EXPOSURE TO COPPER OXIDE NANOPARTICLES: BIOLOGICAL IMPACTS

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Abstract

Copper (Cu) is a biogenic element that plays an important role in many biological processes. Cu is also used in various industrial products and its nanosized form has antibacterial and antiviral properties. Cu nanoparticles (NPs) are further applied in industry, chemistry and electronic devices. When compared with other metal oxide NPs, Cu NPs are less commonly used. However, the potential of copper oxide NPs (CuO NPs) to induce cytotoxicity and DNA damage *in vitro* was higher than that of other tested NPs. Despite this fact, studies on CuO NPs toxicity are relatively scarce and their effects on human health have never been investigated. *In vitro* tests in model cell lines showed the ability of CuO NPs to cause oxidative stress, but induction of apoptosis and cell cycle arrest were also observed. In animal studies, inflammatory responses in lungs were most common, but some studies revealed damage to other organs, including the brain. Considering toxicity of CuO NPs and the fact that these NPs are routinely manufactured and used in various products, more research is needed to elucidate mechanisms of their negative health effects in humans.

Keywords: CuO nanoparticles, toxicity, experimental systems

1. INTRODUCTION

As other metal-based nanoparticles, copper oxide nanoparticles (CuO NPs) may enter body either by inhalation, dermal or ingestion route. For human exposure, the inhalation route is usually the most significant one, particularly for occupational exposures. In the body, nanoparticles (NPs) may reach lungs, where they can be deposited, but also other organs, including the central nervous system. Their presence usually causes oxidative stress or inflammatory responses.

Copper (Cu) is a biogenic element with many biological functions. Cu is necessary for the proper growth, development and maintenance of bones, connective tissues and various organs [1]. It is involved in the stimulation of immune system in the protection against infection, in repair of injuries and healing. It plays an important role as a cofactor in some enzymes (e.g. superoxide dismutase, cytochrome oxidase), has roles in cell signaling and cell proliferation. In mollusks and some arthropods Cu is responsible for oxygen transport. Also, its metabolism is critical for tumor progression [2]. Nanosized Cu has antimicrobial and antiviral properties and is therefore used is face masks, wound dressings and socks [3]. It is

