Toxicology 290 (2011) 208–217

Contents lists available at SciVerse ScienceDirect

Toxicology

journal homepage: www.elsevier.com/locate/toxicol

Nano-copper induces oxidative stress and apoptosis in kidney via both extrinsic and intrinsic pathways

Abhijit Sarkar, Joydeep Das, Prasenjit Manna, Parames C. Sil*

Division of Molecular Medicine, Bose Institute, P-1/12, CIT Scheme VIIM, Calcutta 700054, West Bengal, India

ARTICLE INFO

Article history:
Received 20 July 2011
Received in revised form
27 September 2011
Accepted 28 September 2011
Available online 5 October 2011

Keywords:
Nano copper
Kidney
Oxidative stress
Nitrosative stress
Apoptosis

ABSTRACT

In the modern medicine nano particle has been used as a power tool but recently it has been established that nano particle pathophysiologically affects different organs. Recently, we have investigated the role of copper nano particle in liver dysfunction. In the literature, practically little is known about the nano-copper induced renal dysfunction. We, therefore, conducted the present study as a continuation of our earlier one to investigate the molecular mechanism in nano-copper induced kidney dysfunction. Nano-copper exposure increased the production of reactive oxygen species (ROS), reactive nitrogen species (RNS) and altered the levels of oxidative stress related biomarkers in kidney tissue. Signal transduction mechanism studies showed that nano copper exposure reciprocally regulated Bcl-2 family protein expression, disturbed mitochondrial membrane potential and subsequently helped releasing cytochrome *c* from mitochondria to cytosol. Apoptotic nature of cell death is confirmed by activation of caspases 3 which is also supported by histological study. In addition, we also observed the activation of Fas, caspase 8 and tBid in kidney tissue in this pathophysiology, suggesting the involvement of extrinsic pathways. Combining all, results suggest that nano copper can trigger both intrinsic and extrinsic apoptotic pathways in oxidative stress mediated kidney dysfunction.

1. Introduction

Nanoparticle research is currently an area of great scientific interest due to a wide variety of applications in medical fields. Ultrafine particles covering a range between 1 and 100 nm are known as nano particles. There are some unique physiochemical properties of nanoparticles like ultra small size and large surface area to mass ratio (Oberdorster et al., 2005, 2007). These properties can be used to overcome some of the limitations found in traditional therapeutic and diagnostic agents. Nanotechnology has emerged as a therapeutic field in bio medical sciences. Recent years of study have shown that nano medicine has incredible potential for developing the medical science. They not only act as therapeutic agents but also deliver themselves to specific regions or tissues in the body (Kipen and Laskin, 2005). Another advantage of nano technology is that, very small quantity is needed comparison to usual drugs. While benefits of nanotechnology are widely used, public concerns begin to emerge on the adverse effects of the nanomaterials. After exposure and absorption, nanoparticles enter into the cells of different organs and cause organ pathophysiology by interacting with protein or DNA or RNA.

Copper is an essential trace element and its deficiency leads to several diseases in human. On the other hand, it acts as a catalytic co-factor in a number of redox enzymes required in a wide range of metabolic processes (Georgopoulos et al., 2001). When the intake of copper exceeds the tolerable limit, it exerts toxic effects leading to cell death (Nawaz et al., 2006). Copper overload can cause cellular damage through the formation of free radicals (Ozcelik et al., 2003). Recent studies indicate that in vivo copper overload induces a set of toxicological activities such as oxidative stress, changes in lipid profile and hepatic dysfunction. Now-a-days nano copper particles are industrially produced and are commercially available. They are used as the additive in lubricants, polymers/plastics, metallic coating and inks, etc. The nano-sized copper-fluoro polymer is used as bioactive coatings that inhibit the growth of microorganisms (Cioffi et al., 2005). Earlier studies have been done on different organs such as liver and respiratory organs where nano particles have found to exhibit toxicity. Recently Chen et al. (2006) reported that nanoparticles induced toxicological effects and severe injuries on kidney, liver and spleen of experimental mice while micro-copper particles did not show any toxic effect. Nano-copper-induced hepatotoxicity

Abbreviations: CAT, catalase; CDNB, 1-chloro-2,4-dinitrobenzene; DCFDA, 2,7-dichlorofluorescein diacetate; DNPH, 2,4-dinitro phenyl hydrazine; DTNB, 5,5′-dithiobis (2-nitrobenzoic acid); GSH, glutathione; GSSG, glutathione disulfide; GST, glutathione S-transferase; GPx, glutathione peroxidase; GR, glutathione reductase; MDA, malonaldehyde; NBT, nitro blue tetrazolium chloride; NEM, N-ethylmaleimide; ROS, reactive oxygen species; RNS, reactive nitrogen species; SOD, superoxide dismutase; TBA, thiobarbituric acid; TCA, tricholoro acetic acid.

* Corresponding author. Tel.: +91 33 2569 3243; fax: +91 33 2350 6790.
E-mail addresses: parames@bosemain.boseinst.ac.in, parames_95@yahoo.co.in (P.C. Sil).

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doi:10.1016/j.tox.2011.09.086

and nephrotoxicity was also showed by Lei et al. (2008). However, till now there is no detailed mechanistic study that suggests the toxicity of copper nano particles on renal pathophysiology through oxidative stress. As kidney is a vital organ with several functions, so, in the present study we aimed to investigate the role of stress responsive signaling pathways in copper nano particles induced kidney dysfunction.

2. Materials and methods

2.1. Chemicals

Anti cytochrome *c*, anti Apaf1, anti Fas, anti cleaved caspases-9, anti cleaved caspases-8, anti cleaved caspase-3, anti PI3K and anti phospho Akt antibodies were purchased from Sigma–Aldrich Chemical Company (St. Louis, USA). Kits for creatinine and blood urea nitrogen (BUN) measurements were purchased from Span diagnostic Ltd., India. Anti Bax, anti Bcl-2, anti Fas and anti tBid were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Other chemicals and reagents were of the highest analytical grade and were bought from Sisco Research Laboratory (Mumbai, India).

2.2. Animals

Swiss albino male mice, weighing approximately 20–22 g were acclimatized under laboratory condition for two weeks prior to the experiments. They were maintained under standard conditions of temperature (23 ± 2 °C) and humidity (50 ± 10%) with an alternating 12 h light/dark cycles. All the experiments with animals were carried out according to the guidelines of the institutional animal ethical committee.

