Research Article

Stress Induced by Cadmium: Its Effects on Growth Respiratory Metabolism, Antioxidant Enzymes and Reactive Oxygen Species (ROS) of *Paramecium sp.*

Meryem Benlaifa*[1], Mohamed Reda Djebar[1], Hajira Berredjem[2], Maroua Benamara[1], Kheireddine Ouali[3], Houria Djebar[1]
[1]Laboratory of Cellular Toxicology, Department of Biology, University of Badji Mokhtar, BP12 Sidi Amar, Annaba, Algeria.
[2]Department of Biochemistry, University of Badji Mokhtar, BP12 Sidi Amar, Annaba, Algeria.
[3]Laboratory of Environmental Biosurveillance, Department of Biology, University of Badji Mokhtar, BP12 Sidi Amar, Annaba, Algeria.
***Corresponding author's E-mail:** meryem.benlaifa@yahoo.fr

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ABSTRACT

The Heavy metal cadmium (Cd) pollution is a major concern worldwide. It is a dangerous environmental toxicant that can be lethal to humans and other organisms. The current study was designed to investigate the toxicity of Cd on the oxidative stress biomarkers and the respiratory metabolism of an alternative unicellular model, *Paramecium sp.* Four Cd concentrations were used: 0.5; 1; 2 and 5 mM. Stress generated by the metal was assessed on cellular growth, respiratory metabolism, antioxidant enzymatic activity (GSH, GST and Cat) and reactive oxygen species variations (ROS). The outcomes of this investigation revealed that Cd is lethal to the protozoan *Paramecium sp.* since the first lowest concentration, showing a concentration dependent decrease of the cell number. Our results highlight an inhibitory effect of *Paramecium sp.* respiratory metabolism. A time dependent increase in antioxidant enzymes, including catalase (CAT) and glutathione-s-transferase (GST) associated with ROS production, and a time dependent decrease in reduced glutathione (GSH) was observed. In summary, our study shows, that the Cd is involved in the induction of an oxidizing stress expressed by, a cell growth disturbance, a respiratory metabolism disruption and a stimulation of ROS production. The results also highlight significant antioxidant capacity induced in *Paramecium sp.*

Keywords: cadmium, *Paramecium sp.*, oxidative stress, ROS, GST, CAT.

INTRODUCTION

The Human activities, especially the industrial ones, are the most important sources of direct and indirect heavy metals discharges into the environment. These heavy metals are an integral part of the earth's crust and form the structure of various rocks making them uncontrollable environmental elements through their persistence, their toxicity and their accumulative powers.[1,2]

According to their effects, heavy metals have several categories such as Fe, Cu, Zn and Cr which are considered essential for humans; however, others heavy metals such as Hg, Pb and Cd are considered non-essential and might cause toxically effects even at very low concentration.[3,4] Among these xenobiotics, Cd is considered as the most dangerous ETM which was detected in several environments and particularly in the aquatic one.[5] The heavy metal Cd is one of the most harmful ETM in the ecosystem.[6] This Cd property makes it highly absorbable by the environment and consequently quickly toxic.

Many ciliated species intervene in the trophic chain as important elements in the energy transfer (sugar). Thus, these species represent different levels of tolerance to xenobiotics among which is the ETM.[2] Simultaneously, these species are considered as alternative models widely used in research for their easy culture and their relatively short cycle.[7,8]

Due to its features as a single-cell eukaryotic organism, widely occurring in freshwater ecosystems, *Paramecium* has been proposed as a bioethical and excellent test organism for standardized laboratory procedures to evaluate environmental quality and the effects of xenobiotic compounds on a simple alternative biosystem.[9] The sensitivity of protozoa is due to their simple cell organization which exposes their receptors to the external environment, making them respond directly to environmental stimuli. Moreover, thanks to their short cell-cycles it is possible to study the effects of pollutants over a short period of time.[10] For this reason, *Paramecium* have been used either as bioindicator of pollution or bioassays to evaluate the effects of toxic compounds.[11-13]

These toxic effects, related to oxidative stress condition, induce cell injury due to the increased cellular free radicals. The main parts of free radicals are Reactive Oxygen Species (ROS) which are mainly generated in cellular respiration. Increase in amount of ROS during pathological conditions such as oxygen toxicity, ionization radiation, drugs, chemicals, toxins, etc can disturb mitochondrial function and make cellular damage.[14,15] The endogenous enzymatic defenses such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), glutathione S-transferase (GST) and non-enzymatic components like reduced glutathione (GSH), ascorbic acid, vitamin E, etc[16] allowed the cells to fight oxidative stress.

