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GENOTOXIC EFFECTS OF MERCURY AND ZINC ON *Acanthamoeba* sp., A FREE –LIVING AMOEBA FROM SETIU WETLANDS WATER: A LABORATORY STUDY

NAKISAH MAT AMIN[1], SYAFAZ-SYAZWANI SIDEK[1] AND ANTONINA ABDULLAH[2]

[1]*Department of Biological Sciences, Faculty of Science and Technology,*
[2]*Department of Marine Science, Faculty of Maritime and Marine Studies,*
Universiti Malaysia Terengganu, 21030 Kuala Terengganu, Terengganu, Malaysia.

** Corresponding author e-mail address: nakisah@umt.edu.my*

Abstract: Heavy metals are stable in the environment and cannot be degraded or destroyed so they tend to build up in the atmosphere, soils, sediments and water. Excessive levels of metals in our environment will contribute pollution and pose a risk to humans and other living things, including *Acanthamoeba. Acanthamoeba* spp are free-living amoebae that are in abundance, especially in the aquatic environment. Their role as mainly a bacterial consumer indicates their importance in the food-web cycle of the ecosystem. Previous studies showed that heavy metals, like cadmium, lead, mercury and zinc, inhibited the growth of *Acanthamoeba* spp. Therefore, the objectives of the present study were to examine further the effects of mercury and zinc on *Acanthamoeba* sp, an amoeba isolated from water in Setiu Wetlands, by looking at the level of DNA damage in the amoeba cells. The amoebae were exposed to five different concentrations of the metals for 72h before the cytotoxic and genotoxic effects on the amoebae were observed. The IC_{50} values of mercury and zinc against the amoeba obtained in this study were 1.10 ppm and 39.00 ppm, respectively. The DNA damage with various score levels in *Acanthamoeba* cells by different concentrations of mercury and zinc treatment are presented and discussed.

KEYWORDS: Genotoxic, free-living amoebae, *Acanthamoeba*, comet assay.

Introduction

Free-living amoebae, including *Acanthamoeba* spp, are a diverse group of ubiquitous unicellular organisms. In the natural environment, these amoebae act as predators that control bacterial population. Even though they are mainly bacterial consumers, some species belonging to genus *Acanthamoeba* have been reported to cause serious human diseases, including painful eye keratitis resulting in blindness and fatal granulomatus encephalitis (Cabral and Cabral, 2003). Due to ubiquitous presence and their relevant ecological function, these amoebae can be used in bio-monitoring for environmental health. Furthermore, these amoebae have been reported to be quite sensitive to diverse environmental pollutants since their trophozoites do not posses the cell wall such as has been observed in bacteria and yeasts (Diaz et al., 2006).

Heavy metals are natural component of the earth's crust that cannot be degraded and destroyed but accumulate through the food chain and produce potential human health risks and ecological disturbances. Being one of the most persistent pollutants in the environment, these metals are dangerous and can lead to poisoning at higher concentrations to living organisms including the amoebae. Previous study conducted by Rainee (2005), showed that heavy metals, such as mercury, lead, cadmium and zinc, have very strong cytotoxic effects on *Acanthamoeba* spp. Further effect of mercury and zinc on *Acanthamoeba* at the DNA level was investigated in the present study using

Comet assay. This assay is also known as the alkaline version of the single-cell electrophoresis that is used for a wide range of applications, including DNA damage and repair studies, genetic toxicology, radiation biology, and environmental monitoring (Cotelle and Ferard, 1999).

Materials and Methods

Sources and sample preparation

Acanthamoeba sp. is an environmental isolate from water in Setiu Wetlands, Terengganu. The amoeba were cultivated axenically in polypeptone medium at 30ºC before they were harvested at log-phase growth for treatment with both heavy metals.

Mercury sulfates and zinc chloride powder were provided by Dr Antonina Abdullah, Faculty of Marine Sciences and Maritime, Universiti Malaysia Terengganu. Mercury (Hg) and zinc (Zn) stock solutions (1000ppm) were prepared by dissolving 0.1353g of mercury sulfate and 0.1340g of zinc chloride in 100mL of distilled water respectively. Heavy metal's toxicity test was conducted in 24-well plates. Five different concentrations of mercury and zinc were tested on this amoeba. To obtain the desired concentration of heavy metals, appropriate volume of stock solution of Zn and Hg was transferred into each well containing fresh amoeba culture medium. Ten microliter of amoeba suspension, containing 5.0 x 10^5 cell/mL was added into each well. Appropriate volume of fresh amoeba culture medium was added into the wells in order to get the final volume of 1000µL (except for zinc solution). The plates were wrapped with parafilm and incubated at 30ºC for 72 hours before analysis on the effects of both metals on *Acanthamoeba* was made.