2.3. Preparation of nano copper (Cu) particles

Cu nanoparticle preparation was done following the method of Zhu et al. (2005). At first, 20 ml solution of $CuSO_4{\cdot}5H_2O$ in ethylene glycol (EG) was prepared. Twenty milliliter solution of NaOH and $N_2H_4{\cdot}H_2O$ in EG was also prepared. Now these two solutions were mixed under magnetic stirring. The molar ratio of N_2H_4, $H_2O/CuSO_4{\cdot}5H_2O$ was 1.5 and the molar ratio of $NaOH/CuSO_4{\cdot}5H_2O$ was 0.05. The mixed solution was placed in a microwave oven (2.45 GHz) and allowed to react under medium power (750 W) for 3 min. After irradiation for about 30 s, the mixture turned from light blue to black; at about 90 s, the mixture boiled at about 196 °C. Then the mixture was irradiated for another 2 min. After cooling to room temperature, copper nanoparticles were obtained by centrifugation and the precipitate was washed several times with ethanol. Scanning electron microscope (SEM), dynamic light scattering (DLS) and energy dispersive X-ray spectroscopy (EDX) were used to characterize the synthesized copper nanoparticles.

2.4. Animal treatment

The animals were divided into five groups each consisted of six mice. Group 1 animals were treated as normal control and received only normal diet. Animals of the other groups, group 2 (Cu-1), group 3 (Cu-2) and group 4 (Cu-3) received Cu nanoparticles via oral gavage at a dose of 200, 413 and 600 mg/kg body weight for 3 days respectively. Group 5 animals received copper chloride at a dose of 110 mg/kg body weight for 3 days and served as copper ion group (Cu^{2+}). Based on the earlier reports of Chen et al. (2007) and the dose dependent study, the doses of Cu nanoparticles and copper ion have been selected for all other experiments.

After 3 days of exposure, the animals were euthanized first and then sacrificed. From the sacrificed animals, blood was collected in tubes containing a mixture of potassium oxalate and sodium fluoride (1:3) for the estimation of serum parameters related to nephrotoxicity. The kidney tissue was also collected from the sacrificed animals, weighed, stored at 4 °C and used for the various experiments as needed.

2.5. Determination of dose by blood urea nitrogen and creatinine assay

To establish the dose, mice were treated with six different doses (100, 200, 300, 413, 500 and 600 mg/kg body weight) of nano copper for 3 days. As Chen et al. (2007) already determined the LD_{50} of nano copper, so here these doses were taken in our experimental model. After three days, all mice were sacrificed. Blood samples collected from puncturing mice hearts were kept overnight to clot and then centrifuged at 3000 × *g* for 10 min to separate the serum.

2.6. Estimation of serum specific markers creatinine and blood urea nitrogen (BUN)

The blood urea nitrogen (BUN) and creatinine levels of serum were estimated by using standard kits (Span diagnostic Ltd., India).

2.7. Preparation of kidney tissue homogenates

About 200 mg kidney tissue was homogenized using glass homogenizer in 100 mM phosphate buffer (pH 7.4) containing 1 mM EDTA, PMSF (proteinase inhibitor, 1 mM) and phosphatase inhibitor cocktail (1:100 dilution). The mixture was centrifuged at 12,000 × *g* for 30 min at 4 °C. The supernatant was collected and used for the experiments.

2.8. Determination of protein content

The protein contents of kidney tissue homogenates of the experimental animals were measured by the method of Bradford using crystalline BSA as standard (Bradford, 1976).

2.9. Measurement of intracellular ROS production and serum NO levels

Intracellular ROS production was estimated by using 2,7-dichlorofluorescein diacetate (DCFDA) as a probe using the method of LeBel and Bondy (1990) followed by the modification introduced by Kim et al. (1996). The formation of DCF was measured at the excitation wavelength of 488 nm and emission wavelength of 525 nm for 10 min by using fluorescence spectrometer (HITACHI, Model No. F4500) equipped with a FITC filter.

The levels of serum NO have been measured by estimating the concentration of nitrate and nitrite with a modified nitrate reductase and Griess reaction method (Hevel and Marietta, 1994). Nitrate was reduced to nitrite by the action of nitrate-reductase in the presence of NADPH. Excess NADPH was then consumed and the nitrite concentration was measured by the addition of equal volumes of 1% sulfanilamide in 5% concentrated phosphoric acid and 0.1% N-(1-naphthyl) ethylene diaminedihydrochloride. The Griess colour reaction forms a pink azo dye with an absorbance wavelength of 540 nm. The assay was standardised using various dilutions of sodium nitrate and nitrite. Vitamin E was used as a positive control in the study.

2.10. Assay of oxidative stress related biochemical parameters in renal tissue

The levels of the lipid peroxidation and protein carbonylation in kidney tissue of experimental animals were determined following the methods of Esterbauer and Cheeseman (1990). The activities of the antioxidant enzymes (SOD, CAT, GST, GR and GPx) were estimated according to the methods of Manna et al. (2009) and Das et al. (2010). The levels of GSH, GSSG and total thiol in the kidney tissue homogenates of the experimental animals were determined following the methods of Hissin and Hilf (1976) and Sedlak and Lindsay (1968) respectively. Vitamin E was used as a positive control in the study.

2.11. TUNEL assay

TdT-mediated dUTP nick-end labeling (TUNEL) detection of apoptotic cells was performed in formalin-fixed, paraffin-embedded renal tissue sections according to the manufacturer instruction (Invitrogen, USA).

2.12. Isolation of mitochondria from renal tissue and determination of mitochondrial membrane potential ($\Delta\psi_m$)

Mitochondria were isolated from kidney tissue of experimental mice. Briefly, renal tissue was homogenized in ice-cold isolation buffer (10 ml Tris–MOPS [0.1 M; pH7.4], sucrose [1 M], and EGTA–Tris buffer [0.1 M; pH 7.4]). The homogenates were centrifuged at 600 × *g* at 4 °C for 10 min, and then the supernatants were centrifuged at 7000 × *g* at 4 °C for 10 min, after which the supernatants were kept as cytosolic fraction for future use. The pellets were washed with isolation buffer, and the centrifugation steps were repeated twice. After the three-step centrifugation, the supernatants were discarded, and the pellets were suspended in isolation buffer and used for further analysis (Chandrasekaran et al., 2009). The purity of mitochondria was checked by the activity of succinate dehydrogenase. Mitochondrial membrane potential was measured by adding exogenous succinate and ADP to all sets of mitochondria isolated from experimental animal (Toorn et al., 2007). Mitochondrial membrane potential ($\Delta\psi_m$) was estimated (Francesc et al., 2002) on the basis of cell retention of the fluorescent cationic probe rhodamine 123.

