Optimal Conditions for the Bioassay Using Sperms of a Starfish, *Asterias amurensis*

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아무르불가사리 (*Asterias amurensis*) 정자를 이용한 생물검정의 최적조건

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요 약

성게의 정자를 이용한 생물검정은 해양환경의 독성평가에 널리 사용되고 있다. 그러나, 국내산 성게류의 경우 산란기가 3~11월로 겨울에는 생물검정 실험이 불가능한 단점이 있다. 이를 보완하기 위하여 겨울에 산란이 가능한 아무르불가사리 (*Asterias amurensis*)를 생물검정용으로 개발하였다. 불가사리류의 생식과 발생과정은 성게류와 유사하므로 성게류를 이용한 실험 방법을 아무르불가사리에 적용할 수 있다. 그러나, 생물검정 실험 결과의 유효성은 실험조건에 의하여 달라질 수 있으므로, 본 연구에서는 실험 결과에 큰 영향을 미칠 수 있는 조건들 즉, 수정막의 발달 시간, 정자와 알의 비율, 정자의 활력이 유지되는 시간, 정자의 노출 시간, 그리고 시료의 염분 범위 등을 최적화 하고자 하였다. 수정막의 발달이 완전하게 이루어지는 최소 시간은 60분이었고, 시료에 주입할 정자와 알의 비율은 3,000 이상일 때 수정률이 높게 유지되었다 (70% 이상). 정자의 활력은 해수에 희석한 후 3시간 이상이 경과하면 급격히 감소하였다. 정자의 노출시간은 20~60분의 범위에서 구리에 대한 EC_{50}이 크게 달라지지 않았다 (변동계수: 17%). 실험에 적절한 시료의 염분 범위는 26~38 psu이었다. 아무르불가사리 정자의 민감도는, 일반적으로 해양환경 평가에 흔히 사용되는 생물들의 민감도 범위의 중간 정도에 해당하였다. 따라서 아무르불가사리의 정자는 해양환경 평가를 위한 생물검정용으로 적합한 것으로 여겨진다.

주요어 : 아무르불가사리 (*Asterias amurensis*), 정자, 생물검정, 최적조건

INTRODUCTION

The sperm cell bioassay of echinoids has high sensitivity (Nacci *et al.*, 1986), simple test procedure (Dinnel *et al.*, 1987), short exposure duration (USEPA, 1995), and needs relatively small volume

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of sample (USEPA, 1995). Therefore, echinoid sperms are widely used in bioassays for quality assessments of marine environments (Zúñiga *et al.*, 1995; Carr *et al.*, 1996; Riveros *et al.*, 1996). The standard protocols prepared by the USEPA (1989; 1994; 1995) for conducting toxicity tests using marine organisms include some echinoid species in north America (*Arbacia punctulata, Strongylocentrotus purpuratus, Dendraster excentricus*). OECD (1998) adopted the

methods by the USEPA (1994; 1995) and suggested the use of these protocols for testing pesticides and industrial chemicals. In Korea, several studies were performed using the gametes of sea urchins (Yang *et al.*, 1996; Yu, 1998; Lee, 2000; Won, 2000).

The disadvantage of sperm cell bioassay is that tests can be conducted during only the spawning seasons (at most 3 months). Lee (2000) listed sea urchin species utilizable as bioassay organisms in Korea with their spawning seasons, which are different from one another. The overall range of spawning seasons of these species is from March to November. None of them spawns during winter. But, the starfish, *Asterias amurensis* (Class Asteroidea, Family Asteriidae) is known as a winter spawner (Hatanaka and Kosaka, 1959; Byrne *et al.*, 1997). In addition, *A. amurensis* has a wide range of distribution along the coasts of Korea (Shin and Rho, 1996), and abound in shallow subtidal and intertidal area of rocky shores. So, they can be easily collected at shorelines during low tides. The reproductive system and processes of gametogenesis, fertilization, and development of asteroids are similar to those of echinoids (Chia and Walker, 1991). Therefore, toxicity test protocols for echinoids can be similarly applied to asteroids. But, acceptable ranges of test conditions for asteroids may not be the same as those for echinoids.

