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Evaluating the effect of green synthesised copper oxide nanoparticles on oxidative stress and mitochondrial function using murine model

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Abstract: Green synthesis of metal nanoparticles (NPs) has now received the attention of researchers due to ease of preparation and its potential to overcome hazards of these chemicals for an eco-friendly milieu. In this study, copper oxide (CuO) NPs were synthesised via *Desmodium gangeticum* aqueous root extract and standard chemical method, further characterised by UV–visible spectroscopy, Fourier transform infrared spectroscopy, X-ray diffraction, Thermogravimetric analysis and scanning electron microscopy. The nephrotoxicity of the NP obtained from two routes were compared and evaluated at subcellular level in Wistar rat, renal proximal epithelial cells (LLC PK1 cell lines) and isolated renal mitochondria. CuO NP synthesised by chemical route showed prominent nephrotoxicity measured via adverse cytotoxicity to LLC PK1 cells, elevated renal oxidative stress and damage to renal tissue (determined by impaired alanine transaminase, aspartate transaminase, urea, uric acid and creatinine in the blood). However, at the level of cell organelle, CuO NP from both routes are non-toxic to mitochondrial functional activity. The authors' finding suggests that CuO NP synthesised by chemical route may induce nephrotoxicity, but may be overcome by co-administration of antioxidants, as it is not mito-toxic.

1 Introduction

Over the past few years, copper oxide nanoparticles (CuO NPs) have generated considerable interest due to their potential applications in diverse fields including catalysis, semiconductors, microelectronics and sensors and also have been used as magnetic devices, as additives in lubricants, as nanoprobes in the bio-medicine analysis [1]. Due to the natural and unavoidable exposure of CuO NPs via above mentioned frequently used gadgets, the possibility of its intake either directly or indirectly through water, food, plants and animals, into the human system cannot be ruled out, and its potential risk from exposure must be further evaluated.

According to the literature, the various reducing and/or capping agents involved in the synthesis of copper nanomaterials possess adverse effect on human health. The traces of materials/solvent accumulated along with the product during the different steps of NPs synthesis process might be responsible for the toxicity [2]. In fact, previous findings supported that CuNPs showed size- and concentration-dependent toxicity in cell and genome [3, 4].

Among the diverse biosynthetic approaches, the use of plant extracts has raised the investigators' attention towards the green synthetic route, as they are safe to handle, readily available and have a broad variability of metabolites [5]. However, little attention has been paid to evaluate its toxicity and efficacy against chemically prepared NPs to replace them for safe applications.

Medicinal plants are widely used in traditional medical practice since prehistoric times, primarily due to its potential to synthesise hundreds of chemical compounds to defence against different microbial attack and herbivorous mammals, which was utilised by human beings for their self-defence. Desmodium species are one such medicinal plants that include *Desmodium latifolium*, *Desmodium triflorum* and *Desmodium gangeticum (DG)* and are well studied for the health benefit and have been exploited extensively for the biosynthesis of NPs. The previous study from our lab had shown that aqueous extract of DG root can be utilised to synthesise CuO NPs [6]. Similarly, we have shown that DG can protect renal cells from calcium oxalate mediated stone formation in renal tissue by reducing oxidative stress and inflammation [7, 8].

Pharmacodynamics studies have shown that CuNPs possess broad bio-distribution, enable them to get accumulated in different organ systems like kidney, liver, brain, heart, spleen and lungs, for 4 weeks upon a single exposure of CuNPs, which could activate toxic responses in respective tissue. In align to these observations, few studies have shown renal epithelial damage [9]. Thus in this study, the nephro-toxic effect of CuNPs prepared by both chemical and green routes (through an aqueous extract of DG) are evaluated by using three different models: (i) in vivo (Wistar rats), (ii) in-vitro proximal rat renal epithelial (LLC-PK1) cells and (iii) isolated renal mitochondria. This study provides information regarding biocompatability of CuO NPs for their further application.

2 Materials and methods

Copper sulphate ($CuSO_4$), polyvinylpyrrolidone (PVP), sodium borohydride ($NaBH_4$) and sodium hydroxide (NaOH) were purchased from Sigma-Aldrich, India. All chemicals used were of analytical grade. DG roots were collected, chopped and ground coarsely using mechanical pulveriser. Soxhlet extraction was carried out to obtain a crude aqueous extract. The obtained sample was freeze dried (lyophilised) for further use.

