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Morphological analysis on the toxic effect of manganese on *Acanthamoeba* sp. isolated from Setiu Wetland, Terengganu: An *in vitro* study

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Abstract

Manganese (Mn) is one of the common pollutants found in aquatic environment that are difficult to destroy and very persistent in the water body. Excessive levels of Mn in the aquatic environment have threatened some of the aquatic organisms including free-living *Acanthamoeba* sp. In this study, Mn was assessed for its cytotoxicity and genotoxicity on *Acanthamoeba* sp. isolated from Setiu Wetland, Terengganu. IC_{50} value of Mn obtained was 24 ppm while morphological observation indicates that *Acanthamoeba* sp. underwent apoptotic and necrotic mode of cell death after exposure to Mn for 24 hours at 30°C. This metal is also able to induce mild genotoxicity towards the DNA of *Acanthamoeba* sp.

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Keywords: Cytotoxicity; genotoxicity; alkaline comet assay; heavy metal; scanning electron microscopy

1. Introduction

Nowadays, human health and the ecosystem of the water bodies were adversely being affected by the majority or river system that are polluted due to the mixture of domestic waste, industrial effluents and many other pollutants [1]. In addition, Lah et al. [2] also stated that these activities are the major source of surface water pollution as they

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give off variety of genotoxic compounds.

Direct toxic effects can occur from the contaminants such as petroleum hydrocarbon, heavy metals and pesticides once released to the environment [3]. Carcinogenic and oxidative potential of metal pollution are considered hazardous to biological system [4] and the exposure of pollutants may directly and/or indirectly affect the populations and communities abundances with the presence of the toxicants [3]. Mn is one of the common metal found in the aquatic environment as it may exist in different forms either as compound, or complexes with organic compounds. It is very difficult to be removed from the water body since it will only precipitate out if the pH is raised to 10.0 [5]. Mn persists in the environments and has raise concern on how elevated Mn affects the aquatic life as there are not as many information on their toxicity towards aquatic organism compared to other metals. *Acanthamoeba* sp. is a common protist that is found in diverse environment, including aquatic environment. Due to its ubiquitousness, their properties of being a single cell organism with their uncovered cell wall of trophozoites makes them sensitive to any change in the environment [6,7]. Thus, this study evaluates the cytotoxicity and genotoxicity effect of Mn on this free-living amoebae by observation based on their morphological characteristics. The objectives of this study is to determine the IC_{50} value of Mn towards *Acanthamoeba* sp. and to observe morphological changes in *Acanthamoeba* sp. as well as genotoxicity evaluation of Mn after exposed to *Acanthamoeba* sp. DNA.

2. Materials and method

2.1 MTT (2 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay to determine the IC_{50} value of Mn on Acanthamoeba sp.

Acanthamoeba sp. (environmental isolate) was used in all experiments. The trophozoites were maintained in axenic conditions in 4% protease-yeast-glucose medium and were subcultured every 4 days. MTT assay by Mossman [8] was used in this study to determine the IC_{50} value of Mn on *Acanthamoeba* sp.

2.2 Morphological observation of Acanthamoeba treated with Mn by light and scanning electron microscopy.

For morphological observation of *Acanthamoeba* after exposure to Mn, the amoeba were first seeded in a six-well plate and incubated at 37°C in the absence and presence of Mn at their IC_{50} concentration. After 24h of incubation, the morphological changes of *Acanthamoeba* were observed under light microscopy. Scanning electron microscopy observation was carried out following the method by Fatimah et al. [9].

2.3 Mode of cell death determination by fluorescence microscopy - Acridine orange/ propidium iodide (AO/PI) staining.

For AO/PI staining, the Mn-treated and untreated *Acanthamoeba* cells were harvested and washed with PBS and then incubated with 5µl of acridine orange (10µg/ml) and propidium iodide (10µg/ml) at a ratio of 1:1 in 1 ml of cells and centrifuged at 1000 rpm/15 min. After centrifuge, supernatant was removed leaving 50 µl of residual supernatant with pellet. The pellet was resuspended and 10 µl was pipetted on the slide before putting on the cover slip. Within 30 min, the slide was analyzed using fluorescence microscope (Leica, Germany).