In the standard protocol for sperm cell bioassay with echinoids (USEPA, 1995), sperms are exposed to toxicant solutions for 20 min, eggs are added, and after additional 20 min, tests are terminated by injection of fixatives. The toxicity is evaluated by the presence or absence of fertilization membrane. The acceptability of test is judged by the fertilization rates in control, which should be more than 70% (ASTM, 1995; USEPA, 1995). Here, we attempted to establish the optimal experimental conditions, which could make the results of bioassay acceptable. We considered 5 conditions as crucial to the acceptability of tests, i.e. (1) fertilization time, (2) sperm to egg ratio, (3) sperm viability after dilution in seawater, (4) exposure time, and (5) salinity of test solution.

Experimental data were evaluated by statistical procedures and optimal conditions were suggested. This study will provide basic information for preparing standard bioassay protocols with marine organisms in Korean waters.

MATERIALS AND METHODS

Preparation of gametes

Adult specimens (radius > 10 cm) were collected at shallow subtidal areas (ca. 1 m depth) along the rocky coast of Geoje Island from February to March 2003. The surface seawater temperature during collection was $8 \sim 12°C$. Spawning was induced prior to each experiment by injecting 1 mL of 100 μM 1-methyladenine (Aldrich) to the coelomic cavity (Strathmann, 1987). Six to eight individuals were used in each spawning induction. Mature starfishes shed their gametes within 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°C) 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°C) before use. Experiments began within 30 min after the collection of both gametes.

Time course experiment for the formation of fertilization membrane

This experiment was designed to determine the fertilization time of the bioassay. Approximately 5.0 × 10⁴ eggs were transferred to a 1-L polycarbonate

bottle (Nalgene) filled with 500 mL of FSW. Then, ca. 2.5×10^8 sperms were added to the bottle. The densities of eggs and sperms were determined using a Sedgwick-Rafter counting chamber and a Neubauer hemocytometer, respectively. After injection of sperms, 10-mL aliquots of subsamples were taken and fixed with formaldehyde at 10 min interval for 2 hr. Fertilization rate was measured by examining 100 eggs under an inverted microscope ($\times$ 100). Fertilized eggs were distinguished by the presence of fertilization membrane around the egg mass. Twenty fertilized eggs were randomly chosen from each subsample and the diameter of fertilized egg was measured with a micrometer to the nearest 1 μm.

Determination of effect of sperm to egg ratio on fertilization rate

This experiment was designed to determine the optimal range of sperm to egg (S : E) ratio for the bioassay. Experiments were repeated 5 times with different pairs of male and female. At each trial, 12 to 15 treatments were set up with S : E ratios ranging from 4 to 167,608. Each treatment was triplicated. Experiments were carried out using 24-well plates (polystyrene, Corning) as test chambers. Wells were filled with 1 mL of sperm suspensions with target S : E ratios, allowed for 20 min, and then 200 eggs were injected to each well. After 60 min, experiment was terminated by injecting 50 μL of formaldehyde. The microscopic observation and the measurement of the fertilization rate were the same as the above.

Experiment for sperm viability test after dilution in seawater

This experiment was designed to know the viability of sperm after dilution in seawater and to determine the acceptable time in preparing the sperm suspension before the bioassay. Basic experimental design was similar to the experiment for S : E ratio, except for the duration of sperms in seawater after dilution. Six treatments of durations, namely 1, 2, 3, 4, 5, and 6 hr were set up. For each treatment, 8 dif-

ferent S : E ratios were prepared ranging from 83 to 16,506. Triplicate wells were filled with 1 mL of sperm suspensions with target S : E ratios. Two hundred eggs were injected to each well after the specified time for each treatment. Additional 60 min after the injection of eggs, experiments were terminated by formaldehyde. The fertilization rate was measured the same as the above.

