Original Article

Protective effect of sulphoraphane against oxidative stress mediated toxicity induced by CuO nanoparticles in mouse embryonic fibroblasts BALB 3T3

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ABSTRACT — Despite the great interest in nanoparticles (NPs) safety, no comprehensive test paradigm has been developed. Oxidative stress has been implicated as an explanation behind the toxicity of NPs. It is reported that sulphoraphane (SFN) present in cruciferous vegetables like cauliflower and broccoli has potential to protect cells from oxidative damage and inflammation. However, protective role of SFN in nanotoxicity is not explored. We investigated the protective effect of SFN against the toxic response of copper oxide (CuO) NPs in mouse embryonic fibroblasts (BALB 3T3). Results showed that CuO NPs induced dose-dependent (5-15 µg/ml) cytotoxicity in BALB 3T3 cells demonstrated by MTT and lactate dehydrogenase (LDH) assays. CuO NPs were also found to induce oxidative stress in dose-dependent manner indicated by induction of reactive oxygen species (ROS) and lipid peroxidation (LPO) and depletion of glutathione and glutathione reductase. Co-treatment of BALB 3T3 cells with SFN (6 µM) significantly attenuated the cytotoxicity, ROS generation and oxidative stress caused by CuO NPs. Moreover, we found that co-treatment of another antioxidant N-acetyl-cysteine (NAC) (2 mM) also significantly attenuated glutathione depletion caused by CuO NPs but protection from the loss of cell viability due to CuO NPs exposure was not significant. We believe this is the first report showing that SFN significantly protected the BALB 3T3 cells from CuO NPs toxicity, which is mediated through generation of oxidants and depletion of antioxidants. Consequently, protective mechanism of SFN against CuO NPs toxicity was different from NAC that should be further investigated.

Key words: CuO Nanoparticles, Cytotoxicity, ROS, Oxidative stress, Sulforaphane, Protection

INTRODUCTION

Nanotechnology broadly refers to the manipulation and manufacture of materials and devices in the size range 1-100 nm. Nanoparticles (NPs) are used in a wide-range of products including food, clothing, electronic, cosmetics, medicine and agriculture (Karthikeyan *et al.*, 2008). Metal oxide NPs such as copper oxide (CuO) NPs are of great interest in nanotechnology because they offer intriguing new properties for applications in current and future technologies (Song *et al.*, 2008; Karthikeyan *et al.*, 2008; Liu *et al.*, 2008; Shende *et al.*, 2008). These broad applications, however, increase the environmental and

human exposure and thus the potential risk related to their hazardous effects.

There are some important studies reporting the toxicity of CuO NPs (Fahmy and Cormier, 2009; Kahru and Dubourguier, 2010). Among the possible mechanisms of nanoparticle toxicity, reactive oxygen species (ROS) generation and oxidative stress remain an important area of consideration (Nel *et al.*, 2006; Ahamed *et al.*, 2010a, 2010b, 2010c, 2011a, 2011b and 2011c; Akhtar *et al.*, 2010a, 2010b). Our previous publication also demonstrated the oxidative stress mediated genotoxicity of CuO NPs in human lung epithelial (A549) cells (Ahamed *et al.*, 2010c). Consequently, investigation into the mechanisms

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of ROS generation and their biological consequences could serve as a paradigm for potential safety against the toxicity of NPs.

Chemoprevention with natural agents offers an attractive approach to boost the body's defenses toward the environmental and endogenous insults (Talalay and Fahey, 2001; Surh, 2003). Sulphoraphane [1-isothiocyanato-4-(methylsulfinyl)butane], a naturally occurring isothiocyanate derived from cruciferous vegetables is a highly potent inducer of phase 2 cytoprotective enzymes and can protect against electrophiles including carcinogens, oxidative damage and inflammation (Juge *et al.*, 2007). A broad series of cellular, animal and human studies have demonstrated the protective potential of SFN against cancer, retinal disease, skin damage, UV irradiation (Cornblatt *et al.*, 2007; Dinkova-Kostova *et al.*, 2007; Talalay *et al.*, 2007; Kong *et al.*, 2009). However, protective effect of SFN against the toxicity of NPs is not fully explored.

