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MORPHOLOGICAL CHANGES AND DNA DAMAGE IN *Chlorella vulgaris* (UMT-M1) INDUCED BY Hg^{2+}

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ABSTRACT

This study reported the effects of Hg^{2+} on the morphology and DNA of a microalga, *Chlorella vulgaris* (UMT-M1). Morphological changes and DNA damage in cells were analysed using scanning electron microscope (SEM) and Comet assay, respectively. The half maximal inhibitory concentration (i.e. IC_{50}) of Hg^{2+} on the growth of *C. vulgaris* obtained from the dose-response curve was 0.72 mg/L. Under SEM, it was observed that Hg^{2+} treated cells become smaller in size (i.e. 2.1±0.1 µm) compared to normal cells (i.e. 3.2±0.1 µm). The morphology of cells changed from an intact microalgal cells with smooth spherical surface to a slightly roughened surface and shrivelled shape. Apoptotic bodies were also observed under 0.001, 0.01 and 0.1 mg/L of Hg^{2+} but not in 1.0 mg/L Hg^{2+}. These results indicate that Hg^{2+} may induce apoptotic cell death at low concentrations but not at the highest concentration of 1.0 mg/L. The highest percentage of cells with comet level of 4 was observed in 1.0 mg/L Hg^{2+}. However, Hg^{2+} is genotoxic to the microalga even at low concentration. In conclusion, Hg^{2+} can exert its toxic effects on *C. vulgaris* by changing the microalgal morphology, damaging the microalgal DNA and inducing cell death in the microalgal cells using different pathways at different concentrations.

Key words: Microalgae, heavy metals, comet assay, genotoxic effect, cell death

INTRODUCTION

Mercury (Hg) is widely known as one of the most toxic man-made contaminants to date. It is poisonous in any of its existing forms as either elemental Hg, inorganic salts or organic compounds (Bernhoft, 2012). Due to its persistent nature, it is not easily degraded nor destroyed, it can exert adverse effects on living organisms (Bernhoft, 2012). To make matter worse, it can also accumulate inside the organisms that uptake the Hg from their environment which then eventually reach us, humans. Being a nonessential micronutrient to organisms, its toxic effect can be induced even at low concentrations. For example, Hg in the environment has profound effects on photosynthetic activity and pigment content of marine macroalgae (Luqman *et al.*, 2015), swimming activity and risks taken by shrimps (Harayashiki *et al.*, 2016) as well as on pancreatic beta (β) cell development and function, resulting in insulin resistance and hyperglycaemia (Schumacher & Abbott, 2017). Toxic metals can also trigger cellular signalling pathways that can either be death or survival signals leading to the programmed cell death modes such as 'self-suicidal' apoptosis and necrosis as well as 'self-eating' autophagy (Chatterjee *et al.*, 2014). Inorganic Hg, for instance, induced cellular apoptosis and autophagocytosis in a marine copepod, *Calanus finmarchicus* (Tollefson *et al.*, 2017). In human hepatic cell line, Hg exposure renders the organelles dysfunctional leading to cell death through apoptosis and autophagy (Vergilio *et al.*, 2015). The above responses, therefore, may be used as biomarkers or bioindicators for Hg contamination.

Fortunately, Hg can be removed from polluted areas through phytoremediation which include the application of microalgae (Kumar *et al.*, 2015). Microalgae are ubiquitous organisms that can be found anywhere, whether in the terrestrial or aquatic ecosystems. Microalgae like any other photoautotrophs required inorganic nutrients for growth. The fast-growth rate of some microalgae species can account for rapid nutrient removal from water bodies. Most of them are able to immobilize

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the metals to make them less toxic (Sánchez-Rodriguez *et al.*, 2001). They also have the ability to adsorb and metabolize trace metals due to their large surface to volume ratios, the presence of high-affinity as well as metal-binding groups on their cell surfaces and efficient metal uptake and storage systems (Rajamani *et al.*, 2007). In addition, microalgae plays a major role in marine ecosystems. As the first organism in marine food chains, they not only provide nutrients and energy but also shelter and habitat for many coastal animals. Thus, it is important to study what happens in the microalgae to obtain more information on how they can survive and adapt to their harsh environmental conditions. In addition, the microalgae can be used as efficient tool for metal bioremediation and bioindication.