Determination of effect of exposure time on fertilization rate

This experiment was designed to determine the optimal exposure time of sperm to toxicant solution. Six different exposure times were set up as 10, 20, 30, 40, 50, and 60 min. For each treatment of exposure time, 5 nominal concentrations of Cu (as $CuCl_2 \cdot 2H_2O$, Sigma) solutions (25, 50, 100, 200, and 400 μg/L) plus a control (FSW only) were prepared. Triplicate wells were filled with 1 mL of each Cu solution. Approximately 1.0×10^6 sperms were injected to each well and exposed for the specified time for each treatment. The rest of experimental procedure was the same as the above.

Determination of salinity effect on fertilization rate

This experiment was designed to know the acceptable range of salinity for the bioassay. Thirteen different treatments of salinities ranging from 17 to 43 psu were set up. Each solution was prepared by diluting the hypersaline brine (HSB) with deionized water. HSB (43 psu) was prepared by partial freezing and thawing of FSW (USEPA, 1995). Triplicate wells were filled with 1 mL of each solution of target salinity. Approximately 1.0×10^6 sperms were added to each well and allowed for 20 min. The rest of experimental procedure was the same as the above.

Data analyses

Statistical analyses were performed using the SPSS program. Fertilization rate data for different treat-

ments in each experiment were compared by one-way analysis of variance (ANOVA). Before the analyses, data were tested for normality (Shapiro-Wilk's test) and homogeneity of variance (Bartlett's test). If one of the above ANOVA requirements was not met, the data were $\log_{10}$ transformed, then ANOVA was repeated. For all analyses, a significance level of $\alpha = 0.05$ was used. If significant F values were found in any of ANOVA tests, multiple comparisons were conducted using Tukey's HSD (Zar, 1984) to determine which means were significantly different from one another.

Fertilization rate data at each Cu concentration from the experiment for exposure time were corrected to those at controls (FSW) with Abbott's formula (ASTM, 1995):

$$E = 100 \times (A - M) / (100 - M)$$

Where, E is the corrected percentage of the unfertilized eggs, A is the percentage of the unfertilized eggs before correction, and M is the average percentage of the unfertilized eggs in the control. One-way Dunnett's t-test (Sokal and Rholf, 1981) was used to determine the arcsine square root transformed data at each Cu concentration were significantly different from that of control. The median effective concentration (EC_{50}) of Cu for each exposure time was estimated by trimmed Spearman-Karber method (Hamilton et al., 1977) using the TOXSTAT program.

RESULTS

Time for the formation of fertilization membrane

The fertilization membrane of *A. amurensis* could be observed at all times during the experiment. The fertilization rate was $90.3 \pm 4.7\%$ (mean $\pm$ SD, n = 3) after 10 min. It slightly increased as the fertilization time increased (Table 1), but the change was not statistically significant (F = 2.102, P = 0.062). However, the diameter of fertilization membrane changed markedly as the fertilization time increased (F =

Table 1. Changes in the fertilization rate (mean $\pm$ SD, n = 3) and the diameter of fertilization membrane (mean $\pm$ SD, n = 20) of *Asterias amurensis* with increasing fertilization time

Time (min)	Fertilization rate (%)	Diameter[1] (µm)
0	–	120.6 ± 7.7^a
10	90.3 ± 4.7^a	130.7 ± 10.2^a
20	91.7 ± 1.5^a	142.7 ± 12.2^b
30	92.0 ± 2.0^a	143.3 ± 12.9^{bc}
40	92.7 ± 3.8^a	153.8 ± 8.0^{cd}
50	92.7 ± 3.8^a	154.8 ± 8.6^d
60	93.3 ± 2.1^a	156.3 ± 10.4^{de}
70	93.7 ± 3.5^a	156.5 ± 9.5^{de}
80	94.0 ± 3.6^a	156.6 ± 11.1^{de}
90	94.7 ± 3.8^a	158.8 ± 10.4^{de}
100	94.7 ± 1.2^a	161.6 ± 10.0^{de}
110	98.3 ± 1.5^a	161.9 ± 10.1^{de}
120	98.7 ± 1.2^a	165.8 ± 11.4^e

[1] The value at time 0 represents the diameter of unfertilized egg. Values with the same superscripts in the same column are not significantly different (P > 0.05).

