Mechanism of nanotoxicity in *Chlorella vulgaris* exposed to zinc and iron oxide

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**A R T I C L E   I N F O**

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**A B S T R A C T**

Usage of nanoparticle in various products has increased tremendously in the recent past. Toxicity of these nanoparticles can have a huge impact on aquatic ecosystem. Algae are the ideal organism of the aquatic ecosystem to understand the toxicity impact of nanoparticles. The present study focuses on the toxicity evaluation of zinc oxide (ZnO) and iron oxide (Fe_{3}O_{4}) nanoparticles towards freshwater microalgae, *Chlorella vulgaris*. The dose dependent growth retardation in *Chlorella vulgaris* is observed under ZnO and Fe_{3}O_{4} nanoparticles and nanoform attributed more toxicity than their bulk counterparts. The IC_{50} values of ZnO and Fe_{3}O_{4} nanoparticles was reported at 0.258 mg L^{-1} and 12.99 mg L^{-1} whereas, for the bulk-form, it was 1.255 mg L^{-1} and 17.88 mg L^{-1}, respectively. The significant decline in chlorophyll content and increase in proline content, activity of superoxide dismutase and catalase, indicated the stressful physiological state of microalgae. An increased lactate dehydrogenase level in treated samples suggested membrane disintegration by ZnO and Fe_{3}O_{4} nanoparticles. Compound microscopy, scanning electron microscopy and transmission electron microscopy confirm cell entrapment, deposition of nanoparticles on the cell surface and disintegration of algal cell wall. Higher toxicity of nanoform in comparison to bulk chemistry is a point of concern.

1. Introduction

Nanotechnology based-products inventory is growing rapidly. Current trend of using nanoparticles (NPs) has increased in almost every utility sector of mankind use [1]. Among the new promising metallic oxide NPs, zinc oxide (ZnO) has marked its place due to its amazing piezoelectric and pyroelectric characteristic properties [2]. Moreover, it possesses great excitation binding energy and a large band gap, wurtzite structure without center of symmetry which attributes for unique physiochemical properties [3]. Adding on greater surface per volume in its nano form also enhances its exotic properties and makes it viable and resourceful for multiple usage at larger scale. Presently, ZnO NPs are in uses as UV-filters [4], antimicrobial agent [5], bio-remediating agent [6], nano-fertilizers component [7], food-packaging purposes [8], in electronics, in textiles [9], for biomedical purposes [10] and so on. Earlier studies revealed that the ZnO NPs are kinetically active and undergoes different transformations which in returns causes toxicological issues. Dissolution, aggregation, agglomeration, adsorptions are the prime factors associated with the ZnO NPs toxicity. Still a clear-cut relationship about the mechanism of toxicity has not been established.

**Abbreviations:** ANOVA, analysis of variance; BG-11, blue green-11; BSA, bovine serum albumin; CAT, catalase; CDH, central drug house; DDW, double distilled water; ICSO, half maximal inhibitory concentration; Fe_{3}O_{4}, ferric oxide; FTIR, fourier-transform infrared spectroscopy; JCPDS, Joint Committee on Powder Diffraction Standards; LDH, lactate dehydrogenase; MDA, malondialdehyde assay; NADH, nicotinamide adenine dinucleotide (reduced form); NCBI, national center for biotechnology information; OD, optical density; NPs, nanoparticles; PBS, phosphate-buffered saline; PDI, polydispersity index; ROS, reactive oxygen species; SD, standard deviation; SEM, scanning electron microscopy; SOD, superoxide dismutase; TEM, transmission electron microscopy; UV, ultra violet; XRD, X-ray diffraction; ZnO, zinc oxide.