The main objectives of this study are the evaluation of the stress generated by Cd in *Paramecium sp.*, and the search of the ETM impact on the respiratory metabolism,

the antioxidant enzyme potential, including CAT, GSH and GST, and the ROS production.

MATERIALS AND METHODS

Paramecium sp. Treatment

The unicellular micro-organism *Paramecium sp.* was used in all our experiments. *Paramecium* culture was performed in a lettuce medium at 28 ± 2°C, pH 6,5 as described by Azzouz.[17]

The cells were transplanted every three days to maintain a good culture.[18] Cd, in the form of cadmium chloride ($CdCl_2$), was used in this study.

A series of four test-concentration solutions was made, through of serial dilutions, using distilled water. Cd was then added to different cell cultures into 10 ml tubes.

The cells were incubated at the final concentration of 0.5, 1, 2 and 5 mM. The control was prepared without exposing the cells to Cd.

Growth kinetic determination

In order to develop a dosage Growth kinetic curve, toxic response of *Paramecium* to Cd was examined at various concentrations of $CdCl_2$ ranging from 0,5M to 5mM, which had been added to the growth media. Kinetics growth were performed daily (24, 48, 72, 96 and 120 h) in 10 ml tubes of 10^3 cells/ml initial number of *Paramecium sp* by counting cells after Lugol fixation, using an optical microscope (Leica DM 1000)[19]. Cell growth was determined by the formula:

N= log Nt – log N0/log 2

N0: Initial Number of cells.

Nt: Number of population over time.

Respiratory Metabolism

The *Paramecium* respiratory activity was carried out by a HANSATECH oxygen electrode, used to measure the oxygen production or consumption by the cells during the experimentation.[20]

Determination of glutathione (GSH)

Reduced glutathione (GSH) dosage is based on the method of Weckberker and Cory (1988).[21] The principle is based on the colorimetric measurement of 2-nitro-5mercapturic acid, resulting from the reduction of 5-5-dithio-bis-2-nitrobenzoic acid (DTNB) by the thiol (–SH) glutathione measured at a wave-length of 412 nm. Cells were mixed in 1ml ethylene diamine tetra-acetique (EDTA) (0.02M). 0.2 ml of acide sulfosalicylique (ASS) was added to 0.8 ml of homogenate. After agitation, the homogenate was centrifuged (10000 rpm/5min). The assay mixture contains 1 ml tris/EDTA buffer (0.02M, pH 9.6), 0.025 ml of 5,5'-diyhiobis-2-nitrobenzoic acid (DTNB) and the *Paramecium*. The reaction was monitored at 412 nm and the amount of GSH was expressed as µmol/mg of proteins.

Determination of Glutathione S-Transferase (GST) activity

Glutathione S-Transférase (GST) measurement was performed according to the method of Regoli and Principato (1995)[22] based on the conjugation of GSH with CDNB. The homogenization of samples was done in 1 ml of phosphate buffer (0.1M, pH 6) and centrifuged at 14000 rpm/30 min. The final reaction contains 1.2 ml CDNB (1 mM)/GSH (5 mM) and the sample. The absorbance was measured spectrophotometrically at 340 nm for 5 min. The result was expressed as µmol/min/mg of proteins.

Determination of Catalase (CAT) activity

The catalase activity was determined according to the method of Palanivelu.[23] The decrease in absorbance at 240 nm was monitored spectrophotometrically for about three minutes at 25°C by calculating the rate of degradation of H_2O_2. Samples were mixed in 1 ml of phosphate buffer then centrifuged at 15000 rpm/30 min. Finally, we added 0.75 ml of phosphate buffer and H_2O_2 at 0.025 ml of supernatant. The result was expressed as µmol/min/mg of proteins.

