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CuO nanoparticles induce apoptosis by impairing the antioxidant defense and detoxification systems in the mouse hippocampal HT22 cell line: Protective effect of crocetin

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ABSTRACT

Several studies have reported that CuO nanoparticles (CuONPs) have the capacity to cross the blood brain barrier and exert a toxic effect. The aims of our study were to investigate mechanisms underlying CuONPs-induced neurotoxicity *in vitro* and neuroprotective effects of crocetin. We investigated the toxicological effects of exposure of HT22 hippocampal cells to CuONPs (31 nm) in the presence or absence of crocetin. Crocetin is a carotenoid with wide spectrum of pharmacological effects and the ability to cross blood-brain barrier. Exposure of HT22 cells to CuONPs resulted in: (1) increased cell death in a time- and concentration-dependent manner, with a LC_{50} of 25.9 μg/ml after 24 h; (2) decreased antioxidant/detoxification enzymes activities: glutathione peroxidase (GPx), superoxide dismutase (SOD), glutathione S-tranferase (GST), and reduced glutathione (GSH) levels; (3) decreased gene expression of GPx and SOD; (4) reactive oxygen species (ROS) generation; (5) enhanced apoptosis; and (6) up-regulation of the pro-apoptotic genes Bax, and down-regulation of anti-apoptotic genes Bcl-2. Importantly, all these effects were significantly attenuated by co-incubation of hippocampal cells with 5 μM crocetin.

1. Introduction

Nanotechnology is widely used across a range of industries, including the food, military, energy, pharmaceutical, medical, and cosmetic industries. While there is growing excitement about the potential applications of nanomaterials, there is a shortage of data regarding the potential harmful effects of these materials. NPs have been shown to exert systemic effects via the blood stream after being inhaled or swallowed, with some NPs being capable of directly penetrating the skin (Yildirimer et al., 2011). The brain is one of the key targets for possible toxic effects of NPs and these can result from environmental, occupational or medicinal exposure (Gwinn and Vallyathan, 2006; Win-Shwe and Fujimaki, 2011). Once within the blood, NPs may cross biological barriers in the body. Sharma and Sharma (2007) found that intravenous, intraperitoneal, or intracerebral administration of Ag, Cu, or AlNPs (50–60 nm) disrupts the blood-brain barrier, as indicated by staining with albumin-bound Evans blue. It has also been noticed that metal NPs that come into contact with the olfactory epithelium can be transported to the brain by olfactory neurons (Tjalve and Henriksson, 1999; Yang et al., 2010). Although the neurotoxicity induced by various NPs has been demonstrated, very little is known about molecular mechanisms underlying the brain damage. It was indicated and hypothesized that NPs may induce oxidative stress by releasing various mediators from microglia and astrocyte and/or altering the expression level of genes related to oxidative stress (Prabhu et al., 2010; Shimizu et al., 2009; Win-Shwe and Fujimaki, 2011).

A deeper understanding of the neurotoxic effects of NPs might help in the development of safety guidelines in order to advance nanotechnological applications while minimizing health hazards and risks.

Metal oxide nanoparticles are among the most widely used types of engineered nanomaterials (Ray et al., 2009). They are used in catalysis, environmental remediation, as sensors, and in the production of personal care products. Among various metal oxide NPs, CuO nanoparticles (CuONPs) have attracted particular attention because they are simple and show a range of useful physical properties such as high temperature superconductivity, electron correlation effects, and spin dynamics, as well as a low cost preparation

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(Suleiman et al., 2013). CuONPs are used in antimicrobial preparations, heat transfer fluids or intrauterine contraceptive devices (Aruoja et al., 2009). However, studies have shown that CuONPs may cross the blood-brain barrier and exert some neurotoxic effects by modulating neurotransmitters levels and affecting neurophysiologic signaling (Li et al., 2012; Xu et al., 2009; Yang et al., 2010; Wang et al., 2009; Sharma and Sharma, 2012).

We used mouse hippocampal HT22 cells as an experimental model to study the effects of CuONPs. The hippocampus is a vertebrate brain structure responsible for consolidating short-term memories into long-term memories and damage to this structure underlies some of the pathology associated with Alzheimer's disease (Oishi and Lyketsos, 2014).

We hypothesized that CuONPs affect the integrity of hippocampal neuronal cells. Many studies have shown that oxidative stress is a key mechanism responsible for NPs-induced cytotoxicity (Manke et al., 2013; Sarkar et al., 2014). Also, we have found in our previous studies that the generation of free radicals was implicated in AgNPs and SiO_2NPs toxicity in HGF-1and Calu-3 cells (Inkielewicz-Stepniak et al., 2014; McCarthy et al., 2012). Thus, it was of interest to investigate the contribution of oxidative stress to CuONPs-induced damage in hippocampal HT22 cells.

Epidemiologic investigations as well as *in vitro* and *in vivo* laboratory studies indicated that crocetin (8,8′-diapocarotene-8,8′-dioic acid) – a natural carotenoid compound of gardenia fruits and saffron has potent antioxidant properties (Schmidt et al., 2007; Song et al., 2013; Yoshino et al., 2011). This yellow compound has been used as an important spice and natural food colorant in various parts of the world. Additionally, saffron and gardenia fruits have been used as traditional medicine and crocetin is one of the major active compounds of these herbal medicines. This unique carotenoid exerts antioxidant, anti-inflammatory, antiplatelet, antidepressant and anticancer effects (Hosseinzadeh et al., 2012; Nam et al., 2010; Schmidt et al., 2007). It was also demonstrated that crocetin effectively crosses the blood-brain barrier and reduces the risk of neurodegenerative diseases (Yoshino et al., 2011).

