

Short-Term Cytotoxicity of Zinc Oxide Nanoparticles on *Chlorella vulgaris* (Ketoksikan Jangka Pendek Zarah Nano Zink Oksida ke atas *Chlorella vulgaris*)

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ABSTRACT

Zinc oxide nanoparticles (ZnO NPs) are widely used in industrial and personal care products. The use of these nanoparticles (NPs) has created residues that contaminate the environment, thus cytotoxicity studies of the NPs in biological system is required. Most of the recent cytotoxicity studies has however focused on long-term exposure of the NPs to the biological system. In this study, the cytotoxicity effects of short-term exposure of ZnO NPs to *Chlorella vulgaris* are reported. The algal cells were exposed to 10, 50, 100, 150, and 200 mg/L of ZnO NPs for 12 h. The toxicity effects of ZnO NPs were then determined through the changes in fluorescence emission of chlorophyll, algal biomass and the viable cell count. The results showed a decrease in the chlorophyll content, algal biomass and cell viability after treatment with ZnO NPs as compared with control. Through this study, the effects of ZnO NPs to *C. vulgaris* were confirmed. The significant responses of the algal cells to ZnO NPs in a short duration of exposure reflect the potential of the algal cells to be used as bioindicators of ZnO NPs in the aquatic environment.

Keywords: Algal cells; *Chlorella vulgaris*; short-term cytotoxicity; zinc oxide nanoparticles

ABSTRACT

Zarah nano zink oksida (ZnO NP) digunakan secara meluas dalam produk industri dan penjagaan peribadi. Penggunaan zarah nano tersebut telah menghasilkan residu yang membawa pencemaran kepada alam sekitar. Oleh itu, kajian kesan kesitotoksikan ZnO NP terhadap sistem biologi amat diperlukan. Namun, kajian kesan kesitotoksikan pada masa ini lebih tertumpu kepada kesan jangka panjang terhadap sistem biologi. Oleh itu, dalam kajian ini, kesan kesitotoksikan jangka masa pendek ZnO NP terhadap *Chlorella vulgaris* telah dilaporkan. Sel alga tersebut telah didedahkan kepada ZnO NP dengan kepekatan yang berbeza (10, 50, 100, 150 dan 200 mg/L) untuk jangka masa sehingga 12 jam. Kesan kesitotoksikan ZnO NP pada sel alga telah dipantau melalui perubahan pendarfluoran klorofil, perubahan biojisim dan kemandirian sel alga. Pendedahan sel alga kepada ZnO NP telah mengakibatkan pengurangan kandungan klorofil, biojisim dan kemandirian sel alga. Melalui kajian ini, kesan ZnO NP terhadap *C. vulgaris* telah dikenal pasti. Keupayaan *C. vulgaris* bertindak balas dengan ZnO NP dalam jangka masa yang pendek menampilkan potensi alga tersebut digunakan sebagai petunjuk biologi untuk penilaian ketoksikan ZnO NP dalam persekitaran akuatik.

Kata kunci: *Chlorella vulgaris*; kesitotoksikan jangka pendek; sel alga; zarah nano zink oksida

INTRODUCTION

The field of nanotechnology is expanding rapidly and the usage of nanoparticles in industry brings great impact to the society, economy and environment (Ji et al. 2011). Zinc oxide nanoparticles represent a type of nanoparticle most widely used in consumer products due to its unique characteristics. For instance, the presence of ZnO NPs provides a clear transparent appearance that allows them to be used in skin care products (Popov et al. 2005). ZnO NPs are also commonly used in the electronics, rubber and food industries (Dastjerdi & Montazer 2010; Song et al. 2010) due to their chemical stability, adsorption ability and antimicrobial property (Osmond & Mccall 2010). However, use of ZnO NPs may bring negative impact to the environment and to human health (Adams et al. 2006; Moore 2006; Wiesner et al. 2006; Zhao & Castranova 2011; Zhou et al. 2014). Previous cytotoxicity studies of ZnO NPs using fresh water microalgae *C. vulgaris* were

mostly focused on long-term exposure to these NPs (Chen et al. 2012; Suman et al. 2015; Zhou et al. 2014). However, no study has been conducted to study the short-term cytotoxicity of ZnO NPs on *C. vulgaris*. Thus, the present study involves cytotoxicity assessment based on short-term exposure of ZnO NPs to *C. vulgaris*.

MATERIALS AND METHODS

PREPARATION OF ALGAL CULTURE AND SOLUTION OF ZnO NPs

Fresh water microalgae *C. vulgaris* was obtained from the Culture Collection of Algae and Protozoa, United Kingdom. The algal cells were cultured with Bold Basal Medium (BBM, Sigma-Aldrich, Malaysia) at room temperature ($22 \pm 1^\circ\text{C}$) with light and dark conditions and maintained for 16 and 8 h, respectively. Continuous

agitation was carried out at 120 revolutions per minute using orbital shaker (A3446, Smith, Malaysia). The growth pattern of the algal cells was determined based on cell count, biomass and chlorophyll content.

