Pakistan J. Zool., vol. 43(3), pp. 569-574, 2011.

Response of Glutathione Level in a Protozoan Ciliate, *Stylonychia mytilus*, to Increasing Uptake of and Tolerance to Nickel and Zinc in the Medium

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Abstract.- A ciliate protozoan, *Stylonychia mytilus*, has been shown to tolerate Zn^{2+} up to 29 µg/mL and Ni^{2+} up to 16 µg/mL. *S. mytilus* could uptake 28% Ni^{2+} and 40% Zn^{2+} from the medium containing 5 mg/L of Ni^{2+} and 16 µg/mL of Zn^{2+} after 48 hrs of incubation. Ni and Zn uptake resulted in an increase in glutathione (GSH) level by 148% and 102% and non-protein thiols level by 49% and 37%, respectively at 5 mg/L. An increased GSH level correlates with metal tolerance and hence this high GSH level may be used as a marker for metal stress.

Key Words: Metal tolerance, metal uptake, glutathione, *Stylonychia mytilus*

INTRODUCTION

Microbial detoxification of metal ions is achieved by several mechanisms including regulation of uptake, transformation into less toxic species and intracellular immobilization (Gharieb and Gadd, 1998). Glutathione is widely spread in bacteria, plants and animals (Meister and Anderson 1983) and has been shown to play numerous roles, such as protection against oxidative stress, detoxification, transport and enzymatic catalysis (Penninckx, 2002; Kim *et al*., 2005). This tripeptide has been reported to be the first line of defence against metal toxicity in animals, and Protists (Singhal *et al*., 1987).

In view of the hazardous effects of heavy metal, their removal from the contaminated environment has been a challenge for a long time (Honjoh *et al*., 1997). Because traditional clean up processes of heavy metal contaminated soils and waters are expensive and practical only in small areas, new cost effective technologies that include the use of microorganisms, biomass, and live plants need to be investigated (Ebbs and Kochian, 1997).

Nickel, a major environmental pollutant, is known for its clastogenic, toxic, and carcinogenic potential (Ross, 1995; Hartwig and Schwerdtle, 2002). The carcinogenic potential of nickel while particulate nickel compounds like Ni_3S_2 or NiO are strong carcinogens, the soluble nickel (II) salts exert weaker effects (Dunnick *et al*., 1995). This may be due to differences in bioavailability. Water soluble nickel salts are taken up only slowly by cells, while particulate nickel compounds are phagocytosed and, due to the low pH, are gradually dissolved in lysosomes, yielding high concentrations of nickel ions in the nucleus (Costa *et al*., 1981).

Zinc is a major inorganic pollutant, which has inhibitory and stimulating effects on the growth along with accumulation in plants (Kumar, 1989). Seedling growth and enzyme activities have been found inhibited by zinc in *Phaseolus aureus* cv. R-851 (Veer, 1989). Zinc inhibits transporter-mediated glutamate uptake (Vandenberg *et al*., 1998), and depending on concentration, can inhibit or potentiate glycine receptors (Han and Wu, 1999). It is also known that zinc is toxic to neurons. Studies in animal models suggest that endogenous zinc mediates neurodegeneration resulting from ischemia (Koh *et al*., 1996) and seizure (Suh *et al*., 1996). It has been suggested that increased intracellular zinc may result in mitochondrial impairment and generation of reactive oxygen species (Dineley *et al*., 2003).

Shakoori *et al*. (2004) have reported 99% and 48% uptake of Zn^{2+} and Cr^{6+} by *Vorticella microstoma* after 96 hrs from the medium,

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0030-9923/2011/0003-0569 $ 8.00/0

respectively. These microorganisms actively contribute to the amelioration of the effluent quality, since the majority of them feed upon dispersed bacteria (Madoni, 2000). Heavy metal uptake processes by biological cells are known under the general term of biosorption. These phenomena include both passive adsorption of heavy metals to the cell walls and metabolically mediated uptake by the cells (Gadd, 1990).

The present study reports data on the survival of a ciliate protozoan, *Stylonychia mytilus,* in media containing Zn^{2+} and Ni^{2+} and correlates metal tolerance and accumulation with glutathione and non-protein thiol levels with an objective to consider its candidacy for bioremediation of metal contaminated wastewater.