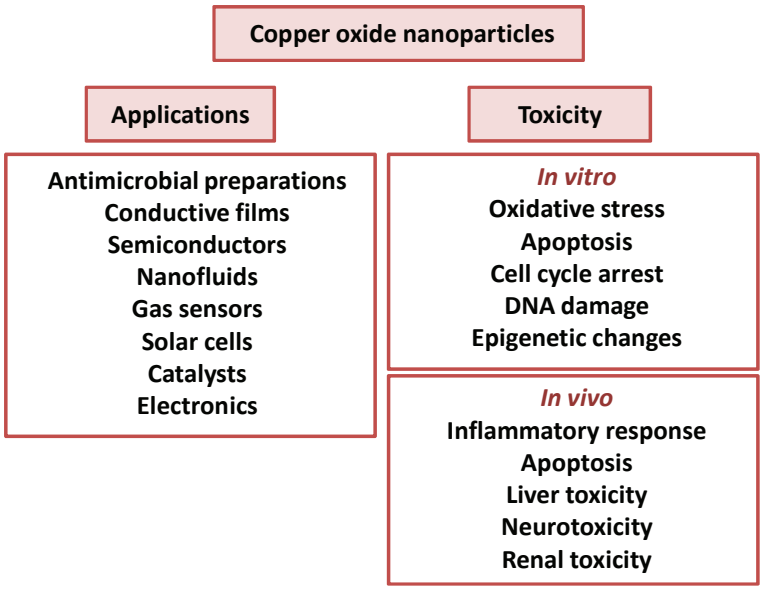

Figure 1 Overview of application and toxic effects of copper oxide nanoparticles

further applied in conductive films, lubrication, nanofluids, gas sensors, solar cells, lithium batteries and catalysts. Cu-based nanostructures are also used in various electronic devices, e.g. inkjet printers [4] (**Figure 1**). Unlike some other nanomaterials, Cu-based materials are relatively cheap. The global production of CuO nanoparticles (NPs) was 570 tons in 2014 and it is estimated to increase to 1600 tons in 2025 [5]. In comparison with other metal-based NPs (e.g. Ag or Zn), Cu-based NPs are less commonly used. Similar to other metal-based NPs, toxicity of Cu-based NPs depends on solubility of the material. In general, solubility depends on pH, dissolved organic carbon content and water hardness [3]. Acidic pH increases solubility; similarly, organic material may coat and disperse NPs thus supporting solubility.

2. TOXICITY OF CuO NANOPARTICLES IN VITRO

The production of CuO NPs is relatively low when compared with other metal oxide NPs. Nevertheless, CuO NPs properties and behavior in biological systems make them an important subject for toxicological investigation. Theoretical mechanisms of CuO NPs toxicity include (1) release of Cu ions from the surface of NPs, (2) oxidative damage mediated by Fenton-type reactions [reactive oxygen species (ROS) generation] induced by particles and (3) cell death associated with autophagy caused by CuO NP exposure. Autophagy is manifested by formation of autophagosomes that engulf and degrade defective proteins and organelles thus protecting the cells from damaged structures and helping the cells to survive. However, autophagy may also lead to cell death that is independent from apoptosis.

Although metal oxide NPs (e.g. Cu, Zn, Ti, Fe, Si) share similarities in their biological effects often mediated by ROS production, CuO NPs have been shown to have the highest potential to induce cytotoxicity and DNA damage in vitro [6]. This seems to be related to the ability of CuO NPs to better overwhelm antioxidant defenses of the organism. Toxicity of metal-based NPs may be caused by both ions and the particles themselves. These effects depend on the ability of NPs to release ions in the aqueous environment. CuO NPs have been shown to release Cu ions in culture medium but not in water [7], but the results on negative biological effects of the released ions are conflicting (reviewed in [6]): Cu ions accounted for part of the cytotoxicity of CuO NP in A549 [7] and BEAS-2B cells; however, other authors did not observe any contribution of Cu ions to toxic effects of CuO NPs in HEp-2 cells and A549 cells concluding that particles themselves are mostly responsible for toxicity of CuO NPs.

Toxicity of CuO NPs has been investigated in various cell lines in several studies; most of them observed oxidative stress-related response (for overview of in vitro effects see **Figure 1**). Thus, damage to mitochondria leading to increased production of ROS has been observed in A549 cells exposed to CuO NPs suggesting induction of oxidative damage [8]. CuO NPs have been further shown to induce lipid peroxidation, trigger intracellular signaling resulting in oxidative stress response and induction of apoptosis. In another study, CuO NPs generated ROS that subsequently induced the expression of p38 and p53 indicating induction of DNA damage in A549 cells [9]. CuO NPs have been further shown to cause oxidative stress induction measured by increased levels of 8-isoprostane and glutathione levels in human airway epithelial cells. Oxidative stress may induce autophagy; there are reports indicating that CuO NPs exposure is linked to this process in A549 cells [7]. On the other hand, CuO NPs may cause autophagic dysfunction by affecting lysosomal enzymes or disrupting vesicle trafficking [10].

Other processes potentially mediating toxic effects of CuO NPs have also been investigated. Apoptosis as a mechanism induced by CuO NPs was described (reviewed in [10]). The response is initiated by ROS generation resulting in destruction of mitochondrial membrane. This process results in activation of p53 which increases Bax/Bcl2 ratio. CuO NPs cause cell cycle arrest and DNA damage that was manifested by γ-H2AX formation [11]. CuO NPs may also induce IL-8 production which may consequently cause activation of NF-κB pathway [11]. CuO NPs may be associated with selective damage to cellular membranes because they release some amino acids, mostly glutamate, and cellular K^+ [10]. CuO NPs have been shown to bind to proteins via hydrogen bonds [12]. This may result in changes of protein phosphorylation and ubiquitination which plays an important role in cell signaling.

