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Oxidative Stress Induced by CuO Nanoparticles (CuO NPs) to Human Hepatocarcinoma (HepG2) Cells

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Abstract

The toxicity of CuO nanoparticles (CuO NPs) to human hepatocarcinoma (HepG2) cells was investigated in this study. CuO NPs (1 - 40 mg/L) had significant toxicity to HepG2 cells. The antioxidant N-acetyl-L-cysteine (NAC) significantly reduces the cytotoxicity induced by the CuO NPs, supporting the hypothesis that oxidative stress contributes to the cytotoxicity of CuO NPs. To further explore the oxidative mechanisms of cytotoxicity, we examined CuO NPs-induced production of reactive oxygen species (ROS) in HepG2 cells. CuO NPs generated intracellular ROS in HepG2 cells in a concentration-dependent manner.

Keywords

CuO NPs, ROS, Cell Viability

1. Introduction

Metal and metal oxide nanoparticles (NPs) are widely applied in personal care products [1], catalysis [2], optical and recording devices [3], and water purification [4]. Copper oxide nanoparticles (CuO NPs) are wildly used as industrial catalysts in manufacturing processes and are heavily utilized in semiconductor devices, gas sensor, solar energy converter and heat transfer fluids [5] [6]. The increasing production of CuO NPs has led to major concerns regarding the potential toxicity to the environment and human health.

Recently, the toxicity of metal and metal oxide NPs on mammalian cells has been studied, and a number of researchers have found that metal and metal oxide NPs can generate oxidative stress and promote cytotoxicity [7] [8]. Then, recent studies have shown the oxidative stress mediated toxicity of CuO NPs in different human cell lines, such as human lung epithelial (A549), human cardiac microvascular endothelial, kidney and neuronal cells

[9]-[14]. It is also reported in the scientific literature that liver is one of the target organs for nanoparticles after they gain entry into the body through any of the possible routes [15] [16]. Wang *et al*. [17] observer that cytoxicity of CuO NPs in HepG2 cells was found the relationship between reactive oxygen species (ROS) generation and gene expression during CuO NPs exposure. Piret *et al*. [18] (2012) found that CuO NPs induce ROS mediated cytotoxicity and pro-inflammatory response in HepG2 cells. Then, Maqsood *et al*. [19] (2013) focused on the underlying mechanism of apoptosis in HepG2 cells due to CuO NPs exposure. Oxidative stress is believed to be one of the most important mechanisms of cytotoxicity of CuO NPs due to the formation of reactive oxygen species.

In this study, we focused on the cytotoxicity of CuO NPs. Recent studies have shown that CuO NPs display significant toxicity to algae [20], bacteria [21], and fish [22]. For human cells, Karlsson et al reported that CuO NPs were significantly more toxic than ZnO, Fe_2O_3, TiO_2 NPs and CuO NPs resulted in oxidative stress for human cells [23].

2. Materials and Methods

2.1. Cell Culture and CuO NPs Suspension Characterization

The HepG2 cell line was purchased from American Type Culture Collection (ATCCHB-8065). Cell were cultured in DMEM (DMEM. Gibco, United States) supplemented with 10% fetal bovine serum (FBS. Hyclone, United States), 2 mM L-glutamine (Amresco Int. United States), and 100 U/ml penicillin streptomycin (Tianlin Hao Yang Biological Manufacture Co. LTE). Cell were maintained at 37˚C in an incubactor with 5% CO_2.

CuO NPs were purchased from Beijing Nachen S&T Ltd. Dry power of CuO NPs was suspended in DMEM-FBS medium of 1000 mg/L, and then sonicated for 30 min before aseptic addition to the tissue culture plates. The number of cell added in each well was identical (3×10^4 cells/well). Then, different volumes of CuO NPs stock solution were added to the cell culture to achieve predetermined concentrations of CuO NPs (1, 5, 10, 20 and 40 mg/L) for cell incubation. The Zeta potential of CuO NPs at 8 mg/L in the DMEM-FBS medium and ultrapure water were determined using a Nanosizer (Nano S90. Malvern Instruments Ltd. United Kingdom). The particle size of CuO NPs was also evaluated by a transmission electron microscopy (TEM, JEM-2100.JEOL.Japan). The crystalline nature of CuO NPs was carried out by taking X-ray diffraction (XRD) pattern. The XRD pattern of CuO NPs was acquired at room temperature with the help of PANalytical X'Pert X-ray diffractometer equipped with a Ni filtered using Cu Kα (λ = 1.54056 Å) radiations as X-ray source.

