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OXIDATIVE STRESS OF MICROALGAE *Chlorella vulgaris* BY ZINC OXIDE NANOPARTICLES

(Tekanan Oksidatif Terhadap Mikroalga *Chlorella vulgaris* oleh Zarah Nano Zink Oksida)

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Abstract

Nanoparticles, such as zinc oxide nanoparticles (ZnO NPs) have wide range of applications in the industrial and personal care products, but at the same time contaminates the environment. The presence of the nanoparticles causes negative impact to the aquatic ecosystem and the organisms within. In this study, the cytotoxic effects of ZnO NPs on the fresh water microalgae *Chlorella vulgaris* is reported. *C. vulgaris* cells were treated with 10 mg/L, 50 mg/L, 100 mg/L, 150 mg/L and 200 mg/L of ZnO NPs for 24, 48 and 72 hours. The cytotoxicity effect of ZnO NPs was assessed by measuring the fluorescence emission of chlorophyll using fluorescent spectrophotometer, algal biomass by spectrophotometer and cell viability through counting viable cells with cell count. The exposure to ZnO NPs caused decrease in chlorophyll emission, algal biomass and cell viability. The toxicity increased as the concentration and exposure duration of ZnO NPs increased. The toxicity of ZnO NPs was indicated by the deterioration of photosynthetic II reaction center (PSII) due to the production of reactive oxygen species through the oxidative stress induced by ZnO NPs on the tested cells. The impairment of photosynthesis and cell division resulted in reduced cell growth and chlorophyll production. This study showed the potential of *C. vulgaris* to be the bioindicator for ZnO NPs' toxicity.

Keywords: zinc oxide nanoparticles, *Chlorella vulgaris,* oxidative stress, bioindicator

Abstrak

Zarah-zarah bersaiz nano seperti zarah nano zink oksida (ZnO NPs) mempunyai pelbagai kegunaan di dalam bidang industri dan di dalam produk-produk penjagaan, tetapi pada masa yang sama memudaratkan alam sekitar. Kehadiran zarah nano tersebut memberi kesan negatif kepada ekosistem akuatik and organisma-organisma yang hidup di dalam ekosistem tersebut. Dalam kajian ini, kesan sitotoksik ZnO NPs terhadap mikroalga air tawar *Chlorella vulgaris* dilaporkan. *C. vulgaris* didedahkan kepada 10 mg/L, 50 mg/L, 100 mg/L, 150 nm/L, dan 200 mg/L ZnO NPs selama 24 jam, 48 jam, dan 72 jam. Kesan sitotoksik disukat melalui pendarflouran klorofil dengan menggunakan spektrofotometer pendarflour, biojisim dengan menggunakan spektrofotometer, dan daya hidup sel. Pendedahan sel kepada ZnO NPs mengurangkan pancaran pendarflour klorofil, biojisim sel, dan juga daya hidup sel. Kesan sitotoksik meningkat dengan kepekatan ZnO NPs. Ketoksikan ZnO NPs membawa kepada kerosakan pusat tindak balas II dalam fotosintesis dengan menghasilkan tekanan oksidatif yang membawa kepada penghasilan spesis oksigen reaktif di dalam sel. Kegagalan berfotosintesis dan pembahagian sel mengurangkan perkembangan sel dan klorofil. Kajian ini menunjukkan potensi *C. vulgaris* sebagai penunjuk biologi bagi ketoksikan ZnO NPs.

Kata kunci: zarah bersaiz nano zink oksida, *Chlorella vulgaris*, tekanan oksidatif, penunjuk biologi

Introduction

Nanotechnology involves the manufacturing of wide varieties of nanoparticles (NPs) with broad range of industrial applications in recent years. Zinc oxide nanoparticles (ZnO NPs) are widely used nanoparticles among the rapidly expanding list of engineered nanoparticles in the industrial and commercial products [1]. ZnO NPs are most commonly utilized in the production of pigments, semiconductors, rubber, solar cells, chemical fibers, electronic devices, sunscreens, food additives [2, 3] because of their chemical stability and adsorption ability [4]. The extensive application of ZnO NPs in the cosmetic industries results in the release of these particles into the aquatic environment through sewage of the industries, leading to concerns of their potential toxicity to human and environmental health [5]. Several studies have shown that ZnO NPs are toxic to algae [6], bacteria [7], crustaceans [8] and fish [9]. The large surface area of ZnO NPs endows them with high electron density and high reactivity to interact with biomolecules which contributes the high bio-toxicity. In addition to the physiochemical properties , ZnO NPs can also release free zinc ions which grounds the major toxic effects [10]. The chemical reactions occurring during the interaction of ZnO NPs with living cells cause oxidative stress and result in the increased formation of reactive oxygen species (ROS) [11]. Since microalgae are sensitive to the metallic contaminants than fish and invertebrates, they are the important organisms for monitoring water quality and aquatic toxicity [12]. Investigating the toxicity of ZnO NPs on algae is of greater importance and can potentially lead to strategies to assess the potential adverse effects of engineered NPs in the environment [13]. Hence in this study, the authors used *C. vulgaris* as a model organism for evaluating the toxic effects of ZnO NPs from 24 to 72 hours by investigating the variations in cell viability, algal biomass, and chlorophyll content before and after the treatment with NPs.

