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Short Communication
Copper Oxide Nanoparticles Synthesized at Different pH Pose Varied Genotoxic Effects in *Allium cepa*

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Abstract

Background and Objective: Metallic nanoparticles including copper oxide nanoparticles (CuO NPs) are being used in different fields of research. The pH of precursor solution during NP synthesis may alter their morphology and final toxicity. In the present study, CuO NPs synthesized at different pH were evaluated for their toxicity using *Allium cepa* as test model. **Materials and Methods:** The CuO Nps were synthesized at pH 7 and 10 of the precursor solution. The nanoparticles were characterized for their morphology using scanning electron microscope (SEM), transmission electron microscope (TEM) and X-ray diffraction (XRD) analysis for their particle sizes. Different nanoparticle concentrations (0.1, 0.01 and 0.001 g/100 mL) were made and used for exposure treatments using *Allium cepa* to assess their genotoxic effects. **Results:** The CuO nanoparticles showed a dose dependent toxicity in *Allium cepa* root cytological analysis test. Significantly higher chromosomal damage in the form of clumped chromosomes, chromosomal bridges and diagonal mitotic spindles was observed in exposed roots as compared to controls ($p<0.05$). Moreover, nanoparticles synthesized at pH10 posed higher toxicity than the sample prepared with pH7 at both the observed exposure durations. **Conclusion:** The CuO NPs were found to pose genotoxic effects in *Allium cepa* root analysis test. Moreover, nanoparticles synthesized at pH10 were found to be more toxic as compared to pH7 samples. Hence, pH of the precursor solution may play an important role in determining the crystallite size of nanoparticles and hence their terminal toxicity.

Key words: Copper oxide nanoparticles, metal oxide, genotoxicity, chromosomal aberrations, *Allium cepa*, toxicity

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

There has been a rapid global escalation in the manufacture of functionally divergent nanoparticles in recent times. As production and research in the field of nanoparticles is increasing day by day, the studies in the field of toxicology of various nanoparticles are also emerging[1-3]. These researches are the need of the hour as these may be helpful in finding out the possible health hazards caused by nanoparticles. The present study is a part of the same research chain to find out the toxicological aspects of copper oxide nanoparticles (CuO NPs) employing *Allium cepa*.

Developments in shaping the nanoparticles for better performance is increasing due to their wide applications in various fields including sensing technologies, sieving properties, electronics and biomedical applications[1]. Their exposure is also increasing to humans as well as animals. Nanoparticles used in industry, medicines and research ultimately become a part of the environment and undergo different physical and chemical changes. These nanoparticles present in the environment may enter the human body through different routes including skin, oral route or inhalation[4]. This entry of nanoparticles into the human body may lead to toxicity in the form of protein misfolding, DNA damage, mitochondrial damage, membrane damage or production of reactive oxygen species. Due to large surface area, nanoparticles pose greater toxicities as compared to their larger counterparts.

Metallic nanoparticles are a class of nanoparticles synthesized for their particular functions in the field of semiconductors, thermoelectric materials or biomedical fields[5-8]. Nanoparticles have also been used due to their antibacterial property[3]. These have been employed in the field of drug delivery systems to increase the efficiency of medicines[9]. Metallic and metal oxides based nanoparticles have been found to pose detrimental effects on the cells including DNA breaks, DNA oxidation, mutations, apoptosis, necrosis and reduced cell viability. These nanoparticles can cross biological membranes and can have access to the cellular contents. CuO NPs are one of the intensively used metal oxide nanoparticles[10-13]. These nanoparticles have been utilized in superconducting materials, glass, sensing materials and antimicrobial applications[1]. Extensive mass production and the uses of CuO NPs increases the chances of their exposure and subsequent hazardous effects to various animals including humans. Various studies have reported the toxic effects of CuO NPs on different organisms[14-19]. In this study, an attempt has been made to find out the toxicity of synthesized CuO NPs in the form of chromosomal aberrations using *Allium cepa* root cell analysis. Moreover, toxic effects of CuO NPs synthesized at different pH of precursor solution were also compared.

MATERIALS AND METHODS

The present study was conducted at the Department of Zoology and Physics, Khalsa College, Amritsar. The study took 14 months from March, 2017 to April, 2018 which included synthesis and characterization of nanoparticles, their toxicological assessment and statistical analysis.