2.1 Synthesis of CuO NPs

CuO NPs (CuNP DG) were prepared using $CuSO_4$ (10 mM) as a precursor and aqueous DG root extract (25 mg/ml) as a bio-reducer in the ratio 1:4 and heated at 80°C to yield green CuO NPs (CuNP DG). The colour change from brown to black was observed confirming the formation of CuO NPs. Thus, obtained NPs were dried and stored [10]. In the synthesis of chemical CuO NPs, $NaBH_4$ (100 mM) was used as a reducing agent with PVP (20 mM) and NaOH (100 mM) as capping and stabilising agent, respectively [11]. The mixture was centrifuged to yield black coloured NPs and then dried for further use.

2.2 Characterisation of CuO NPs

The obtained CuO NPs were dried, characterised using UV–visible spectroscopy, X-ray diffraction, Fourier transform infrared spectroscopy (FTIR) and scanning electron microscopy [6]. The

Table 1 Blood and urine chemistry

Parameters	Normal	CuNP Chem	CuNP DG
serum urea, mg/dl	16.3 ± 1.2	38.7 ± 3.2*	48.0 ± 3.4*
serum creatinine, mg/dl	0.45 ± 0.05	0.16 ± 0.02*	0.16 ± 0.01*
serum uric acid, mg/dl	3.1 ± 1.1	0.20 ± 0.002*	0.14 ± 0.001*
AST, U/l	53.9 ± 3	50 ± 4	52 ± 6
ALT, U/l	49.1 ± 7	28.8 ± 3*	27.5 ± 3*
ALP, U/l	81 ± 5	67 ± 6	75 ± 7
seum urea/creatinine	36 ± 2	241 ± 24*	300 ± 28*
serum uric acid/creatinine	7 ± 0.9	1.2 ± 0.1*	0.8 ± 0.07*
urea clearance, ml/min/kg bw	8.45 ± 1.1	4.2 ± 1.1*	12.6 ± 1.0*
creatinine clearance, ml/min/kg bw	3.9 ± 0.2	2.2 ± 0.9	3.6 ± 0.4

Values are represented as mean _SD, $n = 3$.

(*) – $P < 0.05$ versus normal control; (#) – $P < 0.05$ CuO Chem treated group. ALT-Alanine transaminase; AST-Aspartate transaminase; ALP-Alkaline phosphatase.

Table 2 Antioxidant analysis in renal tissue, cytotoxicity analysis and mitochondrial enzyme analysis

Parameters	Normal	CuNP Chem	CuNP DG
oxidative stress markers			
TBARS, nmoles MDA/mg	0.1 ± 0.01	30.1 ± 0.01*	0.05 ± 0.01**
catalase, mU/mg	35 ± 1	17 ± 1.59*	27 ± 0.86**
SOD, mIU/mg	14 ± 0.7	9 ± 0.75*	14.4 ± 0.7**
GPx, nM GSH/mg	8 ± 0.4	3.9 ± 0.19*	7.2 ± 0.06**
mitochondria dysfunction			
MDH	25 ± 1.1	7 ± 1.2*	13 ± 1.96**
SDH	18 ± 0.9	8 ± 1.1*	17 ± 1.1**
NADH	40 ± 3.0	24 ± 3.3*	34 ± 3.5**
cytotoxicity			
LDH	128.6 ± 6.4	228.6 ± 3*	142 ± 7**
MTT (% cell viability)	96 ± 2	28 ± 5*	85 ± 4
mito-toxicity			
swelling (Δ A540)	0.20 ± 0.05	0.03 ± 0.001*	0.15 ± 0.01*
isolated mitochondria toxicity-SDH activity, U/ml			
	1 mg/ml	0.5 mg/ml	0.25 mg/ml
CuNP Chem	8 ± 1.1*	5 ± 0.5*	3 ± 0.2*
CuNP DG	17 ± 2.1**	14 ± 1.3**	11 ± 0.6**
control		17.1 ± 0.5	

Values are represented as mean _SD, ($n = 6$).

Activity is expressed as mol of succinate oxidized per min per mg protein for SDH; mol of NADH oxidized per min per mg proteins for MDH and mol of NADH oxidized per min per mg protein for NADH dehydrogenase.

(*) – $P < 0.05$ vs Normal control; (#) – $P < 0.05$ vs CuNP Chem.

absorption spectrum of the CuO NPs was recorded in the wavelength range of 300–800 nm, using UV–visible spectrophotometer (Perkin-Elmer Lambda 2). The crystallinity of synthesised CuO NPs was examined using X-ray diffractometry (Ultima IV, Rigako). Diffraction pattern was recorded in a wide range of Bragg angles (θ) using optimum operating parameters (40 kV and 30 mA) with the scanning rate of 2°min^{-1} and Cu Kα target (λ−1.5405 Å). To identify the functional groups interacting with CuO NPs, FTIR spectrum was recorded using the Perkin-Elmer FTIR system (Spectrum GX mode) in the frequency range of 400–4000 cm^{-1} at a resolution of 4 cm^{-1}.