2.2. Cell Viability Assay

Cell viability was determined by cell Counting Kit-8 (Beyotime Institute of Biotechnology). Briefly, 3×10^4 cells were spread onto each 96-multiwell plate. Cell in each well were treated with different concentrations of CuO NPs (1, 5, 10, 20 and 40 mg/L) at 37˚C for 8, 12, 16 and 20 h. The cells were then incubated with 10 µl of cholecystokinin octapeptide (CCK-8) for 4 h, and the absorbance was measured at 450 nm. The mean absorbance of nonexposed cells served as the reference value for calculating percentage of cellular viability. Finally, the half maximal inhibitory concentration (IC_{50}) was calculated according to cell viability from various CuO NPs exposures.

2.3. Determination of ROS

ROS production was determined using 2'7'-dichlorofluorescein diacetate (DCFH-DA) (Beyotime Institute of Biotechnology) as described elsewhere [24]. Cells were seeded into 24-well plates at a density of 4×10^3 cells/500 µL/well 24 h before treatment. Treated with different concentrations of CuO NPs for 16 h. Briefly, the cells were incubated with media containing 25 µM DCFH-DA at 37˚C for 45 min. After removing the DCFH-DA solution, the cells were washed twice with phosphate buffered saline (PBS; Invitrogen), then, the plate was monitored in a Biological fluorescence inverted microscope.

3. Results and Discussion

3.1. Physicochemical Characterization of CuO Nanoparticles

Then, characterize for the CuO nanoparticles. **Figure 1(a)** shows the XRD pattern of prepared CuO NPs that

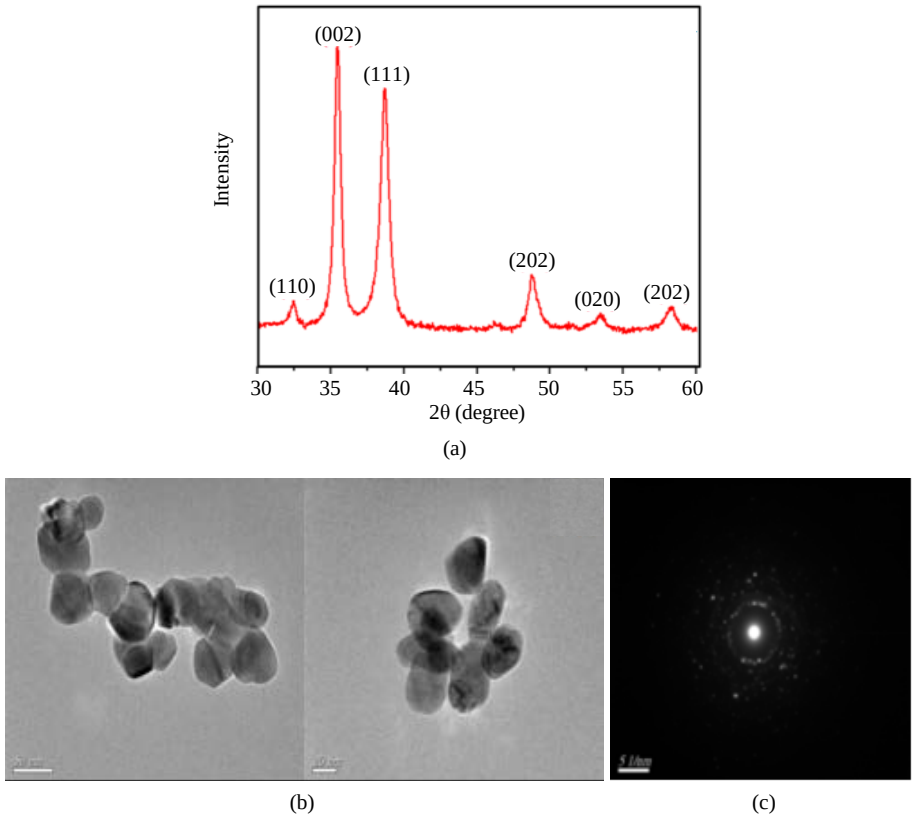

Figure 1. The characterization of CuO NPs. (a) XRD pattern; (b)Transmission electron microscope (TEM) image of CuO NPs; (c) Electron diffraction pattern of CuO NPs from aggregates in (a) image.