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with respect to algae [11]. The testified toxicity is changed with respect to the species [12,13], particle nature [13] and test methods [14]. Albeit toxicological studies regarding the effects of ZnO NPs had been performed on different algae earlier but not in term of growth kinetics of algae [15–20]. Applications of iron oxide (Fe$_2$O$_3$) NPs are also increasing due to its use for different purposes like in agriculture as fertilizers [21], for waste-water management [22,23] for number of biomedical applications [24,25]. Large amount of usage inevitably enhances the release of higher quantity of iron-based NPs into the environment [26]. The ample amount of usage of these NPs will ultimately sink into the aquatic environment. They are entering into the aquatic ecosystem and induces toxicity to the flora and fauna [27]. Overall potential impact of these NPs release into the aquatic environment is still unknown which escalates the concern to evaluate the possible risks regarding the aquatic entities [28]. As algae constitute the key source of biomass production which supports all higher trophic levels of the aquatic body, therefore, they are an ideal model organism to assess the impact of these metal oxide NPs [12,13]. In addition to that, algae have low nutritional requirements and easy in handling. It also allows whole life-cycle assessment within short period of time. For that reason, to assess the impact of NPs, algae seem to be an epitomic monitoring tool [29,30]. The aim of this study is to do comparative analysis of toxicity of NPs and bulk form exposure on algal growth kinetics. In order to investigate the unresolved mechanism of toxicity of NPs, impact in terms of morphology, physiology and biochemistry has been evaluated after treating the algal cultures with different concentrations of respective NPs and their bulk counterpart.

2. Materials and methods

2.1. Algal culture establishment

Algal sample was collected from the Fatehsagar Lake, Udaipur, Rajasthan, India. Further, *Chlorella vulgaris* alga is isolated, purified and axenic culture is established through serial dilution and plating in BG-11 medium with 7.4 pH [31]. Algal cultures were maintained in 250 mL Erlenmeyer flasks at 25 ±1 °C under 14.5 W m$^{-2}$ light intensity. Algal cultures were sustained through frequent sub-culturing in fresh media after harvesting of samples during exponential phase of previous cultures after measuring the protein value. All the experiments were performed under the same culture conditions in triplicates.

2.2. Nanoparticle characterization and dispersion

ZnO and Fe$_2$O$_3$ NPs were purchased from Sigma-Aldrich (CAS No. 1314-13-2–030-013–007; CAS No. 1317-61-9) and their bulk counterparts from Central Drug House Private Limited (CAS: 1314-13-2; CAS No. 1309-37-1). Stock solutions for both nano and bulk form of the metal oxides (10 mg each) were prepared in 100 mL deionized water. Stock suspension of ZnO nanoparticles was sonicated for 30 min at 40 Hz, and for Fe$_2$O$_3$ NPs, sonication was performed for 45 min at 60 Hz by using Probe sonicator (Q-500, Qsonica, USA). From these stock solutions, working solutions were made in BG-11 medium for toxicological assessment as per the tolerance range of the algae. NPs with-in the BG-11 media were also caracterized using dynamic light scattering (DLS) to estimate the particle size distribution and zeta potential (ZS90, Malvern, Instruments, UK)

2.3. Algae growth kinetics

Different concentrations of NPs and their bulk counterpart dispersed within the BG-11 medium were inoculated with algal inoculum (100 μg mL$^{-1}$ of protein value). Algal growth was evaluated on the basis of protein quantification [32,33] using bovine serum albumin as standard and absorbance of the samples were taken at 650 nm using UV–vis Spectrophotometer (Hitachi U2900). Protein content of treated cultures was measured at intermittent interval starting from 5th day of inoculation, and after every fifth day.

2.4. Proline content

Algal suspensions were harvested after 25th days of treatment and suspended into 10 mL sulhpo-salicylic acid [34]. Samples were centrifuged and 2 mL ninhydrin and 2 mL glacial acetic acid were added in 4 mL supernatant and placed on water bath for half an hour at 100 °C. Lastly, 4 mL of toluene solution were added in each sample and shaken well. Red chromophore layer of toluene was formed at upper surface which was used to take OD at 520 nm using UV–vis Spectrophotometer maintaining toluene as control.

2.5. Chlorophyll and carotenoids content

Total chlorophyll content was measured according to Porra et al. [35] and carotenoids content were estimated through Lichtenthaler and Wellburn [36]. In brief, algal cells (10 mL) were harvested after 25th days of treatment from cultures after centrifugation at 7000 rpm for 10 min. Then supernatant was discarded and pellets were washed with DDW. After washing, 10 mL methanol were added in and shaken thoroughly. Then, samples were placed on water bath at 60 °C for 15 min. Again, samples were centrifuged (4000 rpm for 5 min) and supernatant were collected to take OD at 663 nm and 645 nm. Remaining pellets were mixed in 10 mL DDW and placed at 4 °C for 1 h and centrifuged (4000 rpm for 5 min). Supernatant was collected and OD is taken at 470 nm. Methanol was considered as blank during all above estimation. Further pigment content is calculated using following equations: Total chlorophyll = 17.76 (A$_{664,6}$) + 7.34 (A$_{660,6}$), Carotenoids = (1000 x O. D$_{470}$ – 2.86 x Chl$_a$-129.2Chl$_b$/245) μg/mg.