In this study, a strain of a green microalga species, *Chlorella vulgaris* was treated with different concentrations of $Hg(NO_3)_2$ to assess the Hg^{2+} toxic effects on the microalgal morphology and DNA damage. Half maximal inhibitory concentration (IC_{50}) of the metal on the microalga was also determined. From these observations, we can make conclusions as to whether the microalga can be used as bioindication tool for Hg^{2+} contamination in aquatic ecosystem.

MATERIALS AND METHODS

Culture and treatments of *Chlorella vulgaris* (UMT-M1)

A stock culture of the microalga, *Chlorella vulgaris* (UMT-M1) was obtained from Associate Prof. Dr. Cha Thye San from the Institute of Marine Biotechnology, UMT. The microalga was further cultured in F2 medium (Guillard, 1975) under the light intensity of ~80 μmol m^{-2} at 25°C with 24h constant light. The cell growth was monitored for 1 week and harvested for treatments when the growth reached the log phase.

The harvested microalgae (at a cell density of 10^6) was then treated with four different concentrations of $Hg(NO_3)_2$: 0.001 mg/L, 0.01 mg/L, 0.1 mg/L and 1.0 mg/L. About 250 mL of treated cultures was kept in 500 mL flasks for 48h in similar conditions as during the culturing. Each treatment was done in triplicates while treatment without the $Hg(NO_3)_2$ was set as control. After 48h, IC_{50} value of $Hg(NO_3)_2$ against the microalga was determined from a dose-response curve. The treated microalgae was also subjected to morphological changes and genotoxicity analyses as stated in the methods below.

Morphological and Genotoxicity Analyses

Morphological changes of treated and control microalgae was observed using the scanning electron microscopy (SEM) technique. The standard method of SEM was followed according to that supplied by the Institute of Oceanography and Environment (INOS), UMT. The samples were first prepared by subjecting them to a series of fixation, washing and dehydration steps. In the fixation steps, a Karnovsky's fixative solution (i.e. 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer) and 0.1% osmium tetroxide in 0.1 M sodium cacodylate buffer were used. Both fixation steps were carried out for 2h. In between the fixation steps as well as after the second fixation step, the samples were washed thrice with 0.1 M sodium cacodylate buffer. In the dehydration steps, the samples were gradually subjected to a series of alcohol (35% – 100%) and finally were air-dried. In the last step, the samples were sputter-coated with gold and observed under a SEM (JEOL 6310, Japan) at an accelerating voltage of 10 or 15 kV under 5000x magnification. About 50 cells from each treatment were selected to determine their cellular size and surface morphology changes.

For assessment of genotoxic effects of Hg^{2+} on the microalgae, an alkaline comet assay was performed according to the method stated in Tice *et al.* (2000). About $1x10^4$ cells in 5 μL medium were first suspended in a warm 0.5% low-melting agarose (LMA). The cells were then quickly pipetted and evenly spread onto a frosted slide which was coated with 0.5% normal-melting agarose (NMA). To solidify the LMA, the slide was left on ice for about 5 min. Next, the slide was spread with another layer of 0.5% LMA. The three-layered slide was placed in a Coplin jar containing chilled lysis solution (i.e. 10% DMSO, 1% Triton X in an alkaline lysis buffer) at pH>13 for 1h. Next, the slide was placed in a horizontal gel electrophoresis unit filled with cold electrophoresis buffer of 10 mM NaOH and 200 mM Na_2EDTA at pH 13. Electrophoresis was carried out at 4°C for 20 min at 15 V and 300 mA. Following a dropwise neutralization (Tris-HCl, pH 7.5) for 5 min, cells were stained with 1X ethidium bromide. To assess the extent of DNA damage, the comet's tail length of about 100 cells was measured with an epi-fluorescence microscope. The tail length measured was then compared with comet score level between 0 and 4 as stated by Collins (2004). Score level of 0 shows no tail was observed with an intact DNA; score level of 1 shows about 25% DNA migration observed in the tail; score level of 2 shows between

25 and 50% migration; score level of 3 shows between 50 and 75% migration; and, score level of 4 shows higher than 75% migration.