2.13. Immunoblotting

Samples containing 50 μg proteins were subjected to 10% SDS-PAGE and transferred to nitrocellulose membrane. Membranes were blocked at room temperature for 2 h in blocking buffer containing 5% non-fat dry milk to prevent non specific binding and then incubated with primary antibodies overnight at 4 °C. The primary antibodies used in the present study were anti Bax, anti Bcl-2, anti cytochrome C, anti cleaved caspase 3, anti caspase 9 and anti Apaf1, anti caspase 8, anti tBid, anti Fas, anti iNos, anti PI3K, anti phospho Akt, anti phospho Bad antibodies (all 1:1000 dilution). The membranes were washed in TBST (50 mmol/L Tris–HCl, pH 7.6, 150 mmol/L NaCl, 0.1% Tween 20) for 30 min and incubated with appropriate

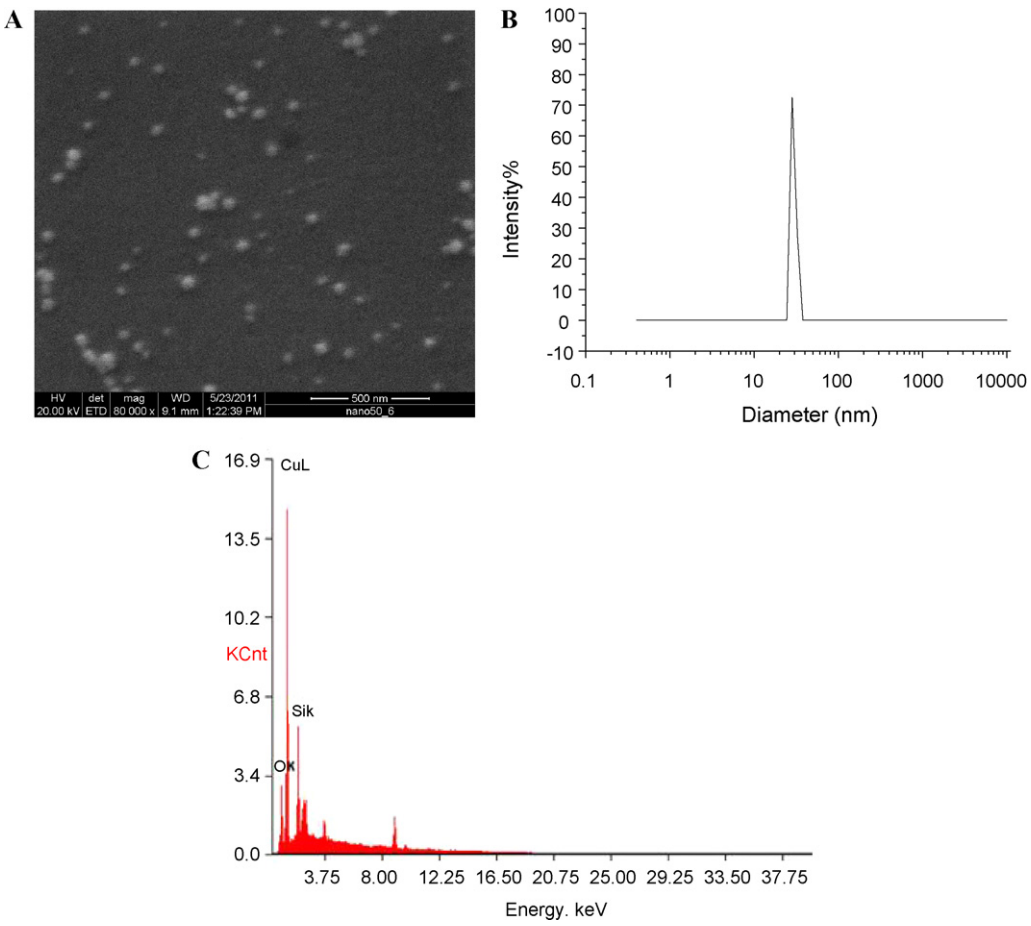

Fig. 1. (Panel A) SEM image of nano copper particles. The scale bar used here is 500 nm. The average size of the particle is 25–40 nm. (Panel B) EDX spectroscopy of nano-copper particles. (Panel C) Size distribution image of nano-copper particles obtained from DLS experiment. Peak indicates the particles with 30 nm in diameter with maximum intensity.

HRP conjugated secondary antibody (1:2000) for 2 h at room temperature and developed by the HRP substrate 3,3′-diaminobenzidine tetrahydrochloride (DAB) system (Bangalore, India).

2.14. Histological studies

Kidneys from the normal and experimental animals were fixed in 10% buffered formalin and were processed for paraffin sectioning. Sections of about 5 μm thickness were stained with haematoxylin and eosin to evaluate under light microscope.

2.15. Statistical analysis

All the values are expressed as mean ± SD ($n = 6$). Significant differences between the groups were determined with SPSS 10.0 software (SPSS Inc., Chicago, IL, USA) for Windows using one-way analysis of variance (ANOVA) and the group means were compared by Student–Newman–Keuls post hoc tests. A difference was considered significant at the $p < 0.05$ level.

3. Results

3.1. Characterizations of nano particles

The sizes of the copper nano-particles were determined by scanning electron microscope (SEM). The SEM image showed that average size of nano-copper particles is 25–40 nm in diameter (Fig. 1, panel A). The size distribution was also determined by a dynamic light scattering (DLS) experiment. The peak observed from DLS experiment showed that most of the nano particles are of 30 nm (Fig. 1, panel B). Finally, the identities of the copper nano-particles were established by energy dispersive X-ray spectroscopy (EDX). Appearance of a large sharp symmetrical peak of copper in EDX spectroscopy supports the identity of the copper nano-particles (Fig. 1, panel C).

3.2. Dose dependent assay

Dose dependent effect of nano-copper has been represented in Fig. 2. Nano-copper intoxication significantly increased the BUN and creatinine level at dose higher than 300 mg/kg body weight.

3.3. Effects on serum biochemical parameters

Blood biochemical parameters (BUN, creatinine) that reflect the renal functions were investigated (Fig. 3). No statistically significant difference was observed between the control and Cu-1 exposed animal groups but increased level of BUN and creatinine was observed in case of Cu-2 and Cu-3 exposed animals (Chen et al., 2006).

3.4. Effects on the activities of antioxidant enzymes

Intracellular antioxidant enzymes protect biological macromolecules from oxidative stress induced organ pathophysiology. To investigate the status of the intracellular antioxidant defense machineries, we measured the activities of antioxidant enzymes, SOD, CAT, GST, GR and GPx. Table 1 represents the activities of the antioxidant enzymes in kidney tissue of the experimental animals. Our study reveals that nano copper administration dose dependently reduced the activities of the antioxidant enzymes.