ZnO NPs with the particle size of 40 - 50 nm were purchased from Zhejiang Hongsheng Material Technology Co., China. A stock solution of 400 mg/L ZnO NPs was prepared with BBM. The colloidal solution was sonicated for 30 min before the exposure test.

EXPOSURE OF ALGAL CELLS TO ZnO NPs

BBM with different concentrations of ZnO NPs (200, 150, 100, 50, and 10 mg/L) were prepared through serial dilutions from the stock solution. *C. vulgaris* cells from a day-3 culture with approximate cell density of 1×10^6 cells/mL were exposed to the different concentrations of ZnO NPs, respectively, for 12 h. Negative control consisted of cells cultured in BBM without ZnO NPs. All exposure tests were conducted in triplicates.

DETERMINATION OF CELL VIABILITY AND ALGAL BIOMASS

The number of viable *C. vulgaris* cells was determined using hemocytometer (Neubauer, Marienfeld, Germany) after the cells were exposed to ZnO NPs for 2, 4, 8, and 12 h, respectively. The cells without any distortion in shape and size and with intact membrane were identified as viable cells. The percentage of growth inhibition of *C. vulgaris* was calculated using (1) (Li et al. 2016).

$$\% I = (\mu C - \mu T) / \mu C \times 100. \quad (1)$$

where % I is the percentage of growth inhibition or cell death; μC is the mean value of viable cells in negative control; and μT is the mean value of viable cells in treatment.

The algal biomass was determined using spectrophotometer (GeneQuant 1300, GE, United States of America) at $\lambda = 680$ nm. BBM without algal cells was used as blank and the suspension of ZnO NPs without algal cells was used as positive control. The biomass yield in the treated samples was calculated by subtracting the absorbance value of positive control from the treated samples in order to take into account interference from ZnO NPs at 680 nm (Sadiq et al. 2011). The percentage of reduction in biomass yield (%Iy) was calculated using (2).

$$\% Iy = (bC - bT) / bC \times 100. \quad (2)$$

where % Iy is the percentage of reduction in biomass yield; bC is the biomass in negative control; and bT is the biomass in treatment.

DETERMINATION OF CHLOROPHYLL FLUORESCENCE EMISSION

Inhibition of photosynthetic activity by ZnO NPs was estimated by measuring fluorescence emission of

chlorophyll using a fluorometer (Promega Glomax Multi Jr., Promega, United States of America). The excitation and emission wavelengths were set at $\lambda = 430$ nm and $\lambda = 663$ nm, respectively. The percentage of change in the intensity of chlorophyll fluorescence emission was calculated using Equation 3 (Shing et al. 2012).

$$\% fE = fC - fT / fC \times 100. \quad (3)$$

where % fE is the percentage of change in chlorophyll fluorescence emission; fC is the fluorescence emission from negative control; and fT is the fluorescence emission from treatment.

RESULTS AND DISCUSSION

ALGAL GROWTH INHIBITION

The number of viable cell was affected by the presence of ZnO NPs. The growth inhibition in negative control was fixed as 0% for the exposure tests. The number of viable cells decreased when the duration of exposure and the concentration of ZnO NPs were increased (Figure 1). Suman et al. (2015) reported a similar trend on cytotoxicity effects of ZnO NPs on *C. vulgaris*. Nano-sized ZnO particles which are easily absorbed through cell surfaces, inhibited the growth of *C. vulgaris* and decreased the number of viable cells. Under microscopic examination, the cell membrane remained intact in untreated algal cells (Figure 2(A)), whereas cell rupture and aggregation were observed in cells treated with ZnO NPs (Figure 2(B)). The ruptured cells appear colorless due to the loss of chlorophyll through membrane damage. The aggregation of algal cells may be due to the secretion of algal exudates, for example exopolysaccharides (EPS) reported to be used in the protective mechanism during oxidative stress caused by the ZnO NPs on algal cells (Bhuvaneshwari et al. 2015; Brayner et al. 2010; Miao et al. 2009).