CuO NPs may affect gene expression through epigenetic mechanisms, specifically by histone modification [13]. They upregulate the expression of DNA repair proteins and directly interact with structural elements of the cell (e.g. cytoskeleton). This may disrupt mitotic spindle and cause aberrant cell division [10]. CuO NPs further directly interact with DNA causing replication arrest possibly leading to genome instability.

Molecular mechanisms of CuO NPs cytotoxicity in A549 cells were assessed using whole genome gene expression analysis. Exposure to CuO NPs caused upregulation of the expression of 648 genes and downregulation of the expression of 562 genes [7]. These genes included e.g. those involved in nucleic acids metabolic processes, response to stress, cell cycle, mitosis, cytokinesis, chromosome segregation, cellular component organization and morphogenesis. Genes encoding heat shock proteins or DNA damage-inducible genes were in the category of response to stress. This suggests that CuO NP stimulate protein denaturation and induce cell cycle arrest in the G1 and G2 phases. On the other hand, p53 protein does not seem to play a major role in the response to CuO NPs treatment. Cu ions alone induced expression of superoxide dismutase 2 and genes encoding metallothionein isomers. Proteins encoded by these genes help to protect the cells against oxidative stress induced e.g. by the presence of Cu ions. Thus, in A549 cells CuO NPs seem to damage both mitochondria and DNA; Cu ions contribute to the mitochondrial damage and lead to cell cycle arrest.

3. TOXICITY OF CuO NANOPARTICLES IN VIVO

Although in vitro tests in model cell lines provide information on cellular mechanisms of CuO NPs exposure and give general hints on their toxicity, experiments in laboratory animals represent a more relevant approach to assess the impact of CuO NPs exposure on human health. Despite this fact, such animal studies are relative scarce. Most of them were conducted in rodents and focused on pulmonary and neurotoxic effects, but also on changes in other organs associated with CuO NPs exposure (**Figure 1**).

Effects of various ways of pulmonary delivery of CuO NPs were investigated in both rats and mice (reviewed in [1]). Most of the studies reported inflammatory responses. Thus, lung instillation of CuO NPs <50 nm induced acute and chronic inflammation in rats. In another study, intratracheal instillation of CuO NPs induced neoplastic lesions [14]. A decrease in levels of antioxidants following intratracheal instillation was detected by another group [6]. Yokohira et al. studied CuO NPs toxicity in F344 male rats after intratracheal instillation and observed numerous negative effects including acute cell death, neutrophil infiltration or edema [15]. Cho et al. reported that intratracheal instillation of CuO NPs caused inflammation in lungs of rats both after 24 h and 4 weeks postinstillation [16]. Gosens et al. focused on pulmonary toxicity of CuO NPs in rats after a short-term inhalation exposure [17]. The animals were exposed to five doses of CuO NPs for five days, 6 hours/day and examined either 1 day or 22 days after exposure. The exposure resulted in inflammation and cytotoxicity detected 24 hours after the treatment. Histopathological examination of the lungs revealed alveolitis, bronchiolitis, vacuolation of the respiratory epithelium and emphysema of the lung. These effects almost completely resolved after a 3-week post exposure period. Thus, clearance of NPs is an important factor affecting their toxicity. Hirano et al. reported that intratracheally-instilled CuO NP had a clearance half-time of 37 h [18].

So far, there is only one study that focused on analysis of whole genome transcriptional changes after inhalation of CuO NPs [19]. The authors exposed rats for 6 hours to two doses (3.3 mg/m^3 and 13.2 mg/m^3) of CuO NPs and analyzed transcriptomics response 1 day post-exposure and after a 22 day recovery period. The exposure resulted in deregulation of about 1000 genes in the high-dose group and about 200 genes in the low-dose group. After the recovery period, the number of deregulated genes dropped to about 20. The main processes affected by the exposure included cell proliferation/survival and inflammation. Interestingly, no oxidative stress related pathways were affected. The authors observed proliferation of alveolar epithelial cells and detected upregulation of CCl2 (monocyte chemoattractant protein 1) both on the level of mRNA and

protein and upregulation of ECT2 oncoprotein. Finally, no aberrant DNA methylation of inflammation-related genes was observed.

