Veridian E-Journal, Science and Technology Silpakorn University
Volume 5 Number 1 January – February 2018 ISSN 2408 - 1248

Single Cell Gel Electrophoresis of Microcrustaceans *Moina macrocopa* Exposed to Cadmium*

Chanya Phromchaloem**
Amonrujee Nakphlaiphan***
Setthawut Piriyamasakul****
Warangkhana Pruksarojanakul*****
Thanit Pewnim******

Abstract

Exposure of microcrustacean *Moina macrocopa* neonates to cadmium chloride at a concentration of 160 µg L^{-1} resulted in significant nuclear damages as judged by single cell gel electrophoresis or Comet Assay. DNA damages were assessed quantitatively by measuring percentage of tail DNA and tail moment. It was found that the % tail DNA and the tail moment of the cadmium treated group were 4.44 ± 1.40 % and 0.17 ± 0.07 as compared to the values of 0.32 ± 0.08 % and 0.00 ± 0.00 in the control group, respectively. The values of both % tail DNA and the tail moment of cadmium treated group were significantly higher than that of the control group, having $p < 0.05$ in both cases. Examination of the morphology of the cell nuclei revealed that the nuclei of most cells in the control group are rounder than the cadmium treated group.

Keywords: *Moina macrocopa*, Cadmium, SCGE, Comet assay, Tail DNA, Tail moment

---

* This manuscript is a part of the research work done during the study towards the degree of Doctor of Philosophy Program.

** Ph.D. student in Bioinorganic Chemistry, Department of Chemistry, Faculty of Science, Silpakorn University, Thailand. e-mail: chanya.phromchaloem@gmail.com

*** Assistant researcher, Department of Chemistry, Faculty of Science, Silpakorn University, Thailand. e-mail: amonrujee.nakphlaiphan@gmail.com

**** Assistant researcher, Department of Chemistry, Faculty of Science, Silpakorn University, Thailand. e-mail: s.piriyamasakul@gmail.com

***** Assistant researcher, Department of Chemistry, Faculty of Science, Silpakorn University, Thailand. e-mail: warangkhana.pruksarojanakul@gmail.com

****** A thesis advisor, Assoc. Prof. Dr. Thanit Pewnim, Department of Chemistry, Faculty of Science, Silpakorn University, Thailand. e-mail: thanit.pewnim@gmail.com

1. Introduction

Heavy metals have been a major concern for the safety of the environments during the past decades due to its uses in several industries. Damages to DNA of living organisms, both plants and animals, have been shown to occur widely upon exposures to those metals (Almeida, Pereira et al., 2013; Duan, Zhai et al., 2013; Prochazkova, Wilhelmova et al., 2013; Fae, Balestrazzi et al., 2014; Kaur, Singh et al., 2014; Gupta, Ahmad et al., 2015; Khisroon, Gul et al., 2015; Santos, Pourrut et al., 2015; Xin, Wenchao et al., 2015; Gasulla, Picco et al., 2016; Singh, Bhagat et al., 2017)

The issue of our special concern is the cadmium contamination in the aquatic environment. There has always been a requirement to find bioindicators for its contamination. Cladocerans are organisms of choice to be used in the study of heavy metals toxicity. This is because the cladocerans are zooplanktons, so they are usually sensitive to the changing in their environments. In this regard, *Daphnia spp.,* particularly *Daphnia magna* seems to be an organism for standard monitoring of the aquatic environment (Barata, Markich et al., 2002; Pellegri, Gorbi et al., 2014). Cadmium is known to be a carcinogen because of its inhibition on DNA repair, apoptosis resistance, induction of oxidative stress, and the cause of aberrant gene expression (Joseph, 2009). The well accepted standard assay is therefore the assay for the damages of genetic materials using the single cell gel electrophoresis (SCGE) or the Comet Assay (Olive and Banath, 2006; Kumaravel, Vilhar et al., 2009; Lorenzo, Costa et al., 2013; Muthukaruppan, 2015; Santos, Pourrut et al., 2015). Comet assay has been shown to be truly a good indicator for genetic damages by pollutants (Azqueta and Collins, 2013).