In this study, we investigated the protective role of SFN against CuO NPs induced cytotoxicity (cell viability and membrane damage) in mouse embryonic fibroblasts (BALB 3T3). To explore the possible mechanism of action through which SFN protect the cells from cytotoxicity we have also evaluated the oxidative stress biomarkers such as ROS, lipid peroxidation (LPO), glutathione (GSH) and glutathione reductase (GR) in BALB 3T3 cells against CuO NPs insult. The selection of BALB 3T3 cells was based on the fact that the present research could be advanced to *in vivo* models like BALB 3T3 mice. The protective efficiency of SFN against CuO NPs induced toxicity was also compared with another antioxidant N-acetyl-cysteine (NAC).

MATERIALS AND METHODS

Synthesis of CuO NPs

CuO NPs are synthesized by aqueous precipitation method using copper acetate [$(\text{Cu}(\text{CH}_3\text{COO})_2 \cdot \text{H}_2\text{O})$] as a precursor and sodium hydroxide (NaOH) as a reducing agent as described elsewhere (Zhu *et al.*, 2011).

CuO NPs characterization

The crystalline nature of CuO NPs was carried out by taking X-ray diffraction (XRD) pattern at room temperature with the help of PANalytical X'Pert X-ray diffractometer equipped with a Ni filtered using Cu K_α ($\lambda = 1.54056 \text{ \AA}$) radiations as X-ray source. Morphology and size of CuO NPs was examined by field emission transmission electron microscopy (FETEM, JEM-2100F, JEOL Inc., Tokyo, Japan) at an accelerating voltage of

200 kV. Briefly, suspension of CuO NPs was sonicated using a sonicator bath at room temperature for 15 min at 40 W to form a homogeneous suspension. Further, a drop of aqueous NPs was placed onto TEM grid, air-dried and taken the image. Energy dispersive X-ray spectroscopy (EDX) was utilized used to see the elemental composition (purity) of synthesized CuO NPs.

Dynamic light scattering (DLS) (Nano-ZetaSizer-HT, Malvern Instrument) was used to determine the hydrodynamic size the CuO NPs in cell culture medium. NPs were suspended in aqueous media, sonicated at room temperature for 10 min at 40 W to form homogeneous mixture and DLS was performed.

Cell culture and exposure with CuO NPs

Mouse embryonic fibroblast (BALB 3T3) cells were obtained from National Centre for Science (NCCS), Pune, India. BALB 3T3 cells were used between passages 10 and 20. Cells were cultured in DMEM/F-12 medium supplemented with 10% FBS and 100 U/ml penicillin-streptomycin at 5% CO_2 and 37°C. At 85% confluence, cells were harvested using 0.25% trypsin and were sub-cultured into 75 cm^2 flasks, 6-well plates or 96-well plates according to selection of experiments. Cells were allowed to attach the surface for 24 hr prior to treatment. CuO NPs were suspended in cell culture medium and diluted to appropriate concentrations. The dilutions of CuO NPs were then sonicated using a sonicator bath at room temperature for 10 min at 40 W to avoid NPs agglomeration prior to administration to the cells.

Treatment of SFN and NAC

Treatment of SFN (6 μM) to cells was performed before 30 min exposure of CuO NPs. In an exposure regime, we also used N-acetyl-cysteine (NAC) (2 mM) to compare with the protective effect with SFN against CuO NPs induced toxicity. The concentrations of SFN and NAC used in this study were according to previous publications (Gao *et al.*, 2001; Dinkova-Kostova *et al.*, 2007; Akhtar *et al.*, 2010a).

Cell viability assay

Viability of BALB 3T3 cells was determined using MTT assay as described by Mosmann (1983) and later modified by Hansen *et al.* (1989). Briefly, 1×10^4 cells/well were seeded in 96-well plates and exposed with CuO NPs at the concentrations of 0, 5, 10 and 15 $\mu\text{g/ml}$ for 48 hr. At the end of exposure, culture media was removed from each well and replaced with new medium containing MTT solution (0.5 mg/ml) and incubated for 3 hr at 37°C to get a purple colored formazan product. The

resulting formazan product was dissolved in acidified isopropanol. Further, 96-well plate was centrifuged at 2,500 rpm for 5 min to settle down the remaining NPs. Then, a 100 μ l supernatant was transferred to other fresh wells of 96-well plate and absorbance was measured at 570 nm by a microplate reader (Omega Fluostar, Germany).