Copper oxide nanoparticles: CuO Nps were synthesized using sol-gel auto combustion method in the form of powder using hydrated cupric nitrate ($Cu(NO_3)_2.3H_2O$) and citric acid monohydrate ($C_6H_8O_7.H_2O$) at pH 7 and 10 of the precursor solution. The CuO powder samples were designated as CU1 and CU2 corresponding to sample synthesized at pH value of 7 and 10, respectively.

Characterization of nanoparticles

X-Ray diffraction analysis: The phase identification of the powder samples for pure CuO phase was performed by X-ray diffraction (XRD) on a X' Pert Panalytical diffractometer using Cu K_α radiation ($\lambda = 1.5405$ Å, 30mA , 40 kV) in 2θ range from 30-80°.

FESEM and TEM analysis: The surface topography of CuO powder samples was studied by scanning electron micrographs taken using JEOL JSM-6700F with a beam voltage of 30 KV. TEM images were taken using transmission electron microscope system (HRTEM, model FEI Technai 30) operated at 300 kV.

Treatment sample preparation: Three concentration groups per CuO powder samples, CU1 and CU2 were made and named as per the Table 1.

Allium cepa root test

Test material: Onion bulbs (*Allium cepa* L.) of average size (20-30 mm diameter) were purchased from the local market. The onion bulbs were sun-dried for 2 weeks. The roots of the dried bulbs were shaved off from the base with a sharp blade. This exposed the fresh meristematic tissues and the bulbs were placed in distilled water to protect the primodials from drying up.

Table 1: Formulation of different treatment concentrations of CuO nanoparticles solutions

CuO powder sample	Concentration (g/100 mL)	Sample annotation
CuO pH7 (CU1)	0.001	CU1C1
	0.01	CU1C2
	0.1	CU1C3
CuO pH10 (CU2)	0.001	CU2C1
	0.01	CU2C2
	0.1	CU2C3

Treatment of test material: Before exposing the onion bulbs to nanoparticle samples, their bases were dipped in tap water for 5 days which allowed normal root growth. After 5 days, excess water was removed with a blotting paper and the bases of the onion bulbs were dipped in solutions of all the test nanoparticle samples as described in Table 1. The treatment in the experiment was run for 7 days in dark. A series of 7 onion bulbs were used for each sample concentration and control (tap water). After the exposure durations of 3 and 7 days, out of the seven exposed onion bulbs, best 5 onions in terms of average root length were chosen for cytological analysis.

Cytological analysis: For the analysis of chromosomal aberrations, the tips of the emerged roots from the onion bulbs exposed to different sample concentrations were cut and fixed in ethanol: Glacial acetic acid (3:1, v/v). This procedure was done for all the five selected onion bulbs in each category of treated and control samples after 3rd and 7th day. After fixation, the root tips were hydrolyzed in 1N HCl at 60°C for 5 min and were washed with double distilled water. Root tips were squashed on a microscopic slide and stained with aceto-carmine for 10-15 min. The stained slides were covered with cover slips and were sealed to prevent moisture loss. Slides were analyzed at 1000X magnification on a trinocular microscope (Olympus, CH21) fitted with a digital camera (Olympus, MIPS). Five slides for all the five chosen onions in each treatment category were observed for presence of any chromosomal damage.

Statistical analysis: The difference in various cytological parameters between the control and exposed groups was analyzed using Mann-Whitney U test. Mean±SE were found using descriptive analysis and $p<0.05$ was considered as the significant level of the statistical analysis. All statistical analyses were performed using the program Minitab version 16.1.0 (Minitab Inc.) for windows.

RESULTS

XRD analysis: The XRD diffractograms of the CuO samples (CU1 and CU2) are shown in Fig. 1. The diffractogram reveals the polycrystalline nature of both the samples. Among the various diffraction peaks, two prominent peaks from (002) and (111) atomic planes of CuO lattice and it reveals the probable grain growth direction, as well as the most stable minimum energy growth phases of the crystal. No other peak corresponding to any undesired phase of Cu or Cu_2O has been noticed.