In a tropical country like Thailand, *Daphnia spp.* are not common in nature. However, another cladocerans *Moina macrocopa* are abundant and ubiquitous. Only few studies have been carried out on cadmium toxicity in *Moina macrocopa*. For example, a study locally showed that cadmium can enter *Moina macrocopa* through a food chain. Cadmium was shown to find its route from the phytoplankton *C. vulgaris* to the zooplankton *M. macrocopa* and eventually to the catfish *C. macrocephalus* and *C. gariepinus* (Ruangsomboon and Wongrat, 2006). Another study showed that exposure of *M. macrocopa* to $CdCl_2$ at 0.08, 0.16 and 0.32 L^{-1} had negative effects on survivorship and reproduction of the microcrustaceans (Gama-Flores, Sarma et al., 2007). However, the work did not include the study on damages of cell nuclei. It therefore becomes the objective of this research to study the effect of cadmium chloride on cell nuclei morphology as well as to assess the damages of

Veridian E-Journal, Science and Technology Silpakorn University
Volume 5 Number 1 January – February 2018 ISSN 2408 - 1248

cell nuclei in *Moina macrocopa* by single cell gel electrophoresis (SCGE) or also known as the Comet Assay.

2. Materials and methods

2.1. Animals and growth conditions

Microcrustaceans *Moina macrocopa* were purchased from an aquaculture farm in the eastern part of Bangkok, Thailand. The microcrustaceans were acclimated to laboratory conditions at 30 ± 2 ^{0}C under a light-dark cycle of 12:12 for one day with continuous aeration. The animals were fed daily using a commercial yeast *Saccharomyces cerevisiae.* Neonates of less than 24 hour were separated from adults and used in the experiments.

2.2 $CdCl_2$ treatment

A control group consisted of approximately 100 microcrustaceans *Moina macrocopa* neonates raised in a 250 mL-size beaker containing 50 mL of filtered water. A cadmium-treated group contained the same number of neonates, raised in 50 mL filtered water but containing 160 µg L^{-1} of $CdCl_2$. The neonates were raised in a light: dark cycle of 12:12 hours at temperature 30 ± 2 ^{0}C with gentle aeration. Neonates were fed with the yeast once a day. After 48 hours, the neonates were washed with filtered water three times before being used in the experiment

2.3 Examination of microcrustaceans Moina macrocopa under a light and fluorescence microscope.

It is a common practice to assess the health of the microcrustaceans *Moina macrocopa* obtained from the farm before being used in the study. It is generally done by placing fresh specimens on a glass slide. After a coverslip was placed over the specimens, photomicrographs were taken by an Olympus ix71 inverted fluorescence & phase contrast microscope equipped with a DIC system (Olympus Co, Japan). The first set of photomicrographs were taken under visible light. When the water between the coverslip and the glass slide was nearly dried out, a small amount of DAPI (4',6-diamidino-2-phenylindole) solution at a concentration of 2 µg mL^{-1} was placed to seep through between the cover slip and the glass slide.

2.4 Comet assay or Single cell gel electrophoresis (SCGE)

Neonate samples in a 1.5 mL microcentrifuge tube were homogenized by a blue plastic pestle after a solution of 500 µL of buffer P (1 mL of PBS, 8 mL of 0.1 M EDTA and 1 mL of 10% (v/v) DMSO) was added. The homogenate was filtered through a 45 µm nylon filter and the filtrate was centrifuged at 16,000 x g for 4 min in 1.5 mL microcentrifuge tubes. After

the supernatant was removed and discarded, the precipitate was kept cold in the dark for further processing

A volume of 500 µL of 2% (w/v) low melting point agarose (LMA) in PBS buffer was added to the precipitate and mixed gently. The slurry agarose-cell mixture was layered onto microscope glass slides that were previously coated with 1% (w/v) LMA (in PBS). When the gel started to set, 120 µL of 1% (w/v) LMA was layered on top of the agarose-cell mixture. The whole process was carried out in a dark room. The glass slides were incubated in a lysis solution for 24 hr at 4 ^{0}C. The lysis solution consisted of 2.5 M NaCl, 0.1 M Na_2EDTA, 10 mM Tris-HCl, adjusted to pH 10, and just before use, Triton-X100 and DMSO were added to have a final concentration of 1% (v/v) and 20% (v/v), respectively.