2.3 Analysis of DNA damage by Alkaline Comet Assay

Analysis of DNA damage by alkaline comet assay for *Acanthamoeba* sp. was performed after 2 h treatment with Mn in six-well plates containing trophozoites (10^4 cell/ml) at their IC_{25} concentration. The alkaline comet assay protocol described by Lah et al. [2] was followed. One hundred cells on each slide with three replicates were viewed, classified and quantified based on descriptions by Collins [10]. Kruskal-Wallis Test was used to analyse the data obtained from the comet scoring.

3. Results and discussion

Anthropogenic activities have led to metal pollution in the water bodies as it threatened some of the aquatic

organism and this includes the free-living amoebae. Decreasing population of amoebae due to the presence of heavy metal will leads to imbalance of the natural ecosystem as amoebae act as predator for bacteria.
The presence of heavy metal in the aquatic environments makes them a suitable toxicant to be evaluated for their effects towards amoebae. To date, there are only a few reports on cytotoxic and genotoxic effects of heavy metal on amoebae like cadmium, mercury, lead and zinc on these free-living amoebae [7]. However, there are no study focusing the effect of Mn on *Acanthamoeba*. Therefore, it is important to evaluate the effect of Mn towards *Acanthamoeba* as it would indicate the presence of exceed level of Mn in aquatic environment.

3.1 Determination of Mn Fifty percent inhibition concentration (IC_{50}) on Acanthamoeba sp.

In this study, *Acanthamoeba* sp. were exposed to various concentrations of Mn i.e 0, 30, 60, 90, 120, 150 and 180 ppm for 24 hours at 30°C in a 96-well plate to determine the cytotoxicity of the metal. The MTT assay was carried out to assess the viability of *Acanthamoeba* sp. Fig.1 shows the graph of viability of cell against different concentrations of Mn. Based on the graph, the IC_{50} value obtained was 24 ppm and *Acanthamoeba* sp. showed the reduction in viability upon exposure to increasing in Mn concentration.

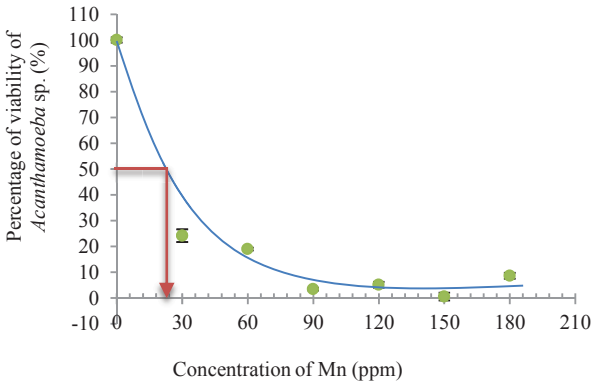

Fig 1. Percentage of viability of *Acanthamoeba* sp. cells assessed by MTT assay exposure to Mn for 24 hours. IC_{50} value obtained was 24 ppm