Reactive Oxygen Species Determination (ROS)

Reactive Oxygen Species (ROS) production was performed by a photometric method adapted by Djebar.[24] The principle of this test is based on the responses of ROMs (ROS) on a specific chromogenic buffer N,N-diethylparaphenylendiamine (DEPPD) giving a red coloration. The ROS formed in the samples were measured by spectrophotometer at 505 nm.

Statistical analysis

All the experiments were done in triplicate and the results were expressed as mean ± SE.

Data statistical analysis for comparisons was performed using the Student's t test Minitab. Statistical significance was considered at $P < 0.05$.

RESULTS

The effect of cadmium on kinetics growth

Parameciums were tested for their viability at various concentrations of $CdCl_2$. As can be seen in Figure 1, cells exhibited a toxic response to $CdCl_2$.

In fact, we noticed a strong depletion of the treated cells number that persists until the end of the treatment and reaches, at the highest concentrations (2 and 5 mM), 900 cells after 96 and 120 h of exposure.

In parallel, we observed a normal growth of the control *Paramecium* which initial number was 4500 cells at t_0. On the 3rd day, the number of cells reaches 12000.

These data demonstrate that increased levels of $CdCl_2$ resulted in rapid death of *Paramecium* in a concentration-dependent manner.

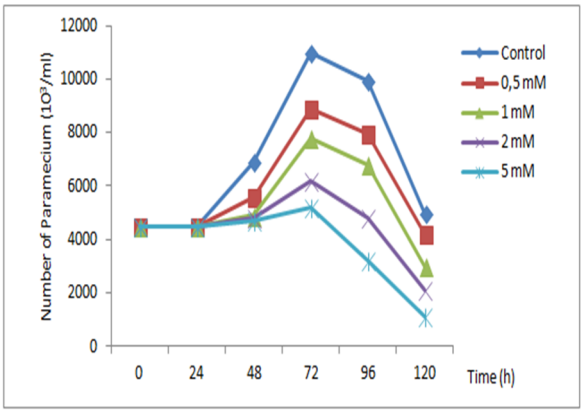

Figure 1: Effect of Cd on kinetics growth in *Paramecium sp*.

Cadmium effect on respiratory metabolism

As shown in Figure 2, treatment with high Cd concentrations tends to reduce, by half, the respiratory metabolism of the cells. When the Cd concentration was increased, the respiratory metabolism was strongly inhibited (approximately 95% for the highest concentration 5 mM).

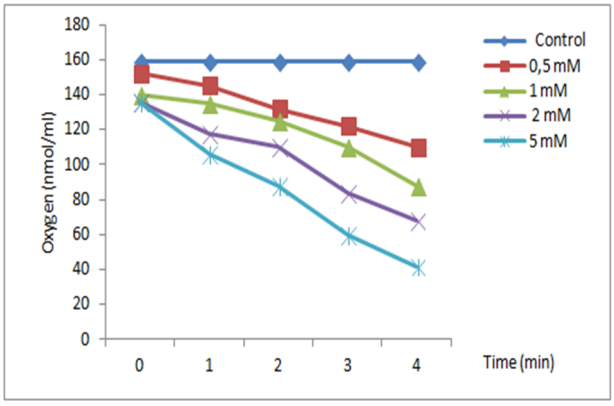

Figure 2: Effect of Cd on the respiratory metabolism of *Paramecium sp*.

Estimation of glutathione content (GSH)

A reduced dose-dependent GSH levels according to time exposure (5 times lower than the control value) was observed after *Paramecium* exposure to Cd (Fig. 3). The statistical studies indicate a highly significant ($p<0.05$) GSH decrease in cells treated with Cd than the control.