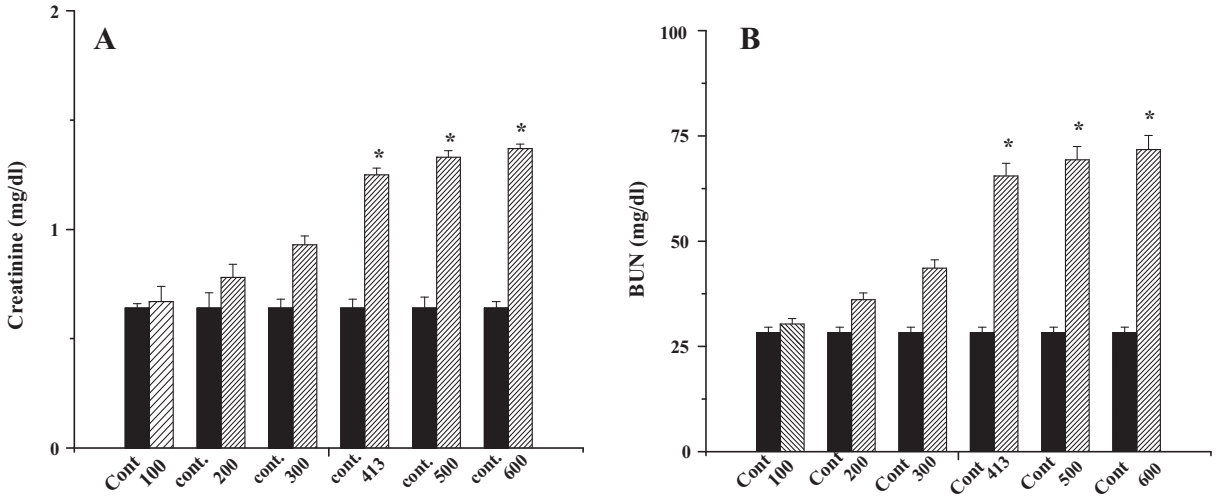

Fig. 2. Dose dependent study by blood urea nitrogen and creatinine assay. Cont: normal mice, 100, 200, 300, 413, 500, 600 mg/kg body weight nano-copper was given. Data are mean ± SD, for 6 animals per group and were analyzed by one-way ANOVA, with Student–Newman–Keuls post hoc tests. Differences were attributed at $p < 0.05$, and homogeneous subgroups share common superscripted letters.

3.5. Effects on intracellular ROS and NO production

Several investigations support the fact that increased production of ROS and RNS plays an important role in copper induced organ dysfunction. Exposure to nano sized copper particles caused alteration in the levels of intracellular ROS as well as NO production (measured by nitrite level) in our present study (Table 2).

We next determined the expression of inducible NO synthase (iNOS) in the kidney tissue after nano-copper exposure. Western blot analysis revealed that iNOS expression was increased in case of Cu-2 and Cu-3 compared to normal whereas Cu-1 showed expression close to normal (Fig. 4).

3.6. Effects on lipid peroxidation and protein carbonyl content

Cell membrane damage and modification of proteins due to oxidative stress were measured by lipid peroxidation and protein carbonyl content. In the present study the lipid peroxidation has been measured by estimating the concentration of MDA (lipid peroxidation end product). Exposure with nano copper particles increased the levels of MDA and protein carbonylation in the kidney tissue of the experimental animals.

3.7. Effect on GSH/GSSG ratios

Thiol based antioxidant system plays an important role in cellular defense against reactive free radicals. It scavenges intracellular reactive intermediates. A large amount of GSH is consumed to accomplish this task. Whenever GSH level decreases than its normal level, the concentration of reactive intermediates increases, causes oxidative stress and decreases the GSH/GSSG ratio. Exposure with nano copper particles in the present study increased the levels of GSSG, decreased the levels of GSH and thereby decreased the GSH/GSSG ratio in the kidney tissue of the experimental animals.

3.8. Effect of copper-nano on DNA strand break

TUNEL assay is one of the reliable methods for detection of apoptosis. Fig. 4 represents the result of TUNEL assay. From the result of this assay it is evident that nano-copper exposure significantly increased tunel positive nuclei.

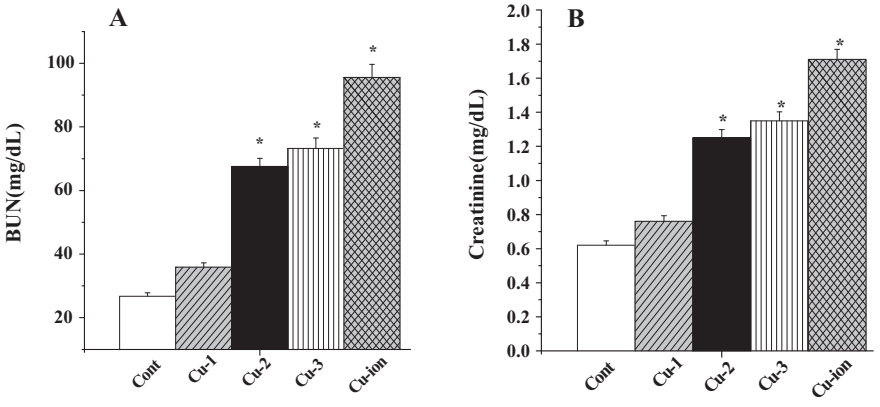

Fig. 3. (Panels A and B) BUN (blood urea nitrogen) and creatinine level in serum following nano-copper treatment. Cont: normal mice; Cu-1, Cu-2 and Cu-3: animals administered with nano copper particles at a dose of 200, 413 and 600 mg/kg body weight and Cu^{2+}: animals administered with copper chloride at a dose of 110 mg/kg body weight. Data are mean ± SD, for 6 animals per group and were analyzed by one-way ANOVA, with Student–Newman–Keuls post hoc tests. Differences were attributed at $p < 0.05$, and homogeneous subgroups share common superscripted letters.

Table 1
Effect of nano Cu particles on antioxidant enzymes.

Name of the antioxidant enzymes	Activities of the antioxidant enzymes				Cu-ion exposed
	Normal	Cu-1 exposed	Cu-2 exposed	Cu-3 exposed	
SOD (Unit/mg protein)	162.47 ± 7.81	152.32 ± 7.32	115.75 ± 5.49*	111.24 ± 5.36*	102.1 ± 4.93*
CAT (μmol/min/mg protein)	152.94 ± 7.36	143.39 ± 6.96	106.47 ± 5.12*	101.51 ± 4.90*	94.92 ± 4.54*
GST (μmol/min/mg protein)	3.11 ± 0.14	2.83 ± 0.13	1.96 ± 0.09*	1.79 ± 0.08*	1.61 ± 0.07*
GR (nmol/min/mg protein)	38.29 ± 1.79	35.82 ± 1.65	23.66 ± 1.07*	21.17 ± 0.97*	18.59 ± 0.82*
GPx (nmol/min/mg protein)	81.66 ± 3.98	77.24 ± 3.72	51.58 ± 2.42*	48.33 ± 2.28*	43.14 ± 2.01*

Values are expressed as mean ± SD, for 6 animals in each group.
* Values differs significantly from control ($p < 0.05$).

Table 2
Effect of copper nano, copper ion and copper nano + vitamin E on oxidative stress related biomarkers.