IC_{50} value was obtained by using MTT assay with a slight modification on its protocol. After the cells were treated with Mn at varying concentrations for 24 hours at 30°C in a 96-well plate, the medium in the plate was discarded. The plate was washed with PBS solution 3 times before loading the MTT solution. This is to ensure that the glucose content in the medium was completely removed. Presence of glucose in the medium will reduce the efficiency of MTT reaction. MTT specific activity are reduced by cells that are extensively metabolized D-glucose [11]. Cytotoxicity of Mn on *Acanthamoeba* sp. was determined based on the percentage of viable *Acanthamoeba* sp. after exposure to Mn for 24 hours at 30°C. In general, the viability of *Acanthamoeba* sp. decreased with increasing concentrations of Mn (Fig. 1). This shows that the growth of the cells was affected once exposed to Mn. The concentration of Mn that inhibit 50% of the cell population was 24 ppm. The lower IC_{50} of Mn shows that it has higher toxicity to *Acanthamoeba* sp. compared to Zn which showed an IC_{50} of 39 ppm [7]. This shows that Mn have higher toxicity compares to Zn since the IC_{50} of Mn was lower than Zn. Based on the periodic table of element, Mn have a less stable electron configuration which is $[Ar]4s^2\ 3d^5$ compares to Zn with electronic configuration of $[Ar]4s^2\ 3d^{10}$. This is supported by Martin-Gonzalez et al. [12] by stating that toxicity of heavy metals decrease with increased stability of electron configuration. In addition, heavy metals have high attraction to negatively-charged group of protein and thiol group [13]. Any alteration that occurs in these proteins will lead to dysfunction of certain enzymes in which may affect certain metabolic reactions in the cell. This phenomenon may have also occurred in *Acanthamoeba* sp.

3.2 Morphological observation of Acanthamoeba sp. treated with Mn by light and scanning electron microscopy.

To date, many techniques have been developed to determine the mode of cell death after exposure to xenobiotics and in this study, morphological observation due to effect of Mn on *Acanthamoeba* sp. was assessed by using light

and scanning electron microscopy after incubation of the *Acanthamoeba* with Mn at their IC_{50} value. Fig. 2 shows a comparison between viable (Fig. 2a) and non-viable Mn-treated *Acanthamoeba* cells (Fig. 2b) are rounded and underwent apoptotic and necrotic type of cell death.

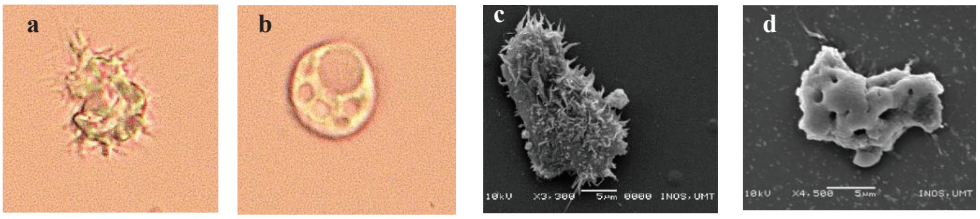

Fig 2. Image of *Acanthamoeba* sp. under light microscope of untreated *Acanthamoeba* sp. (a) and *Acanthamoeba* sp. after treatment with IC_{50} value of Mn (b). Scanning electron micrographs of untreated (c) and Mn-treated *Acanthamoeba* sp. (d). Prominent acanthapodia can be seen in untreated *Acanthamoeba* whereas Mn- treated *Acanthamoeba* appear with severe damage on the cell surface with decreased number of acanthapodia structure.

Based on the results obtained, untreated *Acanthamoeba* sp. shows the presence of acanthapodia with irregular shape of *Acanthamoeba* cells (Fig. 2a). Whereas the treated *Acanthamoeba* sp. shows the absence of acanthapodia with formation of vacuoles compared to the untreated *Acanthamoeba* cell (Fig. 2b). Mn- treated trophozoites of *Acanthamoeba* in the present study became rounded and floating in the culture medium perhaps as result of shortened or loss of acanthapodia (Fig. 2b). Contractile vacuoles were undetectable. The morphological change consequently inhibited the ability of the trophozoites to attach to the surface of the culture plate; in fact, the nearly spherical shape of trophozoite suggested the amoebae were beginning to become inactive and stressed out. Mn-treated *Acanthamoeba* became rounded in shape and underwent an encystment process due to unfavourable condition in the presence on Mn in its environment.