32.817, P < 0.001). The diameter of unfertilized eggs (at time 0 in Table 1) was 120.6 ± 7.7 µm (mean $\pm$ SD, n = 20). The membrane diameter increased rapidly and reached 153.8 ± 8.0 µm until 40 min after fertilization. After then, it increased slowly but continuously to 165.8 ± 11.4 µm until 120 min. Multiple comparisons showed that there was no significant difference in membrane diameter when the fertilization time was 60 ~ 120 min (P = 0.147).

Effect of S : E ratio on fertilization rate

The S : E ratio affected significantly the fertilization rate of *A. amurensis* in all of 5 trials (P < 0.001). In trial 1, fertilization rate was low (< 10%) when S : E ratio was less than 106. It increased rapidly when S : E ratio increased from 106 to 1,690. At S : E ratios above 1,690, fertilization rate was higher than 70%. The general trends of fertilization rates with increasing S : E ratio in other trials were more or less similar to that in trial 1. The variations in fertilization rates among different trials were observed when the S : E ratio ranged between 10 and 2,000. Multiple comparisons showed that there were no significant
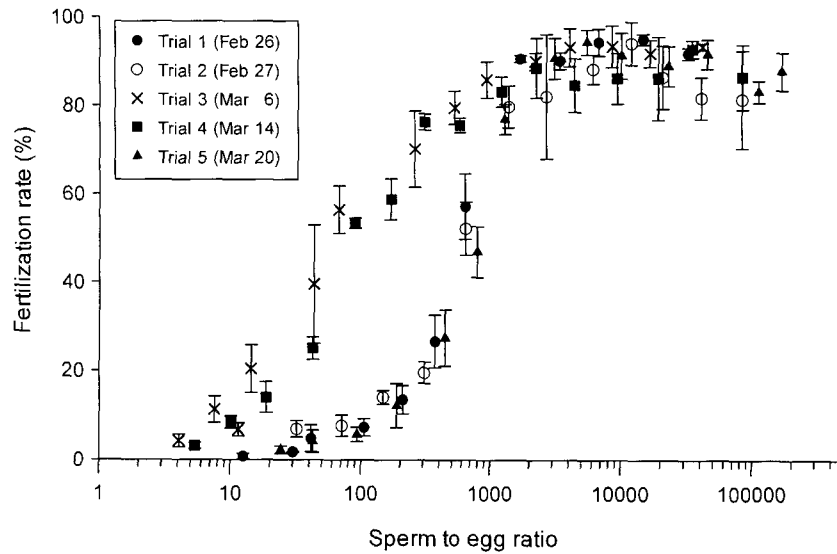
Fig. 1. Relationship between the sperm to egg (S : E) ratio and the fertilization rate of *Asterias amurensis* in filtered seawater from 5 trials. Symbols represent treatment mean ± SD (n = 3).

differences in fertilization rates in all 5 trials when the S : E ratios were higher than 3,000 (P = 0.791, 0.282, 0.154, 0.465, and 0.084 for trial 1, 2, 3, 4 and 5, respectively).

Sperm viability after dilution in seawater

Sperm viability of *A. amurensis* was strongly affected by the elapsed time after dilution with seawater at all the 8 S : E ratios (P < 0.001). When the S : E ratio was 16,506, fertilization rate was higher than 70% until 5 hr (Fig. 2A), but it decreased rapidly to 48.7 ± 4.9% (mean ± SD, n = 3) at 6 hr. As the S : E ratio became lower, sperm viability decreased more rapidly. The threshold times for fertilization rate to become less then 70% were 3 hr when the S : E ratio was 8,253 or 4,126 (Figs. 2B, C), 2 hr when the S : E ratio was 1,651 (Fig. 2D), and only 1 hr when the S : E ratio was between 825 and 83 (Figs. 2E–H). Multiple comparisons showed that there were no significant differences in fertilization rates with values higher than 70% when the elapsed time after sperm dilution was from 1 to 4 hr for S : E = 16,506 (P = 0.232), 1 to 3 hr for S : E = 8,253 and 4,126 (P = 0.266

and 0.496, respectively).