2.6. Lipid peroxidation (Malondialdehyde Assay)

Lipid peroxidation was estimated by spectrophotometer method as described earlier [37]. In brief, 1.0 mL of algal cell suspension (after 25th days of treatment) was added in to 2 mL of trichloroacetic acid (20 %) and centrifuged for 45 min at 7000 rpm. Supernatant was collected and further added to 3 mL of 2-thiobarbituric acid (0.5 %) and heated for 10 min in boiling water bath. After cooling, absorbance was measured at 532 nm using UV–vis spectrophotometer.

2.7. Superoxide dismutase (SOD) enzyme activity

SOD activity was measured by adding 2 mL PBS buffer solution (0.5 M; pH 7.5) in 50 mg biomass of algal samples collected after 25th days of treatment. Samples were centrifuged for 10 min at 4°C at 13000 rpm and 100 μL supernatant was mixed with reaction mixture. Further, samples were incubated for 10 min at 37°C and absorbance were recorded at 560 nm [38].

2.8. Catalase (CAT) enzyme activity

To measure CAT activity after 25th day of treatment, 50 mg interacted algal biomass was mix in 2 mL PBS buffer (0.5 M; 7.5 pH). Samples were centrifuged at 12000 rpm at 4°C for 30 min and 100 μL supernatant was mixed in reaction mixture [39], containing 1.6 mL PBS (0.1 M, 7.8 pH), 100 μL EDTA (3 mM), 200 μL H$_2$O$_2$ (0.3 %). Absorbance was taken at 240 nm using UV–vis spectrophotometer and the activity was represented in terms of % decrease with respect to the control.

2.9. Lactate dehydrogenase (LDH) enzyme activity

Algal suspension after 25th day of treatment was centrifuged at 7000 rpm for 10 min and 100 μL supernatant was suspended in 100 μL of sodium pyruvate (30 mM) followed by addition of 2.8 mL of Tris–HCl
(0.2 M). Just before measuring the decrease in absorbance, about 100 μL of NADH (6.6 mM) was added and ten readings were measured at 340 nm using UV–vis spectrophotometer [40].

2.10. Microscopic analysis

To confirm the cytological damages, TEM (Tecnai, G-20 (FEI), USA) and SEM (Zeiss, Germany) analysis was performed. Algal cells treated with 5 mgL⁻¹ of ZnO and 25 mgL⁻¹ Fe₂O₃ NPs, bulk forms and control were harvested after 25 days, and used for TEM and SEM analyses. Ultrathin sections of the samples were obtained from ultramicrotome and shifted to copper grid for TEM analysis. For SEM study, air dried algal samples were subjected to gold sputter coating and analyzed.

2.11. Statistical analysis

All the experiments were conducted in triplicates. Mean, standard deviations, correlation and regression parameters were calculated using MS-Excel (office version 10.0). Statistically significant difference between control and treatment were analyzed using one-way ANOVA with the help of SPSS software version 17, with probability of error (P values) is taken as less than 0.5, for determining the significant differences.

3. Results and discussion

3.1. Nanoparticle characterization

Transmission electron microscopy (TEM) investigation uncovered polymorphic shaped ≤ 100 nm sized ZnO NPs, while, Fe₂O₃ NPs exhibited 50 nm size with nearly spherical shape (Fig. 1). XRD peaks of the NPs and bulk counterpart were observed in accordance with the standard graphs database of Joint Committee on Powder Diffraction Standards (JCPDS) (Fig. 1). In DLS study, hydrodynamic size of ZnO and Fe₂O₃ NPs were 249.1 nm and 900 nm, respectively, whereas, zeta potential was -25.5 mV and -19.9 mV for ZnO and Fe₂O₃ NPs in the working media. Results indicated that NPs possess enough charge on them to keep themselves suspended with in the media and avoid agglomeration.