To further evaluate the effects of Mn on the morphology of *Acanthamoeba* sp., the cells were viewed under scanning electron microscope. Fig. 2(c) shows untreated *Acanthamoeba* cell while Fig. 2(d) shows the Mn-treated cell. The untreated *Acanthamoeba* sp. showed the presence of acanthapodia, whereas the Mn-treated cell showed that the acanthapodia which is very important for adhesion to surface or substrate and for feeding [14], were almost absent. Collectively, the trophozoites became reduced in size, cystic cell appearance, and sunken food cups, loss of acanthapodia structure as well as wrinkle on the upper side of cell surface (Fig. 2d). In addition, thickened, broadened and elongated acanthapodia that are tightly attached to the surface of the substratum were also observed. This observation provides convincing evidence that Mn generate alterations of the *Acanthamoeba* sp. external morphology. *Acanthameoba* sp. turns to cyst stage when the cells are metabolically inactive as the environments are not conducive for them. Muller [15]) also stated that large numbers of vacuoles formed in early encystations as pinocytic activity are reduced. The treated *Acanthamoeba* sp. showed reduction in size with less acanthapodia present compared to the untreated cell. The external morphology also has been damaged. Amoebae that are having inactive throphozoites will later differentiate into cyst stage in which they are defending themselves from the unfavourable condition [7]. Siddiqui et al. [16] also stated that in cyst stage, the cell enclosed itself within a resistant shell and the trophozoites become metabolically inactive (minimal metabolic activity) after an initial burst of metabolic activity. Therefore, it can be concluded that Mn are toxic towards the *Acanthamoeba* cell.

3.3 Mode of cell death determination by fluorescence microscopy - Acridine orange/ propidium iodide (AOPI) staining.

AOPI staining able to differentiate between viable and non-viable cell as well as the mode of cell death either apoptosis or necrosis. It was employed on *Acanthamoeba* cells after being incubated with Mn at its IC_{50} value. The stage of apoptosis either early or late apoptosis can be determined by the coloration of the cytoplasm. Green cytoplasm with orange stained nucleus indicates that the cell had undergone early apoptosis. Whereas for late apoptosis, both of the cytoplasm and the nucleus appear orange in colour as a results of the breakage of the nuclear envelop that led nuclear destruction [7]. Results obtained in this study shows that Mn played a role in inducing apoptosis or programmed cell death of *Acanthamoeba* sp. (Fig. 3).

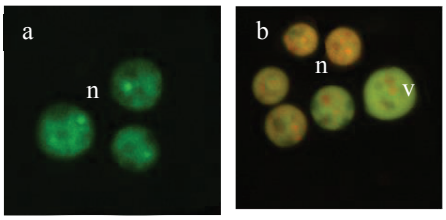

Fig 3. Green fluorescence of AOPI stained untreated *Acanthamoeba* sp. (a) and apoptotic and necrotic *Acanthamoeba* sp. after exposed to Mn (b). (v: vacuole, n: nucleus) (Mag : 400×)

Fig. 3 (a) shows cell with green cytoplasm with bright green fluorescent of intact nucleus and indicates that the cell were viable as the membrane-permeable AO dyes which are cationic are able to stain the negatively charged DNA in the nucleus and thus producing such colouration. Fig. 3(b) shows green colouration of cell with orange-stained nucleus to indicate the *Acanthamoeba* cell were non-viable. The *Acanthamoeba* cell that undergoes apoptosis are characterized by the green to orange of the cytoplasm with orange nucleus. PI dyes that are membrane impermeable are able to penetrate the cell and thus stain the nucleus with orange colour. This has indicated that the membrane of *Acanthamoeba* sp. has broken which allows the penetration on the PI dye. Nuclei of these cells are substantially fragmented and condensed to suggest the function of their membrane are disturbed even though the cells are coloured with green as in viable cells [7].