The crystallite size in CuO samples was evaluated using the full width at half maximum β (FWHM) from the most prominent (002) peak by using the Scherrer's formula as:

$$D = \frac{0.9\lambda}{\beta\cos\theta} \quad (1)$$

where, $\lambda = 1.5405$ Å for Cu K_α radiations and θ is the Bragg's angle. The values of lattice parameters calculated for both the nanoparticle samples are tabulated in Table 2. The crystallite size in samples was found to be less (28.24 nm) in case of pH 10 precursor solution (CU2) as compared to CU1 (66.32 nm). Thus increase in pH of the precursor solution suggested a decrease in crystallite size. The lattice parameters of the CuO samples ($a \neq b \neq c$, $\alpha = \gamma = 90° \neq \beta$ for monoclinic structure) have been calculated using the relation:

$$\frac{1}{d^2} = \frac{1}{\sin^2\beta}\left(\frac{h^2}{a^2} + \frac{k^2\sin^2\beta}{b^2} + \frac{l^2}{c^2} - \frac{2hl\cos\beta}{ac}\right) \quad (2)$$

where, d is the inter planar spacing, h, k, l are the Miller indices of the crystal planes, a, b, c, β are the lattice parameters. The values of the lattice parameters are recorded in Table 2.

SEM and TEM analysis: The SEM and TEM images of both the CuO samples are shown in Fig. 2 and 3, respectively. The agglomeration of particles in the CU1 sample (Fig. 2a) is higher as compared to the CU2 sample. The decrease in agglomeration in case of CU2 as observed in the SEM images (Fig. 2b) of the sample prepared at pH value of 10, may be attributed to the large quantity of the gas evolved in the exothermic auto combustion reaction. A high pH value of the precursor solution results inloosely agglomerated with sharp particle distribution in the resulted sample as revealed by the TEM images embedded in Fig. 3.

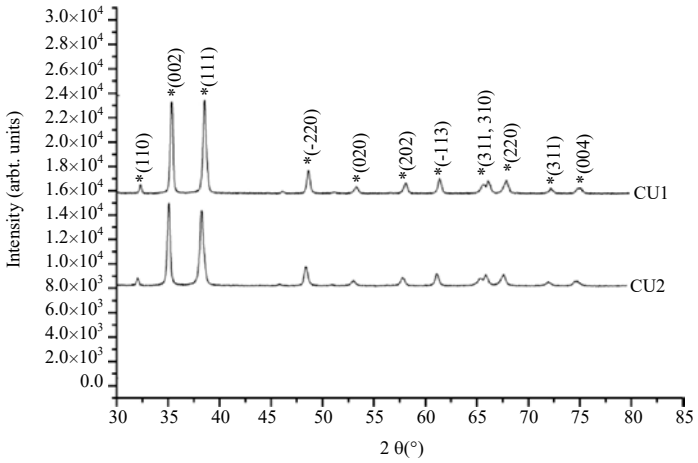

Fig. 1: XRD spectrum of CuO samples synthesized at pH7: CU1 and pH10: CU2

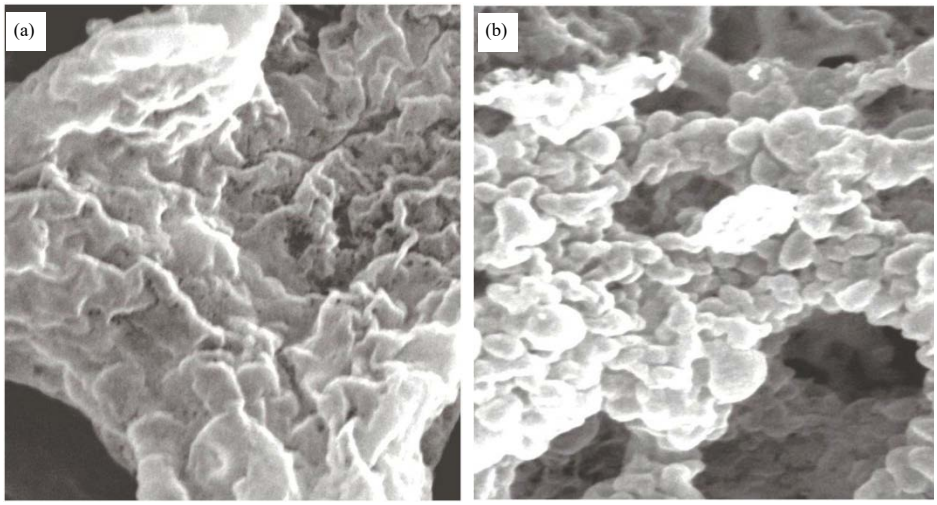

Fig. 2(a-b): SEM images of CuO nanoparticle samples (a) SEM image of CuO NPs synthesized at pH7 of precursor solution (CU1) and (b) SEM image of CuO NPs synthesized at pH10 of precursor solution (CU2)

Table 2: Values of the lattice parameters and crystallite size calculated from X-ray diffraction analysis and TEM measurement for CU1 and CU2 samples