clearly exhibits the crystalline nature of this material. The Peaks at 2θ = 32.62˚, 35.61˚, 38.80˚, 48.75˚, 53.68˚ and 58.42˚ were assigned to (110), (002), (111), (202), (020) and (113) of CuO NPs. The crystallite size of CuO NPs was found to be around to be 30 - 50 nm. TEM image (**Figure 1(b)**) showed that the majority of CuO NPs were in spherical shape. These pictures exhibit that the majority of the particles were spherical sharped with smooth surface. The Zeta potential of water by DLS (Nano-Zetasizer-HT. Malvern Instruments. Malvern. UK) was −20.4 mv and −8.6 mv, respectively. The specific surface area of CuO NPs determined by BET was 15.31 m^2/g. The physicochemical characteristics of CuO NPs were listed in **Table 1**.

The free Cu^{2+} ions concentration dissolved from CuO NPs into the cell medium was determined using a modified procedure by Navarro *et al*. The CuO NPs (40 mg/L) in the DMEM medium were allowed to equilibrate for 2, 4, 6, 8, 10, 12, 14 and 16 h (5% CO_2, 37˚C). CuO NPs suspensions were then centrifuged for 30 min at 1500 g using ultrafiltrate centrifugal tubes (3 kDa, Millipore). The Cu concentration in the filtrate was determined using flame atomic absorption. No Cu^{2+} ions in the medium were observed from 2 h until 16 h. These date suggest only a neglectable role of copper ions in our experiment design, even during the longest incubation times.

3.2. CuO Nanoparticle-Induced Cytotoxicity

HepG2 cells were exposed to CuO NPs at the concentrations of 1, 5, 10, 20 and 40 mg/L for 8, 12, 16 and 20 h and cytotoxicity was determined using CCK-8 assay. Results showed that CuO NPs significantly decreased the cell viability in dosedependent manner. CuO NPs at low concentrations (1 and 5 mg/L) has insignificant toxicity to HepG2 cells after exposure for 8 h, which is likely the results of Cu being a required micronutrient. The toxicity of CuO NPs at moderate concentrations (10 and 20 mg/L) began to appear after exposure for 12 h become significantly greater in the subsequent 20 h. For high concentration of CuO NPs (40 mg/L) toxicity to HepG2 cells was the greatest at 20 h, and the cell viability dropped to 4.5% (**Figure 2(a)**). The toxicity of CuO NPs

Table 1. Physicochemical properties of CuO NPs.

Parameters	Values
Purity (%)	>99.9
Size from TEM (nm)	30 - 50 nm
Surface area from BET ($m^2 \cdot g^{-1}$)	15.31
Zeta potential distilled water (-mV)	20.4
Zeta potential culture medium (-mV)	8.6

