduced cells and vacuolation in cytoplasm were exhibited by thymus, hepatic tissues represented sinusoid dilation, mononuclear cell infiltration, deteriorated or binucleated liver cells, tubule dilation, cell fragments with purple or pink pigmented tubular casts, while nephrotic cells displayed disintegrated tubule cells along with inflammatory cell infiltration. Lei *et al.* used 50, 100 and 200 mg/kg of nano-Cu and examined their pathological effects on kidney and liver [102]. They found that 200 mg/kg of nano-Cu caused toxic effects including prevalent necrosis in proximal tubule, cell debris present in tubule's lumen where deposition of orange crystalline material was seen in the renal tissues while scattered dot necrosis in liver cells were also observed at same dose. Moreover, other doses (50 and 100 mg/kg) caused proximal tubule swelling in renal tissues and did not exhibited any toxic sign

Table 3 Histological alteration induced by CuO NPs in animal models

Organism	Concentration, mg/kg	Organs	Histological observations	References
rats	10, 100 and 300	liver	vasculature in central veins and portal triad vessels, loss of hexagonal lobules	[105]
		lungs	thickening of air sacs, increase in fibrous tissues	
rats	100 and 200	liver	necrosis of tissues	[102]
		kidney	necrosis in proximal renal tubule, cellular fragments around tubule lumen, swelling of proximal tubule	
rats	1250 and 2500	liver	slight inflammatory cell's infiltration, dilation in sinusoids vacuolation	[103]
		spleen	multinucleated giant cell, decline in cell's white pulp	
		kidney	inflammatory cells infiltration, tubular dilation, renal glomerulus atrophy	
rats	CuO NPs and conjugated with quercetin	liver	lobular hepatic architecture, hepatocyte with ballooning, binucleated hepatocytes, sinusoidal dilatation, lymphocytes aggregates	[71]
rat	nano- and micro-Cu	spleen	appearance of atrophic white pulp, increased yellow pigmentation reduction of cellularity, functional and structural damage	[100]
		thymus,	disrupted demarcation of medulla/cortex, decreased cellularity of medulla/cortex, cytoplasmic vacuolation	
		liver	mononuclear cell infiltration, dilated sinusoid, degenerated hepatocytes, binucleated hepatocytes	
		kidney	dilated tubules, degenerated tubular cells, inflammatory cell infiltration	
rats	50, 100 and 200	kidney	necrosis in proximal tubule, cell debris present in tubule's lumen, proximal tubule swelling	[81]
		liver	no changes were observed in liver	
rat		kidney	swelling of glomerulus, glomerulonephritis, deterioration in the lumen of Bowman's capsule	[106]
mice	nano and micro Cu	kidney	proximal tubule injuries, swollen glomerulus, dwindling in Bowman's capsule lumen, deterioration of epithelial cells of proximal convoluted tubules	[48]
mice	CuO NPs and conjugated with thiamine	ovary	degeneration of granulosa cells, disintegration of corpus luteum	[75]