Properties	CU1	CU2
a (Å)	4.578 (0.0005)	4.682 (0.0011)
b (Å)	3.414 (0.0018)	3.434 (0.0021)
c (Å)	5.225 (0.0040)	5.114 (0.0053)
β (Degree)	98.120 (0.0018)	99.102 (0.0017)
Dcrystallite size (nm) XRD (Scherrer's formula)	66.320	28.240
Crystallite size (nm) TEM	52.000	26.000

Cytological analysis: For the analysis of chromosomal aberrations, root tips were squashed on a microscopic slide and stained with aceto-carmine. The stained slides were analyzed at 1000X magnification on a trinocular microscope (Olympus, CH21) fitted with a digital camera (Olympus, MIPS). The slides for all the 5 chosen samples in each category were observed for chromosomal damage after 3 and 7 days of treatment.

3rd day observations: The *Allium cepa* root analysis showed a significant mean chromosomal clumping with exposures to both the nanoparticle samples types, CU1 and CU2 (Table 3).

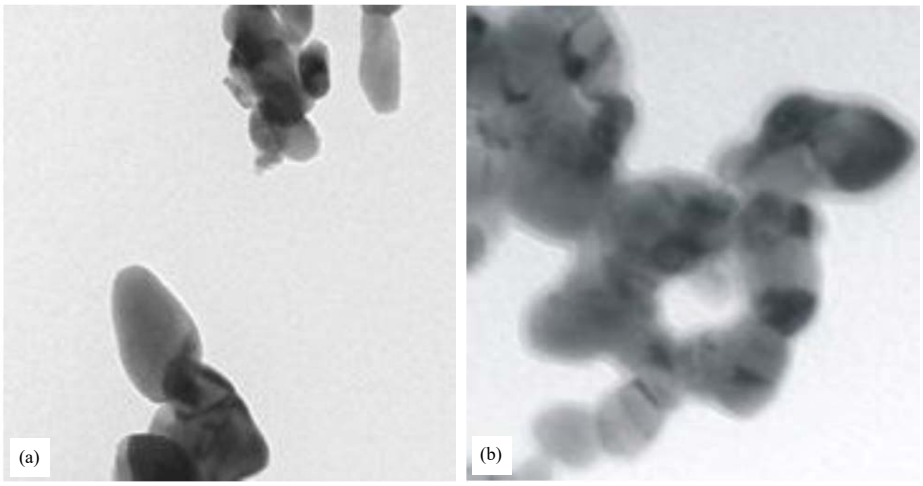

Fig. 3(a-b): TEM images of CuO nanoparticle samples (a) TEM image of CuO NPs synthesized at pH7 of precursor solution (CU1) and (b) TEM image of CuO NPs synthesized at pH10 of precursor solution (CU2)

Table 3: Mean chromosomal damage indices in different treatment concentration groups after 3 and 7 days exposure periods

Exposure period	Concentration treatment group	Chromosomal clumping	Chromosomal bridges	Diagonal spindles	Total abnormalities
3 days	CU1C1	3.6±0.40	2.2±0.52	3.1±0.62	8.9
	CU1C2	3.7±0.24	3.2±0.47	3.8±0.43	10.7
	CU1C3	5.8±0.37	3.6±0.43	4.6±0.67	14.0
	CU2C1	5.2±0.42*	2.6±0.57	3.8±0.51	11.6
	CU2C2	6.8±0.35*	3.5±0.51	4.2±0.36	14.5
	CU2C3	8.2±0.42*	4.2±0.52*	5.4±0.25	17.8
7 days	CU1C1	5.5±0.63	2.8±0.47	3.9±0.39	12.2
	CU1C2	7.3±0.65	3.6±0.36	4.7±0.35	15.6
	CU1C3	11.2±0.82	3.9±0.37	6.4±0.53	21.5
	CU2C1	8.3±0.42*	3.2±0.54	5.7±0.52*	17.2
	CU2C2	8.6±0.26*	3.8±0.47	6.8±0.57*	19.2
	CU2C3	13.7±0.45*	4.6±0.48	8.4±0.48*	26.7

*p<0.05, Significant difference as compared to corresponding CU1 sample on same exposure period. Total slides observed: 05, Total cells observed per slide: 100

At day 3rd, the minimum mean clumping was found in CU1C1 group (3.6±0.40) and the extent of chromosomal clumps increased with increasing concentration of nanoparticle samples. The maximum clumping was shown to be in group CU2C3 (8.2±0.42). The Cu NPs synthesized at pH10 of the precursor solution (CU2) showed a significant increased clumping at 3rd day as compared to the corresponding CU1 treatment groups. At maximum concentration, C3, the magnitude of mean clumping was found to be significantly higher in CU2 group as compared to CU1 (8.2±0.42 vs. 5.8±0.37, p<0.05, Fig. 4a).

