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Effect of Nickel on Embryo Development and Expression of Metallothionein Gene in the Sea Urchin (*Hemicentrotus pulcherrimus*)

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Sea urchin embryo has been used to monitor pollutants in marine environments. Nickel (Nickel chloride, Ni), as a heavy metal, is a chemical element with the chemical formula $NiCl_2$–$6H_2O$. It may cause harmful effects on the central nervous system and growth. Metallothionein (MT) is a metal binding protein and it play a regulatory role in the homeostasis and detoxification of heavy metals. In this study, we examined the gametotoxic and embryotoxic effects of Ni at various concentrations (0, 10, 25, 50, 100, 500 ppb) in the sea urchin *Hemicentrotus pulcherrimus*. Spawning was induced by injecting 1 ml of 0.5 M KCl into coelomic cavity. Experiment was begun within 30 min the collection of both gametes. The fertilization and embryo development rates test were performed for 10 min and 64 h after fertilization, respectively. Results of this study, the fertilization rates in the control condition (not including Ni) and experimental group were not significantly changed. However, the embryo development rates were significantly decreased with concentration dependent manner. Especially, 50 ppb Ni treatment was significantly lower than vehicle control at 64 h onward. The normal embryogenesis rate was significantly inhibited in exposed to Ni (EC_{50}=34.19 ppb, 95% Cl=29.56–38.09 ppb). The NOEC and LOEC of normal embryogenesis rate were <10 ppb and 25 ppb, respectively. The expression of MT mRNA gene was significantly increased with concentration dependent manner. These results suggest that the early embryo stages of *H. pulcherrimus* have toxic effect at greater than 25 ppb of Ni concentration and MT mRNA gene may be used as a biomarker gene for risk assessment on contamination of Ni.

Key words: 95% Cl, EC_{50}, *Hemicentrotus pulcherrimus*, Metallothionein, Nickel

INTRODUCTION

Pollution associated with urbanization and industrialization endanger marine ecosystems. Heavy metals such as nickel, cadmium, zinc, lithium, and the major constituents of industrial wastes, they affect the marine ecosystem (Gopalakrishnan *et al*., 2008). These heavy metals have embryotoxic/teratogenic effects by directly or indirectly (Calevro *et al*., 1998). Nickel, as a heavy metal, the chemical symbol Ni and atomic number 28, should mainly target on active sites of enzymes. In embryos testing of Ni, a number of studies showed that Ni affect toxicity (Léonard *et al*., 1981; Lu *et al*., 1979; Luo *et al*., 1993; Saillenfait *et al*., 1991; 1993; Sunderman *et al*., 1980).

Metallothionein (MT) is a low molecular weight cysteine–rich metal–binding proteins, is present in many phylogenetically diverse organisms, and is induced by many chemicals, including metals, glutathione depletors, and hydrogen peroxide (Dalton *et al*., 1994; Klaassen *et al*., 1999; Sato *et al*., 1995; Zhu and Thiele, 1996). In previous reported that MT proteins play a regulatory role in the homeostasis of zinc and copper (Cosson, 1994; Cousins, 1985; Fukushima *et al*., 1988; Olsson *et al*., 1989), and detoxification of cadmium and mercury (Debec *et al*., 1985; Din and Frazier, 1985; Kay *et al*., 1987; Kito *et al*., 1982;). Also, MT was decreased the chemical carcinogenesis (Dalal and Bhattacharya, 1991; Takaishi *et al*., 2009).

Sea urchin embryo larval development has been studied and used to monitor pollutants in marine environments over the last several decades (Flammang *et al*., 1997; Kobayashi, 1971; 1994; Okubo and Okubo, 1962; Tabata, 1956). In the two main life stages, the embryo larval and adult stages were generally studied and used in testing (Bielmyer *et al*., 2005). In particular, the early life stages of sea urchins have been shown to be sensitive to metals (Kobayashi, 1973; 1980; Phillips *et al*., 2003).

The present study was conducted to evaluate the acute toxicities of Ni in sea urchin (*H. pulcherrimus*) embryo and to predict these toxicities in the marine environment.

MATERIALS AND METHODS

Sample and collection

The organisms that were used for this study are sea urchins (*H. pulcherrimus*) which belong to the marine invertebrate classification Echinodermata that inhabit the rock areas of the entire Korean coast. The developed *H. pulcherrimus* can be easily collected at any time, as they have different spawning periods that depend on water temperature prevalent across the intertidal zones.

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Test organisms were collected from the intertidal zone of the Buan Fish Hatchery in Gyeokpo–ri, Byeonsan–myeon, Buan–gun, Jeollabuk–do between March and April 2011, the season for the main spawning period. The collected sea urchins were kept in a laboratory with a water temperature of about 9±1˚C for over a week before the test.