2.5 Electrophoresis

Single cell gel electrophoresis of microcrustaceans *Moina macrocopa* was carried out in a cold room. After the removal of the lysis solution, the glass slides were placed on the sample stage of HU10 electrophoresis equipment (Scie-Plas, United Kingdom). After adding electrophoresis buffer (0.3 N NaOH, 1 mM Na_2EDTA, pH 13) to cover the glass slides and let it equilibrate for 5 min, a current of 300 mA having a voltage of 25 V was applied for 15 min. The glass slides were treated with a neutralization buffer (0.4 M Tris base, pH 7.5) for 5 min. The glass slides were stained by 10 µg L^{-1} of ethidium bromide for 10 min. Wash the glass slide several times to remove the dye. Photos were taken by a fluorescent microscope (Nikon Company, Japan). % Tail DNA and Tail moment of 100 cells were analyzed by LUCIA program.

2.6 Statistical analysis

% Tail DNA and Tail moment were analyzed by using the Brown-Forsythe test at $P < 0.05$ of SPSS 18 (IBM) to test for equality of means. Normality of data was tested by the Kolmogorov–Smirnov test. Homogeneity of variances of the data was assessed by the Levene's test.

3. Results and discussion

Light microscopy and fluorescent microscopy of the Microcrustaceans *Moina macrocopa* samples were carried out to check if the adults and neonates were healthy prior to be used in the experiment. Typical photomicrographs of the healthy microcrustaceans are shown in Fig.1 for both the adult and the neonates. It is evident from light microscopy (Fig. 1A) that the healthy adult would have a gut full of feeds while the fluorescent microscopy (Fig.

Veridian E-Journal, Science and Technology Silpakorn University
Volume 5 Number 1 January – February 2018 ISSN 2408 - 1248

1B) shows that both adults and the neonates contained healthy cell nuclei well stained with DNA intercalating dye, the DAPI. The neonates have cells packed together at high density, ready to be isolated for single cell gel electrophoresis, the Comet Assay.

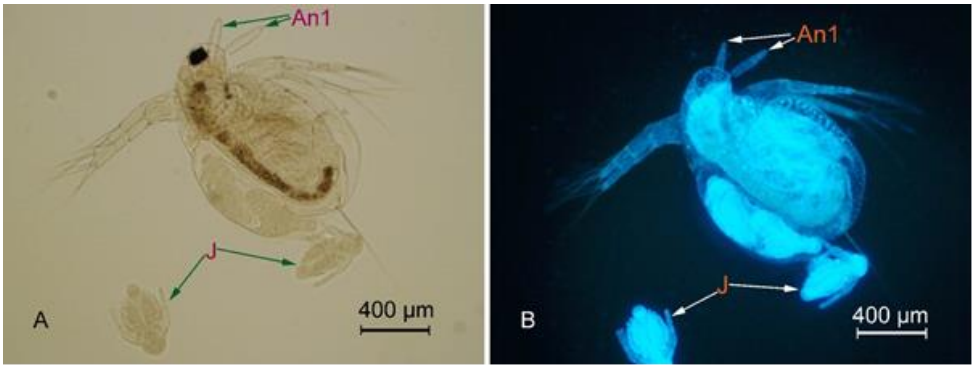

Fig. 1. A DAPI-stained female *Moina macrocopa* with juveniles (J) under a light microscope (A), and under an Epi-fluorescence microscope (B) showing overall morphology of the microcrustaceans under different light conditions. A paired first antennae (An1) is shown on top of the figure.

Nuclei of cells migrated in electric field for 15 min are shown in Fig. 2. From general observation, the nuclei of most cells in the control group are rounder than the cadmium treated group. A typical cell nucleus of the control group is shown in Fig. 2A, while a nucleus with a comet tail of a typical cell from the Cd treated group is shown in Fig. 2B. It is apparent that the comet tail is rather short and not as long and pronounce as those demonstrated in other species studied. The comparative species are, for examples, isolated marine muscle cells (Zhang, Chen et al., 2017), human liver carcinoma (HepG2) cells (Skipper, Sims et al., 2016), and Daphnia magna (Pellegri, Gorbi et al., 2014). However, the above comparison cannot be taken as a concrete differentiation because the conditions of the experiments in all cases were not the same.