Similarly, slight higher values for occurrence chromosomal bridges have been observed in CU2 group as compared to CU1. At lowest concentration, CU2 group shows higher minimum mean bridges (2.6±0.57) as compared to CU1 group (2.2±0.52) but failed to reach statistical significance (p>0.05). At highest concentration treatment, a statistically significant increase was found in chromosomal bridging (3.6±0.43 vs. 4.2±0.52, p<0.05, Fig. 4b). After 3 days treatment, a non-significant increase has been observed in occurrence of diagonal spindles among all the concentration groups (Table 1). Even at highest concentration the difference failed to reach statistical significance (4.6±0.67 vs. 5.4±0.25, p>0.05, Fig. 4c). On the whole, the observations after 3 days treatment of both types of nanoparticles revealed a dose dependent toxicity. The nanoparticles synthesized at pH10 (CU2) revealed higher values of chromosomal clumping, bridging and malformed spindles as compared to nanoparticles synthesized at pH7 (CU1). Figure 5 shows various chromosomal abnormalities on exposure to nanoparticle samples.

7th day observations: A significant chromosomal clumping was recorded with exposures to both the nanoparticle

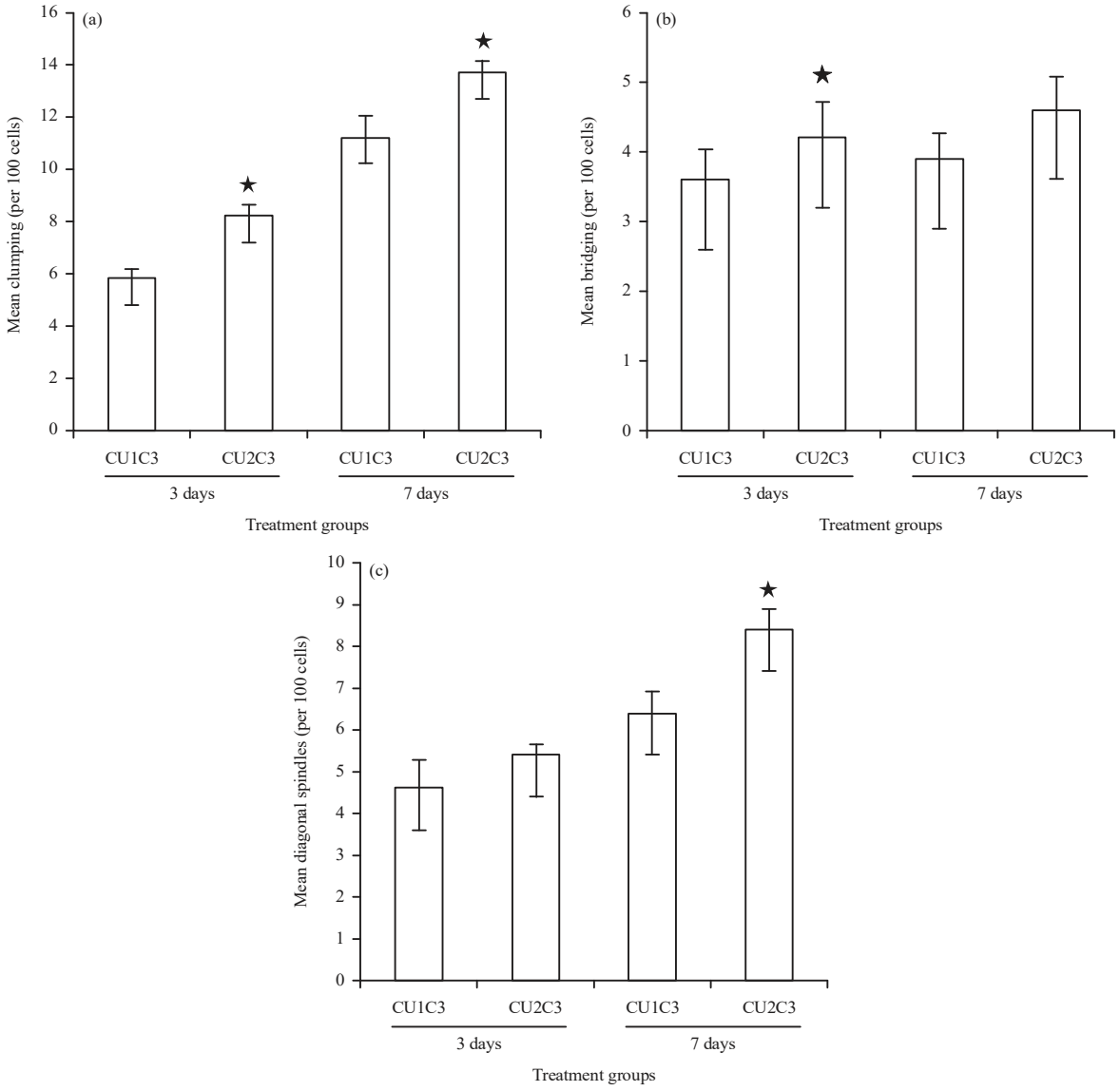

Fig. 4(a-c): Comparative mean chromosomal damage indices after exposure to both types of CuO nanoparticles (CU1 and CU2) at highest concentrations with respect to different exposure durations (a) Mean clumped chromosomes observed among highest concentration groups, (b) Mean chromosomal bridges observed at highest concentrations and (c) Mean diagonal spindles observed among highest concentration groups

samples types (CU1 and CU2) (Table 3). At day 7th, the minimum clumping was found in CU1C1 group (5.5±0.63) and the extent of chromosomal clumps increased with increasing concentration of nanoparticle samples. The maximum clumping was shown to be in group CU2C3. The Cu NPs synthesized at pH10 of the precursor solution (CU2) showed a significant increased clumping at 7th day as compared to the corresponding CU1 treatment groups. At maximum concentration, C3, the magnitude of mean clumping was found to be significantly higher in CU2 group as compared to CU1 (13.7±0.45 vs. 11.2±0.82, $p<0.05$, Fig. 4a).

Similarly, slightly higher values for occurrence chromosomal bridges have been observed in CU2 group as compared to CU1. At lowest concentration, CU2 group shows higher minimum mean bridges (3.2±0.54) as compared to CU1 group (2.8±0.47) but failed to reach statistical significance ($p>0.05$). Even at highest concentration treatment, a non-significant increase was found in chromosomal bridging (3.9±0.37 vs. 4.6±0.48, $p>0.05$,