Prepared sperm and ovum

Sterilized sea water was used to remove the protozoa or other foreign matters that might be on the sea urchin surface. Eighteen of this species, with 3.5 cm or more diameters, were used for spawning. The natural sea water was filtered (using a membrane filter with a pore size of 0.45 μm) and sterilized before it was used in the test. A 100 ml beaker was filled with the filtered natural sea water to sufficiently soak the genital pore, and 1 ml 0.5 M KCl solution was injected in the coelomic cavity. Gametes were obtained from the 30 min spawning. The sperm solution was washed once, and the ovum solution was washed thrice before they were used for the test (Fujisawa, 1989).

Nickel treatment and fertile eggs–embryos testing

Nickel (Nickel chloride, Ni, $NiCl_2$–$6H_2O$, CAS No. 7718–54–9) was obtained from Shamchun Chemicals (Korea) and dissolved in sterilized sea water. Fertilized eggs and embryos were obtained from at least three different male/female pairs for each bioassay. Sperms were exposed to varying concentration of nickel (0, 10, 25, 50, 100 and 500 ppb in sterilized sea water) for 30 min; after which, the ova were injected. 10 min later, the fertilization rates were identified by checking whether the fertilization membrane was formed. They were transferred in the cap tube, fixed with formalin solution (3%), and observed using an optical microscope (Fig. 1). To examine the normal embryogenesis rate, 64 h after adding the ova, the specimens were fixed with formalin solution (3%); they were then observed using an optical microscope that would help classify them into normal and abnormal pluteus, that is, small in size and deformed (Pagano *et al.*, 1985a; b) (Fig. 1). The test medium was not changed.

Experiment was replicated three times, while 100 or more embryos exposed to the test solution were also repeatedly counted three times to determine the ratio of normal embryos to fixed embryos in percentage. To assess the toxicity of the Ni using the results, the probit analysis method was used to analyze the 50% effective concentration (EC_{50}) and the 95% confidence limit (95%

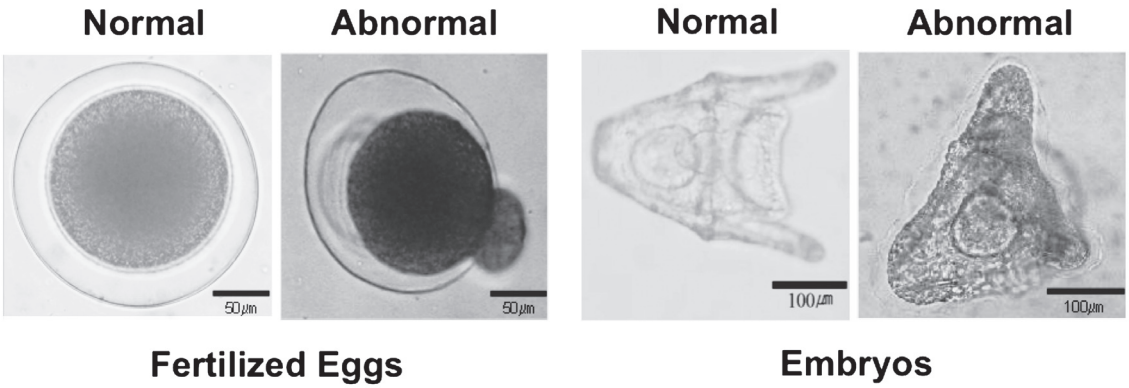

Fig. 1. Normal and abnormal forms in fertilized eggs and embryos of sea urchin (*H. pulcherrimus*).

Table 1. Experimental culture conditions using the sea urchin (*H. pulcherrimus*)

Test parameters	Conditions
Culture type	Static non– renewal 10 min – 64 h toxicity test
Photoperiod	Ambient light condition and 8L:16D periods
Temperature	16 ± 0.5˚C
pH	7.8 – 8.2
Salinity	32 ± 1.0
Chamber volume	80 ml glass
Solution	Filtered (0.4 μm) and sterilized seawater
Solution exchange	None
Experiment period	10 min – 64 h
Investigation item	Fertilization, larval development rates
Acceptability criterion	> 90% fertilized eggs and pluteus larvae at control

Cl) of the fertilization and normal embryogenesis rate. In addition, no observed effective concentration (NOEC) and lowest observed effective concentration (LOEC) were analyzed using the Dunnett's test.

The embryos were cultured in an incubator (LMI–3004PL, DAIHAN Lab Tech, Korea) at 16±0.5˚C for 64 h. The pH of the culture fluid was kept constant at 7.8–8.2. Via the preliminary test for optimal sperm addition, sperms were diluted by 2,000–2,500 times, and 1,500–2,000 fertilized eggs were added to 1 ml of test water used for the culturing process based on the conditions in Table 1.