3.2. Algal growth kinetics

Growth kinetics of Chlorella vulgaris were evaluated under ZnO and Fe₂O₃ treatments (both NPs and bulk) by monitoring the algal growth up to 35 days on the basis of protein value. Analysis revealed that the nano form of ZnO and Fe₂O₃ particles were more toxic than their bulk counterparts as observed in change of growth kinetics (Fig. 2). Moreover, perversiveness was found directly proportional to the concentration under bulk as well as nano treatments in both ZnO and Fe₂O₃ [41, 42]. In case of ZnO, at the highest concentration i.e., 5 mgL⁻¹, 42 % reduction in protein-based growth-rate was observed under NPs exposure, while nearly 35 % reduction is observed under bulk chemical, in comparison to control on the 25th day. At lower dose, i.e., at 2.5 mgL⁻¹ concentration, reduction in growth were almost same in both (nano and bulk form) which was about 21 % and 20 %, respectively. Whereas, at the lowest concentration of treatment, reduction was not found significant under nano as well as bulk. IC₅₀ in case of nanoform was found to
be 25th day = 0.258 mg L\(^{-1}\) and in case of bulk, IC\(_{50}\) values was 1.255 mgL\(^{-1}\). It is also found from the data that the inhibitory concentration was lowered down, with the respect to increase in the time of exposure, indicating that the time also plays a crucial role in attributing toxicity. Under Fe\(_2\)O\(_3\) treatment, it is observed that 25 mgL\(^{-1}\) concentration as sub-lethal for Chlorella vulgaris, as 26 % of the algal growth was found suppressed in nano-form, whereas, 25.44 % reduction is observed under bulk treatment in comparison to the control. Even at the lowest dosage of concentration i.e., 5 mgL\(^{-1}\), 11 % and 9.4 % reduction in algal growth rates were examined. Likewise, at 10 mgL\(^{-1}\), 12.5 % reduction under nano and 11 % less growth under bulk form were found. IC\(_{50}\) values was observed at 12.99 mg L\(^{-1}\) in case of nano and 17.88 mg L\(^{-1}\) in case of bulk form of Fe\(_2\)O\(_3\) treatment.

Under both metal oxide treatments, NPs exerted more toxicity and that was due to more interaction of NPs to algal surfaces as NPs having more surface area than its bulk forms [43]. Moreover, interactions were found dosage dependent as higher concentration increases the probability of more interactions of NPs towards algal cell surfaces [44,45]. In the case of ZnO, particles tend to lose the repulsive force with time, hence, tend to aggregate. Similar conceptions were also reported by few other studies performed under ZnO NPs [46–48]. Moreover, delayed in the onset of the exponential phase can also be accredited to the dissolution of ZnO NPs and its bulk form with in the algal culture media along with the aggregation under both of the cases. As the initial hours after the exposure, dissolution of zinc ions contributed toxicity within algal cells [49,50]. The dissolution of ZnO NPs is previously reported to be dependent on the initial exposure concentration [51]. Similarly, under Fe\(_2\)O\(_3\) NPs treatment, NPs adhered to the algal cell surface and established the primary contact which might have hindered the ion exchange of the cell membrane. Due to this interference, it might have affected the ion transport and damaged the membrane. Due to the presence of particles over the algal cells, it obstructed the nutrient exchange from the medium, which ultimately suppresses the metabolic activities and attributed to the toxicity [52,53]. Moreover, these interactions could play a vital role in developing oxidative stress (discussed below). Aggregation and adherence of NPs developed stronger interactions between the algal cell surface and particles due to which oxidative stress was observed within the treated algal cells [54]. The oxidative stress was mediated by the production and accumulation of ROS and later on confirmed through the various biochemical analysis and algal growth kinetics [55].

3.3. Proline content

Proline is known to play a role of antioxidant by mitigating ROS [56]. As ZnO NPs were reported to possess strong protein adsorption potential [57] which could lead to the elevated intracellular concentration of ZnO NPs within the algal cells and subsequently triggered the ROS production [58]. Increased level of proline content marked their role as ROS scavenger. Moreover, proline content increased in dose-dependent manner under both form (Table 1). Likewise, under the Fe\(_2\)O\(_3\) NP treatments, much higher levels of proline content were examined in comparison to bulk counterpart (Table 2). Results clearly revealed that the size of the particle plays a vital role in attributing toxicity as far as proline parameter is concerned [59]. Increased proline abates the oxidative stress generated by ROS within the treated algal cells, which was clearly distinguished from untreated algal cells in both forms.

3.4. Chlorophyll and carotenoid content

Chlorophyll content was found significantly reduced under both bulk
Biochemical parameters analysis of *Chlorella vulgaris* under the treatment of ZnO NPs and its bulk counterpart.