Name of the parameters	Normal	Cu-1 exposed	Cu-2 exposed	Cu-3 exposed	Cu-ion exposed	Cu-2 + vitamin E treated
MDA (nmol/mg protein)	2.66 ± 0.12	3.41 ± 0.15	5.24 ± 0.23*	5.69 ± 0.25*	5.92 ± 0.28*	3.96 ± 0.16
Protein carbonyl (nmol/mg protein)	4.70 ± 0.21	5.96 ± 0.27	9.23 ± 0.42*	10.31 ± 0.46*	10.89 ± 0.51*	6.47 ± 0.28
GSH (nmol/mg protein)	11.12 ± 0.51	10.53 ± 0.49	7.44 ± 0.34*	6.97 ± 0.31*	6.64 ± 0.29*	9.11 ± 0.40
GSSG (nmol/mg protein)	1.61 ± 0.07	1.73 ± 0.08	2.52 ± 0.11*	2.66 ± 0.12*	2.81 ± 0.14*	2.13 ± 0.09
Total thiol (nmol/mg protein)	139.34 ± 6.6	130.79 ± 6.3	95.50 ± 4.5*	87.34 ± 4.16*	81.67 ± 3.97*	118.3 ± 4.6
ROS (% over control)	100	121.17 ± 4.9	244.3 ± 9.3*	255.28 ± 9.6*	284.51 ± 11.2*	172.39 ± 6.41
NO production (μM)	50.86 ± 2.45	54.72 ± 2.60	82.43 ± 3.9*	86.69 ± 4.15*	92.91 ± 4.52*	66.82 ± 3.14

Values are expressed as mean ± SD, for 6 animals in each group.
* Values differs significantly from control ($p < 0.05$).

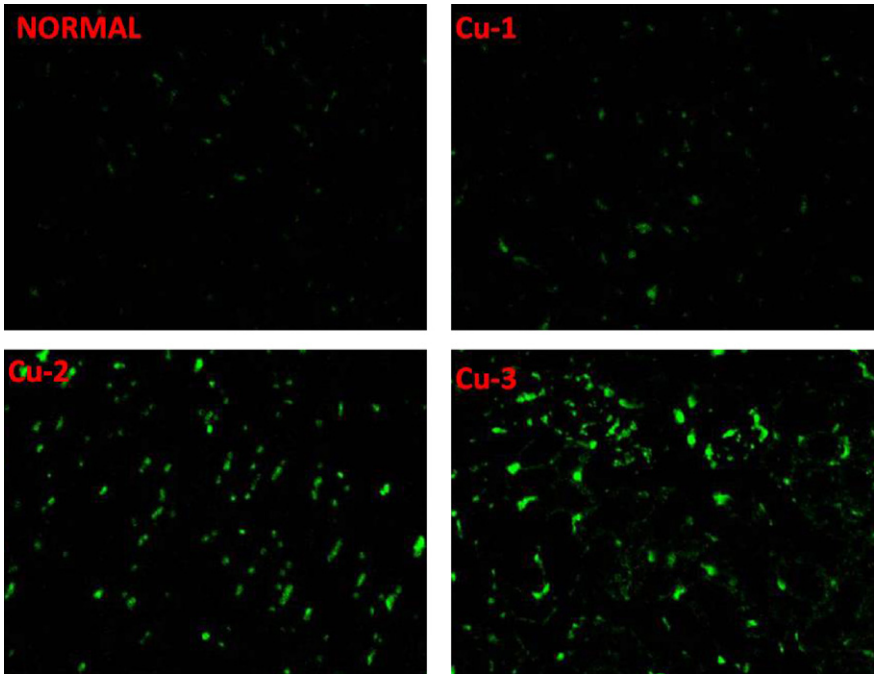

Fig. 4. TUNEL assay showing tunel positive cell in kidney tissue, treated with nano-copper. Cu-2 and Cu-3 exposed mice, showing greater number of tunel positive nuclei whereas normal and Cu-1 exposed mice, showing very less amount of tunel positive nuclei.

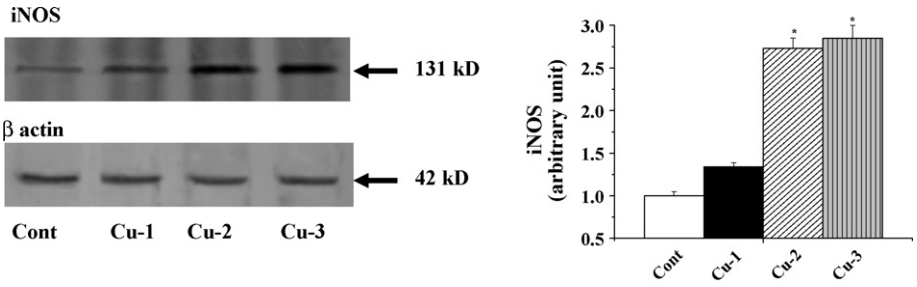

Fig. 5. Western blot analysis showing renal expression of inducible nitric oxide synthase (iNOS) in mice treated with nano-copper. β-Actin was used as loading control. The relative intensities of bands were determined using NIH-image software and the control band was given an arbitrary value of 1. Differences were attributed at $p < 0.05$, and homogeneous subgroups share common superscripted letters.