Therefore, the purpose of the present study was to unravel the mechanisms of CuONPs-induced cytotoxicity by studying cell viability, apoptosis, reactive oxygen species (ROS) and antioxidant/detoxification defense systems in hippocampal cells. Finally, we have also examined the effects of crocetin on CuONPs-induced neurotoxicity.

2. Materials and methods

2.1. Chemicals

The concentrations of CuONPs and crocetin used in these experiments were carefully selected according to literature data for *in vitro* experiments (Siddiqui et al., 2013; Song et al., 2013; Yoshino et al., 2014) and a preliminary concentration-response study (data not shown).

Crocetin (TRC, Canada, CAS: 27876-94-4) was dissolved in dimethylsulfoxide (DMSO, Sigma, USA). CuONPs (31 nm) were purchased from Alfa Aesar (US).

2.2. Characterization of NPs

2.2.1. Dynamic Light Scattering (DLS)

CuONPs size distributions and zeta potential were measured using a Zetasizer Nano ZS (Malvern Instruments, UK). Measurements were performed six times for concentration 50 μg/ml in a serum free (SF) culture medium in the presence or absence of crocetin (5 μM) at room temperature.

2.3. Cell culture

An immortalized mouse hippocampal cell line HT22 was a kind gift from Prof. Tillman Grune (Friedrich Schiller University, Germany). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Sigma Aldrich), 100 U/ml penicillin and 100 μg/ml streptomycin B at 37 °C under 5% CO_2. For all experiments, cells were grown to 80–90% confluence.

2.4. Treatments

CuONPs were sonicated using a sonicator bath at room temperature for 20 min to reduce nanoparticle agglomeration. CuONPs were then suspended in a SF cell culture medium and diluted to appropriate concentrations at the time of addition to the cells. HT22 cells were then incubated with CuONPs (1, 5, 10, 25, 50 and 80 μg/ml) for 6, 18 and 24 h. In another set of experiments, crocetin (5 μM) was added 1 h prior to the treatment with CuONPs (5, 10, 25 μg/ml) and the incubation was carried out for 24 h.

2.5. Measurement of cytotoxicity

Cell viability was measured using an MTT assay (PROMEGA, Poland). HT22 cells were seeded at a density of 10^4 cells/100 μl of cell culture medium into 96-well plates. The following day, HT22 cells were treated with CuONPs under SF conditions (1, 5, 10, 25, 50 and 80 μg/ml) at time points 6, 18 and 24 h. In another set of experiments different concentrations of crocetin (1, 5, 10 and 15 μM) was added 1 h prior to the treatment with two concentrations of CuONPs (25 and 50 μg/ml). In some experiments the effects of crocetin (1, 5, 10 and 15 μM) on HT22 cell viability were tested at increasing concentrations for 24 h (data not shown).

Mitochondrial activity assay as an index of cell growth and cell death was performed by adding an optimized dye solution: 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to culture wells (final concentration MTT was 33 μg/well). The MTT enters the cell and passes into the mitochondria where it is reduced to an insoluble and colored (dark purple) formazan product. When cells die, they rapidly lose the ability to convert the substrate to product. Absorbance was read at 570 nm (ASYS Hitech GmbH microplate reader, Biogenet). Results from the treatment groups were calculated as a percentage of control values (unexposed cells) according to the following equation:

$$\%\ \text{cytotoxity} = \frac{\text{experimental abs}_{570\text{nm}}\ \text{of exposed cells} - \text{backround experimental abs}_{570\text{nm}}}{\text{experimental abs}_{570\text{nm}}\ \text{of unexposed cells}} \cdot 100\%$$

Absorbance values were corrected for background (NPs blank used for each concentration).

2.6. Flow cytometry analysis of apoptosis

The Annexin V-FITC apoptosis detection kit (BD Pharmingen) was used to measure apoptosis according to the manufacturer's instructions. HT22 cells (10^6) were seeded into 25-cm^2 tissue culture flasks. 48 h after seeding, cells were treated with CuONPs (1, 5, 10, 25, 50 and 80 μg/ml) for 24 h. In other experiments, crocetin (5 μM) was added 1 h prior to the treatment with CuONPs. The cells were collected and washed twice with cold phosphate-buffered saline (PBS) (NaCl 0.138 M; KCl 0.0027 M; pH 7.4). 5 μL of Annexin V and 2.5 μL of propidium iodide (PI) were added to the cells after being resuspended in binding buffer (50 mM HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 700 mM NaCl, 12.5 mM $CaCl_2$, pH 7.4). The cells were gently shaken and incubated in the dark for 15 min at room temperature. Following incubation they were analyzed by flow cytometry (BD FACSCalibur; US) within 1 h as previously described (Inkielewicz-Stepniak et al., 2014; McCarthy et al., 2012). Twenty thousand specific events were analyzed and data were expressed as a percentage of total population.

2.7. Detection of ROS

ROS generation was measured using flow cytometry. Briefly, HT22 cells were seeded into 12-well plates at a concentration of 10^6 cells/well and 24 h after seeding, cells were treated as indicated in Section 2.4, then collected and incubated with 10 μM of 2,7-dichlorofluorescein diacetate (DCF-DA) for 30 min at 37 °C in a humidified atmosphere containing 5% CO_2/95% air to measure ROS-mediated oxidation of DCF-DA to the fluorescent compound 2,7-dichlorofluorescin (DCF). Fluorescence of oxidized DCF was measured at an excitation wavelength of 480 nm and an emission wavelength of 525 nm using a flow cytometer (BD FACSCalibur; US). Data were presented as a percentage of control.

