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Research Paper

Distinct toxicological characteristics and mechanisms of Hg^{2+} and MeHg in *Tetrahymena* under low concentration exposure

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ABSTRACT

Inorganic divalent mercury complexes (Hg^{2+}) and monomethylmercury complexes (MeHg) are the main mercury species in aquatic systems and their toxicity to aquatic organisms is of great concern. *Tetrahymena* is a type of unicellular eukaryotic protozoa located at the bottom of food chain that plays a fundamental role in the biomagnification of mercury. In this work, the dynamic accumulation properties, toxicological characteristics and mechanisms of Hg^{2+} and MeHg in five *Tetrahymena* species were evaluated in detail. The results showed that both Hg^{2+} and MeHg were ingested and exhibited inhibitory effects on the proliferation or survival of *Tetrahymena* species. However, the ingestion rate of MeHg was significantly higher than that of Hg^{2+}. The mechanisms responsible for the toxicity of MeHg and Hg^{2+} were different, although both chemicals altered mitochondrial membrane potential (MMP). MeHg disrupted the integrity of membranes while Hg^{2+} had detrimental effects on *Tetrahymena* as a result of the increased generation of reactive oxygen species (ROS). In addition, the five *Tetrahymena* species showed different capacities in accumulating Hg^{2+} and MeHg, with *T. corlissi* exhibiting the highest accumulations. The study also found significant growth-promoting effect on *T. corlissi* under low concentration exposure (0.003 and 0.01 μg Hg/mL (15 and 50 nM)), suggesting different effect and mechanism that should be more closely examined when assessing the bioaccumulation and toxicity of mercury in aquatic ecosystems.

1. Introduction

Mercury (Hg) has been defined as a global pollutant as a result of its characteristics of long-range transport, persistence, bioaccumulation and toxicity (Fitzgerald et al., 2007; Jiang et al., 2006; Liu et al., 2012). The toxicity of Hg depends largely on its chemical forms, and monomethylmercury complex (MeHg) is the most toxic species of Hg. In aquatic systems, inorganic divalent mercury complexes (Hg^{2+}) and MeHg are the two main species for Hg. Studies have shown that increased generation of reactive oxygen species (ROS) is a toxic mechanism in cell death and fish mortality induced by Hg^{2+} (Yole et al., 2007; Zhang et al., 2016). However, MeHg causes nervous system damage, especially during the early growth phase of the brain (Clarkson, 1997; Tonazzi et al., 2015). With its high rate of bioaccumulation and biomagnification in the food chain, MeHg could do grievous damage to human beings (Hsu-Kim et al., 2013; Meng et al., 2014; Yan et al., 2010). Determining the biomagnification of Hg species in the food chain is therefore critical to assessing the risks of Hg in the environment.

The mercury uptake of protozoa situated at the bottom of the aquatic food chain can be considered a starting point for mercury accumulation and an essential process affecting the distribution of Hg in food webs (Hammerschmidt et al., 2013; Hsu-Kim et al., 2013; Lin et al., 2013). *Tetrahymena* is a genus of free-living protozoa that is widespread in aquatic systems (Fenchel, 2013). Because of its rapid proliferation, unique nuclear dualism, extensive membrane structure and rapid reaction to external exposure, *Tetrahymena* serves as a useful model organism for the investigation of some exogenous toxic substances. Studies have found that the fatty acid composition of *Tetrahymena thermophila* can be affected by exposure to CuO nanoparticles, and TiO_2 may increase Cd bioaccumulation in *Tetrahymena thermophila* and induce greater joint toxicity (Mortimer et al., 2011; Yang et al.,

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2014).