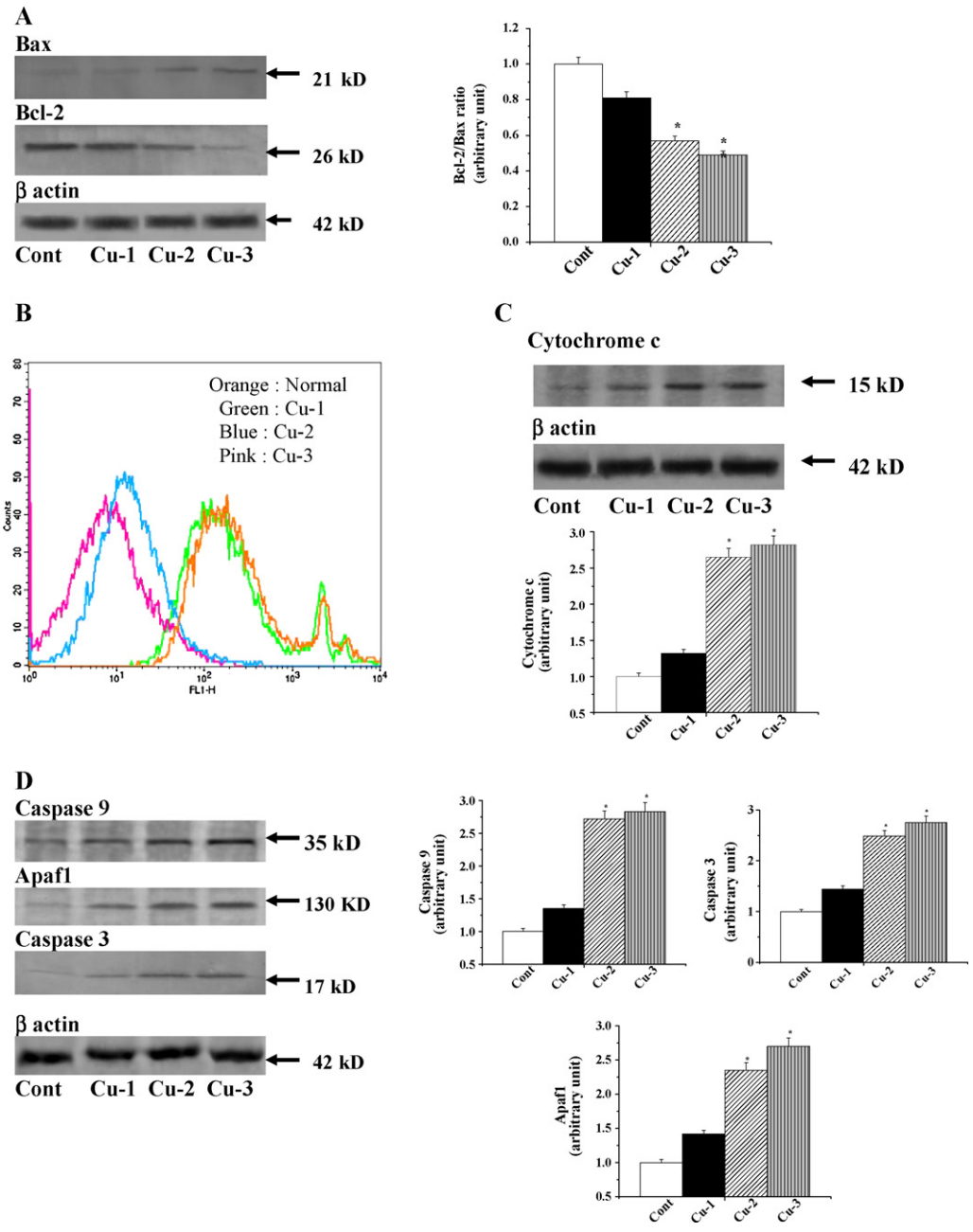

Fig. 6. (Panel A) Western blot analysis of Bax, Bcl-2 in the kidney tissue of nano-copper intoxicated animals. (Panel B) Study on the mitochondrial membrane potential by flow cytometry analysis. Cont: mitochondria isolated from the kidney tissue of normal animals; Cu-1, Cu-2 and Cu-3: mitochondria isolated from the kidney tissue of the animals administered with nano-copper particles at a dose of 200, 413 and 600 mg/kg body weight. Orange, green, blue, pink colour represent the normal, Cu-1, Cu-2, Cu-3 treated mitochondrial membrane potential respectively. (Panel C) Western blot analysis of Cyt *c* in the kidney tissue of nano-copper intoxicated animals. (Panel D) Western blot analysis of Apaf1, caspase 9 and caspase 3 in the kidney tissue of nano-copper intoxicated animals. The relative intensities of bands were determined using NIH-image software and the control band was given an arbitrary value of 1. Cont: normal mice; Cu-1, Cu-2 and Cu-3: animals administered with nano-copper particles at a dose of 200, 413 and 600 mg/kg body weight and Cu^{2+}: animals administered with copper chloride at a dose of 110 mg/kg body weight. Data are mean ± SD, for 6 animals per group and were analyzed by one-way ANOVA, with Student–Newman–Keuls post hoc tests. Differences were attributed at $p < 0.05$, and homogeneous subgroups share common superscripted letters. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

3.9. *Effect of nano-copper on the Bcl-2 and Bax protein and also on the mitochondrial membrane potential*

Bcl-2 proteins are upstream regulator of mitochondrial membrane potential. Fig. 5A represents the expressions of Bcl-2 and Bax proteins. From immunoblotting it has been observed that nano-copper exposure upregulated pro-apoptotic Bax protein and down regulated anti-apoptotic Bcl-2 protein resulting in the reduction of mitochondrial membrane potential. Shifting of peak in Fig. 5B shows the reduction of membrane potential.

3.10. *Involvement of mitochondria-dependent cell death*

Oxidative stress-induced apoptotic cell death is directly related to mitochondrial dysfunction. Disruption of mitochondrial membrane potential, release of cytochrome *c* in cytosol and eventually activation of caspase 3 are the biomarkers of oxidative stress induced cell death via mitochondria dependent pathway. Immunoblot analyses showed that nano-copper intoxication elevated the concentration of cytosolic cytochrome *c*, Apaf1, activated caspase 9 and cleaved caspase 3 (Fig. 6) suggesting the

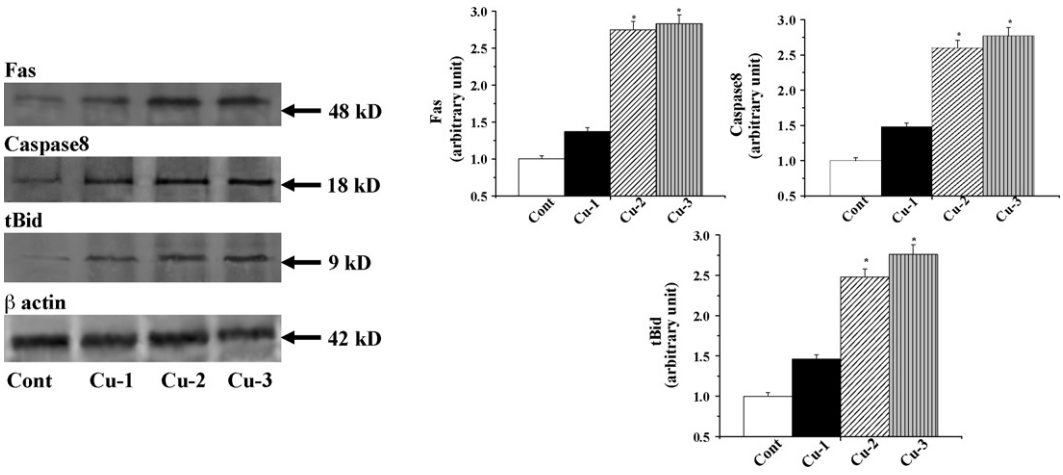

Fig. 7. Western blot analysis of Fas, caspase 8, tBid in the kidney tissue of nano copper intoxicated animals. The relative intensities of bands were determined using NIH-image software and the control band was given an arbitrary value of 1. Cont: normal mice; Cu-1, Cu-2 and Cu-3: animals administered with nano copper particles at a dose of 200, 413 and 600 mg/kg body weight. Data are mean ± SD, for 6 animals per group and were analyzed by one-way ANOVA, with Student–Newman–Keuls post hoc tests. Differences were attributed at $p < 0.05$, and homogeneous subgroups share common superscripted letters.

involvement of the mitochondria dependent signaling cascades in this pathophysiology.

3.11. Involvement of extrinsic apoptotic pathway

To determine whether the extrinsic apoptotic pathway was also involved in nano-copper induced renal pathophysiology, the expressions of various crucial factors which mediate the extrinsic apoptotic pathway, including death receptor Fas, caspase 8 and tBid were examined in the present study. Results showed that nano-copper exposure significantly increased cellular level of Fas (protein), caspase 8 (protein) and tBid (protein). Fig. 7 suggesting the involvement of extrinsic apoptotic signaling pathway in the pathogenic action of nano-copper.