Cytotoxic assay

After 72h incubation in heavy-metal solutions, the viability of *Acanthamoeba* cells in 24 well plates was determined by using eosin staining following technique of Wright et al (1988) and the absorbance reading was done at 490nm, using ELISA-plate reader. The graph of percentage of viable cells against concentration of heavy metals was plotted to determine IC_{50} values for each metal against *Acanthamoeba*.

Genotoxic Assay

The comet assay to assess the DNA damage in *Acanthamoeba* after exposure to the metals was carried out under alkaline conditions, principally as described by Singh et al. (2000). The concentrations of both metals that were exposed to the amoebae were at their IC_{10}, IC_{25}, and IC_{50} against the amoeba.

After 2h incubation in metal solutions, the liquid part in each well was removed while healthy amoebae were attached to the well's surface. The amoeba cells were suspended in Ca^{2+} and Mg^{2+} free PBS and the cell suspension was pelleted by centrifugation (1500rpm/7min). The pellet formed was mixed thoroughly with 80µL of 0.7 % low melting point (LMP) agarose and then the cell mixture was layered onto a slide as a second layer. The first layer was prepared earlier by coating the slide with 200µL of 0.6 % normal melting point (NMP) agarose.

The slides were left on the ice until solidified before they were covered with 0.5% of LMP agarose. Afterwards, the slides were immersed for 1 hour in cold water (4ºC) before the slides were placed in a horizontal gel electrophoresis tank, facing the node. The electrophoresis unit was filled with freshly-prepared buffer (300mM NaOH, 2mM EDTA, pH>13) and the slides were set in this alkaline buffer for 20 minutes to allow DNA unwinding and expression of alkali-labile sites. Denaturation and electrophoresis were performed at 4ºC under dime light or dark. Electrophoresis was carried out for 5 minutes at 25V (300mA).

After electrophoresis, the numbers of intact and lysed cells and the extent of the DNA migration from individual lysed cells (comets) were examined by fluorescence microscopy. The degree of DNA damage from a sample of 50 lysed cells per slide was determined and categorised using a four-stage comet scoring recommended by Collins (2004).

Results and Discussion

Eosin staining was used in the present study to investigate the cytotoxic effects of zinc and mercury on *Acanthamoeba* sp. and the results obtained are presented in Figures 1 and 2. Both metals caused inhibition of amoeba growth *in vitro*. The presence of heavy metal ions in the amoeba culture medium provides unsuitable environment for amoebae, therefore affecting its growth. Heavy metals have high affinity to negatively-charged groups of protein and thiol group (Belyaeva et al., 2006) and this may affect the conformation of protein structure in amoeba in the present study. The enzyme becomes inactivated and the biochemistry process in the amoeba will be disturbed. Heavy metals also can bind easily with the sulfhydril (sulfur-hydrogen-related) group of enzymes that control the speed of metabolic reactions in living things, including amoebae.

The eukaryotic microorganisms, such as amoebae, are quite sensitive to heavy-metal pollutants due to the absence of cell wall for protection in the vegetative stage of amoebae (Diaz, et al., 2006). When cells are exposed to heavy-metal cations, the first reaction between the metal and the cells are with those ligands of the cell membrane for which the metal possesses a chemical affinity. As the metal ion passes through the membrane into the cytoplasm at a rate determined principally by the rate of entry of the metals into the cells, the metals then will react with internal constituents of the cells, depending on the rate of their mixing with the cytoplasm and also on the kinetics of the chemical reactions involved. These effects might include the inhibition of metabolic activities such as respiration, and other cellular activity including cell division (Belyaeva et al., 2007).

The inhibition of growth of *Acanthamoeba* sp. caused by these metals may also occur in the actual environment. However, the IC_{50} value obtained from the study in the actual environment may be different from the value obtained from this study due to the existence of mixture of pollutants in the environment.