As a kind of unicellular eukaryotic organisms in aquatic systems, the response of *Tetrahymena* to pollutants can be observed directly and be analyzed in more detail than other animals, such as fish or rats. Considering the unicellular characteristic of *Tetrahymena*, some analysis methods for cell research may be tentatively applied to assess the effect and potential mechanisms of toxicants on the mentioned protozoa. In addition, *Tetrahymena* species are widespread in freshwater and are essential for the transportation and transformation of mercury species in aquatic environments. Thus, *Tetrahymena* is a potentially valuable model organism for evaluating the toxicity of mercury species. In the past few decades, a few studies have been carried out to target the effects of Hg on *Tetrahymena pyriformis*, suggesting that its generation time or bioassay may be affected under exposure to Hg (Carter and Cameron, 1973; Thrasher and Adams, 1972). Apart from the lack of studies about the toxicity and mechanisms of Hg in *Tetrahymena*, only one species of *Tetrahymena* was used in those previous studies and the differences among *Tetrahymena* species were not investigated. Further, the uptake properties and toxicological mechanisms of different Hg^{2+} and MeHg in *Tetrahymena* species remain unclear.

The aim of this work was to study the toxicological characteristics and mechanisms in *Tetrahymena* under exposure to Hg^{2+} or MeHg, the two main Hg species in aquatic systems. To evaluate the effects of these chemicals, five *Tetrahymena* species (*T. corlissi*, *T. pyriformis*, *T. shanghaiensis*, *T. malaccensis*, and *T. thermophila SB210*) were selected. The bioaccumulation properties and toxic effects of Hg^{2+} and MeHg on *Tetrahymena* species were discussed in detail. In order to comprehensively assess the toxic effects of mercury species in the environment, one of the five *Tetrahymena* species was exposed to low concentrations of Hg^{2+} or MeHg (0.003 and 0.01 μg Hg/mL (15 and 50 nM)).

2. Materials and methods

2.1. Tetrahymena species and cell culture

The five *Tetrahymena* species (*T. corlissi*, *T. pyriformis*, *T. shanghaiensis*, *T. malaccensis* and *T. thermophila SB210*) were provided by Dr. Wei Miao at the Institute of Hydrobiology, Chinese Academy of Sciences (Wuhan, China). The culture medium (SPP medium) used in this study (pH 7.0–7.2) contained 20 g proteose peptone (Becton, Dickinson and Company, USA), 1 g yeast extract (OXOID, Thermo Fisher Scientific, USA), 2 g glucose (Sigma, USA) and 0.03 g Ferric citrate (Sigma, USA) in 1000 mL ultra-pure water (Millipore, Darmstadt, Germany), with 1% (v/v) penicillin-streptomycin solution for preventing infection from bacterium or fungus (10,000 units/mL penicillin and 10,000 mg/L streptomycin, HyClone, GE Healthcare Life Sciences, USA). Considering the living conditions generally suitable for the five *Tetrahymena* species, all *Tetrahymena* cells used in the study were cultured at 28 °C, shaking at 135 rpm (Feng et al., 2007; Gorovsky et al., 1975; Li et al., 2015; Ye et al., 2014).

2.2. Growth curves

Tetrahymena cells were first cultured in medium for about 24 h, until the cells entered the logarithmic growth phase. A portion of these cells was inoculated into 10 mL of fresh medium and cultured in a 20 mL glass bottle (CNW Technologies GmbH, Germany). Experiments were conducted in triplicate. The final cell density for all *Tetrahymena* species was set to be about 3000 cells/mL using a hemocytometer coupled with a microscope following the previous publication (Absher, 1973; Li et al., 2015). Cell densities of all groups were determined and recorded every 3 h using a hemocytometer until the cells entered the decline phase (Absher, 1973; Li et al., 2015; Miao et al., 2006). Before counting, *Tetrahymena* cells were anesthetized by adding a small amount of methanol solution (10%, v/v), in order to immobilize the *Tetrahymena* cells and ensure the accuracy of the cell count.