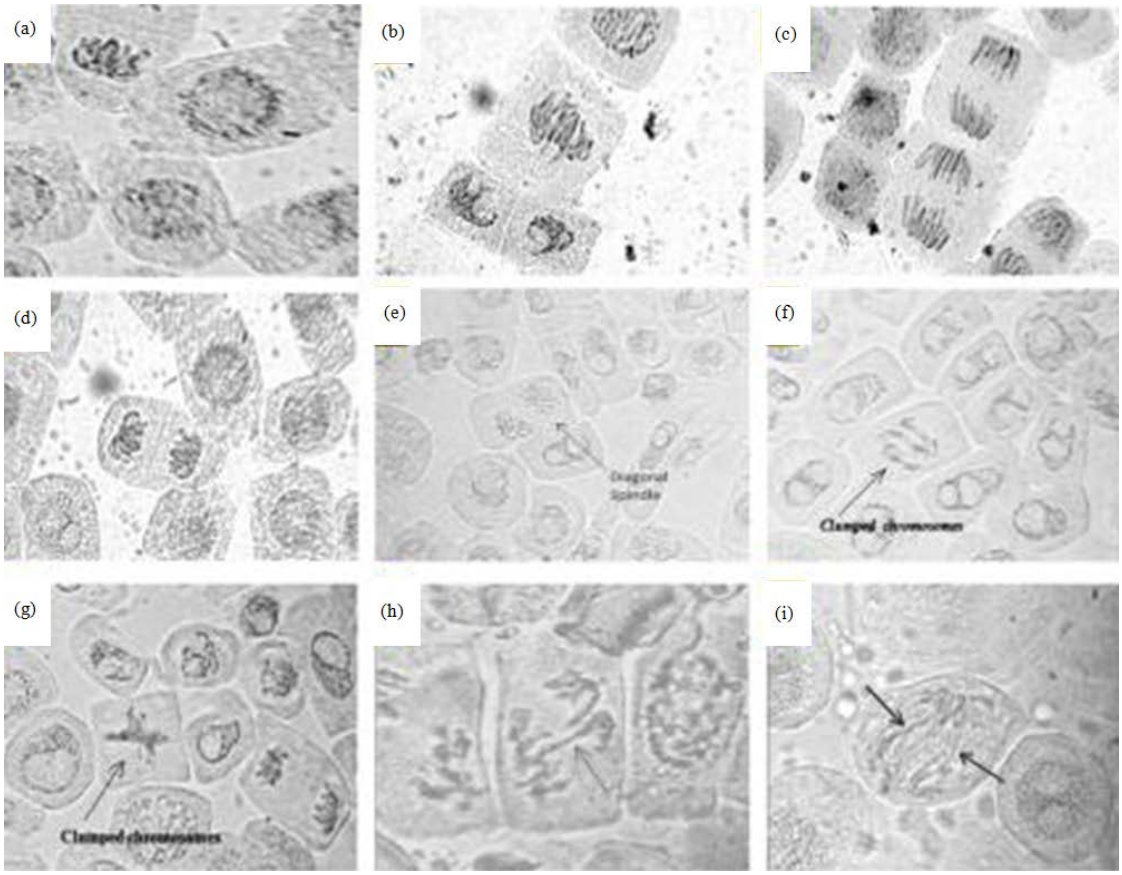

Fig. 5(a-i): Control and exposed cells showing normal and damaged chromosomes, (a) Control cells in prophase, (b) Control cell in metaphase, (c) Control cells in anaphase, (d) Control cell in telophase, (e) An exposed cell with diagonal spindle, (f) Exposed cell showing clumped chromosomes after 3 days exposure treatment, (g) An exposed cell showing clumped chromosomes after 7 day exposure treatment, (h) Exposed cell showing chromosomal bridge after 3 day exposure treatment and (i) Exposed cell showing chromosomal bridges after 7 day exposure treatment

Fig. 4b). After 7 day treatment, a significant increase has been observed in occurrence of diagonal spindles among all the concentration groups (Table 1, Fig. 4c). On the whole, the observations after 7 day treatment of both types of nanoparticles revealed a dose dependent toxicity. The CU2 samples revealed higher values of chromosomal clumping, bridging and malformed spindles than CU1 samples. As discussed earlier, particle size and agglomeration of CU2 samples was lesser in comparison to CU1, these properties might be enhancing the final toxicity of CU2 samples.