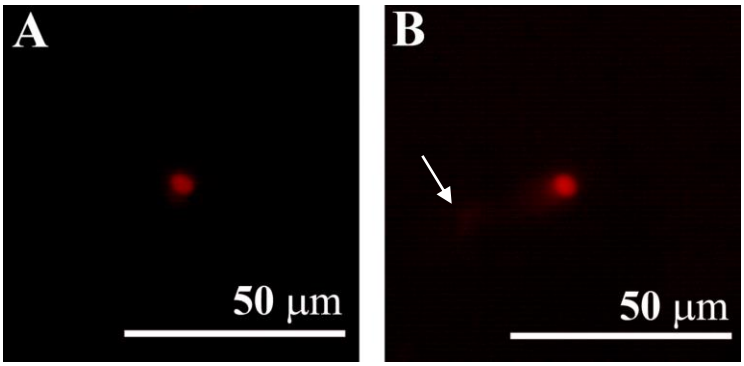

Fig. 2. Fluorescence photomicrographs of comet assay of representative cells of *M. macrocopa* in control and cadmium-treated groups. A is a cell nucleus from a control group. B is a cell nucleus from the 160 µg L^{-1} cadmium treated group where a faint comet tail can be seen in the south-west direction of the figure.

Quantitative analysis of the DNA damage was done by assessing the % Tail DNA (Fig. 3) and the Tail moment (Fig. 4) from a population of 100 cell nuclei for each case of the control group and the cadmium-treated group. It was found that the % tail DNA and the tail moment of the cadmium treated group were 4.44 ± 1.40 % and 0.17 ± 0.07 which are significantly higher than the values of 0.32 ± 0.08 % and 0.00 ± 0.00, respectively in the control group ($p < 0.05$ in both cases). It is evident from Fig. 3 that the % tail DNA of the cadmium treated group is only 4.44 ± 1.40 %. This level of damage is commonly regarded to be a minimum damage because it was a level below 10 % which is classified as minimal (Mitchelmore, Birmelin et al., 1998).

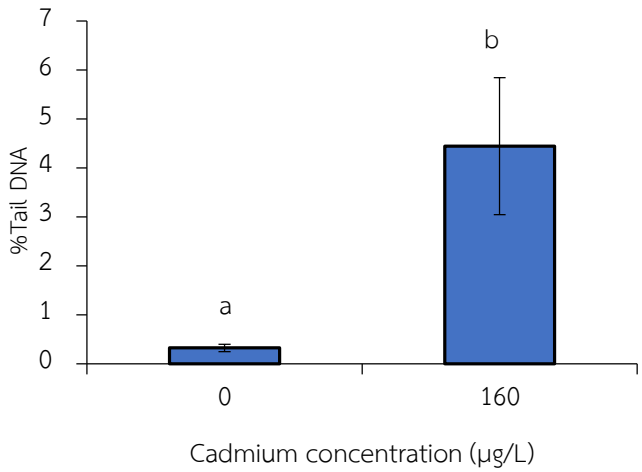

Fig. 3. % tail DNA of cell nuclei from microcrustaceans *Moina macrocopa* neonates exposed to 0 and 160 µg L^{-1} of cadmium for 48 hours. Values of % tail DNA from each histogram represent mean ± SE (n = 100). Different letters show a significant difference at $P < 0.05$.

Our results indicated that microcrustaceans *Moina macrocopa* were less sensitive to cadmium than *Daphnia magna.* In the case of *Moina macrocopa,* treatment with cadmium at a concentration of 160 µg L^{-1} for 48 hr caused damages at a minimum level, while the treatment of *Daphnia magna* with 10 µg L^{-1} of cadmium for only 24 hr did cause substantial damages already (Pellegri, Gorbi et al., 2014). The finding supports the Species Mean Acute

Veridian E-Journal, Science and Technology Silpakorn University
Volume 5 Number 1 January – February 2018 ISSN 2408 - 1248

Value (SMAV) for cadmium set by United States Environmental Protection Agency for *Daphnia magna* to be 40.62 µg L^{-1} and for *Moina macrocopa* to be 86.51 µg L^{-1} (Pellegri, Gorbi et al., 2014).