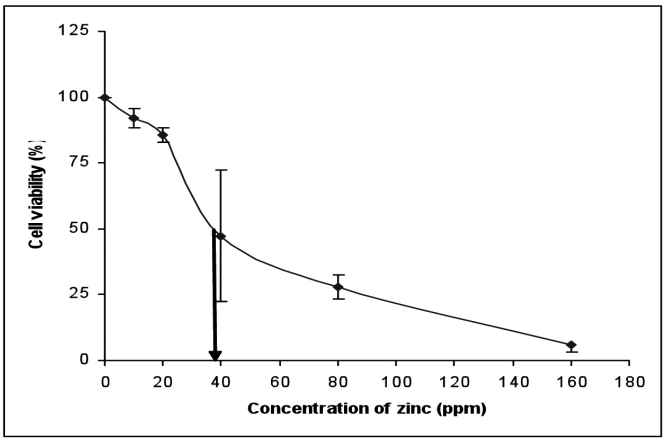

Figure 1. The effects of different concentration of zinc on viability of *Acanthamoeba* sp. The IC_{50} obtained was 39.0 ppm and this value was derived from this graph.

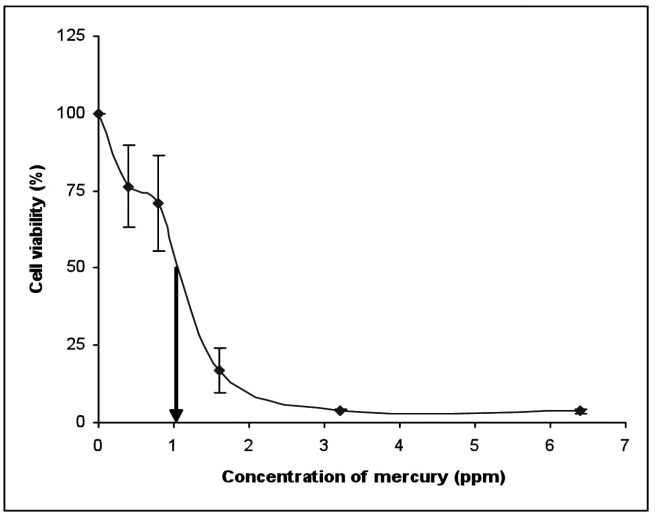

Figure 2. The effects of different concentration of mercury on viability of *Acanthamoeba* sp. The IC_{50} obtained was 1.10 ppm and this value was derived from this graph.

In toxicity test carried out in the present study, mercury has a lower value of IC_{50} which is 1.1ppm compared with zinc (39.0 ppm) to inhibit 50% of cell population of *Acanthamoeba* sp. Higher IC_{50} value for zinc indicates that this metal is less toxic to the amoeba than mercury. According to the period table of the elements, mercury is located at the most electropositive ion while zinc is the most electronegative ion. Toxicity of heavy metals decreases with the increasing stability of electron configuration. Furthermore, zinc is an essential metal which constitutes a catalytic or structural compound of the enzymes (Martin-Gonzalez et al., 2006), therefore it is expected that zinc is less toxic than mercury. Mercury, on the other hand, is known to be the most toxic metal compared with other metals since it can inhibit the cellular respiration completely and also can cause the cell death both by necrosis and apoptosis, as observed by Belyaeva et al., (2007) on rat ascites hepatoma AS-30D cells after treatment with mercury, cadmium and cuprum.

The toxicity of mercury and zinc does not only affect the cellular level of *Acanthamoeba*, but also affects the amoeba's DNA. The quantitative analysis of toxicity effects of mercury and zinc at the DNA level of *Acanthamoeba* sp are presented in Figures 3 and 4. The DNA damage was classified as score 0, 1, 2, 3 and 4 as suggested by Collins (2004). The percentage of DNA damage in *Acanthamoeba* sp. induced by zinc at concentrations of IC_{10}, IC_{25} and IC_{50} are 80%, 100% and 100%, respectively (Figure 3). In control experiment, only a small number of cells (5%) showed the DNA damage in *Acanthamoeba*. 95% of the control cells showed intact DNA (at score 0). At IC_{50}, 48% of the DNA damage is at score 4. At IC_{10}, the percentage of DNA damage at score 1, 2 and 3 are 67%, 13%, and 0%, respectively and at IC_{25}, these scores are 21%, 42%, and 37%, respectively. While the percentage of DNA damage of *Acanthamoeba* sp. induced by mercury at its concentration of IC_{10}, IC_{25} and IC_{50} are 89%, 100% and 100%, respectively (Figure 4)., only 5% of cells in control experiment showed DNA damage, and 95% of cells showed intact DNA (at score 0). At IC_{50} 64% of the DNA damage is at score 4. At IC_{10} the percentage of DNA damage at score 1, 2 and 3 are 43%, 40%, and 6%, respectively and at IC_{25} the percentage for each score are 17%, 44%, and 39%, respectively. Results in this study indicate that the genotoxic effect of the metals to the amoeba DNA are dose-dependent.