2.3 In-vivo toxicity study

Male Wistar rats (260 ± 15 g, 8–10 weeks) were procured from the Central Animal Facility, SASTRA University, India (213/SASTRA/RPP/IAEC). Rats were housed in a humidified condition with 12 h dark/12 h light and were given free access to standard rat chow and water. The rats were then randomly grouped into control and treated, among which treated groups were dosed with (5 mg/kg b.wt; i.p.) CuNP Chem and CuNP DG and kept under observation for any behavioural changes for 2 weeks. On the 14th day, urine and serum were collected and analysed for functional enzymes like alanine transaminase (ALT), aspartate transaminase (AST), urea, uric acid, creatinine and alkaline phosphatase (ALP) using assay kits (SPAN Diagnostics Ltd, Gujarat, INDIA) (Table 1). Later, rats were dissected under anaesthesia [sodium thiopentone (i.p.)-80 mg/kg b.wt.] to excise two kidneys. The kidney was weighed and cut into two parts wherein one part was stored at −80°C for biochemical analysis and the other part was fixed in neutral buffered formalin for haematoxylin and eosin (H&E) staining.

2.4 Biochemical analysis

Renal tissue was analysed for thiobarbituric acid reactive substances (TBARS) [12], antioxidant enzymes like glutathione peroxidase (GPx) [13], catalase [14] and SOD activity [15] to determine the status of oxidative stress caused by exposing rats to CuO NPs.

2.5 In-vitro toxicity assay

Next, we set up an in-vitro experiment to compare the cytotoxicity effect of CuNP Chem and CuNP DG NPs. The kidney epithelial cells (LLC PK1; NCCS, Pune) were cultured in a 48-well plate (0.2×10^5 cells/well) using Dulbecco's modified essential medium (Gibco) supplemented with 10% fetal bovine serum (FBS) (Invitrogen), 1% penicillin/streptomycin (100 U/ml/100 µg/ml; Invitrogen) and maintained at 37°C. Once cells reached its 60% confluence, CuO NPs were added and incubated for 24 h. Then the media was assayed for LDH, a typical cellular damage marker. In contrast to the existing hypothesis, 'green route is safer than the chemical route', our results showed that green synthesised CuO NPs exhibit higher toxicity towards renal epithelial cells than chemically synthesised ones.

2.6 Isolated mitochondria toxicity

In continuation of cellular toxicity analysis, we designed an experiment to determine the toxicity at cell organelle level using mitochondria, as it played a key role in both physiology and pathology. Briefly, mitochondria were isolated from renal tissue and incubated with varying concentration of CuO NPs (CuNP Chem and CuNP DG) for 2 h (Table 2). After incubation, samples were assayed for (i) SDH activity – to assess the functional status of mitochondria [16] and (ii) calcium-induced swelling – to determine the integrity of mitochondria [17].

2.7 Statistical analysis

Values were expressed as the mean ± standard deviation (SD) of three individual experiments. Statistical significance (5%) was evaluated by one-way analysis of variance followed by Tukey test as post-hoc analysis using GraphPad Prism software (San Diego, CA).

3 Results

We observed that green synthesised CuO NPs exhibit sharp SPR band at 415 nm (Fig. 1*a*). The diffraction pattern of CuO NPs was matched with JCPDS pattern (file no. 04-0836) confirming the face-centred cubic structure. FTIR spectrum of CuO NPs is shown in Fig. 1*b*. The spectra showed the primary frequency band at 617 cm^{-1} which corresponds to CuO in Cu_2O phase. The frequency band at 2928 cm^{-1} refers to alkyl C–H stretching, and other frequencies (cm^{-1}) at 3416, 2924, 2854 and 1400 correspond to H–O–H stretching, O–H stretching of alcohols/phenols, alkyl C–H stretching and C–O stretching, respectively. The peak broadening was used to calculate average crystallite diameter of the CuO NPs (Fig. 1*c*). Stability of CuNP DG was determined by thermogravimetric analysis and found to be stable with only loss of water (Fig. 1*d*). Fig. 2 shows the scanning electron micrograph of CuNP DG, suggesting the round shaped CuO NPs.