MATERIALS AND METHODS

For the present study already established axenic culture of *Stylonychia mytilus* in this laboratory was used. *S. mytilus* was grown in 250 mL conical flasks containing 100 mL of Bold-basal salt medium. Glucose as carbon source was added as 1g/L in Bold-basal salt medium (Shakoori *et al*., 2004; Rehman *et al*., 2008). The pH of the medium was adjusted to 7.5. The cultures were maintained in the laboratory at room temperature (25-27°C). The growth of *S. mytilus* was observed in the cultures by counting the number of ciliates at regular intervals.

The effect of Ni^{2+} and Zn^{2+} on the growth of culture was analyzed by counting the number of protozoan cells in the medium. The cells were grown in the salt medium to which Ni^{2+} and Zn^{2+} were added at a concentration of 1µg/mL per day for eight days. At least three counts were taken every day to get a mean for every day. The growth was compared with that of the control culture, which contained no added metal ions. The activity, shape and size of the protozoans were also noted. The size was measured with an ocular micrometer after restricting the movement of the ciliates by putting the culture in methylcellulose and staining with 1% neutral red.

Resistance of *S. mytilus* to two metal ions *i.e*., Ni^{2+} and Zn^{2+} was checked by addition of the respective metal salts *viz*., $NiCl_2$ and $ZnSO_4.7H_2O$ to Bold-basal salt medium. Metals ions were sterilized separately and added to the medium when the temperature of the salt medium was slightly less than 50°C. For Ni^{2+} and Zn^{2+}, the concentration in the medium on the first day was 1µg/mL with an increase of 1µg/mL every day for 29 days for Zn^{2+} and 16 days for Ni^{2+}. Although the death of protozoa is confirmed by the lysis of the cell, movement is considered to be a vital sign of life. When the protozoans became inactive, no more metal was added.

For determining uptake of heavy metals by *S. mytilus*, the ciliates were grown by inoculating 100 mL of Bold-basal medium in 250 mL conical flasks with 10 µL of original laboratory culture (40±2 cells) at 25°C. Zinc was added at a concentration of 10 µg/mL in the medium containing ciliate cells but nickel was added at a concentration of 5 µg/mL in separate flask. The control culture medium, containing the same metal concentrations as the treated samples, was maintained without any ciliates. Five mL samples were removed under sterile conditions after 0, 48, 96 and 144 h, centrifuged at 350 x g for 15 min and used for estimation of Ni^{2+} and Zn^{2+} concentrations by atomic absorption spectrophotometer (Varian, U.S.A) at wavelengths 232.0 and 213.9 nm, respectively. The amount of metal in the supernatants was determined using standard curves, which were prepared by taking various known concentrations of $NiCl_2$ and $ZnSO_4.7H_2O$ in the medium. The percentage decrease in the amount of Ni^{2+} and Zn^{2+} in the medium was calculated.

Reduced glutathione (GSH), oxidized glutathione (GSSG) and the total glutathione contents were determined according to Israr *et al.* (2006).

Total protein concentration was determined according to the Lowry method (Lowry *et al*., 1951), using a standard curve prepared from different concentrations of bovine serum albumin. The SDS-PAGE protein profile of the protozoan was analyzed according to Rehman *et al*. (2009).

All values are an average of three replicates and have been shown as Mean±SEM. For determining significance of differences between the control and the experimental treatments, Student's "t" test was applied.

RESULTS AND DISCUSSION

Figure 1 shows growth of *S. mytilus* in media with and without metal ions. The growth of ciliate, which is indicated by the number of cells/mL, was affected by the presence of metal ions in the culture media. The *S. mytilus* control culture contained $0.1x10^3$ cells/mL on day 1, which increased to $2.3x10^3$ cells/ml after 8 days (23 fold increase). However, in the presence of Ni^{2+} (8 µg/mL) the number of cells increased from $0.095x10^3$ to $1.602x10^3$ cells/mL in 8 days (17 fold increase). In the presence of Zn^{2+} (8 µg/mL) the number of cells increased from $0.091x10^3$ to $1.846x10^3$ cells/mL (20 fold increase). The addition of metal ions in the medium resulted in slower growth and delayed cell division.