2.8. Reduced glutathione levels (GSH)

Reduced glutathione (GSH) levels were measured using a commercially available kit (Sigma–Aldrich, Poland). Briefly, cells were seeded into 6-well plates at a concentration of 3×10^6 cells/well and 24 h after seeding, cells were treated as specified in Section 2.4. The cells were collected, washed with PBS and centrifuged at $600 \times g$ at 4 °C. Following centrifugation 5% 5-sulfosalicylic acid was added to the pellet and vortexed. The resultant suspension was homogenized by freezing and thawing twice (using liquid nitrogen to freeze and a 37 °C bath to thaw) and kept for 5 min at 2–8 °C. Next, the homogenate was centrifuged at $10{,}000 \times g$ for 10 min at 4 °C. The supernatant was collected, measurements and calculations were performed according to supplier's protocol. Absorbance was read at 412 nm using a plate reader (ASYS Hitech GmbH, Biogenet). Data were expressed as a percentage of control.

2.9. Glutathione S-transferase (GST)

Glutathione S-transferase (GST) activity was measured using a commercially available kit (Abcam, Poland). Briefly, the cells were seeded into 6-well plates at a concentration of 3×10^6 cells/well 24 h after seeding, cells were treated as indicated in Section 2.4, then cells were collected and centrifuged. The pellet was sonicated in GST assay buffer (100 mM potassium phosphate, pH 6.5, containing 0.1% Triton X-100) and then centrifuged at $10{,}000 \times g$ for 15 min at 4 °C. The supernatant was collected and changes in absorbance (ΔA340 nm) per minute were read. Calculations were performed according to supplier's protocol, and data were expressed as a percentage of control.

2.10. Measurement of SOD and GPx activity

Superoxide dismutase (SOD) activity was measured using a commercially available kit (OxiSelect). Briefly, the cells were seeded into 6-well plates at a concentration of 3×10^6 cells/well 24 h after seeding, cells were treated as indicated in Section 2.4. The cells were collected, washed once with 3 ml ice-cold PBS, sonicated in 1 ml of a cold lysis buffer (10 mM Tris, pH 7.5, 150 mM NaCl, 0.1 mM EDTA) and subjected to centrifugation at $12{,}000 \times g$ for 10 min at 4 °C. The resultant supernatant was then collected and assayed. All measurements were performed according to supplier's recommendations, and absorbance was read at 490 nm on a plate reader (ASYS Hitech GmbH, Biogenet). Data were expressed as a percentage of control.

Glutathione peroxidase (GPx) activity was measured as described by Paglia and Valentine (1967) with some modifications. Briefly, cells from culture plates were pooled into 1 ml of 0.1 M phosphate buffer, pH 7.4 and sonicated. The homogenate was centrifuged for 25 min at 3000*g* at 4 °C and the supernatant was collected for the measurements of GPx activity. The peroxide used in this study was t-butyl hydroperoxide (0.32 mM), the concentration of GSH was 1.88 mM, and the pH of the assay was 7.6. The unit of GPx activity was defined as 1 mU = 1 nmole NADPH oxidized per min. Data were expressed as a percentage of control.

2.11. Real time quantitative polymerase chain reaction of Bax and Bcl-2, SOD and GPx

HT22 cells were cultured in T-25 cm^2 flasks in complete medium until confirmed to be 80–90% confluent. Cells were treated as indicated in Section 2.4, then medium was aspirated, and the cells were washed with phosphate-buffer saline. DNA-free RNA was isolated using the Ambion® RiboPure™ kit (Huntingdon, UK) according to the supplier's protocol. RNA quantity was assessed spectrophotometrically (Nanodrop ND-1000, Labtech International, Ringmer, Sussex, UK). Thereafter, the RNA in each sample was reverse-transcribed by the Applied Biosystems High Capacity cDNA Reverse Transcription kit (Woolston, UK). Real-time PCR was run, with pre-designed Applied Biosystems TaqMan® Gene Expression Assays for Bax, Bcl-2, SOD-1, SOD-2, GPx-1, GPx-4, and 18S ribosomal ribonucleic acid along with Applied Biosystems TaqMan® Universal PCR Master Mix. Reverse-transcription and real-time PCR reaction were performed using the Life Technologies StepOnePlus Real-Time PCR System. The expression of each gene within each sample was normalized against 18S rRNA (internal control) expression and expressed relative to the control sample using the formula $2^{-(\Delta\Delta Ct)}$, in which ΔΔCt = (Ct mRNA-Ct 18S rRNA)sample−(Ct mRNA-Ct 18S rRNA)control sample (Inkielewicz-Stepniak et al., 2014; McCarthy et al., 2012).

2.12. Statistical analysis

The experimental results were expressed as mean ± SD for triplicate determination of 3-4 separate experiments. The results were analyzed using one-way ANOVA and Tukey's post hoc test and p value < 0.05 was considered statistically significant.

3. Results

We used commercially available CuONPs sized 31 nm as specified by the supplier. The size distribution for CuONPs measured in culture media ranged from 27 to 39 nm with an average NPs size of 33 nm (Fig. 1), and was not significantly changed in the presence of crocetin.

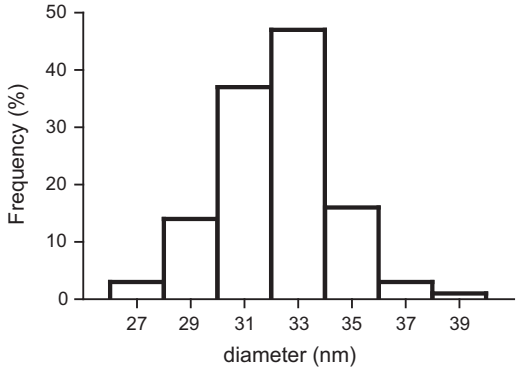

Fig. 1. The histogram shows CuONPs size distribution. The size for CuONPs ranged from 27 to 39 nm with an average particle size of 33 nm. Size distributions were obtained from 6 independent DLS measurements.

Thus, crocetin treatment does not appear to affect aggregation of CuONPs.