### Table 1

| ZnO treatment       | Proline (μg/mL) | Total chlorophyll (μg mL⁻¹) | Carotenoids (μg mL⁻¹) | MDA (μmol/g) | LDH (nmol/min mg prot) | SOD (μ mg⁻¹prot) | CAT (μ mg⁻¹prot) |
|---------------------|----------------|----------------------------|-----------------------|--------------|-----------------------|-----------------|-----------------|
| Control             | 0.04 ± 1       | 13.2674 ± 0.06             | 12.920 ± 0.05         | 0.1005 ± 0.3 | 0.005 ± 0.2           | 2.55 ± 0.3      | 0.05 ± 0.1      |
| ZnO NP (1 mg/L)     | 2.66 ± 0.1     | 9.1940 ± 0.5               | 15.7533 ± 0.08        | 5.3218 ± 0.3 | 10.26 ± 0.2           | 18.88 ± 0.3     | 6.26 ± 0.3      |
| ZnO NP (2.5 mg/L)   | 5.60 ± 0.2     | 6.8437 ± 0.2               | 18.2866 ± 0.06        | 9.4080 ± 0.4 | 12.22 ± 0.3           | 23.47 ± 0.2     | 8.36 ± 0.2      |
| ZnO NP (5 mg/L)     | 6.27 ± 0.1     | 3.6802 ± 0.3               | 22.7266 ± 0.05        | 13.5917 ± 0.3 | 19.69 ± 0.2           | 34.62 ± 0.3     | 11.79 ± 0.3     |
| P value summary     | P < 0.0001 ***| P < 0.0001 ***             | P < 0.0001 ***        | P < 0.0001 *** | P < 0.0001 ***        | P < 0.0001 *** | P < 0.0001 *** |
| Control             | 0.04 ± 0.1     | 13.2674 ± 0.06             | 12.920 ± 0.05         | 0.1005 ± 0.3 | 0.005 ± 0.2           | 2.55 ± 0.3      | 0.05 ± 0.1      |
| ZnO Bulk (1 mg/L)   | 1.75 ± 0.2     | 11.9047 ± 0.08             | 10.7266 ± 0.02        | 3.2280 ± 0.4 | 6.27 ± 0.3            | 10.86 ± 0.2     | 4.77 ± 0.2      |
| ZnO Bulk (2.5 mg/L) | 3.86 ± 0.1     | 9.9621 ± 0.01              | 14.3266 ± 0.03        | 7.3968 ± 0.2 | 9.44 ± 0.2            | 19.62 ± 0.2     | 5.87 ± 0.1      |
| P value summary     | P < 0.0001 ***| P < 0.0001 ***             | P < 0.0001 ***        | P < 0.0001 *** | P < 0.0001 ***        | P < 0.0001 *** | P < 0.0001 *** |

**Statistically highly significant difference.**

**Highly significantly different after analysis of variance.**

3.6. Lactate dehydrogenase (LDH) activity

Higher level of LDH enzyme activity is cue of cellular toxicity and under NPs treatments, higher LDH enzyme activity was observed, revealing that the NPs were more toxic than their bulk counterparts [71]. Membrane damage signifies the toxic effects generated after the exposure of ZnO/Fe₂O₃ NPs and its bulk counterpart [72]. Under NPs exposure, more membrane damage was apparent than the bulk. At similar concentration, NP treated cells was found to have higher LDH activity, in comparison to the both metal oxide bulk [51].

3.7. Superoxide dismutase (SOD) activity

In present investigation, dose dependent SOD activity was observed in the experiments. Even at the lowest concentration of NPs, enzymatic activity was found significantly higher than the bulk (Tables 1 and 2). Increase in SOD activity is directly related with the decrease in ROS content [73,74]. Higher activity of SOD converts O₂⁻ (superoxide) in to H₂O₂ (hydrogen peroxide). However, the amount of activity required to scavenge the ROS was not enough in the treated algae cells [38].