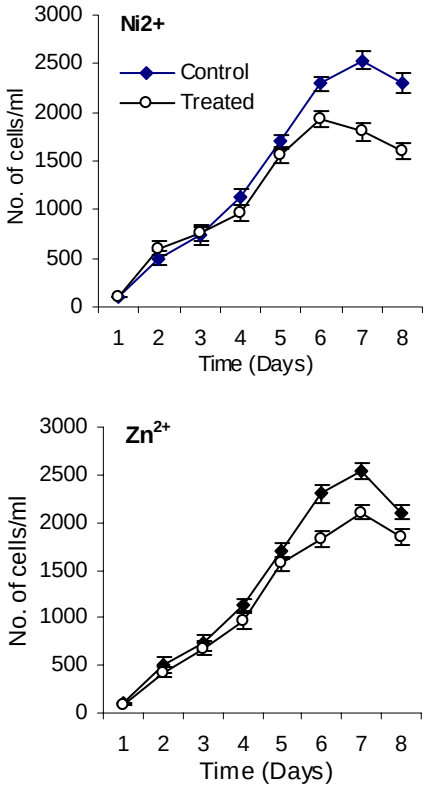

Fig. 1. Growth curves of *Stylonychia mytilus* in Bold-basal medium containing Ni^{2+} and Zn^{2+}. Control cultures did not contain any metal ions.

Maximum number of *S. mytilus* cells in Zn containing medium was achieved on day 7 but for nickel containing medium, it was achieved on day 6. The maximum number of *S. mytilus* cells in control culture was on day 7 (2523.00±1.53) and the maximum number of protozoan cells in Ni^{2+} and Zn^{2+}containing medium was 1602.00±1.53, and 1846.66±1.00, respectively. Growth rate of *S. mytilus* was slower in both metal ions when compared with the control culture containing no metal ions. When the cell population of metal-treated cultures was compared with those of the corresponding control culture on day 8, it was observed that the nickel-treated culture had 30% and the zinc-treated culture had 20% lower cell count when compared with the control culture. Metal resistant protozoa have been reported in wastewaters and metal-polluted environments (Shakoori *et al*., 2004; Madoni and Romeo, 2006; Rehman *et al*., 2007, 2008).

Ciliates are usually found in polluted wastewaters containing less than 10 µg/mL concentrations of toxic metal ions (Shakoori *et al*., 2004). The ciliate, *S. mytilus,* can survive very easily in such polluted waters. The metal removal efficiency of *S. mytilus* is greater than 80% in such metal contaminated wastewaters (Rehman *et al*., 2008) and these ciliates are excellent and convenient bioindicator for evaluating the toxicity of wastewaters polluted by heavy metals (Madoni and Romeo, 2006).

S. mytilus was found to resist Ni^{2+} up to a concentration of 16 µg/mL and Zn^{2+} 29 µg/mL. No reduction in the size of *S. mytilus* cells was observed. Movement was taken as a parameter of effect on growth rate. The hypotrich, *Euplotes patella,* showed the lowest sensitivity for both nickel and most of other tested metals. *E. patella* can resist Ni concentration up to 10 mg/L (24 h LC_{50s}) (Madoni, 2000).

Figure 2 shows the removal of heavy metal ions from the medium by *S. mytilus*. Growing in medium containing nickel (5 µg/mL) could decrease 28% of nickel from the medium after 48 hrs, 51% after 96 hrs and 72% after 144 hrs. It could also remove 40% of zinc after 48 hrs, 55% after 96 hrs and 75% after 144 hrs, respectively from the medium containing 10 µg/mL of zinc.

Rehman *et al*. (2008) reported that *S. mytilu*

grown in the medium containing Ni^{2+} (5 µg/mL) could reduce 49% of nickel from the medium after 48 hrs, 61% after 72 hrs and 73% after 96 hrs. It could also reduce 90% Zn^{2+} after 48 hrs, 94% after 72 hrs, and 98% after 96 hrs from the medium containing Zn^{2+} at a concentration of 10 µg/mL. In the present study *S. mytilus* could remove 72% Ni^{2+} and 75% Zn^{2+} from the medium after 144 hrs of incubation. This clearly indicates that the ciliates actively take up the heavy metals. Metal bioaccumulation has also been reported to be the main mechanism of resistance to heavy metals in ciliates by others (Martin-Gonzalez *et al*., 2006).