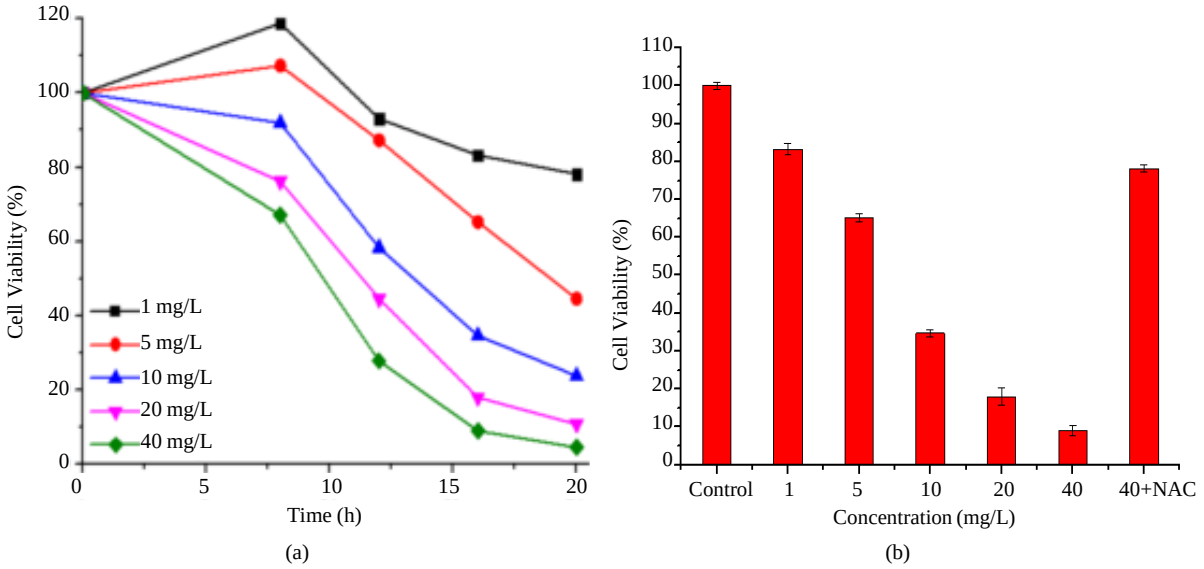

Figure 2. HepG2 cell viability measured with the CCK-8 assay after exposure to different conditions: (a) Cell viability after exposure to 1, 5, 10, 20 and 40 mg/L CuO NPs for 8, 12, 16, and 20 h; and (b) Cell viability after 16 h of exposure to CuO NPs at different concentrations. Data are showed as mean values ± standard deviations, n = 5.

was concentration-dependent (**Figure 2(b)**), and the 16 h IC_{50} was calculated as 8 mg/L. Thus, 8 mg/L was chosen in the following toxicity studies, and chose the low concentration (1 mg/L) and moderate concentration (20 mg/L) for comparison. Thus, CuO NPs had a much higher toxicity potential, a finding supported by Karlsson *et al*. [23], who observed that CuO NPs were more toxic to A549 cells than ZnO and TiO_2 NPs.

3.3. CuO Nanoparticle-Induced Oxidative Stress

NAC act as a normal antioxidant, played an important role in clean the extra free radical to protect the body. We examined the effect of CuO NPs on ROS generation in the presence or absence of anti-oxidant N-acetyl-cystein (NAC). Fluorescent microscopy data revealed that CuO NPs (1, 8, 20 mg/L) induced the intracellular production of ROS in dose-dependent (**Figure 3**). We further observed that coexposure of NAC effectively prevented the ROS generation induced by 20 mg/L of CuO NPs. ROS level was reduced up to control level for CuO NPs in the presence of NAC.

Oxidative stress has been suggested to play an important role in the toxicity mechanisms of nanoparticles [24] [25]. We proposed that CuO NPs were able to induce ROS mediated cytotoxicity in HepG2 cells. Thus, we detected the effect of CuO NPs on cell viability in the presence or absence of the NAC (**Figure 2(b)**). **Figure 2(b)** shows that NAC abolished almost fully the harmful effect of CuO NPs at all concentrations studied.

In summary, this study has demonstrated the relationship between the oxidative stress and the cytotoxicity of the CuO NPs to HepG2 cells, therefore CuO NPs cause significantly reduced viability of HepG2 cells in a concentration-dependent manner. The presence of the antioxidant NAC significantly reduces the cytotoxic effects induced by the CuO NPs. In addition, the CuO NPs can generate intracellular ROS in a concentration-dependent manner and cause oxidative damage to DNA and proteins in HepG2 cells. These results all suggest that an oxidative stress mechanism is involved in the cytotoxic effects determined in HepG2 cells after 16 h CuO NPs.