Effect of exposure time on fertilization rate

The exposure time of sperms affected significantly the fertilization rate of *A. amurensis* at all tested concentrations of Cu and FSW (P < 0.001). In FSW, fertilization rate continuously decreased as the exposure time increased (Fig. 3A). When the exposure time was 10 to 30 min, fertilization rate was higher than 85% and showed no statistical differences (P = 0.143). The general trends in fertilization rate at Cu concentrations of 25 and 50 μg/L were quite similar to that in FSW (Figs. 3B, C). When the exposure time ranged from 10 to 30 min, fertilization rates were higher than 95% in both solutions and were not significantly different (P = 0.783 and 0.984 for 25 and 50 μg/L, respectively). At Cu concentration of 100 μg/L, fertilization rate was 90~97% with no significance (P = 0.397) when the exposure time was 10 to 30 min (Fig. 3D). But, it decreased rapidly as the exposure time increased more than 40 min. The fertilization rate was only 37% at 60 min. At Cu concentrations of 200 and 400 μg/L, decreases in fertiliza-
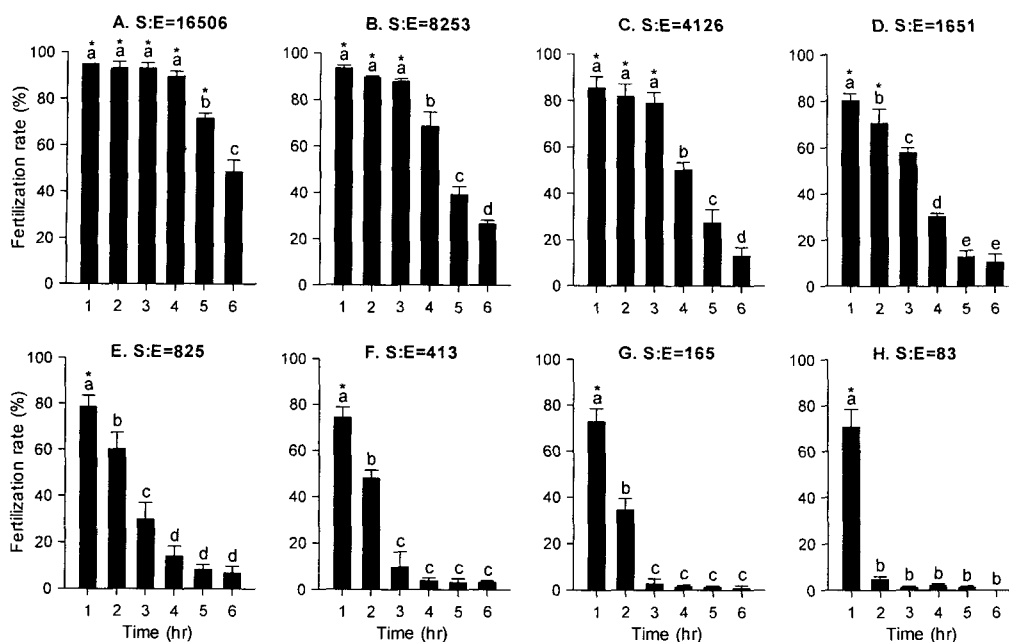
Fig. 2. Changes in the fertilization rate of *Asterias amurensis* with time after dilution of sperms with seawater for each sperm to egg (S : E) ratio. Vertical bar represents SD (n = 3). Values with the same character are not significantly different (P > 0.05). Values with asterisk are acceptable as control in fertilization assay according to USEPA (1995).
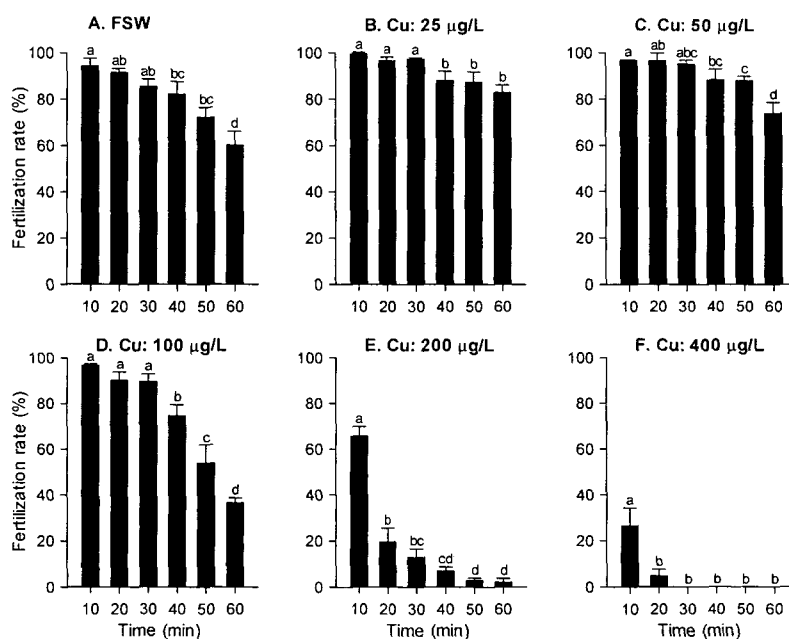
Fig. 3. Changes in the fertilization rate of *Asterias amurensis* with increasing exposure time of sperms in filtered seawater (FSW; A) and 5 concentrations of Cu solutions (B–F). Vertical bar represents SD (n = 3). Values with the same character are not significantly different (P > 0.05).
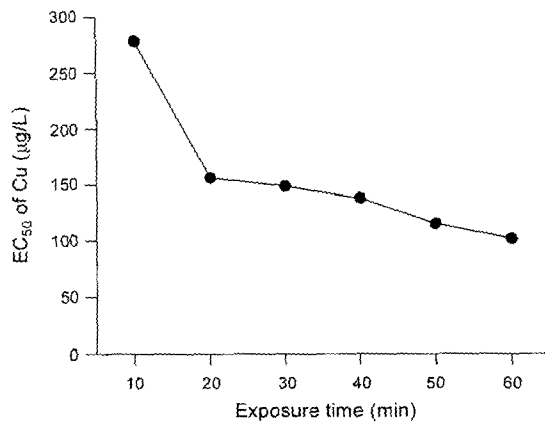
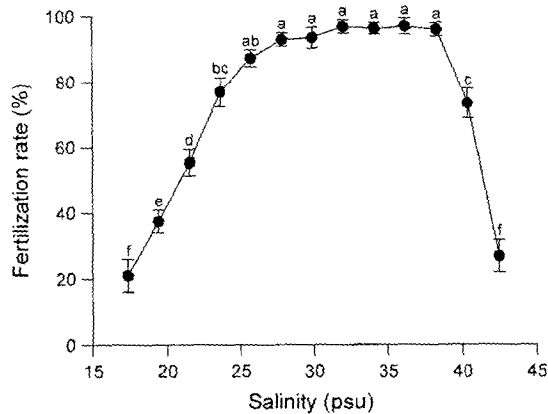Fig. 4. Change in EC_{50} of Cu with increasing exposure time of sperms of *Asterias amurensis*.