in liver. Meng *et al.* [106] reported diverse range of irregularities in kidney including swelling of glomerulus, deterioration in the lumen of Bowman's capsule revealing glomerulonephritis and purplish deposits were also found. Chen *et al.*[124] observed the pathological effects of different doses of nano- and micro-Cu on various organs of both sexes of mice. Mice exposed to micro-Cu did not display any overt effect in any organ of any sex expect at higher dose (5000 mg/kg) in which both male and female mice died and ileus were shown in their intestine. Contrary to these observations, mice exposed to nano-Cu at all concentrations had gravely effected organs. Effect of nano-Cu on tissue damage was dose dependent, i.e. higher dose showed severe damage and vice versa. Exposure to lower dose (108, 158, 232 mg/kg) nano-Cu to mice's kidney caused proximal tubule injuries with swollen glomerulus and dwindling in Bowman's capsule lumen being contributing factors towards glomerulonephritis. Moreover, deterioration of epithelial cells of proximal convoluted tubules occurs at medium dose (341 mg/kg) while extensive necrobiosis was presented at higher dose (1080 mg/kg) mice. Protein fluid filled in renal tubules were seen in which purplish deposits were found in mice kidney treated with medium and higher dose but not observed in lower dose mice. Additionally, nucleus of epithelial cells of renal tubules were seen in lower dose, reduces in medium and ultimately diminished at higher dose. In hepatic tissues, mice exposed to medium and higher dose exhibited steatosis around liver's central vein while nothing was observed in lower dose mice. Spleen of treated mice showed various abnormalities like atrophy, splenic interstitium fibrosis, reduction in splenic units and lymphocytes in mice exposed to various doses (232, 341, 501, 736 and 1080 mg/kg). Fatahian-Dehkordi *et al.* compared the effects of CuO NPs, CuO; and conjugated CuO NPs and CuO with thiamine on the architecture of mice ovary [74]. They observed that both CuO NPs and CuO caused degeneration of granulosa cells, inflammatory cell infiltration especially in lymphocytes raised around blood vessels determining disintegration of corpus luteum while no such abnormalities were observed in mice treated with CuO NPs-thiamine and CuO-thiamine.

8.6 Ultrastructral alterations of cell induced by CuO NPs

The reports of Chen *et al.* [125] focused CuO NPs (using papaya lead extracts) antibacterial activity on soil born pathogenic bacteria (*Ralstonia solanacearum*). The soil born pathogenic bacteria exposed to 250 $\mu g\ ml^{-1}$ concentrations of CuO NPs and the findings showed significant killing of *Ralstonia solanacearum*. The CuO NPs prevented biofilm formation, lowered swarming motility, and disrupted the ATP production by interacting with bacterial cells. After adsorption of CuO NPs, the TEM revealed significant ultrastructural variations in the cytomembrane. The molecular observations elucidated the down-regulations of genes responsible for the mechanisms of pathogenesis and motility.

The cytotoxic effects of surface modified CuO NPs in mouse macrophage cell lines demonstrated that the different surface modifications did not disturb dissolution of the NPs [126]. The CuO NPs showed no ultrastructural changes observed in the exposed cells of macrophages. The internalisation of NPs resulted in agglomeration of NPs in membrane enclosed vesicles. In other subcellular compartments no CuO NPs agglomerates were found. The results of Minigalieva *et al*. [127] demonstrated the ultrastructural alterations in the brain revealed by TEM when the rats exposed to CuO NPs and observed nervous fibre demyelination. CuO NPs also showed minimum ultrastructural damage to the mitochondrial membranes and cristae [128].

The time-dependent changes in the ultrastructure of *Daphnia magna's* midgut epithelium upon exposure to CuO NPs than bulk CuO showed that after 10 min of exposure the dispersion of CuO NPs occurred in the lumen of midgut as compared to bulk CuO where clumps were formed [129]. Upon CuO NPs exposure at the 48 h, subsequent ultrastructural changes observed in the midgut which were protrusion of epithelial cells in the lumen of the midgut, occurrence of circular CuO NPs similar to membrane vesicles from holocrine secretion in the midgut lumen. Surprisingly, bacterial colonisation observed in the midgut upon CuO NPs. The effect of CuO NPs on the ultrastructural alterations in the midgut of D. magna NPs but not to bulk CuO refer to its nanosize-related adverse effects. Similar results have shown 50-