Indeed, zeta potential for CuONPs was: −28.61 ± 2.36 mV v/s −33.43 ± 1.25 mV ($p > 0.05$, $n = 6$) in the presence or absence of crocetin, respectively, thus showing good stability of the NPs (Jiang et al., 2009).

First we evaluated the effects of CuONPs on HT22 cells viability using MTT assay. Incubation with 1, 5, 10, 25, 50 and 80 μg/ml CuONPs for 6, 18 and 24 h resulted in a concentration- and time-dependent decrease in MTT reduction/cell viability (Fig. 2). The LC_{50} for MTT after 24 h of incubation was 25.9 μg/ml.

Based on these results CuONPs at submaximal concentrations 5, 10 and 25 μg/ml and incubation time of 24 h were selected to study molecular mechanisms of neurotoxicity induced by NPs with respect to oxidative stress (Figs. 5–10).

Next, we found that CuONPs (25 and 50 μg/ml)-induced loss of cell viability was attenuated in a concentration-dependent manner by crocetin and this effect was already maximal at 5 μM (Fig. 3).

Therefore, 5 μM crocetin was used for further experiments. Crocetin was determined to be non-toxic for HT22 cells at 1, 5, 10 and 15 μM after 24 h of incubation (data not shown).

Furthermore, we studied whether decreased cellular viability could be linked to apoptosis. We found that exposure to 5, 10, 25, 50 and 80 μg/ml CuONPs did increase apoptosis in HT22 cells (Fig. 4).

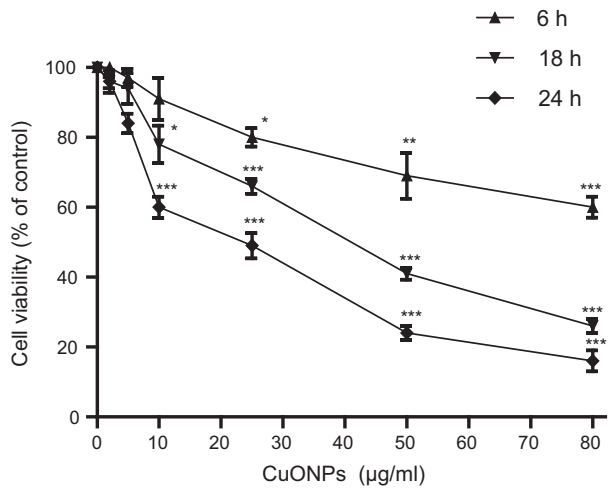

Fig. 2. CuONPs-induced decrease in cell viability. The 6, 18 and 24 h treatments of cells with CuONPs decreased HT22 cell viability. Data are mean ± SD of 3–4 separate determinations. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.01$ compared with control.

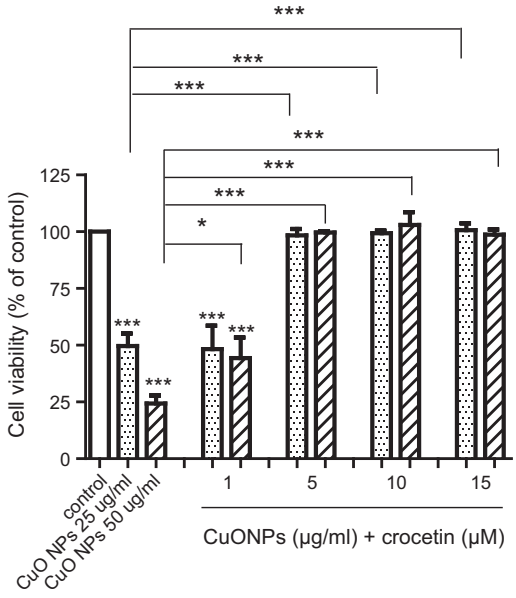

Fig. 3. Crocetin protects hippocampal cells from CuONPs (25 and 50 μg/ml)-induced cytotoxicity following incubation for 24 h. Data are mean ± SD of 3–4 separate determinations. $^{*}p < 0.05$; $^{***}p < 0.001$ treatments v/s control or as indicated.

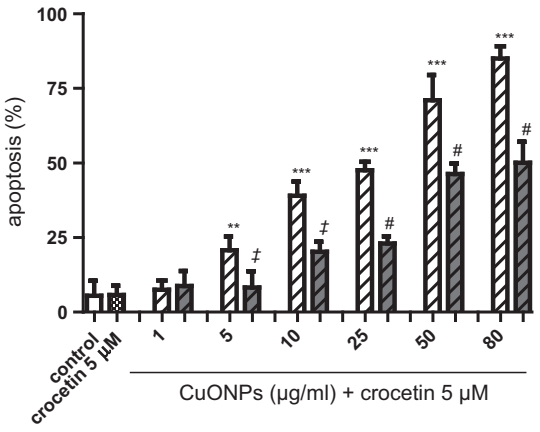

Fig. 4. CuONPs-induced apoptosis in HT22 cells (24 h incubation) and protective effects of crocetin (5 μM). Data are mean ± SD of 3–4 separate determinations. $^{**}p < 0.01$; $^{***}p < 0.001$ treatments v/s control. $^{‡}p < 0.01$; $^{\#}p < 0.001$ CuONPs-treated cells v/s CuONPs-treated cells in the presence of crocetin.

Increased apoptosis was associated with up-regulation of pro-apoptotic Bax (Fig. 5a) and down-regulation of anti-apoptotic Bcl-2 genes (Fig. 5b) in cells treated with 10 and 25 μg/ml CuONPs in a concentration-dependent manner. Interestingly, we found that crocetin at 5 μM attenuated CuONPs-induced apoptosis in HT22 cells (Fig. 4) with the corresponding modulation of Bax and Bcl-2 mRNA (Fig. 5a and b).