Fig. 5. Change in the fertilization rate of *Asterias amurensis* with different salinity of test solution. Symbol represents treatment mean±SD (n = 3). Values with the same character are not significantly different (P > 0.05).

tion rate with the exposure time appeared earlier (Figs. 3E, F). More than half of fertilization rate was reduced as the exposure time increased from 10 to 20 min (from 66 to 20% for 200 μg/L and from 27 to 5% for 400 μg/L). The EC_{50} of Cu also decreased as the exposure time increased (Fig. 4). The EC_{50} was 288 μg/L when sperms were exposed for 10 min. It rapidly decreased to 156 μg/L at 20 min, and then slightly but continuously decreased as the exposure time in-

creased further.

Effect of salinity on fertilization rate

The fertilization rate of *A. amurensis* was strongly affected by salinity (F = 197.681, P < 0.001). The fertilization rate increased rapidly from 21.0 to 87.0% as salinity increased from 17 to 26 psu (Fig. 5). It fluctuated between 92.7 and 96.7% when salinity ranged from 28 to 38 psu, then declined abruptly as salinity increased further. There were no significant differences in fertilization rate when salinity ranged from 26 to 38 psu (P = 0.084).

DISCUSSION

Data from this study revealed that the fertilization rate of *A. amurensis* was strongly affected by various experimental conditions. Therefore, it is necessary to establish standard (or optimum ranges) of experimental conditions for the bioassay to obtain reliable results. In the time course experiment for the formation of fertilization membrane, fertilization time had no effect on fertilization rate, while it affected significantly the diameter of fertilization membrane. These indicate that fertilization time is not a critical factor in the bioassay. However, in practical point of view, fertilization time can affect the efficiency in microscopic observation. If the fertilization membrane is fully developed, it is easy to distinguish between fertilized- and unfertilized eggs. When fertilization time was 60~120 min, the diameter of fertilization membrane ranged between 153.8 and 165.8 μm, which was significantly larger than the diameter of unfertilized eggs (120.6 μm). Here, we suggest the optimum time for the formation of fertilization membrane as 60 min. The optimum fertilization time of *A. amurensis* is longer than 20 min of the purple sea urchin, *Strongylocentrotus nudus* (Lee, 2000).

In the experiment for sperm to egg ratio, the S : E ratio greatly affected the fertilization rate. Although there were variations among trials, the fertilization rate appeared to increase linearly with logarithm of S

Table 2. Comparison of LC_{50} (or EC_{50}) values of Cu among various marine organisms commonly used in aquatic toxicity tests

Group	Species	Endpoint[1]	LC_{50} (or EC_{50}; μg/L)	Reference
Macroalga	*Champia parvula*	R	1.7–4.2	USEPA (1991)
Crustaceans	*Mysidopsis bahia* (juvenile)	S	176–200	USEPA (1991)
	Palaemonetes pugio (embryo)	S	1,820	Rayburn and Fisher (1999)
	Scylla seratta (embryo)	D	80	Ramachandran *et al.* (1997)
Bivalves	*Crassostrea gigas* (embryo)	D	14.7	His *et al.* (1999)
	Crassostrea iradalei (embryo)	D	81	Ramachandran *et al.* (1997)
Fishes	*Cyprinodon variegatus* (larva)	S	323–438	USEPA (1991)
	Menidia beryllina (larva)	S	149–493	USEPA (1991)
	Sparus sarva (fingerling)	S	1,030	Wong *et al.* (1999)
Echinoderms	*Arbacia punctulata* (sperm)	F (60 min)	30–47	USEPA (1994)
	Arbacia spatuligera (sperm)	F (60 min)	8.2	Riveros *et al.* (1996)
	Dendraster excentricus (sperm)	F (60 min)	20–44	USEPA (1995)
	Diadema setosum (sperm)	F (60 min)	70	Ramachandran *et al.* (1997)
	Paracentrotus lividus (sperm)	F (60 min)	16.4	Ghirardini *et al.* (1999)
	Strongylocentrotus purpuratus (sperm)	F (20 min)	14–31	USEPA (1995)
	Strongylocentrotus nudus (sperm)	F (20 min)	34–49	Lee (2000)
	Asterias amurensis (sperm)	F (20 min)	156	Present study

[1] R: reproduction, S: survival, D: development, F: fertilization. Values in parentheses after F are the exposure times of sperms.