Total RNA extraction

Total RNA extraction was carried out with RNAiso Plus (TaKaRa Shuzo Co. Ltd, Japan). Briefly, sample of 1 ml culture embryos were homogenized in 1 ml of RNAiso Plus reagent. Total RNA was separated from DNA and proteins by adding 0.2 ml chloroform, and was precipitated using isopropanol of 1.0 x the volume of the upper layer. The precipitate was washed twice in 75% ethanol, air–dried for 3 min and re–diluted in diethylpycocarbonate (DEPC)–treated distilled water (DW). The amount and purity of extracted RNA were quantitated by Nanovue (SE–75184, Healthcare Bio Sciences, Korea).

Reverse transcription (RT)

For reverse transcription (RT), the AccuPower RT Premix kit (Bioneer Co. Ltd, Daejeon, Korea) was used. The RT Premix tube was prepared for each sample and add to 20 μl DEPC–DW, 50 pmol oligo(dT) primer and 0.3 μg total RNA from each sample. RT was carried out in a TProfessional Thermocycler (Biometra GmbH, Germany) using a program with the following parameters: 42˚C for 60 min, 99˚C for 5 min, and then quenched at 4˚C. After the reaction was completed, samples were stored at –20˚C until the PCR.

Polymerase chain reaction (PCR)

Sequences of oligonucleotide primers for detection of MT and Spz12–1 in sea urchin were obtained from GenBank (MT, Accession: NM214577; Spz12–1, Accession: U19831) and synthesized by Bioneer Co. (Korea). The primer sequences are: MT forward 5–CAAGGATGGAACCTGCTGTG–3; MT reverse 5–GCAGATGCGCTGAGTGACTT–3; SpZ12–1 forward 5–AAAATGACACAGGCGATGGA–3 SpZ12–1 reverse 5–TAATGTTTCTCGCTCCCCCT–3. Spz12–1 mRNA expression was employed as an internal positive control and was detected in all the samples studied, thus assuring the integrity of the RNA extraction and RT–PCR processes. PCR was performed following the protocol suggested by the manufacturer. The reaction was carried out in the AccuPower HotStart PCR Premix kit (Bioneer Co. Ltd, Korea). After an initial denaturation step for 5 min at 95˚C, the amplification consisted of a total 28 cycles for MT and 26 cycles for SpZ12–1 at 95˚C for 30 sec, 60˚C for 30 sec and 72˚C for 30 sec. The reaction was terminated at 72˚C for 5 min and was quenched at 4˚C. 2% agarose gel (SeKem, USA) electrophoresis was carried out in 0.5 x TBE.

Statistical Analysis

Data were analyzed by one–way ANOVA (Fisher PLSD test). Fisher's test was also used to examine the significance of correlation coefficients. Significance was accepted at $*P<0.05$, $**P<0.01$.

RESULTS

When exposed to Ni, fertilized eggs and embryos showed developmental abnormalities including unfertilized eggs, rupture of fertilized eggs and deformed embryos (Fig. 1). Following the Ni treatment the fertilization rates were not changed (Fig. 2) and the rates of normal embryogenesis were gradually decreased in a concentration dependent manner (Fig. 3). The normal embryogenesis rates following 50 ppb Ni treatment was significantly lower than vehicle control at 64 h onward (Fig. 3).

Based on the test results determined from the effects of Ni treatment on the fertilization and normal embryogenesis rate of *H. pulcherrimus*, EC_{50} and 95% Cl were calculated using the probit analysis method, and NOEC and LOEC were calculated using the Dunnett's test (Table 2). The EC_{50} of the rate of normal embryogenesis was 34.19 ppb. The 95% Cl of EC_{50} was 29.56–38.09 ppb for the normal embryogenesis rate. The NOEC and LOEC of normal embryogenesis rate were <10 ppb and 25 ppb, respectively (Table 2).

The effect of Ni on MT mRNA expression is shown in Fig. 4. Ni induced expression of MT mRNA significantly in *H. pulcherrimus*.

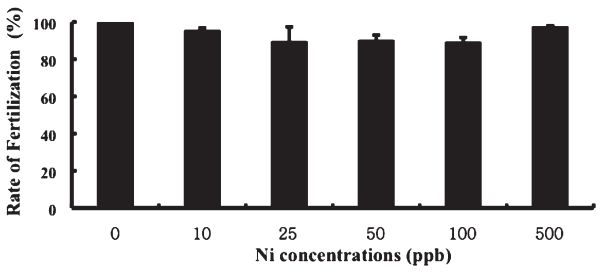

Fig. 2. Fertilization rates of sea urchin (*H. pulcherrimus*) exposed to Ni.