RESULTS AND DISCUSSION

Inhibition concentration (IC) value is often used to determine the effectiveness of any substance in inhibiting a specific biological or biochemical function (Aykul & Martinez-Hackert, 2016). It also can indicate how well the organism can tolerate any heavy metals that they encounter in their cells (Albert *et al.*, 2018). As Hg^{2+} is a nonessential metal to the microalgae, it is understandable that even at low concentration, this metal can exert toxic effect as seen by a 10% reduction in the growth of the microalga after 48h treatment with 0.001 mg/L $Hg(NO_3)_2$ (Figure 1). Thus, in this study, it took 0.72 mg/L $Hg(NO_3)_2$ to inhibit 50% cells population of *C. vulgaris* (UMT-M1).

Presence of Hg^{2+} significantly affects the cell size of *C. vulgaris* (UMT-M1) as shown in Figure 2. The size of the cells shrunk to about 11% in 0.001 mg/L $Hg(NO_3)_2$ which gradually increased to 33% in 1.0 mg/L $Hg(NO_3)_2$. Shrinkage in cell size may be due to retardation or disturbance in normal biochemical processes that occur inside the cells which affect the cell growth. A reduction in cell size may also lead to chlorosis induced by toxic metals (Nam & An, 2015) such as Hg^{2+}. In this study, the treated cells also experienced symptoms of chlorosis or loss of normal green coloration of cells as concentration of $Hg(NO_3)_2$ increases whereby in 1.0 mg/L $Hg(NO_3)_2$, the colour of the cultures became milky white. Chlorosis arise when there is deficiency in the uptake of nutrients such as N, P and S leading to insufficient production of chlorophylls. When chlorophylls are lacking, photosynthetic process will be disturbed. No photosynthesis means no cell growth, hence, there is a reduction in the cell size. Presence of Hg^{2+} may also trigger the production and accumulation of reactive oxygen species (ROS) and other peroxidative products which can lead to reduction in cell growth (Elbaz *et al.*, 2010). Cell shrinkage may also indicate that the cells are getting ready for programmed cell death (Elmore, 2007) as discussed below.

Figure 3 shows the SEM images of *C. vulgaris* (UMT-M1) before and after the treatments. Under the treatment, the intact microalgal cells with smooth spherical surface (Figure 3a) were distorted into an anomalous shape. For instance, apoptotic bodies were observed on the cell's surface under the 0.001, 0.01 and 0.1 mg/L $Hg(NO_3)_2$ which increases in numbers as concentration increases from 0.001 to 0.1 mg/L (Figure 3b-d). The presence of apoptotic bodies indicate that the cells are experiencing the late stage of apoptosis or programmed cell death (Krammer *et al.*, 2007). Hg^{2+} is a highly toxic metal that can exert multiple adverse effects, ultimately leading to programmed cell death. Typical morphological hallmarks of apoptotic pathway are cell shrinkage, nuclear DNA fragmentation and membrane blebbing (Hengartner, 2000). In a study by Bayani *et al.* (2017), presence of Hg^{2+} induced the production of caspase-3/7-like enzyme in the green microalga, *Scenedesmus regularis* which triggers apoptosis. In comparison, under the treatment of 1.0 mg/L $Hg(NO_3)_2$, the apoptotic bodies disappeared and is replaced by a normal but

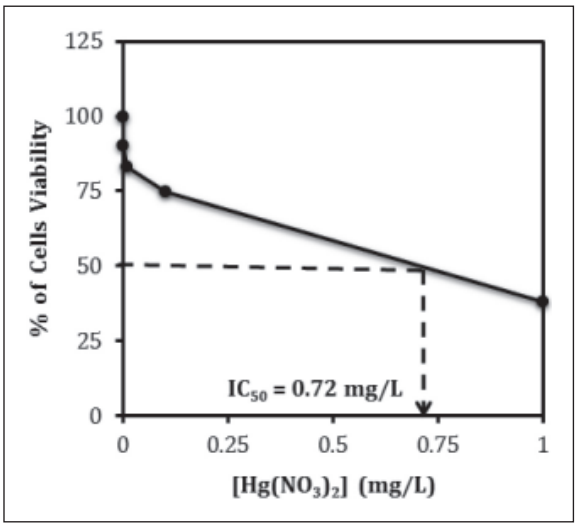

Fig. 1. Dose-response curve of *C. vulgaris* (UMT-M1) treated with various concentrations of $Hg(NO_3)_2$ to determine the IC_{50} value.