Lactate dehydrogenase assay

Lactate dehydrogenase (LDH) is an enzyme widely present in cytosol that converts lactate to pyruvate. When plasma membrane integrity is disrupted, LDH leaks into culture media and its extracellular level is elevated. LDH assay was done with the method described elsewhere (Welder *et al.*, 1991). BALB 3T3 cells were treated with CuO NPs at the concentrations of 0, 5, 10 and 15 μ g/ml for 48 hr. After exposure completed, culture media was collected and centrifuged at 3,000 rpm for 10 min to get supernatant. LDH leakage was measured in 3.0 ml of reaction mixture with 100 μ l supernatant, 100 μ l of Na-pyruvate (2.5 mg/ml), 100 μ l of NADH (2.5 mg/ml) and phosphate buffer. The rate of NADH oxidation was determined following the decrease in absorbance at 340 nm for 3 min at 30 sec interval at 25°C using spectrophotometer (Thermo-Spectronic, Genesys 10). The amount of LDH released is expressed as LDH activity (IU/l) in culture media. To minimize the interference in absorbance caused by the turbidity occurring due to CuO NPs, the 96-well plates were centrifuged as described above.

Intracellular ROS measurement

Intracellular production of ROS was measured using 2,7-dichlorofluorescein diacetate (DCFH-DA) probe (Wang and Joseph, 1999). The DCFH-DA passively enters the cell where it reacts with ROS to form the highly fluorescent compound dichlorofluorescein (DCF). In brief, 10 mM DCFH-DA stock solution was diluted in culture medium without serum to yield a 100 μ M working solution. Cells were treated with CuO NPs at the concentrations of 0, 5, 10 and 15 μ g/ml for 48 hr. At the end of exposure, cells were washed twice with HBSS. Then cells were incubated in 1 ml working solution of DCFH-DA at 37 °C for 30 min. Cells were lysed in alkaline solution and centrifuged at 3,000 rpm for 10 min. A 200 μ l supernatant was transferred to 96-well plate and fluorescence was measured using at 485 nm excitation and 520 nm emission using a microplate reader (Omega Fluostar). The values were expressed as percent of fluorescence intensity relative to control wells.

Measurement of lipid peroxidation

The extent of membrane LPO was estimated by measuring the formation of malondialdehyde (MDA) using the method of Ohkawa *et al.* (1979). MDA is an end product of the membrane LPO of polyunsaturated fatty acids. In brief, cells were washed with physiological buffer. A 200 μ l aliquot of cell suspension was subsequently mixed with 800 μ l of LPO assay cocktail [0.4% (w/v) thiobarbituric acid, 0.5% (w/v) and sodium dodecyl sulphate, 5% (v/v) acetic acid, pH 3.5] and incubated for 60 min at 95°C. The sample was cooled using tap water and centrifuged at 2,500 rpm for 5 min. The absorbance of supernatant was read at 532 nm against the MDA standard curve. LPO was expressed as nmol MDA/mg protein.

Assay of glutathione reductase activity

After CuO NPs exposure, cells were washed twice in cold PBS and lysed in distilled water containing 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) (0.1%), Triton 100 X (0.05%) and sucrose (0.2%) by four cycles of freeze-thaw and centrifuged at 12,000 rpm for 10 min at 4°C. The supernatant (i.e. cell lysate) was used to measure the activity of GR. The GR activity was determined by following the oxidation of reduced nicotinamide adenine dinucleotide (NADPH) to NADP⁺ during the reduction of oxidized glutathione (GSSG) (Goldberg and Spooner, 1983). The reaction was initiated by the addition cell lysate in a quartz cuvette containing 1.5 ml reaction mixture of 0.3 mM NADPH and 3 mM oxidized glutathione in 0.2 M potassium phosphate buffer, pH 7.3. The decrease in absorbance at 340 nm was followed for 3 min on a spectrophotometer (Thermo-Spectronic) and GR activity was calculated by using 6.22, the mM extinction coefficient for NADPH at 340 nm. The GR activity is expressed as nmol NADPH/min/mg protein.

Measurement of glutathione

Intracellular GSH level was quantified by the fluorometric assay as described by Hissin and Hilf (1976). Cells were treated with CuO NPs at the concentrations of 0, 5, 10 and 15 μ g/ml for 48 hr. After exposure completed, cells were washed in ice-cold phosphate buffer saline and lysed in distilled water containing deoxycholic acid and sucrose. Then cell suspension was centrifuged at 10,000 rpm for 10 min at 4°C to get supernatant. The protein of this supernatant was precipitated at 1% perchloric acid and again centrifuged at 10,000 rpm for 10 min at 4°C. A 20 μ l from the protein precipitated sample was mixed with 160 μ l of 0.1 M phosphate buffer containing 5 mM EDTA (pH 8.3) and 20 μ l O-phthalaldehyde

(1 mg/ml) in a black 96-well plate. After 2.5 hr incubation at room temperature in the dark, fluorescence was measured at an emission wavelength of 460 nm and an excitation wavelength of 355 nm along with GSH standard. GSH content was expressed as nmol GSH/mg protein.