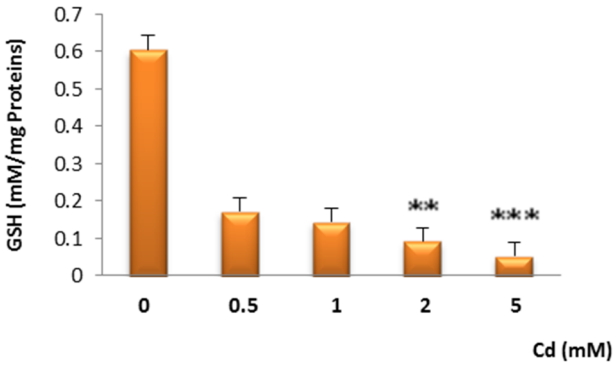

Figure 3: Effect of Cd on GSH changes of *Paramecium sp*.

Cadmium effect on Glutathione S-Transferase activity (GST)

The study findings demonstrated a strong stimulation of GST activity (almost 6 times higher than the control), particularly at high Cd concentrations (Fig. 4). A very highly significant increase ($p < 0.001$) of GST activity was observed according to time different concentrations exposure compared to the control.

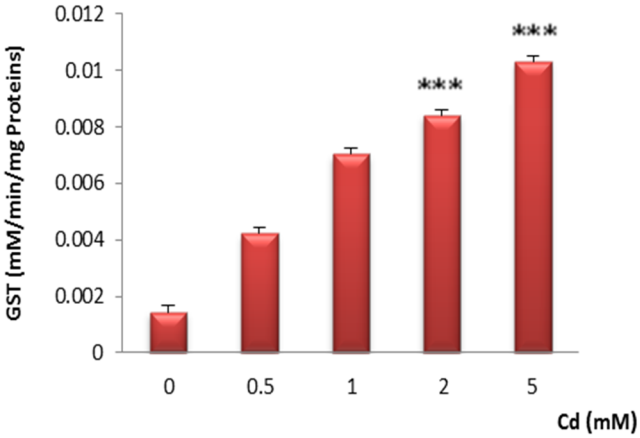

Figure 4: Cd Effect on GST activity variations of *Paramecium sp*.

Cadmium effect on Catalase activity (CAT)

The activity of CAT in paramecium significantly ($p < 0.001$) increased in response to Cd exposure in a dose-dependent manner (Fig. 5). The maximum of CAT stimulation was recorded for the 5 mM concentration (about 80%) (3,558 µM/min/mg Prot) compared to the control ones (0.828 µM/min/mg Prot).

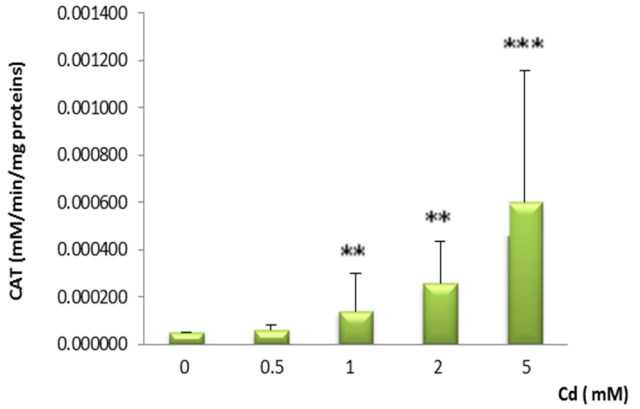

Figure 5: Cd effect on the activity of catalase of *Paramecium sp*.

Cadmium effect on ROS production

As evident in Table 1, the quantitative changes in the ROS production, after the treatment with Cd, results in a strong dose-dependent stimulation of the ROS generation in *Paramecium sp*. In fact, they increased twofold with the highest Cd concentrations.

Table 1: Effect of Cd on ROS production in cells treated with high concentrations.

Cd (mM)	ROS (U carr)
0	12.6
0.5	13.80
1	15.00
2	17.30
5	24.00

DISCUSSION

It is well established that Cd is a principal environmental toxicant that usually finds its way into the ecosystem as a result of human activities. The studies relative to the effects of Cd on ciliates protists are quite recent;[5,22] they all describe Cd as being responsible for significant disturbances strongly causing cellular death. In humans, exposure to cadmium can lead to kidney damage, respiratory disease, neurological disorders and cancer.[25] Recent researches[8,17,26,27] have focused on Cd toxicity through the determination of new parameters such as IC_{50}, energetic metabolism and enzymatic activities, and on the role played by *Paramecium sp.* as an alternative experimental model, and as a bioindicator for ETM induced toxicity.