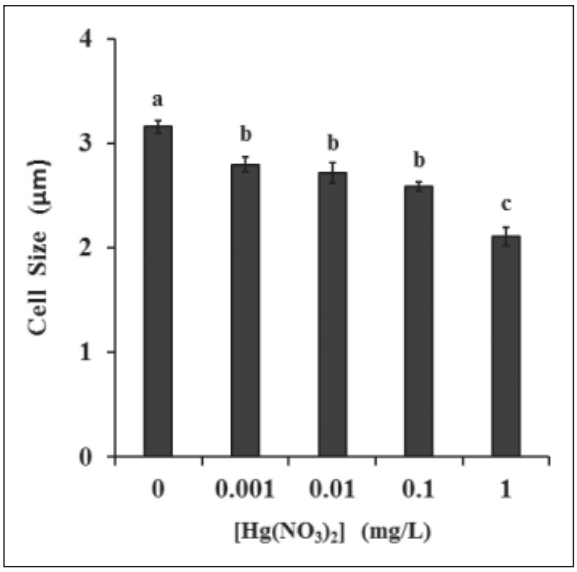

Fig. 2. Changes in the size of *C. vulgaris* (UMT-M1) cells after 48h treatment with various concentrations of $Hg(NO_3)_2$ compared to control (i.e. 0 mg/L).

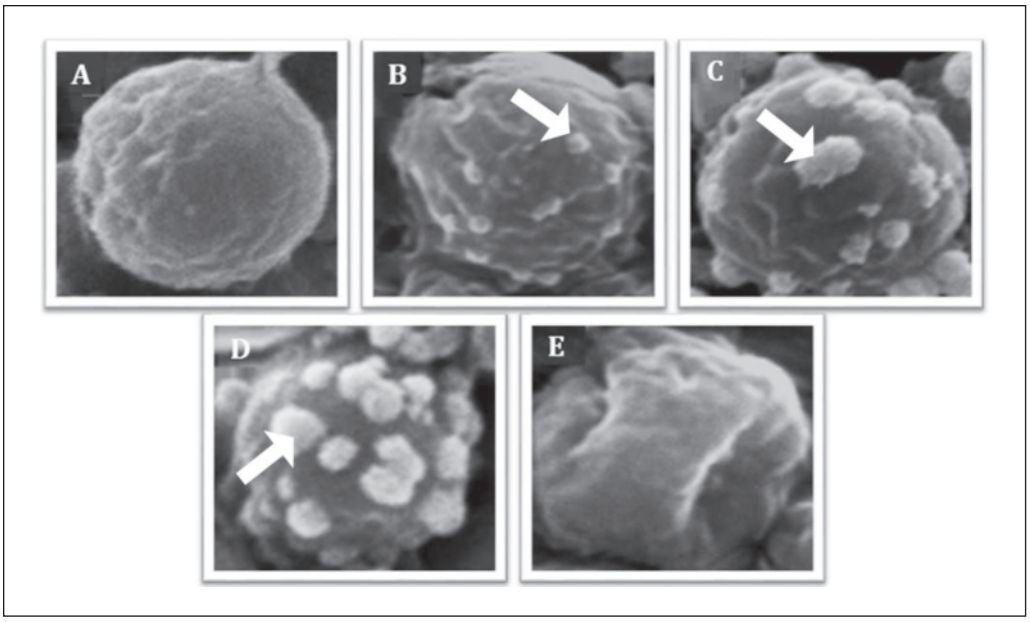

Fig. 3. Scanning electron micrographs of *C. vulgaris* (UMT-M1) in 0.001 mg/mL (*B*), 0.01 mg/mL (*C*), 0.1 mg/mL (*D*) and 1.0 mg/mL (*E*) $Hg(NO_3)_2$ compared to control cells (*A*). Apoptotic bodies are shown by the arrows. (Magnification: 5000x).