3.8. Catalase (CAT) activity

CAT enzyme activity was up-regulated during treatment with NPs and NPs exposure as shown in Tables 1 and 2 [60,61]. Under NPs exposure even at the lowest concentration (1 mgL⁻¹) significant reduction in chlorophyll pigment was observed. Concentration dependent decrease was observed, in both forms, however, in case of NPs more decline of chlorophyll pigment was apparent than the bulk [62]. Similarly, under Fe₂O₃, reduction in chlorophyll content was observed depicting the impairment in photosynthetic machinery in the treated algal cells. As the dose for exposure was increased, decrease in the chlorophyll content was recorded for both forms [63,64]. Contrary, carotenoids content was increased after the exposure under both of the treatments. Carotenoids are known as efficient quenchers of triplet state photosensitizers, singlet oxygen and radicals [65] which strengthen our view that this may be the defense mechanism of algae cells when exposed to metal. Similar observations reported earlier during heavy metal stress in algae [66,17,18].

### Table 2

| Fe₂O₃ Treatment       | Proline (μg/mL) | Total chlorophyll (μg mL⁻¹) | Carotenoids (μg mL⁻¹) | MDA (μmol/g) | LDH (nmol/min mg prot) | SOD (μ mg⁻¹prot) | CAT (μ mg⁻¹prot) |
|-----------------------|----------------|----------------------------|-----------------------|--------------|-----------------------|-----------------|-----------------|
| Control               | 0.108 ± 0.3    | 12.2674 ± 0.06             | 2.920 ± 0.05          | 0.05 ± 0.1   | 0.006 ± 0.2           | 1.95 ± 0.3      | 2.55 ± 0.3      |
| Fe₂O₃ NP (5 mg/L)     | 8.325 ± 0.3    | 8.1940 ± 0.5               | 15.7533 ± 0.08        | 3.26 ± 0.3   | 8.26 ± 0.2            | 8.68 ± 0.3      | 14.88 ± 0.3     |
| Fe₂O₃ NP (10 mg/L)    | 17.448 ± 0.4   | 7.8437 ± 0.2               | 12.2866 ± 0.06        | 7.36 ± 0.2   | 10.22 ± 0.3           | 9.44 ± 0.2      | 17.84 ± 0.2     |
| Fe₂O₃ NP (25 mg/L)    | 25.581 ± 0.3   | 6.6802 ± 0.3               | 17.7266 ± 0.05        | 9.79 ± 0.3   | 12.69 ± 0.2           | 14.62 ± 0.3     | 24.62 ± 0.3     |
| P value summary       | P < 0.0001 ***| P < 0.0001 ***             | P < 0.0001 ***        | P < 0.0001 *** | P < 0.0001 ***        | P < 0.0001 *** | P < 0.0001 *** |
| Summary               | P < 0.0001 ***| P < 0.0001 ***             | P < 0.0001 ***        | P < 0.0001 *** | P < 0.0001 ***        | P < 0.0001 *** | P < 0.0001 *** |

**Statistically highly significant difference.**

**Highly significantly different after analysis of variance.**

3.5. Lipid peroxidation (Malondialdehyde Assay)

Peroxidation of lipid molecules is an indication of oxidative stress. In the present investigation, increase in peroxidation with the increase in concentrations under both bulk and nano form of ZnO is reported (Table 1). Highest value of peroxidation was at 5 mgL⁻¹ under nano and bulk particles of ZnO. Dose-dependent nature of peroxidation was also observed in the previous studies [17,67,18]. Increased in both proline and lipid peroxidation with the increase in concentration pinpointing a correlation between ROS generation and their scavenging by proline. Moreover, lipid peroxidation has been reported to inactivate several essential enzymes and destabilizing the cell membrane which further diminishing the metabolic activity of the cells, therefore, became an elusive reason of growth retardation of algae under treatments [68]. In Fe₂O₃ NPs, more peroxidation was found compared to bulk at the same concentration (Table 2). As explained earlier, higher surface area enables NPs to attach more to surface of algae and endowed membrane destabilization through lipid peroxidation via ROS production [17,69,70,18].
and its bulk counterpart in dose dependent manner as compared to untreated cells (Tables 1 and 2) [75]. Highest activity of CAT was observed at 5 and 25 mgL$^{-1}$ of ZnO and Fe$_2$O$_3$ NPs. Although H$_2$O$_2$ is less toxic compared to O$_2$ and based on the growth retardation of algae, we foresee that higher accumulation of H$_2$O$_2$ may have spurred toxic effect to growing algae cells [76,77]. Thus, higher activity of CAT which we measured in the treated cells not adequate to reduce the oxidative stress of the algae cells in NPs treatment.