To explore mechanisms responsible for CuONPs-induced cytotoxicity, we investigated whether exposure to CuONPs of hippocampal cells could be associated with increased generation of ROS. We observed that CuONPs (10 and 25 μg/ml) induced generation of ROS in these cells, an effect partly attenuated by 5 μM crocetin (Fig. 6).

Therefore, in further study we measured CuONPs effects on the antioxidant/detoxification system and the impact of crocetin on this action of nanoparticles. We found that CuONPs (10 and 25 μg/ml) decreased levels of GSH and preincubation with 5 μM crocetin prevented GSH exhaustion (Fig. 7).

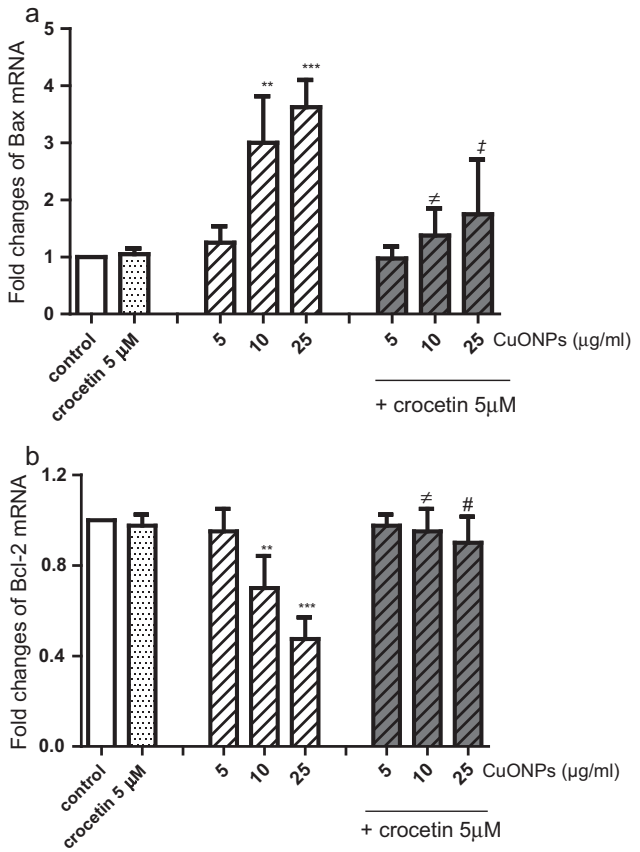

Fig. 5. The exposure of HT22 cells to CuONPs for 24 h increases Bax mRNA levels (a) and decreases Bcl-2 mRNA levels. Crocetin (5 μM) reduces CuONPs-induced Bax mRNA up-regulation (a) and CuONPs-induced Bcl-2 mRNA down-regulation (b) in HT22. Data are as mean ± SD of 3–4 separate determinations. $^{**}p < 0.001$; $^{***}p < 0.001$; treatments v/s control. $^{\neq}p < 0.05$; $^{\ddagger}p < 0.01$; $^{\#}p < 0.001$ CuONPs-treated cells v/s CuONPs-treated cells in the presence of crocetin.

We also found that CuONPs (25 μg/ml)-induced decrease in GSH levels was accompanied by a significant decrease in GST activity (Fig. 8).

The decrease in GST activity was reversed by preincubation of HT22 cells with 5 μM crocetin (Fig. 8).

Our study demonstrates that CuONPs impaired the non-enzymatic and enzymatic antioxidant defense systems in HT22 cell line.

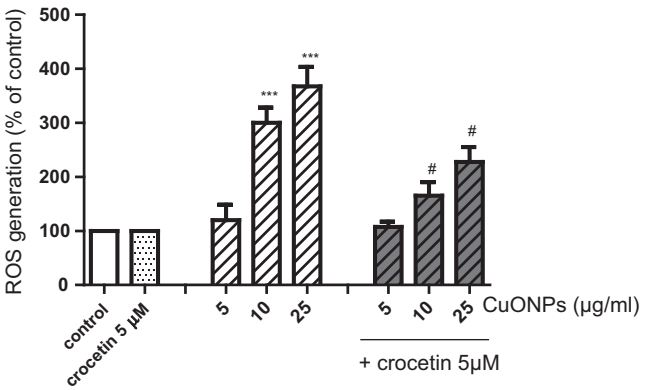

Fig. 6. CuONPs-induced increase in generation of ROS in HT22 cells after incubation for 24 h is reduced by crocetin. Crocetin (5 μM) attenuates CuONPs-induced ROS production in HT22. Data are means ± SD of 3–4 separate determinations. $^{***}p < 0.001$; treatments v/s control. $^{\#}p < 0.001$ CuONPs-treated cells v/s CuONPs-treated cells in the presence of crocetin.

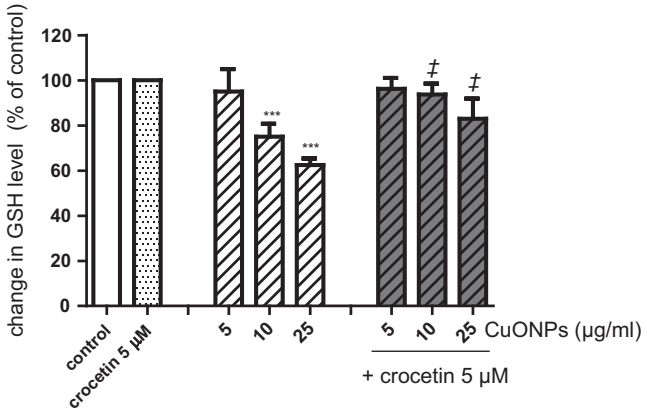

Fig. 7. A decrease in GSH levels following 24 h exposure of cells to CuONPs is attenuated by crocetin (5 μM). Data are mean ± SD of 3–4 separate determinations. $^{***}p < 0.001$; treatments v/s control. $^{\ddagger}p < 0.01$ CuONPs-treated cells v/s CuONPs-treated cells in the presence of crocetin.