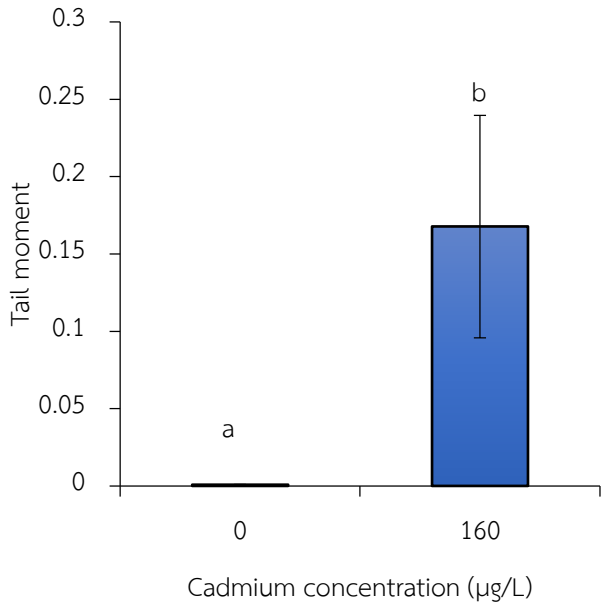

Fig.4. Tail moment of cell nuclei from microcrustaceans *Moina macrocopa* neonates exposed to 0 and 160 µg L^{-1} of cadmium for 48 hr. Values of tail moment from each histogram represent mean ± SE (n = 100). The value for the control group is very low so that the SEM bar is not visible. Different letters show a significant difference at $P < 0.05$.

4. Conclusions

Cell nuclei of microcrustaceans *Moina macrocopa* neonates showed a minimum level of DNA damages upon exposure to 160 µg L^{-1} of cadmium as revealed qualitatively by fluorescence microscopy and quantitatively by the assessment of % tail DNA and tail moment. The scientific basis of the difference in the sensitivity to cadmium in *Moina macrocopa* and *Daphnia magna* warrants further investigation at a molecular level.

Acknowledgements

The authors greatly appreciate the Head of Chemistry, Mrs. Chantana Waivinitpong, Faculty of Science, Silpakorn University for all the supports and encouragement throughout this study. We also would like to thank Dr. Wanwiwa Tumnoi from Department of Biology, Faculty of science, Silpakorn University for rendering helps on several aspects of this research. Supports and kind advice from Professor Stephen S. Tobe of University of Toronto, Canada is

highly appreciated. This research was supported by Ministry of Science and Technology (Thailand) through a Ph.D. fellowship to Ms. Chanya Phromchaloem.

References

Almeida, C., C. G. Pereira, T. Gomes, C. Cardoso, M. J. Bebianno and A. Cravo. (2013). "Genotoxicity in two bivalve species from a coastal lagoon in the south of Portugal." **Mar Environ Res** 89: 29-38.

Azqueta, A. and A. R. Collins. (2013). "The essential comet assay: a comprehensive guide to measuring DNA damage and repair." **Archives of Toxicology** 87(6): 949-968.

Barata, C., S. J. Markich, D. J. Baird and A. Soares. (2002). "The relative importance of water and food as cadmium sources to *Daphnia magna* Straus." **Aquatic Toxicology** 61(3-4): 143-154.

Duan, P. L., T. L. Zhai, C. J. Xu, J. N. Ding and Y. Chen. (2013). "A simple and effective method for detecting toxicity of chromium trioxide on *Vicia faba*." **European Food Research and Technology** 236(3): 517-521.

Fae, M., A. Balestrazzi, M. Confalonieri, M. Dona, A. Macovei, A. Valassi, G. Giraffa and D. Carbonera. (2014). "Copper-mediated genotoxic stress is attenuated by the overexpression of the DNA repair gene *MtTdp2* alpha (tyrosyl-DNA phosphodiesterase 2) in *Medicago truncatula* plants." **Plant Cell Reports** 33(7): 1071-1080.

Gama-Flores, J. L., S. S. S. Sarma and S. Nandini. (2007). "Exposure time-dependent cadmium toxicity to *Moina macrocopa* (Cladocera): a life table demographic study." **Aquatic Ecology** 41(4): 639-648.

Gasulla, J., S. J. Picco, P. Carriquiriborde, F. N. Dulout, A. E. Ronco and J. C. de Luca. (2016). "Genotoxic Effects Induced by Cd^{+2}, Cr^{+6}, Cu^{+2} in the Gill and Liver of *Odontesthes bonariensis* (Piscies, Atherinopsidae)." **Bulletin of Environmental Contamination and Toxicology** 96(5): 591-595.

