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Effect of Silver, Cadmium, Chromium, Copper, and Zinc on the Fertilization of the Northern Pacific Asteroid, *Asterias amurensis*

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The fertilization assay with sea urchin has been widely used in quality assessments of marine environments owing to high sensitivity and short test duration (Nacci *et al.*, 1986; Dinnel *et al.*, 1983; 1989; USEPA, 1995). The disadvantage of sperm cell bioassay is that toxicity test can be conducted during only the spawning period, which is a serious problem in temperate regions in which sea urchin spawns for a limited period (at most 3 months for Korean species). Lee (2000) listed sea urchin species utilizable as bioassay organisms in Korea. The candidate species for bioassay are *Hemicentrotus pulcherrimus*, *Pseudocentrotus depressus*, *Strongylocentrotus nudus*, *S. intermedius*, and *Anthocidaris crassispina*. The spawning periods of these species are different from one another with some overlaps. The overall range covers from March to November. There is no sea urchin species spawning during winter.

Since the reproductive system, the processes of fertilization, and the embryonic development of asteroids are similar to those of echinoids (Chia and Walker, 1991), the bioassay protocol for sperm cell of echinoid can be similarly applied to asteroid. In the course of developing standard bioassay protocols with marine organisms in Korean waters, we found that the northern Pacific asteroid, *Asterias amurensis* Lütken is a winter spawner (Byrne *et al.*, 1997). Since this species is commonly found in shallow subtidal areas along the Korean coast (Shin and Rho, 1996), it can be easily collected during low tides. So, we attempted to make this species a complementary bioassay organism to sea urchins during winter. Lee and Choi (2003) established optimal conditions for the sperm cell bioassay using *A. amurensis*. But information about the sensitivity of this species is still lacking. We set here the purpose of this study to determine the sensitivity of the sperm cell bioassay with *A. amurensis* to five metals (silver, cadmium, chromium, copper, and zinc) of environmental concerns in Korean coastal waters. In this paper, (1) the median effective concentrations (EC_{50}) for each metal from sperm cell bioassay with *A. amurensis* are reported, (2) the sensitivity of sperm cell bioassay with this species is compared with those of other echinoids, and (3) the

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differences between sperm cell bioassay of *A. amurensis* and that of echinoids are described.

MATERIALS AND METHODS

Adults of *Asterias amurensis* (radius >10 cm) were collected at shallow subtidal areas (*ca.* 1 m depth) along the rocky coast of Geoje Island (34° 59' 35" N, 128° 40' 29" E) from February to March, 2003. The surface seawater temperature during collection was 8-12℃. Starfishes were maintained in a 500 L aquarium with constant aeration at 15±1℃. Water was replaced completely every third day. Spawning was induced by injecting 1 mL of 100 μM 1-methyladenine (Aldrich) to the coelomic cavity (Strathmann, 1987). Mature starfishes commenced shedding gametes *ca.* 30 min after the injection. Males released white- or cream-colored sperms and females released yellow- or orange-colored eggs. Sperms released from the gonopores were transferred directly into a 1.5-mL microcentrifuge tube using a Pasteur pipette, then the tube was kept in a refrigerator (5℃) before use. Eggs were collected by placing each female with oral side up on a 1-L beaker filled with GF/F (Whatman) filtered seawater (FSW, salinity: 32 psu) for 30 min. Egg suspension was passed through a 125 μm mesh screen to remove fecal materials and larger particles, then eggs were collected on a 60 μm mesh screen for smaller particles to pass through. Eggs were rinsed with FSW three times, then kept at the experimental temperature (15℃) before use. Experiments began within 30 min after the collection of both gametes.

Experiments were repeated three times with different pairs of male and female. Bioassay procedure was based on the standard protocol for echinoids (USEPA, 1995) with slight modification (Lee and Choi, 2003). Pretests were performed prior to every experiments to determine the optimal sperm to egg (S:E) ratio. Sixteen different S:E ratios were set up ranging from 4:1 to 170,000:1. Triplicate wells of 24-well plates (polystyrene, Corning) were filled with 1 mL of sperm suspension with target S:E ratios, allowed for 20 min, and then 300 eggs were injected into each well. The densities of egg and sperm were determined using a Sedgwick-Rafter counting chamber and a Neubauer hemocytometer, respectively. After 60 min, 50 μL of formaldehyde was injected into each well. Fertilization rate was measured by examining 100 eggs under an inverted microscope (×100, Zeiss). Just like the sea urchin, fertilized eggs were easily distinguished from unfertilized ones by the presence of fertilization membrane around the egg mass. The optimal S:E ratio was determined as the lowest S:E ratio at which the fertilization rate was more than 80%. In all 3 separate experiments the optimal S:E ratio was determined similarly around 3000:1.