Materials and Methods

Establishment of algal culture

Fresh water microalgae *C. vulgaris* was obtained from Culture Collection of Algae and Protozoa (CCAP), United Kingdom. The algal cells were cultured in sterile Bold Basal Medium (BBM) at room temperature (23 ± 1 ^{o}C) in an agitator with 120 rpm under T5 fluorescent light illumination, with dark and light conditions maintained for 8 and 16 hours, respectively. The growth pattern of the algal cells was monitored to recognize the actively multiplying exponential phase of the cells.

Preparation of zinc oxide nanoparticles

Zinc oxide nanoparticles of 40 to 50 nm in diameter were purchased from Zhejiang Hongsheng Material Technology Co., China. The stock solution of ZnO NPs suspensions (500 mg/L) was prepared in algal culture medium BBM and sonicated for 30 minutes to avoid the aggregation of NPs in the solution [1].

Experimental treatment of algal cells

For this experiment, *C. vulgaris* cells were taken from a 3 –day –old culture with the aim of using the cells growing in the exponential growth phase [14] with initial cell density of 1 x 10^6 cells/ml [15]. A stock solution of ZnO NPs was diluted in 125 mL flasks to serial concentrations of 10 mg/L, 50 mg/L, 100 mg/L, 150 mg/L and 200 mg/L with 50 mL culture medium. The cells were cultured in 100 ml BBM in 250 ml Erlenmeyer flask with the presence or absence of ZnO NPs. *C. vulgaris* cells were exposed for 24, 48 and 72 hours to the increasing concentrations of 10 mg/L, 50 mg/L, 100 mg/L, 150 mg/L and 200 mg/L ZnO NPs. The algal cells without test nanoparticles was considered as the control sample. All the five treatments and one control with three replicates for each treatment and control were prepared. The experimental samples were kept in static condition with intermittent shaking at 8 hours' interval to prevent the aggregation of cells. The test samples were analysed for their toxicity after the specific interaction time along with control.

Toxicity assessment

The toxicity of ZnO NPs was assessed by analyzing the final and initial percentage of cell viability, algal biomass, and chlorophyll fluorescence emission after the specific exposure time of ZnO NPs on *C. vulgaris.*

Determination of cell viability and growth inhibition of algal cells

In order to investigate the cytotoxic effect of ZnO NPs on *C. vulgaris*, the growth inhibitory effect was studied using increasing concentrations of ZnO NPs (10-200 mg/L) according to OECD (2006) method. At the end of the

treatment time, aliquots of algal cell suspensions (ZnO NPs treated and untreated) were loaded into the cell count chamber (Neubauer, Marienfeld-Superior, Germany). The cells were counted in all 4 large corner squares under optical microscope (Nikon, Microphot-fxt, Japan) with high power lens (40 X). The number of intact cells without any distortion in the shape and size of algal cells was counted as viable cells [16]. After counting the number of cells and recording the data, the average number of cells was calculated using following formula: Cell density per ml= all cells counted in the large square x 10^4.

Algal biomass

The algal biomass was measured as an increase in absorbance at 685 nm in spectrophotometer (GeneQuant, GE, United States of America) using algal culture medium as blank. The experiments also included positive control (flask containing NPs in BBM medium with no algal cells) and negative control (flask containing algal cells in culture medium with no NPs). The negative control indicated the algal growth in the absence of NPs. The absorbance value of positive control was subtracted from the experimental values (flasks containing algal cells and NPs in culture medium) [17].

Measurement of chlorophyll emission

Inhibition in the photosynthetic activity of the algal cells when treated with nanoparticles was estimated using the fluorescence emission of chlorophyll as the biomarker for nanoparticles' toxicity on the photosynthetic system of the algal cells [18]. The chlorophyll content of the algal cells was determined using spectrofluorometer (GloMax Multi Jr, Pormega Biosystems, United States of America). The intensity of the chlorophyll fluorescence emission was measured at an excitation wavelength 430 nm and an emission wavelength of 663 nm with the algal culture medium as blank.