slightly roughened surface and shrivelled shape (Figure 3e). The cells in this condition may experience an alternative mode of cell death of either necrosis or autophagy. Necrosis is considered to be a toxic process and it refers to the degradative processes that occur after cell death (Elmore, 2007). However, necrosis can be differentiated from apoptosis by the swelling of the cells resulting in the loss of cell membrane integrity and an uncontrolled release of products of cell death into the extracellular space (Proskuryakov *et al.*, 2003). Since in this study, reduction in cell size (Figure 2) was observed instead of cell swelling and no signs of rupture was evident, the theory of necrosis can be ruled out. Autophagy, on the other hand, is thought of as a survival mechanism whereby it plays a housekeeping role in removing misfolded or aggregated proteins as well as clearing damaged organelles caused by biotic and abiotic factors (Glick *et al.*, 2010) including presence of Hg^{2+}. The morphological characteristics of autophagy includes vacuolization, degradation of cytoplasmic contents, and slight chromatin condensation (Fink & Cookson, 2005). The theory of autophagy for this study is yet to be validated. However, in a study by Vergilio *et al.* (2015), both apoptotic and autophagic pathways can be involved in Hg-mediated cell death.

As stated earlier, DNA fragmentation is one of the hallmarks of apoptotic pathway. This is further proven by the tail length of comets formed after the treatments as shown by the Comet assay analysis (Figure 4). The length of comet tail indicates the frequency of DNA breaks (Azqueta & Collins, 2013). For example, in treated *C. vulgaris* cells showing a comet level of 2 (Figure 4c), the tail is shorter than cells showing a comet level of 3 (Figure 4d) which indicates that there is a low frequency of DNA breaks observed. Comparatively, the comet head disappears in cells with comet level of 4 (Figure 4e). As the concentration of $Hg(NO_3)_2$ increases, more cells fall into the comet level of 4, indicating that the DNA was highly damaged (Figure 5). The highest percentage of cells that fall into this level was observed in 1.0 mg/L $Hg(NO_3)_2$. The Comet assay results reveal the genotoxicity of Hg^{2+} on the microalga. Hg^{2+} genotoxicity may be related to the mode of action of this metal. It is known that heavy metals could seriously affect the photosynthetic apparatus by irreversibly binding the components of photosynthetic electron transport chain (Lu *et al.*, 2000; Kukarskikh *et al.*, 2003; Luqman *et al.*, 2015). The effect will eventually lead to generation of reactive oxygen species such as H_2O_2 (Hazlina & Shuhanija, 2013) that could affect cellular DNA (Pieper *et al.*, 2014; Malar *et al.*, 2015). In a study by Pieper *et al.* (2014), Hg strongly disturbed poly(ADP-ribosyl)ation, a signalling reaction induced by DNA strand breaks. Malar *et al.* (2015), on the other hand, reported that following exposure of Hg, disappearance of normal DNA bands and the appearance of new bands were observed indicating the genomic template instability.

In conclusion, Hg^{2+} exerts toxic effects on *C. vulgaris* (UMT-M1) by changing its morphology