Total abnormality: After a treatment for 7 days, the total abnormalities were found to be increasing with higher exposure periods. Total abnormalities have been found to increase both dose dependently as well as with the type of the nanoparticles. The CU2 samples showed higher total abnormalities as compared to CU1 both on day 3rd and 7th. Figure 6 shows total abnormalities observed on treatment at different concentrations prepared.

DISCUSSION

Nanoparticles synthesized at pH10 of precursor solution have been found to pose significantly higher genotoxicity as revealed by higher chromosomal clumping, chromosomal bridges and diagonal spindles as compared to pH7 samples. In line with our results, a few studies also have demonstrated chromosomal abnormalities in *Allium cepa* root analysis[20-22]. CuO NPs have been reported to significantly reduce the mitotic index by 28% in *Allium cepa*[20]. Several types of chromosomal aberration such as bridges, stickiness, vagrant, broken and lag chromosomes were also noticed. In another study, onion roots treated with CuO NPs grew slowly after

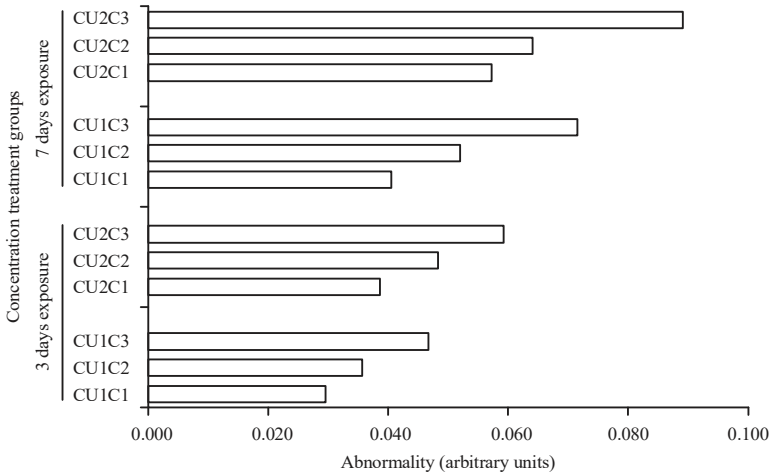

Fig. 6: Comparison of total abnormality among four different concentration treatment groups after 3 and 7 days exposure period