Statistical analysis

One-way analysis of variance (ANOVA) followed by t-test was used to determine the statistical significance of the differences between toxic effects of ZnO NPs in different concentrations and durations. The differences were considered significant, when $p < 0.05$.

Results and Discussion

Algal growth inhibition

The cytotoxic effect of ZnO NPs was assessed using viable cell counts. In the test cultures with the increasing concentration of ZnO NPs, the cell viability was found to have decreased from 24 h until 72 h with gradual increase in the percentage of inhibition of viable cells. The concentration and time dependent growth inhibition was reported as the concentration of ZnO NPs and the duration of exposure was increased. A significant ($p < 0.05$) inhibition of algal cells was observed from 6.44 %, 9.21 %, 14.15 % at 10 mg/L to 32.17 %, 43.79 %, 61.79 % at 200 mg/L for 24 h, 48 h and 72 h respectively. The individual toxicity of increasing concentration of ZnO NPs (10 – 200 mg/L) for the various treatments (24 h - 72 h) was presented in Figure 1.

The reduction in the actual number of viable cells for different treatments was presented in the Figure 2. From the results, it was evident that the number of algal cells died was dependent on the concentration and time of exposure to ZnO NPs. The highest reduction in cell viability was found at the higher concentration of 200 mg/L with the longest exposure duration of 72 hours in our study. A similar phenomenon was reported by Tang et al. [19] and Suman et al. [20] that ZnO NPs exhibited concentration and time dependent cytotoxicity on the cyanobacterium *Anabaena* sp. and *C. vulgaris,* respectively. Lee et al. [21] reported the growth inhibition of ZnO NPs on freshwater algae *Pseudokirchneriella subcapitata* and indicated that the toxicity was solely due to the release of Zn^+ from ZnO NPs. In addition, Manzo et al. [22] revealed the growth inhibitory effect of ZnO NPs on the marine algae *Dunaliella tertiolecta.*

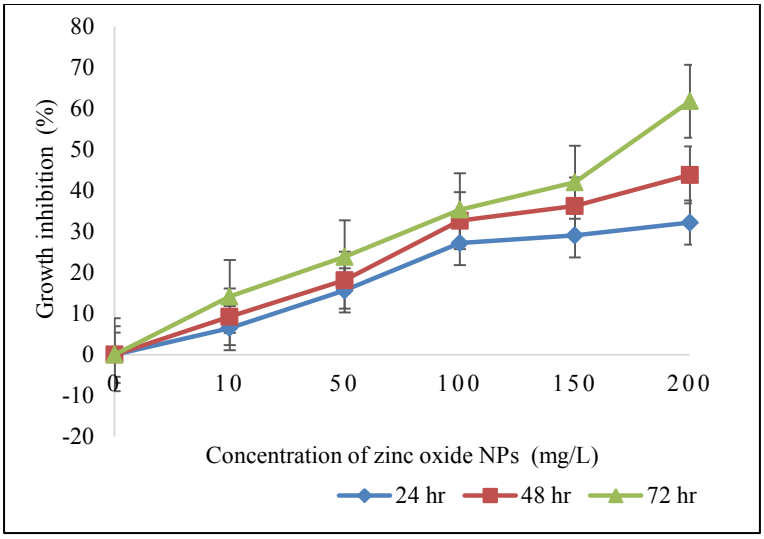

Figure 1. Time and concentration dependent growth inhibition of algal cells by ZnO NPs. The value is in percentage of algal cells' growth inhibition under various treatments with increasing conc. of ZnO NPs from 10 to 200 mg/L for 24, 48 and 72 hours.