We also noticed a decrease in the activity of antioxidant enzymes directly scavenging ROS such as GPx and SOD (Fig. 9a and b).

It was also of interest to investigate whether CuONPs-induced decrease in GPx and SOD activity could be associated with dysregulation of GPx and SOD mRNAs in HT22 cells. Interestingly, we found down-regulation of GPx1 and GPx4, SOD1 and SOD2 genes (Fig. 10a and b).

Pretreatment of HT22 cells with 5 μM crocetin reduced CuONPs-induced effects on the expression and activity of these enzymes (Figs. 9 and 10).

4. Discussion

This is a pioneer study examining the effects of CuONPs on the hippocampal cells focusing specifically on apoptosis, generation of reactive oxygen species (ROS), as well as antioxidant defense and detoxification systems. Additionally, we have found that crocetin exerts beneficial effects on hippocampal cells subjected to the CuONPs-induced cellular damage.

We have performed our study using mouse hippocampus-derived neuronal cell line HT22. Due to the lack of ionotropic glutamate receptors these cells are a sensitive model for monitoring cellular responses to oxidative stress (Sagara and Schubert, 1998). Therefore, HT22 cells are commonly used to study molecular mechanisms involved in neurotoxicity of xenobiotics as well as

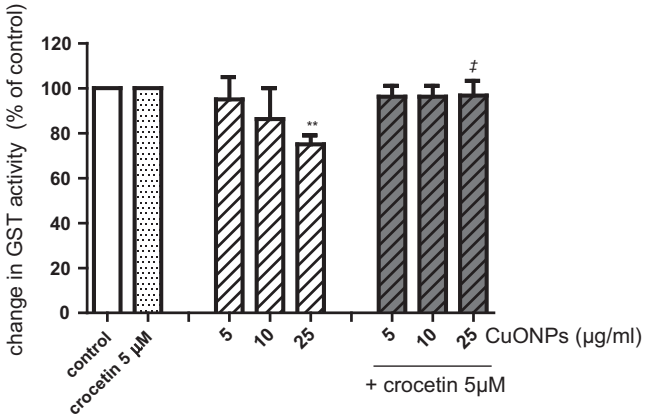

Fig. 8. A decrease in GST activity in cells exposed for 24 h to CuONPs is prevented by crocetin (5 μM). Data are mean ± SD of 3–4 separate determinations. $^{**}p < 0.01$; treatments v/s control. $^{\ddagger}p < 0.01$ CuONPs-treated cells v/s CuONPs-treated cells in the presence of crocetin.

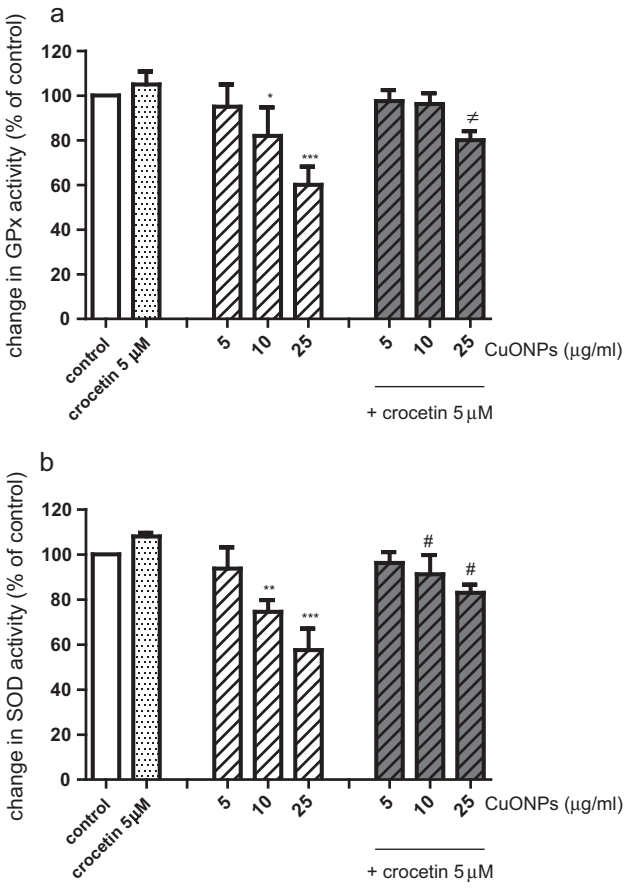

Fig. 9. Decreased GPx (a) and SOD (b) activity in cells exposed to CuONPs for 24 h. Crocetin (5 μM) treatment attenuates the effects of CuONPs. Data are mean ± SD of 3–4 separate determinations. $^{**}p < 0.01$; treatments v/s control. $^{\neq}p < 0.05$; $^{\#}p < 0.001$ CuONPs-treated cells v/s CuONPs-treated cells in the presence of crocetin.