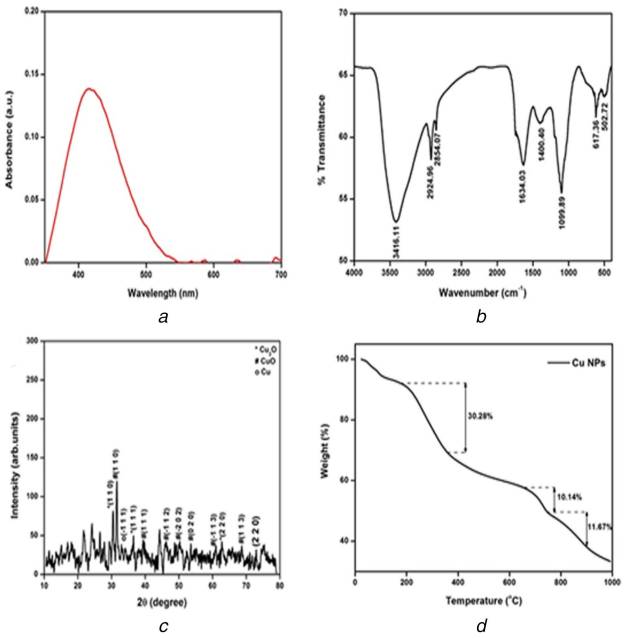

Fig. 1 *Characterisation of DG synthesised CuO NPs*
(a) UV–visible spectrum, ***(b)*** FTIR spectrum, ***(c)*** X-ray diffractometry, ***(d)*** Thermogravimetric analysis

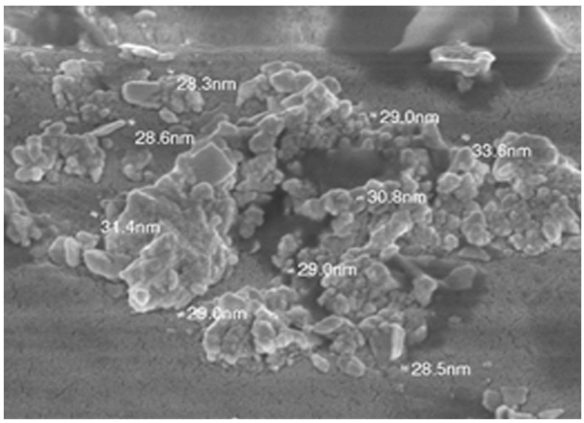

Fig. 2 *Scanning electron micrograph of DG synthesised CuO NPs*

Rats were administered intraperitoneally with a single dose (5 mg/kg b.wt.) of CuNPs synthesised by green and chemical route, and the physiological behaviour was observed for 15 days, and no mortality was reported. Histopathological examination of tissues is useful in identifying the type of lesions caused by xenobiotic and is acknowledged as the most sensitive endpoint for detecting organ toxicity, demonstrated nephrotoxicity for CuNP Chem treated group (Figs. 3*a*–*c*). Organ weights are widely accepted in the evaluation of toxicity and Fig. 3*d* shows the relative kidney weight, and the results suggest that CuNP DG shows a significant increase in kidney weight, whereas these changes were absent in the case of CuNP Chem treated rats. Moving further to confirm the toxicity, serum was analysed for renal markers. Serum ALT level was found to be decreased in both CuNP Chem, and CuNP DG treated rats, whereas AST remains same as that of control (Table 2). Increased urea content (CuNP Chem 57%; CuNP DG 66%) and low level of creatinine (64%) were observed in both CuNP Chem and CuNP DG groups. Rat urine collected on day 14 was analysed for uric acid, urea and creatinine. Clearance of urea was maintained in CuNP DG administered animals as seen in control, which was absent in CuNP Chem treated rats (Table 2). Looking into the creatinine clearance, which serves as an indicator of glomerular filtration rate, was found to be maintained in both CuNP Chem and CuNP DG treated rat kidney (Table 2). Further renal tissue was analysed for oxidative stress and its functional enzymes (Table 1). By the above result, CuNP Chem group showed significant ($P <$

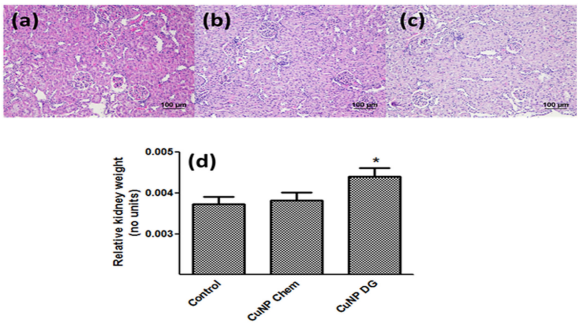

Fig. 3 *Histopathology and morphometric analysis*
(a) H&E stained kidney section of normal control rat, ***(b)*** Histopathology staining of kidney section of CuNP Chem treated rat, ***(c)*** H&E staining of renal tissue section of CuNP DG treated rat, ***(d)*** Relative kidney weight calculated as the renal weight to body weight ratio. * represents statistically different ($P < 0.05$) from the corresponding normal control