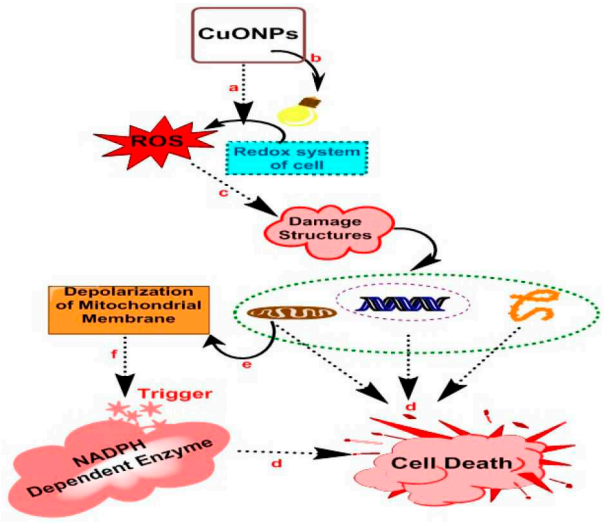

Fig. 2 *Mechanism of action involved in the cytotoxicity of CuO NPs comprises of multiple steps*

(a) Production of ROS directly via exposure of CuO NPs, ***(b)*** Stimulation of redox system of cell that leads to production of ROS, ***(c)*** Generation of ROS ultimately leads to damage structures like mitochondria, DNA, proteins and so on, ***(d)*** Damaged structures promotes cell death, ***(e)*** Damages to mitochondria involve depolarisation of mitochondrial membrane, ***(f)*** Depolarised mitochondrial membrane trigger NADPH-dependent enzymes that cause cell death

fold higher acute toxicity of CuO NPs compared to bulk CuO to *Daphnia magna* [130].

8.7 Morphometric analysis and CuO NPs toxicity

CuO NPs synthesised through green method (Anabaena specie) exposed to fishes exhibited no visual abnormality [131]. The fish (*Channa Punctatus*) showed normal swimming behaviour when exposed to fresh water (control) or CuO NPs. However, after 72 h of CuO NPs (40 µg/ml) exposure resulted 11% mortality. To demonstrate the CuO NPs effects on cell viability, mammalian cell lines (Human gastric epithelium) were treated for 24–48 h at different concentrations. The MTT analyses elucidated that at 2.5% concentration, the cells showed 86% survival whilst at 3% the survival significantly dropped to 55%. The findings of Bhattacharya *et al.* inferred that up to 2.5% CuO NPs did not show much distortion in mammalian cell shapes and morphology and considered as safe for human [131].

The toxicity of CuO NPs in rat's intestine epithelial cells (IEC-6) and human intestinal cells has shown that cell viability was significantly lowered at 4 µg/ml CuO NPs, with a greater response following 24 h exposure (75% cell viability at 4 h and 57% at 24 h). The highest dose, 80 µg/ml, had the greatest effect on viability with ∼25 and 12% of the cells viable at 4 and 24 h, respectively. At 80 µg/ml CuO NPs, the alteration in cellular morphology was noticeable and some cells released cellular contents. To assess the viability under dose and time dependent, the human intestinal cells were exposed to CuO NPs (80 µg/ml) for 4 h. The two-way analysis of variance exhibited noticeable time, dose interaction effects ($p < 0.0001$) on the cells viability. The cells viability significantly lowered at 24 h.

Furthermore, prenatal administration of CuO NPs in mice had significant effect on body weight and mortality during basic development and gestation period. They found that weight gain during gestation period was slower in mothers and pups after delivery. Moreover, mortality rate was also higher in pups as compared to the control [132].

Dose-dependent cytotoxic effects of CuO NPs synthesised through *F. religiosa* on human lung cancer cells (A549) have been observed by Sankar *et al.* (2014). At 50 µg/ml exposure to CuO NPs, A549 cells showed 70% viability whilst the viability noticeably lowered to 6% at 500 µg/ml. The results demonstrated morphological alterations (shape alterations, cell clumping, cell communication inhibition and chromatin condensation) in the cancer cells after 36 h. The cells treated with CuO NPs showed loss in membrane integrity, alterations in mitochondrial membrane potential due to ROS production that leads to apoptosis cascade.

The study conducted by Han *et al.* on *Drosophila melanogaster* showed that Cu NPs effect development, life span and sperm quality. They observed that Cu NPs decreased adult longevity characterised by slow development and reduced sperm competition [133].