3.4 Genotoxicity of Mn on Acanthamoeba sp. assessed by Comet Assay

Alkaline Comet assay was used to determine the genotoxicity of *Acanthamoeba* cell after exposed to Mn for 2 hours at 30°C in IC_{25} value of Mn which was 12 ppm (half of IC_{50} value). The DNA damage was evaluated based on the degree of the DNA damage produced by the cells which were denoted by the scoring of 0,1,2,3 and 4. The scoring was done as shown in Fig. 4.

Fig 4. Image of comet produced by DNA of *Acanthamoeba* sp. after being exposed to IC_{25} concentration of Mn. The scoring was done based on the tail produced by the comet from the score of 0, 1, 2, 3 and 4. Score 0 indicate intact DNA with no tail (a), Score 1 indicate 25 % of DNA at the tail (b), Score 2 indicate 25% to 50% of DNA at the tail (c), Score 3 indicate 50% to 75% of DNA at the tail (d), Score 4 indicate more than 75% of DNA at tail (e) (Collins, 2004). These cells were stained with Ethidium Bromide and viewed under fluorescence microscope. (Magnification: 400×).

A total of 50 cells were randomly chose and scored at IC_{25} concentration of Mn. After the scoring was done, the percentages of cells with their respective scores were calculated (Fig. 4). 44% of the DNA damage were at score 0 and 6% of DNA damage were at score 4. Alkaline comet assay are one of the method developed to detect DNA damage in cell. DNA strand breakages (double, single, alkali-labile sites expressed as single strand breaks) are able to be detected by this comet assay which is also known as single-cell gel electrophoresis test [17]. According to Collins [10], contamination of the environment with genotoxins can be assesed via alkaline comet assay with the suitable organism that can be used as biosensors. In this study, genotoxicity of Mn on *Acanthamoeba* sp. was being evaluated via comet assay. To assess the genotoxicity of this compound, the value of IC_{25} which is 12 ppm (half of IC_{50} value) was used to treat the *Acanthamoeba* sp. cells for 2 hours at 30°C. IC_{25} value was used instead of IC_{50} to determine whether Mn compound is able to induce genotoxic even at low concentration. The damage of the DNA was evaluated according to five types of score which were 0, 1, 2, 3 and 4 (Fig. 4). They are categorized based on

the DNA migration during electrophoresis step. Table 1 shows the percentage of DNA damage with their respective score at the IC_{25} value of Mn.

Table 1 Percentage (%) type of comet of *Acanthamoeba* cells after treatment with Mn for 2h at IC_{25} value.

Score	0	1	2	3	4
% type of comet	43.59±0.333	22.05±1.201	18.46±1.155	10.26±0.882	5.64±0.667

* Data was obtained from three replicates and significantly different among group ($p < 0.05$ in Kruskal-Wallis test).

Score 0 have the highest percentage of damage which are 44%, followed by second highest which are score 1 with 22%. 18% of the DNA damage are at score 2 while score 3 and 4 showed lower percentage of DNA damage which are 10% and 6% respectively. Generally, more than 80% of the comet has noted the score of 0, 1 and 2. Less than 20% of the comet noted the score of 3 and 4. This has shown that Mn are able to induce mild genotoxicity to the *Acanthamoeba* sp. since there are a low percentage of score with 3 and 4 which are considered severe in DNA damage. This is supported by Nakisah et al. [7] by stating that Score 3 and 4 are considered severe and irreversible, compared with score 2, 1 and 0. DNA fragmentation related with necrotic and apoptotic cell death may be featured in increased migration of DNA fragment (comet tail) from the head (nucleus) in comet assay [18]. Though some metal induces cytotoxicity, they do not necessarily induce genotoxicity. In this study, Mn was found to induce both cytotoxicity and also mild genotoxicity even at low concentration.

4. Conclusion

Techniques used in this study proved the toxicity of Mn on *Acanthamoeba* sp. as shown by low IC_{50} value (24 ppm), while the morphological observation on Mn treated-*Acanthamoeba* sp. showed damage in its morphological structure and also has the potential to induce genotoxicity towards *Acanthamoeba* sp. cell.

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