The toxicants used in the experiments were silver (as $AgNO_3$, 99%), cadmium (as $CdCl_2$, 99%), chromium (VI) (as $K_2Cr_2O_7$, 99%), copper (as $CuCl_2$ $2H_2O$, 99%),

and zinc (as $ZnCl_2$, 98%). All toxicants were A.C.S. reagent grade and were purchased from Aldrich. The concentration ranges of test chemicals were pre-determined from the range-finding tests as 0.05-1 mg/L for silver, 10-400 mg/L for cadmium, 20-200 mg/L for chromium, 0.05-0.6 mg/L for copper, and 0.05-1.6 mg/L for zinc. All concentrations in this study are nominal concentrations. Five or seven concentrations of each toxicant solution plus a control (FSW) were prepared. One mL of each test solution was transferred to triplicate wells. Approximately 1.0×10^6 sperms in 50 μL of FSW were injected into each well and sperms were exposed for 20 min, and then 300 eggs in 100 μL of FSW were added. After 60 min, experiment was terminated by injecting 50 μL of formaldehyde. Fertilization rate for each treatment was measured by examining 100 eggs under an inverted microscope.

Fertilization rate data at each treatment were corrected to those at controls with Abbott's formula (ASTM, 1995). The lowest observed effective concentration (LOEC) was determined by Dunnett's t-test and EC_{50} was estimated by trimmed Spearman-Karber method (Hamilton *et al.*, 1977) using the TOXSTAT program (Gulley and WEST, Inc., 1996).

RESULTS AND DISCUSSION

The fertilization rates (mean ± SD, n=15) in control (FSW) were 87.9 ± 4.7% for Test 1, 85.1 ± 7.8% for Test 2, and 82.6 ± 4.1% for Test 3. Therefore control data in all 3 experiments were acceptable (USEPA, 1995).

The LOECs for silver, cadmium, chromium, copper, and zinc were 0.1 mg/L, 80 mg/L, 25 mg/L, 0.05 mg/L, and 0.25 mg/L, respectively. The sensitivity of sperm cell bioassay with *Asterias amurensis* to the toxicants tested was in the following order: copper > silver > zinc > chromium > cadmium.

In general, the fertilization rate appeared to decrease linearly with the logarithm of toxicant concentration within the tested range. EC_{50} values for each metal ranged 0.24-0.52 mg/L for silver, 110-214 mg/L for cadmium, 45-115 mg/L for chromium, 0.13-0.29 mg/L for copper, and 0.41-0.68 for zinc (Table 1). There were some variations in EC_{50} values among tests with different parents. The coefficient of variation (CV) of EC_{50} values ranged from 25 to 45%, which lies between single (24%) and multiple (52%) laboratory precision of an echinoid (*Strongylocentrotus purpuratus*) sperm bioassay (USEPA, 1995). The high variances in some chemicals among the three tests may be due to the use of only one pair of starfish per experiment. So, pooling of gametes from several pairs is recommended to reduce variability among individuals. The toxic order based on the EC_{50} value was the same as that based on the LOEC. *A. amurensis* sperm showed the highest sensitivity to copper, followed by silver, zinc, and chromium.

But it showed lowest sensitivity to cadmium. There was more than 700 fold difference in EC_{50} value between copper and cadmium. The order of sensitivity to the toxicants for *A. amurensis* is similar to that reported for the echinoid *Arbacia spatuligera* (Larrain *et al.*, 1999).

Table 1. Inter-test variability in the EC_{50}s (mg/L) of toxicants from each sperm cell bioassay with *Asterias amurensis* (Values in parentheses are 95% confidence limits).

Toxicant	Test 1	Test 2	Test 3	Mean ± SD	CV
Ag	0.24 (0.22-0.25)	0.52 (0.48-0.56)	0.52 (0.47-0.56)	0.43 ± 0.16	37%
Cd	138 (120-158)	110 (97-125)	214 (207-223)	154 ± 54	35%
Cr	74 (72-77)	115 (111-119)	45 (43-48)	78 ± 35	45%
Cu	0.29 (0.27-0.31)	0.17 (0.16-0.18)	0.13 (0.12-0.14)	0.20 ± 0.09	45%
Zn	0.41 (0.38-0.43)	0.57 (0.53-0.62)	0.68 (0.61-0.75)	0.55 ± 0.14	25%

Table 2. Comparison of EC_{50} values (mg/L) of metals among sperm cell bioassay with *Asterias amurensis* and other echinoid species.