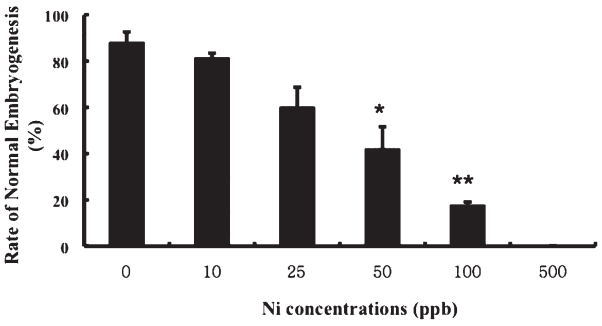

Fig. 3. Normal embryogenesis rates of sea urchin (*H. pulcherrimus*) embryos exposed to Ni. * Significant different from vehicle control by Fisher's exact test ($P<0.05$), ** $P<0.01$.

Table 2. Toxicological estimation using the form of normal embryos in the sea urchin (*H. pulcherrimus*) exposed to Ni. EC_{50}: 50% effecive concentration, 95% CI: 95% confidence limit NOEC: No obseved effective concentration, LOEC: Lowest observed effective concentration

Items	Toxicity (End– points)	Ni (ppb)
EC_{50}	Normal embryos	34.19
95% Cl	Normal embryos	29.56– 38.09
NOEC	Normal embryos	<10
LOEC	Normal embryos	25

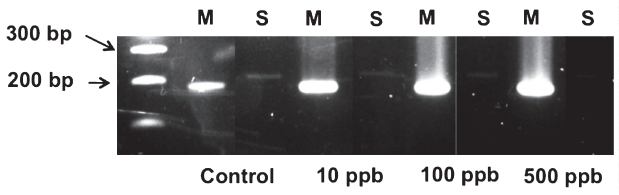

Fig. 4. Expression of MT and Spz12– 1 mRNA genes in embryos exposed to Ni by RT– PCR. M: Metallothionein, S: SpZ12– 1.

DISCUSSION

The sea urchin embryo is an intact developing system, which undergoes events comparable to those of other vertebrates, including mammals. Sea urchin embryo normal embryogenesis and teratogenesis assay is useful because they can rapidly provide information on developmental toxicants.

Ni concentration in seawater generally range from 0.2 to 130 μg/l (DETR, 1998). In exposed to Ni, the normal embryos of *H. pulcherrimus* were decreased with concentration dependent manner. At 50 ppb Ni, normal embryo was significantly decreased compared with vehicle control at 64 h onward. Following Ni treatment, embryos showed developmental abnormalities including unfertilized eggs, rupture of fertilized eggs and deformed embryos. The incidence of developmental abnormalities increased with increasing concentration of Ni. Saillenfait *et al*. (1993) reported that Ni caused growth retardation and brain and caudal abnormalities in rat embryo. Also, in rat embryos, Ni reduced caudal neural tube, branchial arches and dilated optic vesicles (Saillenfait *et al*., 1991). In *Hydroides elegans*, Ni decreased rate of fertilization and delayed or blocked the first mitotic divisions and altered early embryonic development when the sperm and eggs were treated with Ni (Gopalakrishnan *et al*., 2008). The NOEC and LOEC of Ni for normal embryogenesis were each <10 ppb and 25 ppb at 64 h in *H. pulcherrimus*. In the previous studies, Ni has been reported to have toxic effects in sea urchin (*Diadema antillarum*) and showed the 40 h EC_{50} value was 15 μg /l (Bielmyer *et al*., 2005). Our results, in *H. pulcherrimus*, 64 h EC_{50} value was 34.19 ppb, which is higher than *D. antillarum*. The differences in EC_{50} values might be due to the different species and different exposure times in the experiments. Taken together, we suggest that the safe guideline for Ni in marine environment should be below the 25 ppb for embryonic survival of *H. pulcherrimus*.

The induction of MT gene by heavy metals was demonstrated in sea urchin (*Paracentrotus lividus*) by Russo *et al*. (2003). MT gene expression showed an up–regulation by cadmium (Russo *et al*., 2003). In rat kidney cells, MT was up–regulated and concentration dependent by Ni treatment (Lee, 2006). In animal cells, MT gene was rapid induction by exposed to heavy metal ions (Hamer, 1986; Karin *et al*., 1987). In our result, Ni induced expression of MT mRNA gene significantly in *H. pulcherrimus*. These results suggest that MT mRNA gene may be used as biomarker gene for risk assessment on contamination of Ni.

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