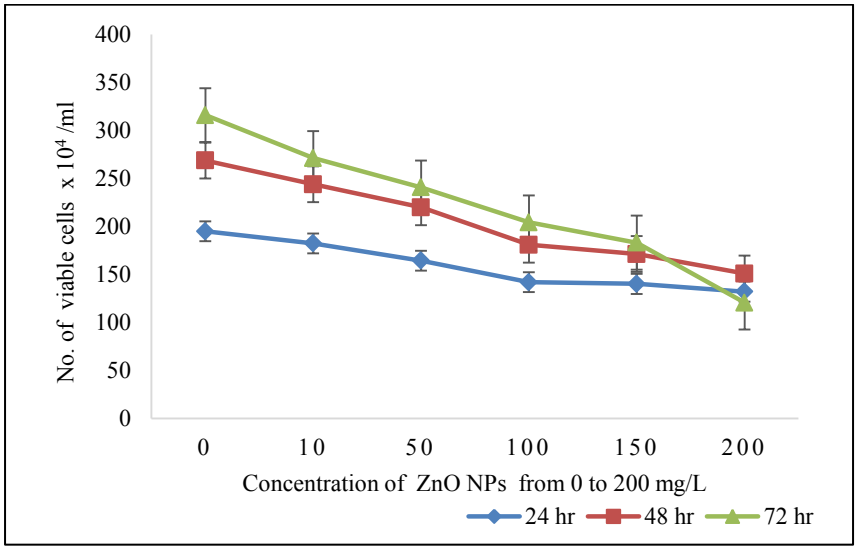

Figure 2. Number of viable cells under various treatments with 0 mg/L, 10 mg/L, 50 mg/L, 100 mg/L, 150 mg/L and 200 mg/L of ZnO NPs for 24, 48 and 72 hours

Reduction in algal biomass

The significant reduction in algal biomass upon the treatments with ZnO NPs was observed as shown in Figure 3. A typical concentration and time dependent inhibitory effect of ZnO NPs on *C. vulgaris* was reported with the higher inhibitory response of 56.24% at the higher concentration of 200 mg/L for the prolonged exposure time of 72 hours. Previous studies have reported the dose and time dependent reduction in biomass of *C. vulgaris* using titanium dioxide NPs [16, 23, 24]. Further, Sadiq et al. [25] demonstrated a significant reduction in biomass of *Chlorella sp*. by aluminum oxide NPs' toxicity.

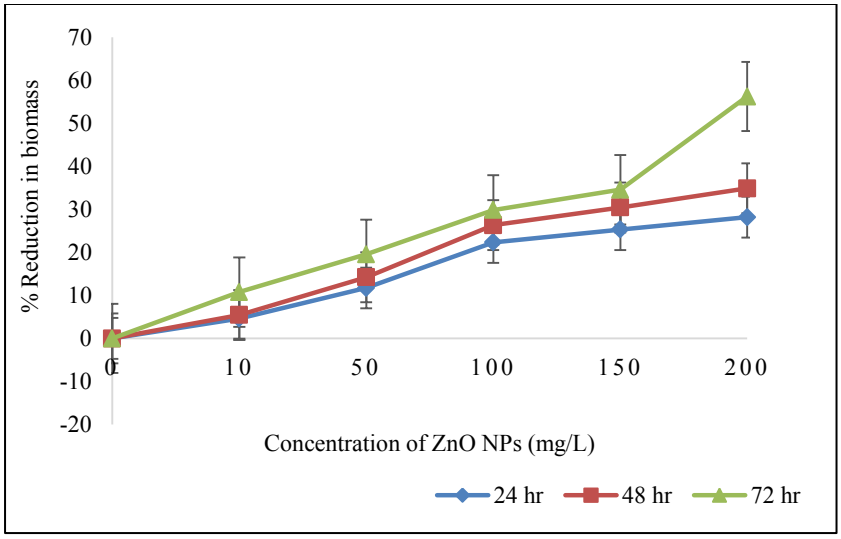

Figure 3. Percentage of reduction in algal biomass under various treatments with the concentration of ZnO NPs from 10 – 200 mg/L for 24, 48 and 72 hours

Reduction in chlorophyll fluorescence emission

The fluorescence emission of the chlorophyll was assessed by the chlorophyll content of the treated algal cells. A concentration and time dependent decrease in the chlorophyll emission was noted, which confirmed the growth inhibitory effect of ZnO NPs with the increasing concentration and exposure duration of ZnO NPs as shown in Figure 4. The strongest effect on the photosynthetic system was reported at 200 mg/L with the percentage of reduction in chlorophyll emission 38.98% (24 hours), 46.6% (48 hours) and 53.22% for 72 hours. Similar findings were reported by Barhoumi and Dewez [18] and Iswarya et al. [16], in their study the strongest effect on photosynthetic electron transport with decreased chlorophyll content was observed on *C. vulgaris* when treated with iron oxide and titanium dioxide NPs respectively. Also the authors recommended the chlorophyll emission measurement can be used as the biomarker for ecotoxicological assessment of NPs toxicity on *C. vulgaris* [18]. Moreover, a study by Gong et al. [26] reported a gradual decrease in chlorophyll content with increasing concentrations of nickel oxide NPs on *C. vulgaris*.