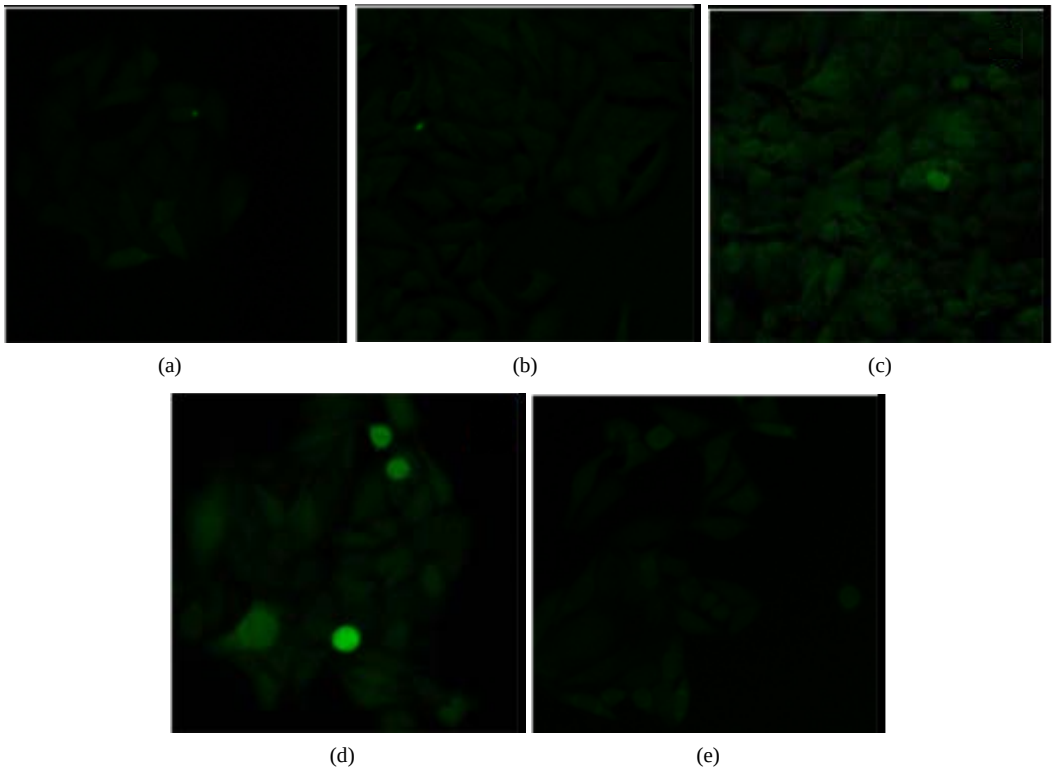

Figure 3. ROS level in HepG2 cells treated with CuO NPs. (a) Control, (b) 1 mg/L, (c) 8 mg/L, (d) 20 mg/L CuO NPs for 16 h, (e) cells were pretreated with 10 mM NAC. The fluorescence intensity was visualized under fluorescence microscopy with a GFP filter, and the images (200×) are representative of the fluorescence levels observed three times.

These results suggest that an ROS-related mechanism at least partially contributes to the CuO NPs-induced cytotoxicity and genotoxicity. These results are useful for developing a better understanding of the potential cellular mechanisms of toxicity of CuO NPs. These data clearly show that increased attention and careful investigations are needed regarding potential exposure, toxicity, and ultimate risk from metal oxide NPs such as CuO. ROS such as superoxide anion (O_2^-), hydroxyl radical (HO^-) and hydrogen peroxide (H_2O_2) elicit a variety of physiological and cellular eventa including inflammation, DNA damage and apoptosis [26]-[28]. ROS can affect many signal transduction pathways by directly reacting with and modifying all major classes of biomolecules, resulting in changes in their structures and functions [29]. In the recent study, we found that with the increasing of ROS induce various diseases including cancer [30] [31].

4. Conclusion

In conclusion, the oxidative stress caused reduced viability of HepG2 cells. In our further study, we should pay more attention to the cytotoxicity of the CuO NPs to HepG2 cells and quantified Comet assay, protein carbonyls, and malondialdehyde (MDA) for the oxidative DNA damage, protein oxidation, and lipid peroxidation, respectively. The findings of this study well provide a better understanding of the potential cellular mechanisms of toxicity of CuO NPs.

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