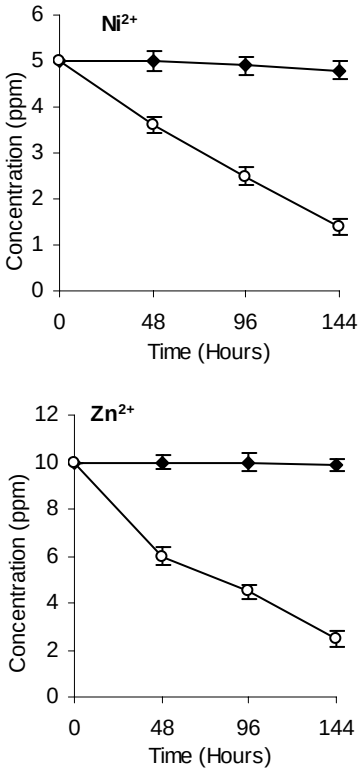

Fig. 2. Uptake of Ni^{2+} and Zn^{2+} by *Stylonychia mytilus* growing in media containing Ni^{2+} and Zn^{2+}. The control did not contain cells of the isolate.

The present investigation clearly demonstrates the importance of glutathione and non protein thiols in Ni^{2+} and Zn^{2+} accumulation and detoxification. Intracellular concentration of GSSG increases at the expense of GSH under stress conditions. Ni and Zn treatment significantly enhanced the GSH/GSSG ratio at 5 mg/L. This indicates the potential of *S. mytilus* to tolerate Ni and Zn stress. Ni and Zn treatment also increased the non-protein thiols content by 49 and 37%, respectively, as against 148% ($P< 0.05$) and 102% ($P< 0.05$) increase in GSH content, compared to the control (Table I). In fact, GSH levels led us to infer that an increase in the synthesis of this tripeptide synthesis is involved in metal tolerance and the presence of increasing GSH concentrations may be a marker for high metal stress in ciliate. Thiols are essential agents in cellular redox signaling and control in animals, plants and fungi (Moran *et al*., 2001; Pócsi *et al*., 2004). The intracellular glutathione in mice functions in protection against Cd^{2+} toxicity, and that this tripeptide provides the first line of defense against metal ions before induction of metallothionein synthesis occurs (Singhal *et al*., 1987).

Table I.- Response of glutathione level in a ciliate *Stylonychia mytilus*, exposed to Ni and Zn at 5 mg/L in the medium.

	Control	Nickle	Zinc
GSH (mM/ g fresh wt)	19.75±0.6*	49.00±0.8[a]	40.00±0.6[a]
GSSG (mM/ g fresh wt)	14.75±1.2	18.00±0.5	16.50±0.5
GSH+GSSG(mM/ g fresh wt)	34.50±1.6	67.00±1.2[a]	56.50±1.6[a]
GSH/GSSG ratio	1.34±0.6	2.7±0.6	2.4±0.6
Non-protein thiols	3.50±0.6	5.20±0.8	4.80±0.8

* Mean± SEM (n=3); [a] ($P< 0.05$)

Polyacrylamide gel analysis showed that a new protein of 18kDa was induced in *S. mytilus* when Zn was added to the culture medium but this band was absent from Ni treated and control samples (Figure not given). Some proteins with molecular weights of 55kDa, 40kDa, 38kDa and 25kDa were present in all samples but their intensity was greater in Zn treated samples as compared to Ni and control samples. In the present investigation 20kDa and 10kDa protein bands were observed in *S. mytilus* against Ni and Zn which indicates that ciliate produces MT like proteins which may be involved in heavy metal handling and detoxification

processes.

In this study we are reporting the multiple heavy metal uptake potential of *S. mytilus* which is resistant to toxic metal ions and is adaptable to the local environmental conditions. Moreover, increased GSH level correlates with metal uptake and hence could be used as a marker for metal toxicity.

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(Received 3 March 2010, revised 10 July 2010)