2.3. Exposure to Hg^{2+} and MeHg at gradient concentrations

The growth responses of five *Tetrahymena* species were observed when exposed to 8 different concentrations of either Hg^{2+} or MeHg for 24 h. Based on the growth curves of the five *Tetrahymena* species, Hg^{2+} or MeHg solutions (GBW08617 and GBW08675, both from National Institute of Metrology, China) were added to the medium early in the logarithmic phase of growth. For the stock solutions, the Hg^{2+} is dissolved in an aqueous nitric acid (3%, v/v) solution and the MeHg is dissolved in methanol. The work solutions were stepwise diluted from the stock solutions (stored at 4 °C) by ultra pure water in brown glass bottles when they would be used. The added concentrations of Hg^{2+} or MeHg were as follows: 0 (the control), 0.03, 0.1, 0.3, 0.6, 1, 3, 6 and 10 μg Hg/mL (0, 0.15, 0.5, 1.5, 3, 5, 15, 30 and 50 μM). To make the work condition more clear and easy for comparison, we expressed each concentration in two representing ways. Three parallel experiments were conducted for each concentration. Following exposure for 24 h, the cell densities of all groups were determined. The concentration producing 50% of the maximum effect (EC_{50}) was calculated according to the growth inhibition of the five *Tetrahymena* cells in terms of the added Hg concentrations, represented by the fold change (%) in growth when compared to the control (Schramm et al., 2011). The medium containing cells was then centrifuged at 5500 rpm, 4 °C for 15 min and the supernatant was quickly discarded. Cell samples at the bottom of the tubes were cleaned with mercury-free SPP medium three times before the cells were recounted. Then the concentrations of total Hg (THg, expressed by the mean Hg mass per 1000 cells) in the samples were analyzed to determine the correlativity between uptake of mercury species and growth inhibition in *Tetrahymena*.

Based on growth curves of the five species tested and EC_{50} values at 24 h, *Tetrahymena* cells were exposed to two relatively low dose (in terms of non-inhibitory effect) of mercury species (0.1 μg Hg/mL (0.5 μM) for Hg^{2+} and 0.03 μg Hg/mL (0.15 μM) for MeHg, respectively) in the early logarithmic phase of growth. Both concentrations had the similar non-inhibitory effects on cell proliferation. Medium containing cells was collected at 12 h, 24 h, and 48 h after exposure. Each treatment was conducted in triplicate. After cleaning and counting, THg concentrations (ng Hg/1000 cells) were analyzed to compare the Hg^{2+} and MeHg bioaccumulation capacity of five *Tetrahymena* species following long-time exposure.

2.4. Hg analysis

The THg concentrations in the cell samples were analyzed using a Hydra II C mercury analyzer (Teledyne Leeman Labs, Hudson, USA) according to the USEPA method 7473 (Shao et al., 2016; USEPA, 1998). Briefly, a 50 μL cell suspension of every treated sample was pipetted into a nickel boat. The boats containing samples were then burned at high temperature to reduce all mercury species to elemental mercury, which was trapped with a gold amalgam. After decomposition, the Hg content of each sample was determined with a Hydra II C mercury analyzer. Finally, the concentrations of THg were calculated as the mean total Hg mass per 1000 cells (ng Hg/1000 cells).

2.5. Measurement of cell death, intracellular reactive oxygen species (ROS) and mitochondrial membrane potential (MMP) using flow cytometry

Cell death (judged through cell membrane permeability), intracellular ROS and MMP were measured using flow cytometry (Novocyte 1040, ACEA, USA). Based on the 24 h EC_{50}, the exposed gradient concentrations of Hg^{2+} and MeHg were relatively low to maintain *Tetrahymena* cell density abundant enough for analysis: 0 (control), 0.03, 0.1, 0.5 μg Hg/mL (0.15, 0.5, 2.5 μM) for Hg^{2+} and 0 (control), 0.03, 0.1 μg Hg/mL (0.15, 0.5 μM) for MeHg. The 0.1 ‰ (v/v) H_2O_2 (diluted from 30% H_2O_2) was used for the positive control. To ensure the viability of *Tetrahymena* cells and for the convenience when