Estimation of Protein

The total protein content was measured according to the method of Bradford (1976) using Bradford reagent (Sigma-Aldrich, MO, USA) and bovine serum albumin as the standard.

Statistical analysis

Statistical significance was determined by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. Significance was ascribed at $p < 0.05$. All analyses were conducted using the Prism software package (GraphPad Software, Version 5.0).

RESULTS

Characterization of CuO NPs

Fig. 1 represents the XRD pattern of CuO NPs. This image clearly exhibits the crystalline nature of CuO NPs. The crystallite size of CuO NPs has been estimated from the XRD pattern using the Scherrer's equation (Patterson, 1939).

$$D = \frac{0.9\lambda}{B \cos \theta}$$

[where D is the grain size, λ the wavelength of X-ray (1.54056 Å), B the full width at half maxima of the diffraction peak (in radian)].

Fig. 1. X-ray diffraction pattern of CuO NPs.

Average crystallite size corresponding to the highest peak observed in XRD pattern was found to be 25.67 ± 5.27 nm (mean $\pm$ S.D. of three values). Fig. 2 A shows the typical TEM image of CuO NPs. TEM average diameter was calculated from measuring over 100 particles in random fields of view. The average TEM size of CuO NPs was 26.35 ± 7.89 nm. Mean value of CuO NPs diameter was in agreement with the XRD results. Fig. 2 B confirms the elemental composition of CuO NPs as measured by energy dispersive X-ray (EDX) spectrum. The average hydrodynamic size of CuO NPs suspension in cell culture medium as determined by DLS was 143.35 ± 11.36 nm. The higher size of NPs in hydrodynamic state as compared to size of TEM and XRD might be due to the tendency of NPs to aggregate in aqueous state. This finding is supported by other investigators (Bai *et al.*, 2009; Sharma *et al.*, 2009) and has been briefly discussed in our previous publication (Ahamed *et al.*, 2010b).

Protective effect of SFN against CuO NPs induced cytotoxicity

Mouse embryonic fibroblast BALB 3T3 cells were exposed to CuO NPs at the concentrations of 0, 5, 10, and 15 μ g/ml for 48 hr and cytotoxicity was determined using MTT and LDH assays. CuO NPs-mediated reduction in cell viability in BALB 3T3 cells was found to be 90, 47, and 22% at the respective concentrations of 5-, 10-, and 15 μ g/ml ($p < 0.05$ for each). Further, co-exposure of cells with SFN significantly attenuated the loss of cell viability (MTT assay) induced by CuO NPs. CuO NPs-mediated reduction in cell viability was found to be 90, 47 and 22% at the concentrations of 5, 10 and 15 μ g/ml whereas in the presence of SFN these values become to 100, 68 and 30% at the concentrations of 5, 10 and 15 μ g/ml respectively (Fig. 3A).

LDH release, a marker of cell membrane damage was also found to increase in a dose-dependent manner due to CuO NPs exposure. CuO NPs induced LDH release was increased by 26, 108 and 213% when compared with control at the concentrations of 5-, 10-, and 15 μ g/ml respectively. However, co-exposure of SFN significantly reduced the leakage of LDH caused by CuO NPs in BALB 3T3 cells (Fig. 3B).

Protective effect of SFN against CuO NPs induced-oxidant generation

The protective potential of SFN against CuO NPs induced oxidant generation was assessed by measuring the ROS and LPO in BALB 3T3 cells. Fig. 4A shows that the CuO NPs significantly induced the intracellular production of ROS in a dose-dependent ($p < 0.05$ for each).

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Fig. 2. Electron microscopy characterization of CuO NPs. (A) FETEM image and (B) EDX spectrum.

Fig. 3. Protective effect of SFN (6 μ M) against CuO NPs induced cytotoxicity in BALB 3T3 cells. (A) MTT assay and (B) LDH leakage. The numbers 5, 10 and 15 represents the concentrations of CuO NPs in μ g/ml. Data represented are mean $\pm$ S.D. of three identical experiments made in three replicate. * Denotes a significant difference from the control ($p < 0.05$ for each). α Indicates the significant protective effect of SFN against CuO NPs induced cytotoxicity.

However, co-exposure of SFN effectively prevented the ROS generation induced by CuO NPs.