3.12. Potential mechanisms activated by nano-copper particles

We investigated the effect of nano-copper on the expression patterns of PI3-K and phospho Akt protein in kidney pathophysiology. Nano-copper significantly decreased PI3-K and phospho Akt protein level and one of its downstream substrate phospho Bad protein (Fig. 8A–C).

3.13. Histological assessment

Histological study of different kidney segments of the normal and experimental animals has been presented in Fig. 9. We observed a loss of integrity around and into the glomerulus suggesting the damage of kidney cells in the nano-copper induced renal dysfunction.

4. Discussion

Along with the beneficial role of nanotechnology, the adverse effect of nanomaterials in humans is a serious growing problem. Research works have been carried out to investigate the optimum dose, routes of exposure as well as the mechanism of the nanoparticles induced organ pathophysiology (Mohanraj and Chen, 2006). While the mechanisms to hepatic injury have been extensively studied (Manna et al., 2011), there are no such data on the molecular mechanisms of nano-copper induced nephro-toxicity. It is reported that after entering into the gastric lumen, nano-copper rapidly reacts with the hydrogen ions of the gastric juice and converts into its ionic states (Chen et al., 2007) leading to copper ion overload and metabolic alkalosis which contribute to nano-copper toxicity (Manna et al., 2011). On the other hand copper chloride itself exists in ionic state, so low dose of copper chloride is more severe than copper nano particle. The strong ionization potential of nano-copper particles helps developing copper ion overload and that in turn damages the cells.

In the present work, a dose dependent study was performed by estimating BUN and creatinine levels (Fig. 2) and the different doses taken for this study are well correlated with the doses taken by Chen et al. (2006). After dose dependent study 200, 413 and 600 mg/kg body weight nano copper were chosen for the further study. Blood urea nitrogen (BUN) and creatinine levels in serum were significantly increased in the group exposed to nano-copper, suggesting renal dysfunction in comparison to the control group. From our study it is also clear that the nano copper particles induce kidney toxicity and cell death via the activation of oxidative and nitrosative stress responsive cell signaling. We observed that exposure to copper nanoparticles dose dependently increased the intracellular ROS and NO production. Both oxidative and nitrosative stress altered the enzymatic and non-enzymatic antioxidant defense. We assayed the activities of the antioxidant enzymes SOD, CAT, GST, GR, GPx and the levels of the non-enzymatic antioxidant molecules GSH and its metabolite GSSG and observed that intracellular nano-copper overload decreased the activities of the antioxidant enzymes and the GSH level along with increased GSSG, lipid peroxidation and protein carbonylation. Since vitamin E treatment can prevent all these alteration related to oxidative stress induced by nano-copper, so it can be concluded that reactive oxygen species play a major role in copper nano induced renal toxicity (Fig. 10).

Increased level of ROS induces a cascade of pathways which in turn activate transcription of several genes; those genes change the cell survival regulatory pathways and ultimately lead to apoptosis. As TUNEL assay is generally considered as a reliable method for the assessment of DNA strand break (apoptosis). We, therefore, performed a TUNEL assay to confirm apoptosis and it found that nano-copper induced apoptotic cell death in kidney tissue.

Apoptosis could be mediated via both mitochondria dependent and independent pathways. Evidence suggest that the alteration in mitochondrial membrane potential is able to switch the committed cells to apoptotic death via oxidative stress responsive signaling cascades (Keeble and Gilmore, 2007), which is a mitochondria dependent pathway. The influence of Bcl-2 family proteins on mitochondria regulates the mitochondria dependent cell death as reported by Verzola et al. (2002). There are two classes of

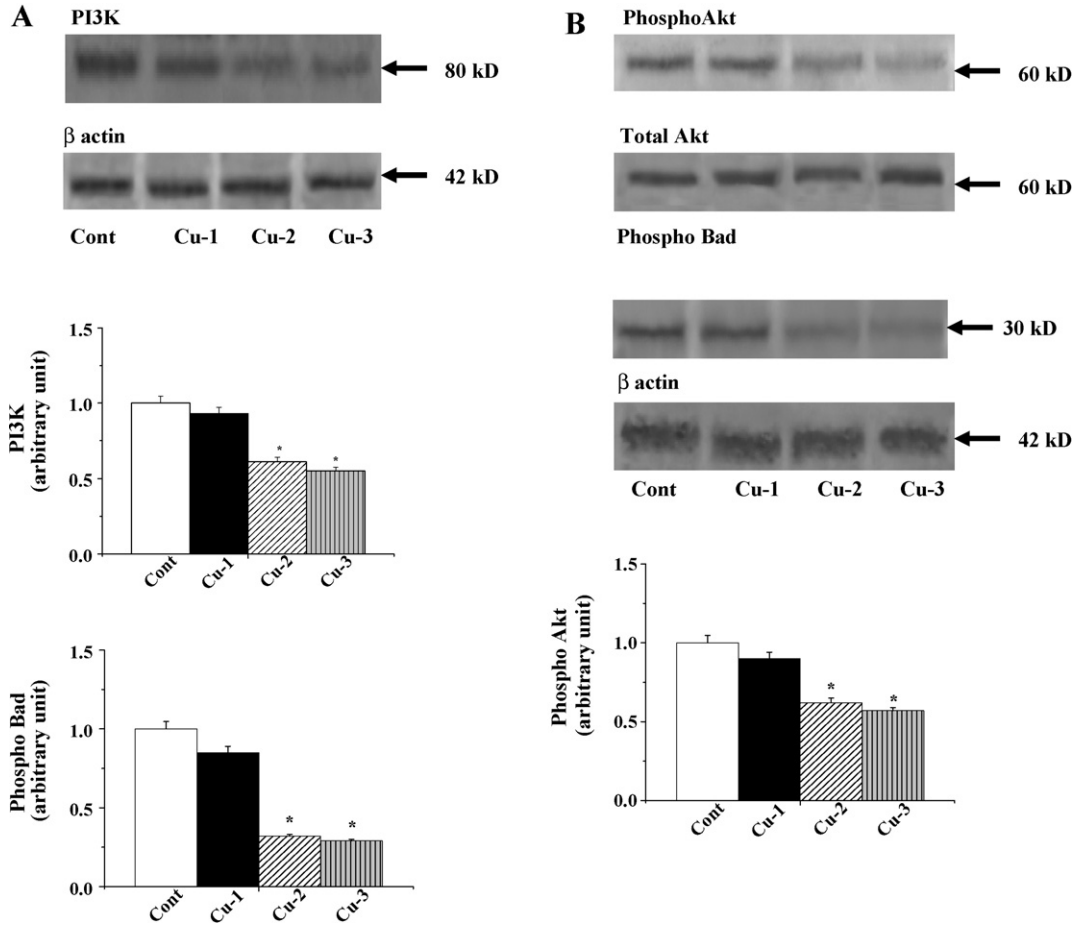

Fig. 8. (Panel A) Western blot analysis showing renal expression of phosphorylated Akt (phospho Akt), Total Akt and phospho Bad in mice treated with nano-copper. β-Actin was used as loading control. (Panel B) Western blot analysis showing renal expression of phosphoinositide 3-kinase (PI3K) in mice treated with nano-copper. β-Actin was used as loading control.