3.9. Microscopic analysis

Compound microscopic images revealed change in shape and colour of treated algal cells in comparison to the untreated and ZnO treatments (Fig. 3). Moreover, aggregation of ZnO NPs as well as bulk particles around the algal cells were also observed. Similarly, under Fe$_2$O$_3$ treatment, aggregation and agglomeration were found in nano and bulk particles (Fig. 3). This phenomenon of shading effect was found to play a prominent role in case of Fe$_2$O$_3$ exposure in attributing toxicity [78,79]. Scanning electron microscopic (SEM) analysis revealed the detrimental effects after the exposure under both nano as well as bulk ZnO particles. Cell lysis and cell membrane damage is observed from the micrographs (Fig. 3). Earlier studies revealed that, with respect to the time, aggregation tendency of ZnO NPs increases as the repulsive force in between the particles tend to decrease [80]. Further, interrupted growth is also due to the reduced availability of nutrients. As the surface area of algal cell is occupied by ZnO particles, interface area for nutrient exchange from the medium is reduced [81,82]. Likewise, under Fe$_2$O$_3$ NPs, cell lysis and cell injury, is observed from micrographs taken under 25 mgL$^{-1}$ treatment of both nano and bulk particles. Transmission electron micrographs (TEM) showed significant changes in cell morphology (Fig. 3). Cell organelles was found collapsed, cell shapes were deformed and plasmolysis is observed and these changes are more pronounced in nano form of treatment. Similar, cellular damages where cell integrity may be persistent but disruption within the cells were prominent have been reported by Xia et al. [83]. Cells were collapsed as the internal machinery was completely found destructed under the exposure due to stress.

3.10. Conclusion

The present investigation uncovered that ZnO and Fe$_2$O$_3$ NPs induces the antioxidant defense system of the cell. However, biochemical defense machinery is not sufficient enough to cope up with the stress at higher doses of treatment. The sub-cellular organelle damage, membrane disintegration, and oxidative stress are the main reasons for toxicity of the NPs to the algal cell at tolerance threshold level. This investigation uncovered that nano form, in general, is more toxic to the C. vulgaris as far as ZnO and Fe$_2$O$_3$ compounds are concerned and it has interceded fundamentally through oxidative stress. The higher interaction of NPs due to more surface area in comparison to the bulk form, led to diminishing in the nutrient exchange interface of the algal cells. This led to a reduction in the growth rate of the algae. The shading effect also contributed to the reduced growth rate of algae. The membrane damage as observed by lipid peroxidation, LDH activity and loss in the chlorophyll content further deteriorated the growth of the algae. Moreover, exposure to these chemicals led to the generation of ROS and oxidative stress.

Fig. 3. Microscopic images of Chlorella vulgaris under control and different treatment of nano/bulk particles after 600 h. Optical microscopic images: (a) Control (b) ZnO NP treated cells (c) ZnO bulk treated cells (d) Fe$_2$O$_3$ NP treated cells (e) Fe$_2$O$_3$ bulk treated cells. Scanning electron microscopic images: (f) Control (g) ZnO NP treated cells (h) ZnO bulk treated cells (i) Fe$_2$O$_3$ NP treated cells (j) Fe$_2$O$_3$ bulk treated cells. Transmission electron microscopic images: (k) control (l) ZnO NP treated cells (m) ZnO bulk treated cells (n) Fe$_2$O$_3$ NP treated cells (o) Fe$_2$O$_3$ bulk treated cells. Treatment level for ZnO was 5 mgL$^{-1}$ concentrations (both NP and bulk) and for Fe$_2$O$_3$ was 25 mgL$^{-1}$ concentrations (both NP and bulk).
stress. Accumulation of proline, increase in activity of SOD and CAT indicated that the extent to which these are needed to cope with oxidative stress are not sufficient in the algae cells treated with NPs. These parameters ascertained the algae cells treated with NPs remained under higher oxidative stress in contrast to bulk treatment. Microscopic images disclosed the structural damages to the subcellular organelle of the algae due to treatment of ZnO and Fe₂O₃, which were more distinct in the nano form than bulk.

Conflict of interest

The authors declare no conflict of interest.

CRediT authorship contribution statement

Pallavi Saxena: Investigation, Methodology, Writing - original draft. Vinod Saharan: Resources, Visualization, Data curation. Prabhat Kumar Baroliji: Resources, Validation. Vinod Singh Gour: Writing - review & editing. Manoj Kumar Rai: Formal analysis, Validation. Harish: Conceptualization, Supervision.

Declaration of Competing Interest

The authors report no declarations of interest.

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