9 Mechanism behind toxicity of CuO NPs

The major toxicological mechanisms of CuO NPs are ROS generation and oxidative stress induction [30]. CuO NPs cause direct toxicity via activation of ROS production and indirect via stimulating redox system of cell which leads to ROS production (Fig. 2). Upon entry through any exposure route (inhalation, ingestion, skin), CuO NPs interact with the acidic environment of lysosomes or with mitochondria (oxidative organelles) leading towards ROS induction that become a persuasive mechanism behind toxicity associated with CuO NPs [16, 63]. The CuO NPs initiated ROS generation that leads to a wide range of biological responses that entirely depends on the abundancy of biochemical factor (ROS), kind of cellular pathways and the antioxidant response elements that are involved in oxidative stress [134]. The CuO NPs act as a pro-oxidant, i.e. they encourage oxidative stress via generating ROS or impeding antioxidants. The ROS collectively constitute hydrogen peroxide, anionic superoxide and hydroxyl free radical. Among the free radicals hydroxyl (OH) toxic effect is considered as one of the most lethal one among the other ROS species. Hence, through direct method the CuO NPs cause their effect through production of ROS inside the cell comprising mitochondrial respiration and triggering NADPH-dependent enzyme systems [124, 135, 136]. Production of ROS also causes inflammatory responses and damages cell membrane, DNA and proteins [137]. Wang *et al.* verified the potential of CuO NPs to induce damages in DNA and mitochondria resulting in cell death when taken up by the cells (A549) via endocytosis as evidently situate inside the cell nucleus and mitochondria [11]. Moreover in indirect method, upon entry of CuO NPS they can also stimulate redox system of the cell most commonly in lungs where alveolar macrophages and neutrophils of immune system direct as ROS inducers through NADPH oxidase enzyme system [124, 138]. These radicals subsequently oxidise biological molecules that lead to substantial oxidative stress and cell death [139]. Both of direct and indirect means of ROS production ultimately leads to damage cellular structures (mitochondria, DNA, protein) which results in cell death. While in case of mitochondria another mechanism participates in cell death via depolarisation of mitochondrial membrane that stimulate NADPH-dependent enzymes and cause cell death. Other leading oxidative stress response is the release of Ca^{2+}, which ends to mitochondrial disruption and cell death. To oxidative stress many lethal diseases are linked such as cardiovascular, autoimmune, lung diseases and ageing [134, 140].

10 Conclusions

CuO NPs have emerged as an important class of nanomaterials for a wide range of applications (medicinal, environmental, industrial) that have potential risks to organisms and environment. In this review, an overview about CuO NPs synthetic methods, their advantages and disadvantages, applications, exposure routes, toxicological evaluations, relationship between ROS production and oxidative stress, immunotoxicity, cytotoxicity and genotoxicity, their testing in in-vitro and in-vivo in different organisms, and factors affecting the toxicity of CuO NPs are discussed. The factors that influence the toxicity of CuO NPs are shape, size, surface modification, morphology and concentration. Conclusively, to ensure that the CuO NPs are safe to environment and organisms, the toxicity of CuO NPs must be reduced to the non-significant level. The objective is to focus on the toxicity factors that will weaken toxicity mechanisms. Innovated work on surface modification, size, dissolution factor, selection of adequate exposure route will minimise the toxicity of metal oxide NPs [30].

As can be seen, these preliminary findings warrant more comprehensive studies to clearly elucidate the mechanisms of CuO NPs induced toxicity. Henceforth, the CuO NPs environmental fate must be determined carefully, and criteria for sustainable applications in different fields must be defined. In future, we recommend that much work should be done in the area of genotoxicity exhibited by CuO NPs as this area is less explored. Research must be carried out in the context of present risk assessments linked with CuO NPs, their applications, distribution and release into the environment.

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