Figure 4B demonstrated that CuO NPs induced 2, 3.6 and 4 fold membrane LPO in BALB 3T3 cells at the concentrations of 5, 10 and 15 μ g/ml respectively. Again, co-exposure of SFN significantly attenuated the membrane LPO caused by CuO NPs.

SFN attenuated the effect of CuO NPs on antioxidant system

Preventive potential of SFN against CuO NPs induced depletion in antioxidant levels was determined by measuring the GSH and GR levels in BALB 3T3 cells. CuO NPs significantly deplete GSH and GR levels in dose-dependent. However, co-exposure of SFN effectively prevented the depletion of GSH and GR levels caused by CuO NPs treatment (Figs. 5 A and B).

Fig. 4. Protective effect of SFN (6 μ M) against CuO NPs induced oxidant generation in BALB 3T3 cells. (A) ROS and (LPO). The numbers 5, 10 and 15 represents the concentrations of CuO NPs in μ g/ml. Data represented are mean $\pm$ S.D. of three identical experiments made in three replicate. * Denotes a significant difference from the control ($p < 0.05$ for each). α Indicates the significant protective effect of SFN against ROS and LPO induced by CuO NPs.

Fig. 5. Protective effect of SFN (6 μ M) against the depletion of GR and GSH induced by CuO NPs in BALB 3T3 cells. (A) GR activity and (B) GSH level. The numbers 5, 10 and 15 represents the concentrations of CuO NPs in μ g/ml. Data represented are mean $\pm$ S.D. of three identical experiments made in three replicate. * Denotes a significant difference from the control ($p < 0.05$ for each). α Indicates the significant protective effect of SFN against GR and GSH depletion caused by CuO NPs.

Effect of NAC on reduction in cell viability and glutathione due to CuO NPs exposure

Fig. 6 describes the GSH replenishing effect of NAC and corresponding preventive potential against CuO NPs induced cytotoxicity. Data shows that NAC is quite effective

in preventing the depletion of GSH induced by CuO NPs (Fig. 6A). However, NAC did not significantly ameliorate the reduction in cell viability caused by CuO NPs (Fig. 6B). This result suggests that GSH is important as antioxidant but as far as the protection from loss of cell

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Fig. 6. Protective effect of NAC (2 mM) against the reduction in GSH and cell viability in BALB 3T3 cells. (A) GSH and (B) cell viability. The numbers 5, 10 and 15 represents the concentrations of CuO NPs in $\mu\text{g/ml}$. Data represented are mean $\pm$ S.D. of three identical experiments made in three replicate. * Denotes a significant difference from the control ($p < 0.05$ for each). α Indicates the significant protective effect of SFN against GSH depletion caused by CuO NPs. However, NAC did not significantly protect the cells from the loss of cell viability induced by CuO NPs.

viability is concerned, an increase of cellular GSH is not sufficient.

DISCUSSION

Recent studies suggested that excessive production of ROS and oxidative stress could be one of the possible mechanisms of NPs toxicity (Nel *et al.*, 2006; Ahamed *et al.*, 2010a, 2010b, 2011a and 2011b; Akhtar *et al.*, 2010a, 2010b). Our results showed that CuO NPs induce dose-dependent cytotoxicity and oxidative stress in mouse embryonic fibroblast BALB 3T3 cells. CuO NPs induced the generation of ROS in exposed cells. MDA level, a biomarker of LPO, was significantly higher while antioxidants GSH and GR levels were significantly lower in CuO NPs treated cells. The depletion of GSH and GR in CuO NPs exposed cells, combined with the increased level of ROS and LPO suggested that oxidative stress might be the primary mechanism for toxicity of CuO NPs in BALB 3T3 cells. Moreover, our results are consistent with previous studies suggesting that cytotoxicity of CuO NPs was mediated through ROS generation and oxidative stress (Griffith *et al.*, 2007; Fahmy and Cormier, 2009; Ahamed *et al.*, 2010c).

Nanomaterials safety is a major challenge and is a considerable hindrance against the full use of nanotechnology and its products. In this study, we found that SFN significantly attenuated the cytotoxicity caused by CuO NPs through the induction of a panel of key antioxidants, GSH

and GR, and depletion of oxidants (e.g. ROS and LPO). It is widely recognized that the up-regulation of these antioxidants and other phase II enzymes is a powerful and highly efficient protective strategy against the damaging effects of ROS and electrophiles (Gao *et al.*, 2001; Gao and Talalay, 2004; Dinkova-Kostova *et al.*, 2007). Recently, Ritz *et al.* (2007) has reported that SFN-stimulated phase II enzyme induction inhibits cytokine production by airway epithelial cells stimulated with diesel extract. Zhu *et al.* (2004) have also reported protective effect of SFN in human keratinocytes challenged with UV-B radiation.