Intranasal instillation of CuO NP caused pulmonary inflammation, induction of apoptosis, collagen accumulation and expression of the progressive fibrosis marker α-SMA in C57BL/6 mice [21]. The animals were exposed to 3 doses of CuO NPs and lung tissue was collected on day 7, 14, 21 and 28. Interestingly, in another nasal instillation study necrosis of hepatocytes in the liver of exposed mice was observed [1]. In these animals, renal glomerulus and olfactory bulb were also affected. Intraperitoneal injection of CuO NPs resulted in increased frequency of micronuclei and induction of oxidative DNA damage in mice [21] indicating genotoxic effects of these NPs. Whole-body inhalation was accompanied by increased levels of inflammation markers in mice. The effects were persistent even 3 weeks post-exposure [1].

Although few investigations have been performed, the current data indicate that CuO NPs are neurotoxic [22]. In animal studies, they altered function of blood-brain barrier, damaged neurons and caused brain edema. Oxidative damage and neuronal apoptosis was also observed [22]. Significant toxic effects of CuO NPs of various sizes and concentrations were detected in dorsal root ganglion neurons of rats [1]. Neurotoxicity of Cu NPs following intraperitoneal, intraveneous, intracarotid and intracerebroventricular administration was detected in mice and rats [1]. Importantly, nasal instillation of CuO NPs affected brain tissue of experimental mice [23]. The exposure caused various pathological changes in neurons, including chromatin congregation and mitochondria shrinkage in olfactory cells, or increase of endoplasmic reticulum and disassociation of ER ribosomes in hippocampus. No change in antioxidant enzymes expression was observed, although malondialdehyde levels significantly increased.

Based on the tests in crustaceans, algae and fish, CuO NPs have been classified as toxic to aquatic organisms [3]. The effect of CuO NPs exposure on changes of expression of selected genes was studied in marine bivalve *Mytilus edulis* [24]. The mussels were exposed to CuO NPs for 24 h and expression of genes involved in immune response, antioxidant activities, cell metabolism, cell transport and cytoskeleton was assessed. The authors also compared the effect of Cu ions with the impact of the particles. The authors observed deregulation of expression of a number of genes, including e.g. β-tubulin, actin, antioxidant enzymes (catalase, SOD) and cell cycle control genes (p53, Gadd45). In general, the effects were more pronounced after treatment with CuO NPs than Cu ions suggesting the toxicity of CuO NPs is not caused solely by Cu ions release.

So far, no study investigated negative health effects of CuO NPs exposure in human populations. Nevertheless, the increased cancer risk among copper smelter workers suggests that Cu NPs exposure is harmful to humans [1]. The only study that used human samples to investigate toxicity of CuO NPs focused on human blood cells [25]. Lymphocytes obtained from blood of healthy volunteers were treated with CuO NPs and several parameters, including cell viability, ROS production, lipid peroxidation, glutathione levels and mitochondrial and lysosomal damaged were analyzed. The results indicated effects on cell viability associated with increased ROS production along with mitochondrial and lysosomal damage.

4. CONCLUSIONS

CuO NPs are used in many industrial products and as antimicrobial agents. Although their production is lower than other metal-based NPs, their toxicity is high. *In vitro* they have been shown to induce oxidative stress, apoptosis and cell cycle arrest. In laboratory animals exposed to CuO NPs inflammation was most commonly detected, but neurotoxicity was also noted. Due to potential negative health effects of exposure in humans, more studies are needed to better characterize mechanisms of CuO NPs toxicity.

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