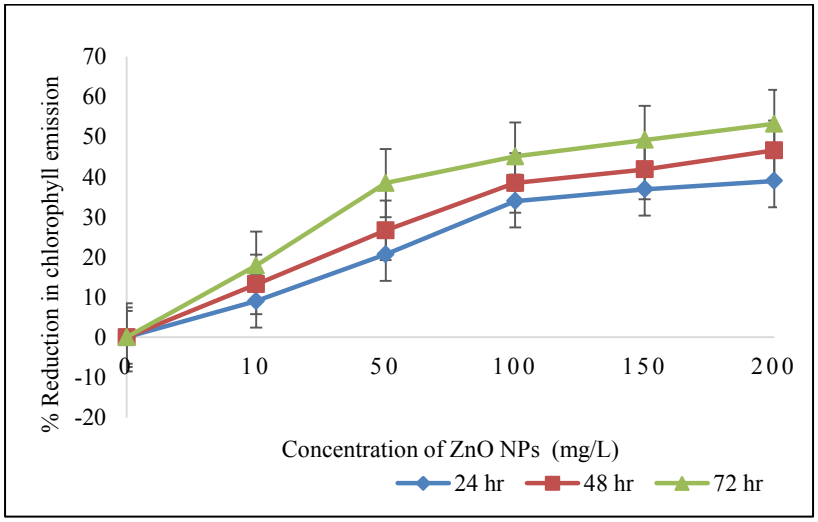

Figure 4. Percentage of reduction in chlorophyll emission under various treatments with the concentration of ZnO NPs from 10 – 200 mg/L for 24, 48 and 72 hours

Mean effective concentration (EC_{50})

Our results indicated that the most effective inhibition of algal cells was occurred at 24 hours when compared to 48 and 72 hours as the lowest concentration of ZnO NPs (54.45 mg/L) caused 50% of algal growth inhibition at 24 hours. The EC_{50} values for 24, 48 and 72 hours were presented in Table 1. The fact behind for less toxicity at 48 and 72 hours may be due to the saturation of Zn^{+} release from ZnO NPs usually at 72 hours [27] and also because of agglomeration NPs by sedimentation in the aqueous solution after 24 hours [21]. Ji et al. [24] reported 20 mg/L of ZnO NPs on *Chlorella sp.* as the EC30 value on day 6, while our results showed 31 mg/L as the EC30 value on *C. vulgaris* on day 3. A study by Sadiq et al. [25] reported EC50 value of 45.40 mg/L on the *chlorella* cells treated with aluminum oxide NPs for 72 h.

Table 1. EC_{50} values of ZnO NPs on *C. vulgaris* for the duration 24, 48 and 72 hours

Duration (hours)	Equation	R^2 Value	EC_{50} (mg/L)
24	$y = -0.0008x^2 + 0.3289x + 1.5621$	0.9892	54.45
48	$y = -0.0009x^2 + 0.3786x + 1.6357$	0.9926	58.85
72	$y = -0.0008x^2 + 0.3627x + 2.661$	0.9809	60.96

The suspension of nanoparticles can directly play a role in the growth inhibitory effect by occupying the surface of the algal cells and decreasing the amount of light reaching the cells, and thus causing the inhibition of photosynthetic activity [28] which results in growth inhibitory effect. Effective absorption of nanoparticles due to its large surface area can trigger greater growth inhibitory effect compared to micro sized particles[25]. The penetration of ZnO NPs into the cell envelope causes disruption of algal cell membrane which attributes to the cell growth inhibition and also the aggregation of ZnO NPs on algal cells could mechanically damage the cell walls and membranes resulting in the release of cellular contents into the extracellular space leading to cell death [29] which eventually result in decreased cell density and biomass. Algal cells develop physiological stress due to the toxicity of NPs and results in the production of free radicals which in turn induce the formation of reactive oxygen species (ROS). The ROS could impair the photosynthetic system II activity leading to decrease in chlorophyll content or emission. The parameters based on fluorescence yield have been proposed to be a useful tool for the toxic evaluation of pollutants [19].

Conclusion

In our study, we used cell viability and the photosynthetic based fluorescence as the biomarkers to characterize the toxicity of ZnO NPs on *C. vulgaris* cells. Reduction in cell viability, biomass and the fluorescent parameter related to photochemical reactions evidenced the potential source of cellular toxicity and proven to be sensitive biomarkers for ZnO NPs toxicity on *C. vulgaris*. This study showed the potential of *C. vulgaris* as the prospective bioindicator for ZnO NPs toxicity with viable cell count, algal biomass and chlorophyll fluorescence emission as the biomarkers for toxicity testing.

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