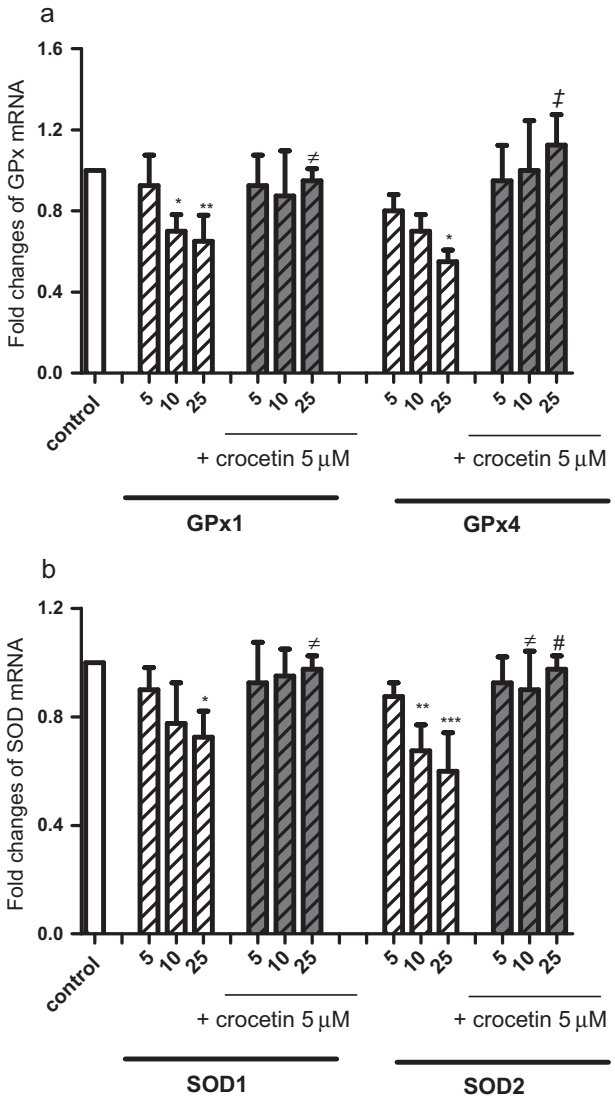

Fig. 10. Decreased GPx-1, GPx-4 mRNA (a) and SOD-1 and SOD-2 mRNA (b) levels in HT22 cells following 24 h exposure to CuONPs and protective effect of crocetin. Crocetin (5 μM) inhibits CuONPs-induced GPx mRNA (a) and SOD mRNA down-regulation (b). Data are mean ± SD of 3–4 separate determinations. $^{*}p < 0.05$; $^{**}p < 0.001$; $^{***}p < 0.001$; treatments v/s control. $^{\neq}p < 0.05$; $^{\ddagger}p < 0.01$; $^{\#}p < 0.001$ CuONPs-treated cells v/s CuO NPs-treated cells in the presence of crocetin.

a screening tool for neuroprotective compounds (Albrecht et al., 2010; Lewerenz et al., 2003; van Leyen et al., 2008).

Our results indicate that the exposure of HT22 cells to CuONPs significantly decreases cellular viability and this effect is attenuated by crocetin. We observed that crocetin at 1 μM had no effect on cell death induced by 25 μg/ml CuONPs, but attenuated cytotoxic effect of 50 μg/ml CuONPs. This maybe due to the fact that the cellular bioavailability of carotenoids such as crocetin is low and the exposure of cells to higher concentrations of CuONPs decreases the integrity of cell membrane, thus increasing the availability of crocetin. In keeping with our findings, Yoshino et al. (2014) found that crocetin at 1–10 μM protected HT22 cells against beta-amyloid ($A\beta_{1\text{-}42}$)-induced neuronal cell death. After oral administration of crocetin in rat (100 mg/kg bw) it reached 0.14 mM and 40 μM levels in plasma and in the brain, respectively (Yoshino et al., 2011). It is noteworthy that in most animal models pharmacological effects of crocetin were observed after administration of relatively high doses of the carotenoid (Yamauchi et al., 2011; Ohno et al., 2012). For example, with saffron extract treatment these amounts were 250 and 400 mg/kg/day (Hosseinzadeh et al., 2012; Fernández-Sánchez et al., 2012). However, the oral administration of saffron extract at doses ranging from 0.1 to 5 g/kg was non-toxic in mice (Abdullaev, 2002). In healthy volunteers taking 400 mg of saffron daily for 7 consecutive days, statistically significant (though clinically irrelevant) increase in serum creatine, sodium, and urea nitrogen levels was observed (Modaghegh et al., 2008). A clinical trial has shown that saffron taken at a dose of 30 mg/per day may be of therapeutic benefit in the treatment of mild to moderate depression (Akhondzadeh et al., 2004). In our cell culture model, crocetin showed a neuroprotective effect at 5 μM. Thus, our results in conjunction with other studies provide evidence that crocetin and/or saffron extract do not result in adverse effects and toxicity in a wide range of doses/concentrations and can be useful in preventing NPs-induced brain cell death. However, further studies will be required to examine the effects of crocetin on NPs-induced brain damage *in vivo*.

Interestingly, we have observed that CuONPs at a relatively low concentration of 5 μg/ml increase apoptosis. This was not associated with a significant impact on the antioxidant/detoxification systems suggesting that increased oxidizing stress could not account for this effect. In contrast, exposure of hippocampal cells to higher concentrations of CuONPs led to the impairment of the antioxidant/detoxification system and consequently increased apoptosis.

Apoptosis was accompanied by a significant dysregulation in Bcl-2 and Bax mRNAs. Bcl-2 is known to inhibit apoptosis induced by a variety of physiologic and pathologic stimuli (Korsmeyer, 1999; Oltvai et al., 1993). Bax has a pro-apoptotic effect and it also counteracts the anti-apoptotic effects of Bcl-2. Apoptosis has been implicated as a major mechanism of cell death caused by NPs-induced oxidative stress (Eom and Choi, 2010; Hsin et al., 2008). Siddiqui et al. (2013) found that 22 nm CuONPs increased apoptosis in HepG2 cell line – a classical hepatic model used to test xenobiotics cytotoxicity. A significant increase in caspase-3 activity, indicating apoptosis was observed during incubation of CuONPs with human skin keratinocytes cells HaCaT (Alarifi et al., 2013).