There is substantial evidence that SFN acts indirectly to increase the antioxidant capacity of animal cells and, thus, increases their abilities to cope with oxidative stress (Fahey and Talalay, 1999; Gao and Talalay, 2004; Zhang *et al.*, 2005). Induction of phase II enzymes is one means by which SFN enhances the cellular antioxidant capacity. To protect themselves from oxidative stress, cells are equipped with highly elaborated antioxidant defense systems including GSH and GR (Meister and Anderson, 1983). GSH is an important tripeptide thiol which in addition to being the substrate for GSH S-transferase and glutathione peroxidase maintains the cellular oxidation-reduction balance and protects cells from ROS. GR plays a critical role in protection from oxidative stress by regenerating reduced GSH from the oxidized form. There are several reports on the differential action of SFN in different cell lines necessitating the detailed research and taking

a lot of precautions before concluding remarks. For example, SFN induces glutathione peroxidase that catalyzes the reduction of organic hydroperoxide and hydrogen peroxide in human Caco-2 cells and mouse Hepa1c1c7 cells ((Banning *et al.*, 2005; Keck and Finley, 2006). In this study, we found that SFN significantly attenuated the GR activity caused by CuO NPs in BALB 3T3 cells. The potential of SFN to induce cellular antioxidant coping oxidants that persists for longer time even after removal of SFN from the cellular systems highlighting the ability of SFN to act as an indirect antioxidant and is able to provide long-lasting protection against oxidative stress (Zhu *et al.*, 2004).

In this study, we also compared the protection efficiency of SFN against CuO NPs toxicity with another antioxidant called NAC. NAC is precursor of GSH biosynthesis. Though, NAC seems to increase GSH level in BALB 3T3 cells, it did not protect cytotoxicity induced by CuO NPs unlike that of SFN. To decipher the role of GSH in cytotoxicity, we treated BALB 3T3 cells with Buthionine-[S,R]-sulfoximine (BSO), a potent inhibitor of GSH synthesis (Shimizu *et al.*, 1997). No cytotoxicity was observed in cells treated with BSO with a significant depletion of GSH when compared with control cells. However, cell viability was highly aggravated by CuO NPs in the presence of BSO (data not shown). This result suggests that GSH is important as antioxidant but as far as the protection from loss of cell viability is concerned, a mere increase of cellular GSH is not sufficient. The protection from toxicity by SFN might not be due to increase in cellular GSH but it might be, we think, due to its ability to modulate a large number of crucial proteins and antioxidant enzymes. Thus, we conclude that increase in cellular GSH by SFN might be a sign of protective action of SFN but the actual pathway leading to protection from toxicity is much more complex. Efficient detoxification of ROS requires, thus, concerted actions of various cellular antioxidants.

Apart from the potential use of SFN as an important antioxidant we further hypothesize the need of rigorous study and search of effective antioxidants, natural or synthetic, particularly in the field of biomedical nanotechnology because there are nanomaterials which are their selves potential anticancer (e.g. ZnO NPs) or antioxidants (e.g. nanocerium). And some nanomaterials, as discussed by Huang *et al.* (2010), may be designed for altered activity from their original activity by forming a shell/core of other drugs/molecules and SFN like compounds could be thought of as another option in complex nanomaterials formulations. The application of nanobiotechnology encompasses development of nanomaterials for deliver-

ing and monitoring biologically active molecules, disease staging, therapeutic planning, surgical guidance, neuro-electronic interfaces, electronic biosensors and new approaches based upon molecular self-assembly developing novel materials with dimensions on the nanoscale (Huang *et al.*, 2010).

In conclusion, our data demonstrated that CuO NPs induce dose-dependent cytotoxicity and oxidative stress in mouse embryonic fibroblast BALB 3T3. Co-treatment of cells with SFN significantly attenuated the cytotoxicity, ROS generation and oxidative stress caused by CuO NPs. We also noted that co-treatment of another antioxidant NAC significantly attenuated glutathione depletion caused by CuO NPs but protection from the loss of cell viability was not significant. Therefore, up-regulation of GR and GSH could one of the possible mechanisms by which SFN exerts its protective effect in BALB 3T3 cell system against CuO NPs toxicity. Protective mechanisms of SFN against CuO NPs induced toxicity should be further investigated.

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