Gupta, A. K., I. Ahmad and M. Ahmad. (2015). "Genotoxicity of refinery waste assessed by some DNA damage tests." **Ecotoxicology and Environmental Safety** 114: 250-256.

Joseph, P. (2009). "Mechanisms of cadmium carcinogenesis." **Toxicology and Applied Pharmacology** 238(3): 272-279.

Kaur, G., H. P. Singh, D. R. Batish and R. K. Kohli. (2014). "Pb-inhibited mitotic activity in onion roots involves DNA damage and disruption of oxidative metabolism." **Ecotoxicology** 23(7): 1292-1304.

Khisroon, M., A. Gul, A. Khan, N. Ali, F. Zaidi, S. B. Rasheed, Ahmadullah and H. Akbar. (2015). "Comet assay based DNA evaluation of fuel filling stations and automobile workshops workers from Khyber Pakhtunkhwa province, Pakistan." **J Occup Med Toxicol** 10: 27.

Kumaravel, T. S., B. Vilhar, S. P. Faux and A. N. Jha. (2009). "Comet Assay measurements: a perspective." **Cell Biol Toxicol** 25(1): 53-64.

Lorenzo, Y., S. Costa, A. R. Collins and A. Azqueta. (2013). "The comet assay, DNA damage, DNA repair and cytotoxicity: hedgehogs are not always dead." **Mutagenesis** 28(4): 427-432.

Mitchelmore, C. L., C. Birmelin, D. R. Livingstone and J. K. Chipman. (1998). "Detection of DNA Strand Breaks in Isolated Mussel (*Mytilus edulis*L.) Digestive Gland Cells Using the "Comet" Assay." **Ecotoxicology and Environmental Safety** 41(1): 51-58.

Muthukaruppan, G. (2015). "Heavy metal induced biomolecule and genotoxic changes in earthworm *Eisenia fetida*." **Isj-Invertebrate Survival Journal** 12: 237-245.

Olive, P. L. and J. P. Banath. (2006). "The comet assay: a method to measure DNA damage in individual cells." **Nat Protoc** 1(1): 23-29.

Pellegri, V., G. Gorbi and A. Buschini. (2014). "Comet Assay on *Daphnia magna* in eco-genotoxicity testing." **Aquatic Toxicology** 155: 261-268.

Prochazkova, D., N. Wilhelmova, D. Pavlikova, J. Szakova and T. Gichner. (2013). "Zinc induces DNA damage in tobacco roots." **Biologia Plantarum** 57(4): 783-787.

Ruangsomboon, S. and L. Wongrat. (2006). "Bioaccumulation of cadmium in an experimental aquatic food chain involving phytoplankton (*Chlorella vulgaris*), zooplankton (*Moina macrocopa*), and the predatory catfish *Clarias macrocephalus*×*C. gariepinus*." **Aquatic Toxicology** 78(1): 15-20.

Santos, C. L. V., B. Pourrut and J. de Oliveira. (2015). "The use of comet assay in plant toxicology: recent advances." **Frontiers in Genetics** 6: 18.

Singh, N., J. Bhagat and B. S. Ingole. (2017). "Genotoxicity of two heavy metal compounds: lead nitrate and cobalt chloride in Polychaete *Perinereis cultrifera*." **Environmental Monitoring and Assessment** 189(7): 11.

Skipper, A., J. N. Sims, C. G. Yedjou and P. B. Tchounwou. (2016). "Cadmium Chloride Induces DNA Damage and Apoptosis of Human Liver Carcinoma Cells via Oxidative Stress." **International Journal of Environmental Research and Public Health** 13(1): 10.

Xin, Z., Z. Wenchao, Y. Zhenguang, H. Yiguo, L. Zhengtao, Y. Xianliang, W. Xiaonan, L. Tingting and Z. Liming. (2015). "Species sensitivity analysis of heavy metals to freshwater organisms." **Ecotoxicology** 24(7-8): 1621-1631.

Zhang, Y. F., S. Y. Chen, M. J. Qu, A. O. Adeleye and Y. N. Di. (2017). "Utilization of isolated marine mussel cells as an in vitro model to assess xenobiotics induced genotoxicity." **Toxicology in Vitro** 44: 219-229.