Our study clearly indicates that crocetin attenuates CuONPs-induced apoptosis in hippocampal cells by regulation of Bcl-2 and Bax. We have found that 5 μM crocetin attenuated the effect of CuONPs on apoptosis by about 50%, whereas its effects on cell viability amounted to the complete protection. It is worth to emphasize that MTT assay reflects only the changes in mitochondrial function, but is not indicative of the manner or stages of cell death (Mosmann, 1983). Thus, reductions in MTT test may result from cell death and/or inhibition of proliferation, whereas apoptosis is only one of types of cell death (Kerr et al., 1972). Moreover, early stage apoptotic cells may be recognized as normal cells by the MTT method.

In keeping with our results, Yamauchi et al. (2011) found that crocetin exerted protective effects against retinal cells apoptosis *in vitro* and *in vivo*, suggesting that the mechanism of action may involve inhibition of caspase-3 and -9 activities after retinal damage. Furthermore, crocetin administration to male Sprague-Dawley rats during resuscitation from shock significantly improved postshock survival rate and reduced apoptosis (Yang et al., 2011). Evidence of reduced hepatic apoptosis was provided by reductions in levels of postshock cytosolic cytochrome c, reduced levels of activated caspase 3, and increases in the levels of protective Bcl-2 protein. Finally, Xu et al. (2007) suggested that crocin, which is metabolized to crocetin could enhance the expression ratio of Bcl-2 to Bax, thus changing the cellular redox state and increasing the intrinsic cytosolic and mitochondrial tolerance against apoptotic triggers.

As mechanisms involved in CuONPs-induced cell death and apoptosis may involve oxidative stress we have evaluated generation of ROS. ROS participate in a several physiological processes, whereas their excessive accumulation causes irreversible oxidative damage to cellular components and ultimately leads to cell death (Birben et al., 2012). As expected, in this study crocetin resulted in a significant decrease of CuONPs-induced generation of ROS. Indeed, Tseng et al. (1995) reported the direct scavenging activity of crocetin against $O_2^{\cdot-}$ while Yoshino et al. (2011) found scavenging effects of crocetin on $HO^{\cdot}$.

Over millions of years of evolution, cells have developed very complex enzymatic and non-enzymatic antioxidant systems, which work synergistically with each other to protect against overproduction of ROS. We decided to study the impact of CuONPs on endogenous antioxidant defense system in HT22 cells: GSH levels and the activity of the most efficient enzymatic antioxidants – SOD and GPx. GSH (gamma-glutamyl-cysteinyl-glycine) is the most abundant low-molecular thiol, and GSH/glutathione disulfide is the major redox couple in mammalian cells (Bains and Shaw, 1997; Lewerenz et al., 2003). It participates in regulating multiple physiological functions, acting as a reducing agent, free-radical scavenger, and is involved in the metabolism and detoxification of xenobiotics.

Besides GSH, glutathione S-transferase (GST) is responsible for the antioxidant and detoxification efficacy of cellular defense. It has been indicated that GST inhibition could contribute to the neuronal cell damage and death induced by different xenobiotics/toxicants (Ishihara et al., 2012). We have observed that preincubation of hippocampal cells with crocetin attenuates CuONPs-induced decrease in GST activity. Similarly, Wang et al. (1991) suggested that the protective effect of crocetin on the aflatoxin B1-cytotoxicity in C3H10T1/2 fibroblast cells might be due to the elevation in GSH content and GST activity.

Glutathione peroxidase (GPx) protects cells from oxidative damage by catalyzing the reduction of both organic and hydrogen peroxides, utilizing glutathione as a reducing agent (Birben et al., 2012). Among seven isomers of GPx in HT22 cells are highly expressed cytosolic and phospholipid hydroperoxide GPx (GPx1 and GPx4, respectively) (Panee et al., 2007). Superoxide dismutases (SOD) are enzymes that catalyze the dismutation of superoxide anion into oxygen and hydrogen peroxide. In our study we investigated influence of CuONPs on SOD1 and SOD2. SOD1 (CuZn-SOD) is a copper and zinc-containing homodimer that is found in cytosol. SOD2 (Mn-SOD) is located in the mitochondrial matrix and exists as a tetramer (Zelko et al., 2002). Our study demonstrates that CuONPs impaired the enzymatic antioxidant defense system in HT22 cell line. We observed a decrease in the activity of antioxidant enzymes directly scavenging ROS: GPx and SOD. Additionally, we found down-regulation of GPx1 and GPx4, SOD1 and SOD2 genes. Oxidative stress occurs when the capacity of antioxidant defense systems are exceeded by ROS generation and resulting in oxidative damage of cellular components: lipids, nucleic acid, and proteins (Birben et al., 2012).

It is known, that the capacity of antioxidant activity of crocetin may involve the activation of the endogenous antioxidant enzymatic activities such as superoxide dismutase (SOD) and glutathione peroxidase (GPx) in various cells and tissues (Shen and Qian, 2006; Yoshino et al., 2011). Ahmad et al. (2005) demonstrated that crocetin might be an effective antioxidant to counter oxidative stress in a hemi-Parkinsonian rat model. Interestingly, in our study we have found that crocetin also counteracts the down-regulation of GPx and SOD mRNA expression. As far as we are concerned, this is the first report to show the link between CuONPs-induced neurotoxicity and impairment of antioxidant defense system, at the transcriptional and enzymatic activity levels in hippocampal cells.

5. Conclusion

Our study provides valuable insights into the mechanisms of CuONPs-dependent neurotoxicity in hippocampal cells. CuONPs were found to induce cytotoxicity in HT22 cells in a concentration- and time-dependent manner. The observed cytotoxicity was associated with induction of apoptosis and dysregulation of Bax and Bcl-2 protein levels. The mechanisms of cytotoxicity may stem from increased ROS production and the impairment of cellular antioxidant/detoxification defense system. Moreover, our study highlights, for the first time, the potential use of crocetin for the prevention of CuONPs-induced damage in hippocampal cells.

Conflict of Interest

The authors declare no conflicts of interest.

Transparency Document

The Transparency document associated with this article can be found in the online version.

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