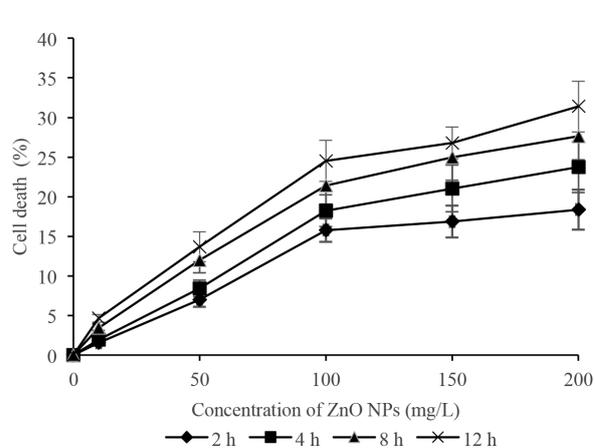


FIGURE 1. Percentage of cell death against different concentrations of ZnO NPs with the exposure of ZnO NPs from 2 to 12 h

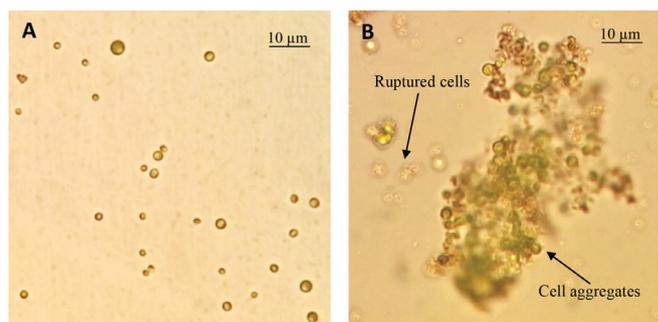


FIGURE 2. Light microscopic image of *C. vulgaris* cells treated with ZnO NPs. Algal cells in the absence of NP (A) and in the presence of ZnO NPs (200 mg/L) at 12 h, (B) showing cell rupture and cell aggregates under 100 × magnification

The algal biomass yield was reduced due to the presence of ZnO NPs (Figure 3). A concentration and time dependent inhibitory effects of ZnO NPs on *C. vulgaris* were observed with the highest inhibition in biomass yield ($26.34 \pm 3.87\%$) at the concentration of 200 mg/L following 12 h of exposure. The results are in agreement with the toxicity effects caused by the presence of aluminum oxide nanoparticles on *Chlorella* sp. (Sadiq et al. 2011).

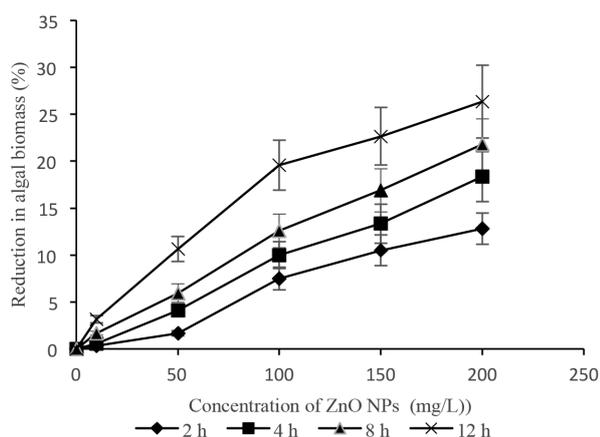


FIGURE 3. The effect of different concentrations of ZnO NPs to the production of biomass from 2 to 12 h

REDUCTION IN CHOROPHYLL CONTENT

The change on chlorophyll content can be used to assess cytotoxicity effect on algal cells (Barhoumi & Dewez 2013; Iswarya et al. 2015). The fluorescence property of chlorophyll has proven to be a useful tool for cytotoxicity evaluation (Shing et al. 2012; Tang et al. 2013). In this study, the fluorescence emission of chlorophyll decreased with the increasing concentrations of ZnO NPs and the duration of exposure to the NPs (Figure 4).

Similar to the cell viability and algal biomass, ZnO NPs exhibited a dosage and time-dependent inhibition on chlorophyll fluorescence emission from *C. vulgaris* cells. At 12 h of inhibition, chlorophyll emission was reduced from $4.48 \pm 0.69\%$ to $25.95 \pm 2.54\%$ as the concentration

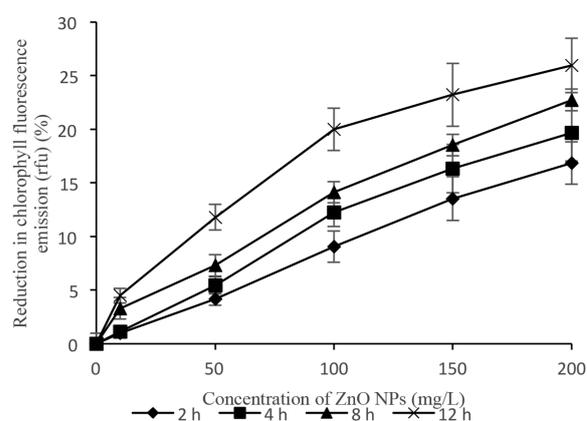


FIGURE 4. Percentage of reduction in chlorophyll content of algal cells treated with different concentrations of ZnO NPs from 2 to 12 h

of the NPs increased from 10 to 200 mg/L. Likewise, with the concentration of 200 mg/L, ZnO NPs caused a time-dependent inhibition with the reduction in chlorophyll emission from $16.83 \pm 1.98\%$ at the exposure time of 2 h to $25.95 \pm 2.54\%$ at 12 h.