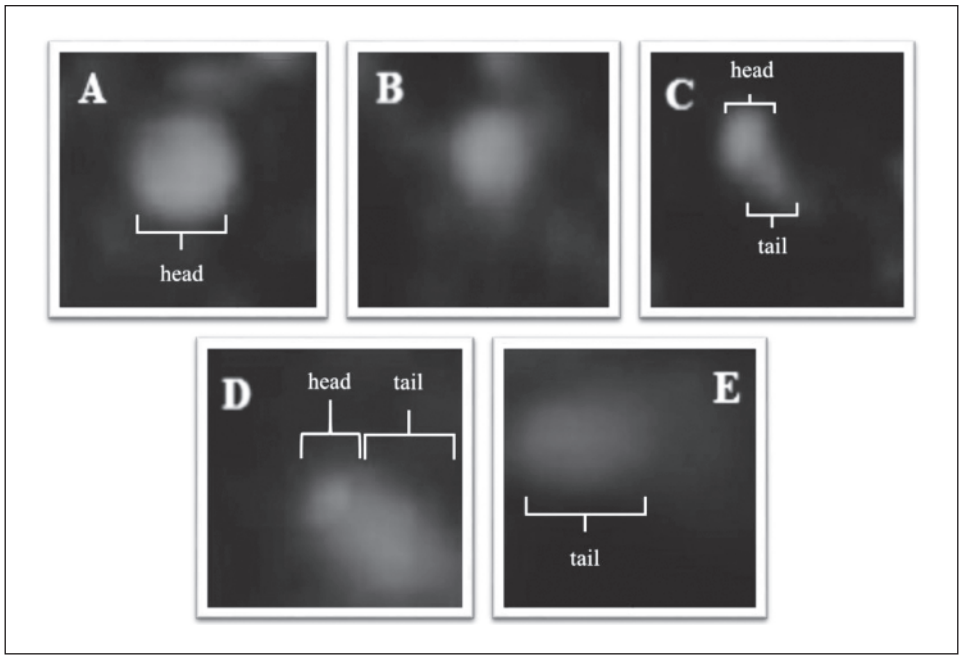

Fig. 4. Images of comets formed in *C. vulgaris* (UMT-M1) cells after 48h treatment with various concentrations of $Hg(NO_3)_2$. (*A*) represents comet of level 0; (*B*) represents comet of level 1; (*C*) represents comet of level 2; (*D*) represents comet of level 3; and, (*E*) represents comet of level 4.

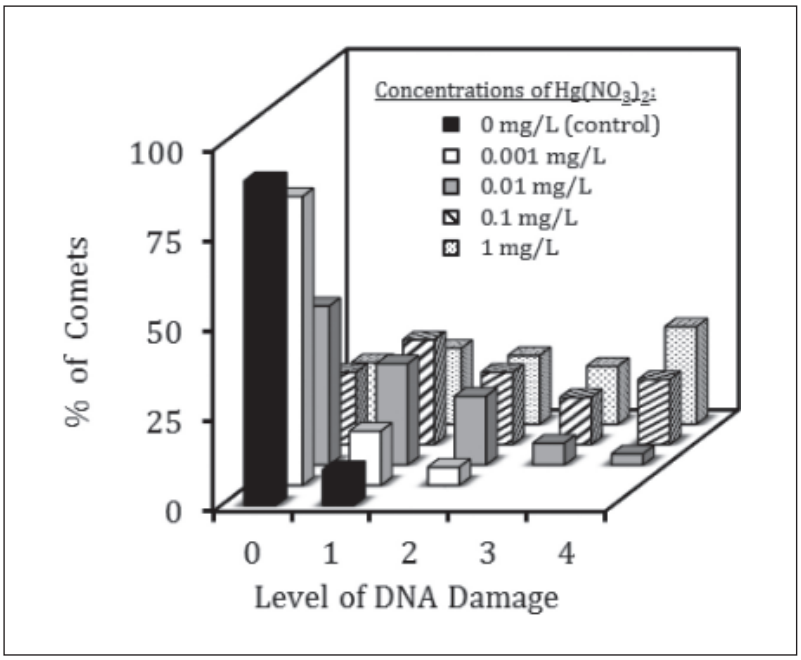

Fig. 5. Level of DNA damaged in *C. vulgaris* (UMT-M1) cells after 48h treatment with various concentrations of $Hg(NO_3)_2$ compared to control (i.e. 0 mg/L) evaluated using the alkaline Comet assay.

and damaging its DNA leading to cell death. At low concentrations of Hg^{2+}, apoptosis is induced inside the cells while at high concentration of Hg^{2+}, autophagy is induced inside the cells as mode of cell death. This is shown by the reduction in cell shrinkage, changes in the shape of cells from a smooth spherical shape into a slightly rougher shrivelled shape, presence of apoptotic bodies on the surface of cells which disappear at the highest concentration studied and the increase in the percentage of DNA damaged within the level of 4. *Chlorella vulgaris* can thus be used as bioindication

tool for Hg^{2+} contamination in aquatic ecosystem. Listed responses can also be used as biomarkers for this metal.

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