Due to their high metabolic ratio, small cell volume, and relatively high surface contact with their environment, ciliates can respond very rapidly to chemical stress. A marked advantage of the respiration test is that the incubation time is very short, only 5 min.[28]

Our study clearly demonstrated that exposure to Cd causes a dose-dependent inhibition of *Paramecium sp.* cellular growth. At the cellular level, Cd is normally chelated by cytoplasmic proteins and transported to lysosomes where it is stored and eventually expelled from the cell.[29] Previous study demonstrated that the constant exposure to elevated heavy metal concentrations leads to apoptosis; however, at lower levels, certain heavy metals, such as Cd, appear to be anti-apoptotic.[30-32] Simultaneously, our results demonstrated that microorganisms subjected to Cd present a highly disturbed respiratory metabolism, resulting from *Paramecium sp.* respiration inhibition, which could be related to an uncoupling of oxydative phosphorylation in the mitochondrial respiratory chain leading to a disturbance of the mitochondrial complex.[17]

Almost all organisms are well protected against reactive species by enzymatic antioxidant defense such as superoxide dismutase (SOD), glutathione peroxidase, catalase etc. When the mechanism of antioxidant protection becomes unbalanced, it leads to the massive production of free radicals, resulting in disease.[33] In the current study, as a result of *Paramecium* exposure to Cd, GSH content have been decreased and the activities of antioxidant enzymes (CAT and GST) have been increased. These results highlight the existence of oxidative stress generated by the exposure to Cd. The GSH maintains the normal structure and function of the cells *via* a redox and detoxification reaction. The imbalance between pro-oxidants and antioxidants in biological systems is defined as oxidative stress.[34] The decreased GSH content during exposure to pollution, maybe due to an increased utilization of GSH, which can be converted into oxidized glutathione, and inefficient GSH regeneration.[7,18,23,35-37] Thus, the significant increase in CAT, and GST could be due to a significant reduction in the activities of GSH with Cd presence.[20,38] Therefore, CAT and GST constitute essential components of the antioxidant system, and their deficiencies result in oxidative stress. This perhaps indicated a consumption of enzyme in the conjugation process of GSH with Cd to attenuate the induced Cd toxicity.

The biological importance of CAT is more evident from various studies due to the fact that H_2O_2 is the main cellular precursor of the hydroxyl radical which is a highly reactive and toxic form of ROS.[39] Therefore the observed increase in CAT activity may indicate an important role to protect cells against H_2O_2 production.[40]

Reactive Oxygen Species (ROS) are produced in normal physiological processes and are catalyzed by antioxidants (GSH, SOD) to prevent cell injury. ROS are free radicals derived from oxygen. Free radicals are atoms or molecules having single unpaired electron in the outer orbital. Energy created by this unstable configuration may lead to chemical reaction with main biological macromolecules (carbohydrate, phospholipids of membranes, proteins and nucleic acids which are the main components of cells) initiating autocatalytic reactions leading to cell death.[41] Although, the antioxidant defense system can counteract the effects of ROS, production of ROS in excessive rate can lead to damage at the cellular level.[42]

Our results suggest that *Paramecium sp.* exposure to different Cd concentrations inhibits the cell growth, generates a real oxydative stress responsible for ROS synthesis and causes disturbance of the mitochondrial respiratory chain that is at the origin of respiratory metabolism which can be caused by ATP-ase complex alteration.[36] In response to this damage, *paramecium sp.* triggers a GSH/GST/CAT antioxidant system that supports some of the produced ROS.

CONCLUSION

Oxidative stress and the antioxidant defense always seek balance in aerobic systems. In this work, the exposure of *Paramecium* to Cd leads to many cellular events, including the induction of oxidative stress and the stimulation of antioxidant defense. The present study underlines the high sensitivity of *Paramecium sp.* to Cd exposure, making it a potential model in studies concerning the ecotoxicological tests, and specifically in the case of contamination of aquatic environments and systems through ETM in general and Cd in particular.

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