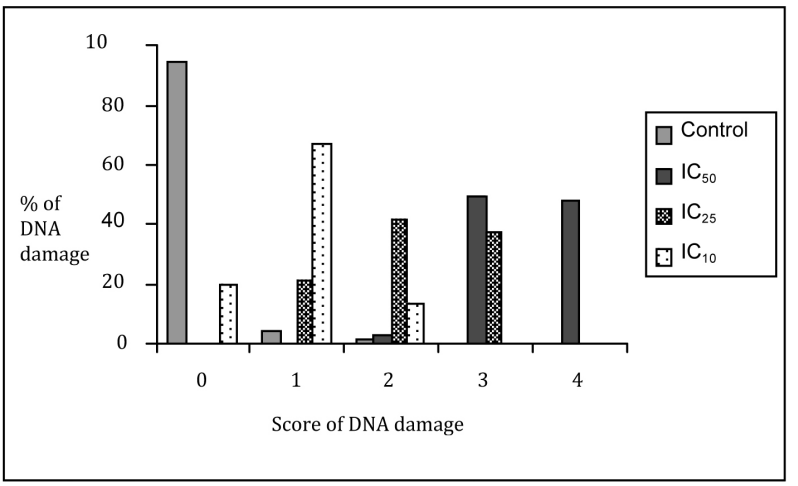

Figure 3. Genotoxic effect of zinc on *Acanthamoeba*'s DNA by comet assay.

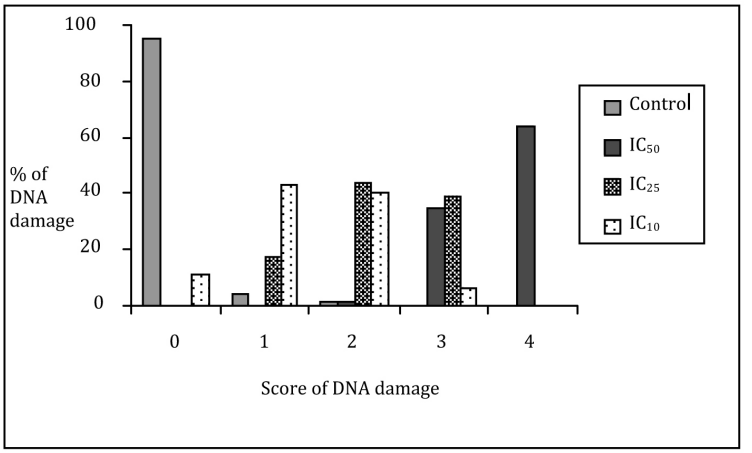

Figure 4. Genotoxic effect of mercury on *Acanthamoeba*'s DNA by comet assay.

The increased in concentration of heavy metals used in the present study caused a higher score of DNA damage in *Acanthamoeba*. The percentage of DNA damage with different scores in *Acanthamoeba* cells varied for both heavy metals observed in the present study. Different scores indicate different degrees of DNA damage (Collins, 2004) and the DNA damage observed in *Acanthamoeba* agrees with the toxicity properties of the metal. The differences in tolerance of the organisms to different toxic pollutants are well-known phenomena and these illustrate the importance of genotype-environmental interactions in the responses of the organisms to the toxicants (Diaz et al., 2006). Since the amoebae are single-celled organisms, the effects of heavy metals, such as mercury and zinc, as conducted in the present study, both at cellular and the DNA level of *Acanthamoeba*, are quite severe. These amoebae, therefore, have the potential to be used for quantitative analysis of the existence of heavy metals, especially mercury and zinc, in the environment by looking at the percentage of their DNA damage. Since these amoebae inhabit most habitats, especially in aquatic ecosystems, the presence of heavy metals in the environment affect their life. This later affects

the entire food-web system since in the natural environment these amoebae control the bacterial population.

Conclusion

The IC_{50} values of mercury and zinc against the amoeba obtained in this study were 1.10 ppm and 39.00 ppm, respectively, indicating that the former metal is more toxic than zinc. Both metals not only exerted cytotoxic effects on *Acanthamoeba* but also caused severe DNA damage in the amoeba. The DNA damage with different scores observed in *Acanthamoeba* are dose-dependant to suggest that these findings can be used for quantitative analysis of mercury's and zinc's presence in water.

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