The reduction in chlorophyll content may be caused by the inhibitory reaction of the NPs on photosynthetic electron transport chain (Barhoumi & Dewez 2013; Sadiq et al. 2011; Xiaoxiao et al. 2012). Entrapment of ZnO NPs by the algal cells caused direct shading effect and thus decreased the light availability and disturbed the energy transduction process. This induced oxidative stress in the cells and affected their growth and chlorophyll content (Iswarya et al. 2015). The decline in chlorophyll content is further explained by the production of free radicals inducing the formation of reactive oxygen species, leading to impairment of the photosynthetic system II activity (Tang et al. 2013).

Findings from the present study showed both dosage- and time-dependent inhibitions by ZnO NPs on cell viability, biomass and chlorophyll fluorescence emission of *C. vulgaris* (Figure 5). The presence of ZnO NPs substantially reduced the viability of *C. vulgaris* cells, biomass and chlorophyll fluorescence emission.

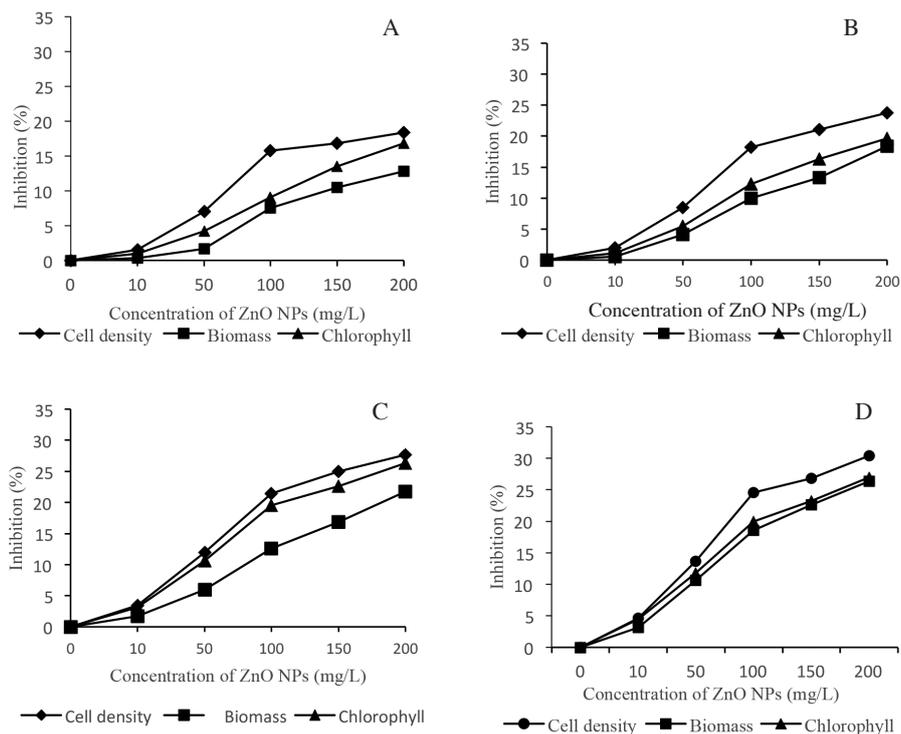


FIGURE 5. Dose response curve of ZnO NPs (10 - 200 mg/L) with the percentage inhibition in cell viability, biomass and chlorophyll fluorescence emission over the different exposure periods of 2 h (A), 4 h (B), 8 h (C) and 12 h (D)

The results obtained here for cytotoxicity studies are in agreement with that obtained in previous studies on *Chlorella* sp. using titanium dioxide (Iswarya et al. 2015) and nickel oxide (Gong et al. 2011) nanoparticles. The similar outcome of the cytotoxicity effects with ZnO NPs were reported for other algae species, such as *Anabaena flos-aquae* (Tang et al. 2013), *Pseudokirchneriella subcapitata* (Aruoja et al. 2009) and *Picochlorum* sp. (Hazeem et al. 2016).

CONCLUSION

The exposure of ZnO NP to *C. vulgaris* from 2 to 12 h has led to significant reductions in cell viability, biomass and chlorophyll fluorescence emission. This study confirmed the sensitivity of *C. vulgaris* to the short-term cytotoxicity of ZnO NPs. Thus, the microalgae *C. vulgaris* may serve as a bioindicator for the presence of ZnO NPs in the aquatic environment.

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