24 h as compared to controls[23]. Surface of the root cap and meristematic zone was damaged and the apical meristem of roots stopped division. Various deformations were also observed as nucleus of meristematic cells was deformed and nucleoli number was found to increase. Cell and nuclear membrane was fractured. With the increase of CuO NPs concentration, the root activity also decreased[23]. *Citrus medica* L. fruit extract-mediated Cu NPs were evaluated for its effect on actively dividing cells of *Allium cepa*. Results revealed that the elevated mitotic index up to the concentration of 20 µg mL^{-1}. A gradual decline in mitotic index and an increase in the abnormality index was observed as the concentration of CuO NPs and treatment duration were increased. Aberrations in chromosomal behavior such as sticky and disturbed chromosomes in metaphase and anaphase, c-metaphase, bridges, laggard, disturbed telophase and vacuolated nucleus were also observed[24].

The effect of CuO NPs on the growth and development of transgenic cotton has also been investigated. The height and the root length were found to decrease by 26.91 and 42.80% after 10-day exposure with 1000 mg L^{-1} CuO NPs[25]. Similarly, the effect of CuO NPs was investigated using *Phaseolus vulgaris* seeds. Seeds were soaked in CuO NPs at different concentrations of 25, 50 and 100 mg mL^{-1} for 15 and 30 min. The effect of nanoparticles on the plant physiological characteristics *in vivo* was detected. Results indicated that the concentration of 50 mg mL^{-1} at 30 min duration affected the physiological parameters including callus induction (%), callus fresh and dry weight[26]. Another study evaluated the effect of Cu NPs on the growth parameters, on the extent of leaves infected by powdery mildew and on spontaneous ectomycorrhizal colonization of English oak (*Quercusrobur* L.) seedlings. The TEM results showed disturbances in the shape of plastids, plastoglobules and the starch content of oak leaves treated with 50 ppm Cu NPs[27]. In another study, seedlings of *Brassica oleracea* var. botrytis and *Solanum lycopersicum* were exposed to 10, 50, 100 and 500 mg L^{-1} concentrations of CuO NPs in the sand medium. Plant exposure to 100 and 500 mg L^{-1} of CuO NPs had resulted in significant reduction of total chlorophyll and sugar content in the two test plants. Augmentation of lipid peroxidation, electrolyte leakage and antioxidant enzyme activity was observed in a dose dependent manner upon plants exposure to CuO NPs[28]. The impact of synthesized CuO NPs on germination and seedling growth of *Vignar adiata* were tested[29]. Germination and seedling growth of the plants were almost unaffected at lower concentrations while significant inhibition was recorded at highest 1000 mg L^{-1} concentration of NPs as compared to control. The amount of sugar and protein registered slight higher value at lower concentration and sharply decreased with increase in CuO NP concentration[29]. Thus, the present study clearly reveals the toxicity associated with CuO NPs at different concentrations. It also presents the effect of pH of precursor solution on the morphology and terminal toxicity of these nano-moieties. Future studies are recommended to find pH effects on nanoparticle synthesis and to endorse their nanotoxicity.

CONCLUSION

Copper oxide nanoparticles (CuO NPs) synthesized at pH7 and pH10 of precursor solution were tested for toxicity using *Allium cepa* root cytological analysis. The CuO NPs showed a significant genotoxicity in terms of chromosomal abnormalities in the exposed test material as compared to

controls. Moreover, nanoparticles synthesized at pH10 of precursor solution posed significantly higher genotoxicity as revealed by the raised chromosomal clumping, chromosomal bridges and diagonal spindles as compared to pH7 samples. Higher pH of the precursor solution may reduce the crystallite size of the nanoparticles resulting in higher genotoxicity. Results of the study clearly demonstrate the significant role of the precursor solution pH in determining the terminal toxicity of CuO NPs.

SIGNIFICANCE STATEMENT

Copper oxide nanoparticles are toxic to different animal and plant models. pH of the precursor solution during synthesis of the nanoparticles may change their morphological properties including crystallite size and agglomeration which may enhance their terminal toxicity. The present paper reveals the effect of precursor solution pH on CuO NP morphology and their geno-toxicological properties.

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