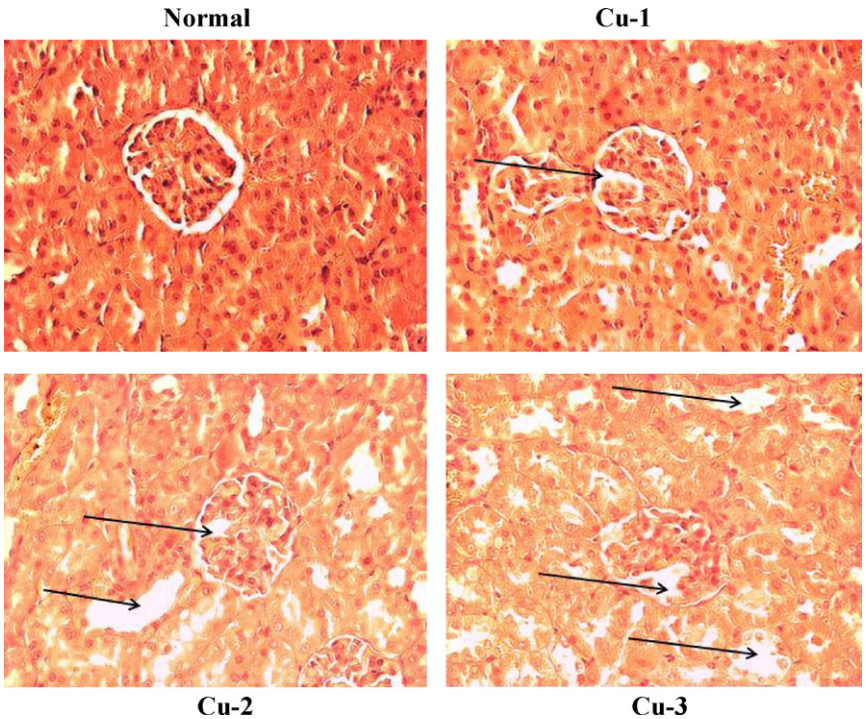

Fig. 9. Haematoxylin and eosin stained kidney section. Kidney section from normal mice showing normal organize appearance of glomeruli. Cu-2 and Cu-3 exposed mice, showing loss of integrity (marked with arrows). Whereas Cu-1 treated mice, showing little damage of glomeruli in kidney.

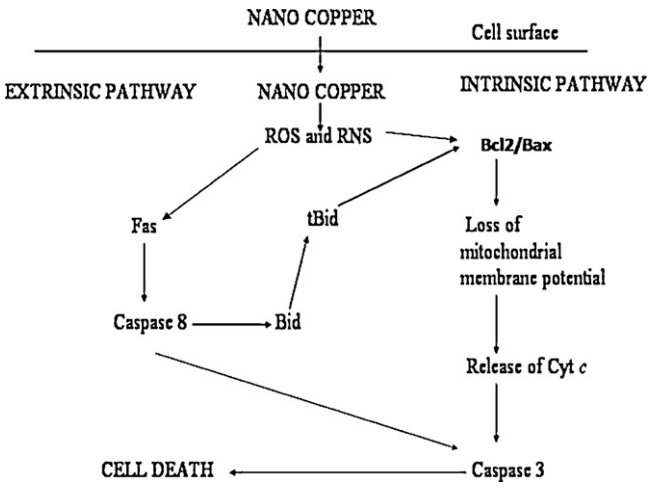

Fig. 10. Schematic diagram of the mechanism of the nano-copper induced nephrotoxicity.

regulatory proteins, anti-apoptotic protein which protects the cells from apoptosis, whereas the pro-apoptotic proteins promote the programmed cell death. When Bcl-2 protein expression is reduced and Bax protein expression is enhanced, there will be a decrease in mitochondrial membrane potential because of disruption of the mitochondrial membrane. This phenomenon helps releasing cytochrome *c* from mitochondria to the cytosol. After this, a cascade reaction takes place which involves cytochrome *c* binding with Apaf1 leading to the activation of active caspase 9 by forming an apoptosome complex that triggers the activation of caspase 3.

Present study demonstrated about the nano-copper particles induced mitochondria dependent cell death. We observed that exposure to nano-copper particles up-regulated the Bax protein and down-regulated Bcl-2 proteins. Alteration in the Bcl-2/Bax ratio caused reduction in mitochondrial membrane potential, leading to release of cytochrome *c* in the cytosol. Nano-copper particles induced loss in mitochondrial membrane potential and the expression levels of cytochrome *c*, Apaf1, caspases 9 and caspase 3 indicate about the intrinsic cell death pathway.

The extrinsic pathway is triggered by death receptor engagement (Fas, TNF, etc.), which initiates a signaling cascade mediated by caspase-8 activation. Active caspase-8 then affects downstream effector caspase which subsequently cleaves specific substrates resulting in cell death. The Bcl-2 family member Bid provides the link between the caspase signaling cascade and the mitochondria. Caspase-8 cleaves Bid to its truncated form (tBID) which in turn translocates to the mitochondria where it acts in combination with the pro-apoptotic Bcl-2 family member proteins to induce the release of cytochrome c and other mitochondrial pro-apoptotic factors like Smac/Diablo into the cytosol. In this study we also investigated the extrinsic pathway. Nano-copper exposure also significantly increased cellular level of Fas (protein), caspase-8 (protein) and tBid, suggesting the involvement of extrinsic pathway (mitochondria independent pathway).

The phosphoinositide 3-kinase (PI3K)/Akt signaling mechanism controls cellular processes such as death and proliferation. The PI3K/Akt cascade was demonstrated to be activated by stressful stimuli like reactive oxygen species (ROS). Normally Akt promotes cell survival by the phosphorylation of Bad protein which makes Bad dissociate from its complex and lose the pro apoptotic function thus helps in the cell survival. In our present study, PI3-kinase, phospho-Akt, phospho Bad levels were reduced in nano-copper exposed animals compared with the control groups which clearly indicate the involvement of these proteins in cell death pathway.

In addition, Western blot studies showed that nano-copper exposure increased NO production in the kidney tissue as evidenced from the over expression of the inducible nitric oxide synthase (iNOS) protein. Though NO can play dual role, either protective or harmful, in organ pathophysiology, we found that increased NO could not decrease the level of oxidative stress biomarkers in our studies. Therefore, these results are in favour of the possibility that overproduction of NO might play an important role in the pathogenesis of nano-copper induced renal damage.

In conclusion present study suggests the involvement of both mitochondria dependent and independent cell death pathways in kidney tissue due to oxidative and nitrosative stress after nano-copper exposure.

Conflict of interest statement

None declared.

Acknowledgement

The authors are grateful to Mr. Prasanta Pal for excellent technical assistance for the study.

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