Species	Ag	Cd	Cr	Cu	Zn
Asterias amurensis[a]	0.43	154	78	0.20	0.55
Arbacia punctulata[b]	0.05	38		0.01	0.12
Arbacia punctulata[c]			342		
Arbacia spatuligera[d]		141	21	0.02	0.12
Diadema setosum[e]		1		0.07	
Dendraster excentricus[f]	0.05	8		0.03	0.03
Strongylocentrotus droebachiensis[f]	0.09	26		0.06	0.38
Strongylocentrotus franciscanus[f]	0.11	12		0.002	0.31
Strongylocentrotus purpuratus[f]	0.12	18		0.03	0.26
Strongylocentrotus nudus[g]	0.12	7		0.08	

[a]Present study, [b]Nacci *et al.* (1986), [c]Jop (1989), [d]Larrain *et al.* (1999), [e]Ramachandran *et al.* (1997), [f]Dinnel *et al.* (1989), [g]Won (2000).

Since there is no data available on the sensitivity of asteroid fertilization bioassay, only comparison with bioassays using echinoids is possible. In general, fertilization bioassay with *A. amurensis* seems less sensitive than those with echinoids (Table 2). There were more than 10-fold differences in the EC_{50} values between *A. amurensis* and the most sensitive echinoid species for silver, cadmium, copper, and zinc. As for chromium, EC_{50} for *A. amurensis* is higher than that for *Arbacia spatuligera*, but lower than that for *Arbacia punctulata*. The higher EC_{50}

values seem to be related to the shorter exposure time. We exposed sperms to toxicants for 20 min according to the standard protocol from USEPA (1995). But data on echinoids in Table 2 were the results from 60 min's exposure. If the exposure time was longer, we could obtain more sensitive results. Therefore, exposure time of 60 min is recommended for further environmental assessment with *A. amurensis* to acquire more compatible data to echinoids.

We performed the sperm cell bioassay with *A. amurensis* based on the standard protocol for echinoids (USEPA, 1995). However, there are several differences in detailed procedure and some conditions. We used 1-methyladenine (1-MA) instead of potassium chloride to induce spawning. In most of echinoids, the germinal vesicle breakdown (GVBD) occurs when vitellogenesis is completed (Pearse and Cameron, 1991). However, in asteroids, GVBD does not occur at the end of vitellogenesis but is caused by maturation-promoting factor (MPF), the synthesis of which is induced by 1-MA (Chia and Walker, 1991). Thus, it requires some time to release gametes after the injection of 1-MA. For *A. amurensis*, it was *ca.* 30 min.

We adopted a micro-scale testing (1 mL test volume) using plastic 24-well plates. In our experiments, both test volume and container types are different from USEPA (1995). In preliminary studies with sea urchin (*Strongylocentrotus nudus*), there were little effects of test volume and container type (1 mL in well plate vs. 5 mL in glass vial) on the results of sperm bioassay when the toxicant was inorganic metal (Lee, 2000). Warnau *et al.* (1996) also used plastic container in testing metal toxicity to sperm and embryo of the echinoid (*Paracentrotus lividus*). Hunt *et al.* (1998) stated some advantages of micro-scale testing. It increases laboratory efficiency, eliminates subsampling and handling bias, produces an acceptable level of precision, and increases the feasibility of environmental assessments, needs less toxicant, and produces less waste. However, micro-scale testing with plastic container can be applied only for inorganic toxicants. Glassware is appropriate when the toxicant is organic substance.

The experimental temperature was 15℃, which was determined by considering the prevailing ambient seawater temperature during the spawning period of *A. amurensis*. The optimal S:E ratio (3000:1) was higher than that of echinoids (USEPA, 1995), which seems to be a difference in biological characteristics between *A. amurensis* and echinoid. At this elevated S:E ratio the fertilization rate in control seawater was over 80%. The duration for complete formation of fertilization membrane was 60 min (Lee and Choi, 2003), which is generally longer than echinoids (less than 30 min). So, after the egg injection, we should wait for 60 min to terminate the tests. These modified conditions (temperature, S:E ratio, time for membrane formation) seem to be related to biological characteristics specific for *A. amurensis*.

It is highly attractive that the gametes of *A. amurensis* can be obtained from November to next April (Lee *et al.*, unpublished data). Therefore, it is possible to perform sperm cell bioassay at any time of a year owing to the addition of *A. amurensis* to the list of bioassay organisms in Korean waters.

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