0.05) increase in MDA level (37%) and decreased catalase (51%), SOD (35%) and GPx (51%) activity, as compared to the control group (Table 1). Increased TBARS level is in agreement with the decreased GPx activity, which indirectly tells the unavailability of reduced glutathione, a potent non-enzymatic antioxidant. Further, to assess the toxicity at the cellular level, we treated renal epithelial cells (LLC PK1), with CuO NPs, in which CuNP Chem was found to be 78% toxic to the cells, by damaging the cell membrane (measured by higher LDH release into the culture medium) and caused 70% decline in cell viability (measured by MTT assay), as compared to the control (Table 1). Relatively, CuNP DG was found to be less toxic towards renal epithelial cells. As mitochondria is a key player in determining the injury; isolated mitochondria were incubated with both chemical and green CuO NPs, and we observed increased SDH activity in CuNP DG as compared to CuNP Chem (Table 1), indicating that CuO NPs prepared by green routes were non-toxic towards mitochondria, as compared to CuNP Chem.

4 Discussion

In this study, we determined the toxic effect of CuO NPs, prepared by both chemical and green routes in the different experimental models such as animal, cell culture and isolated mitochondria. Numerous literature supports the beneficial effect of green synthesised NPs with a protective effect on health care. Present study results show that green synthesised CuO NPs prepared using aqueous DG root extract exhibit significantly lower toxicity than the CuO NPs developed by the conventional chemical route (Table 2). Also, we found that CuNP DG has a better cellular and mitochondrial compatibility (Table 1). DG has been widely used in the Indian system of medicine and has been noted to have cardiotonic, hypolipidemic, anti-inflammatory effects [18], antioxidant activity [19], and is also known to act as a mitochondria protective agent [20]. The kidney is a primary site of chemical excretion, which results in its propensity to exhibit chemically-induced toxicological effects at a higher rate than most other organs. The pathological alterations in the renal architecture (Fig. 2) along with abnormal renal function tests (Table 2) confirm the toxic nature of copper NPs prepared by chemical route. According to Song *et al.* [21], the morphology and ion releasing rate of CuO NPs contributes significantly towards its toxicity. CuO NPs induce oxidative stress and can cause protein damage, DNA damage and cell membrane damage [22]. The toxicity and kinetics of CuO NPs are influenced by the release of Cu from CuO NPs and causes damage to kidney, liver and spleen [18]. It is well established that aqueous DG root extract possesses potent free radical scavenging ability that can reduce oxidative damage to protein, lipid and DNA. Also, our previous study shows that DG synthesised is monodispersive with excellent colloidal stability that prevents the release of Cu ions [6]. Cytoprotective effect of DG had been reported earlier in H9C2 [23], and our results in LLC PK1 cell line also demonstrated significant cell viability with DG synthesised CuO NPs as compared to chemically synthesised ones

(Table 1). This enhanced toxicity observed in CuNP Chem may be attributed to the constituents acting as capping/stabilisation agent used in the preparation period as reported earlier [24, 25]. The minimal toxic response exhibited by renal epithelial cells (LLC-PK1) towards CuNP DG (Table 1) might be attributed to the presence of alkaloids, phenols, sugars in the aqueous extract of DG root that has the potential to act as a capping agent. Having confirmed the less toxic behaviour of green synthesised NPs in both animal and cells (Tables 1 and 2), we further evaluated its deleterious effect on cell organelle such as mitochondria. Mitochondria were chosen, because of its involvement in cell death, mediated by necrosis, apoptosis and even membrane damage. We could not find a deteriorated mitochondrial function in renal tissues obtained from animals treated with DG synthesised CuO NPs as compared to chemical synthesised CuO NPs (Table 1). Aqueous extract of DG is already reported as a cardioprotective agent that can not only reduce the free radicals production but also preserve the mitochondrial enzyme activities. The NP-mediated oxidative stress is dependent on its accumulation in the macrophage [26] and subsequent tissue inflammation and changes in cellular redox balance. The reduced oxidative stress measured by TBARS and GSH level in DG synthesised CuO NPs signify the antioxidant potential of the NPs (Table 1).

5 Conclusion

Based on the above observations, we conclude that DG synthesised NPs are safe towards the renal function as compared to chemically synthesised CuO NPs, which again made a proof for the general notion, 'green synthesised nanoparticles are compatible with a biological system'. Thus, the biogenically prepared CuO NPs (via DG root extract) may be safe for the medical applications, especially for the targeted cancer treatment.

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