: E ratio from 10 to 2,000. The variations in fertilization rates among trials for S : E ratio from 100 to 1,000 seemed to be due to the differences in viability of gametes from different parents. According to the recommendations from the standard protocols using echinoid sperms (USEPA, 1994, 1995), the fertilization rate in control should be more than 70%. In our data, the fertilization rate was higher than 70% when the S : E ratio was more than 3,000, irrespective of parental status. Therefore, we concluded that S : E ratios should be at least 3,000 for a valid sperm bioassay with *A. amurensis*.

In the experiment for sperm viability, the sperm viability changed drastically with the time after dilution in seawater. If we consider all the conditions of S : E ratios, acceptable time after dilution (producing >70% fertilization) is limited to only 1 hr. However, from the experiment for S : E ratio, we found that S : E ratios less than 3,000 are not appropriate. So, we must consider the results for S : E ratios with 4,126, 8,253, and 16,506. In these 3 cases in common, the acceptable range of time after dilution ex-

tends to 3 hr. Therefore, sperms should be used within 3 hr after dilution in seawater.

In the experiment for exposure time, the EC_{50} of Cu decreased as exposure time increased, which is a typical phenomenon in aquatic bioassays (Rand *et al*, 1995). But, the slope of EC_{50} relative to exposure time was not unique (Fig. 4). The changing pattern can be divided into two phases; first, the rapidly decreasing phase (exposure time from 10 to 20 min), and second, the slowly decreasing phase (from 20 to 60 min). Since, the change within the first phase is too large (46% decrease), it is reasonable to select the optimal exposure time within the second phase. During this phase, the CV (coefficient of variation) of EC_{50} is only 17%. In most of studies with echinoid sperms, the exposure time was 20 or 60 min (USEPA, 1995; Riveros *et al.*, 1996; Ramachandran *et al.*, 1997; Ghirardini *et al.*, 1999; Lee, 2000). Therefore, either 20 or 60 min of exposure time was recommended to facilitate the comparison of bioassay results with other echinoid species. Here, we selected the exposure time as 20 min, because prolonged exposure

more than 20 min will not significantly enhance the sensitivity of the bioassay.

In the experiment for salinity, the acceptable range of salinity was from 23 to 40 psu according to USEPA (1995). However, multiple comparisons showed that the fertilization rates at 23 and 40 psu were significantly different from those at salinity from 26 to 38 psu. Therefore, we determined the optimal range of salinity as from 26 to 38 psu, in which the fertilization rate both exceeded 70% and was not different statistically.

We conducted all the experiments at 15°C. The seawater temperature during collection of adults was 8~12°C. So, the experimental temperature of this study was higher than that of natural condition. But, we have the reasonable basis that 15°C is appropriate for the bioassay. We have tested the effect of temperature on the fertilization rate, hatching time, and hatching rate of *A. amurensis* at 3 different temperatures (10, 15, and 20°C). The fertilization rates within temperature range of 10~20°C were not significantly different with values from 95.6 to 96.4% (Lee *et al.*, in preparation). The hatching time at 10°C (36 hr) was longer than those at 15 and 20°C (18 and 15 hr, respectively). The hatching rates were 61, 74, and 21% for 10, 15, and 20°C, respectively. We are preparing standard protocols for bioassays using not only sperms but also embryos and larvae of *A. amurensis*. In these respects, the optimal temperature for the development of *A. amurensis* was 15°C, at which the hatching time was not too long and the hatching rate was high enough to be acceptable as control.

The EC_{50} of Cu was 156 μg/L when sperms of *A. amurensis* were exposed for 20 min. In comparison with other marine organisms commonly used in aquatic toxicity tests (Table 2), *A. amurensis* is more sensitive than the shrimp (*Palaemonetes pugio*), and fishes (*Cyprinodon variegatus*, *Sparus sarva*), comparable to the mysid (*Mysidopsis bahia*) and the fish (*Menidia beryllina*), but less sensitive than the macroalga (*Champia parvula*), bivalves (*Crassostrea* spp.), and sea urchins (*Arbacia* spp., *Strongylocen-*

trotus spp.). Therefore, the sperm bioassay with *A. amurensis* can be satisfactorily applied to ecotoxicological assessments of marine environments.

It is highly attractive that the gametes of *A. amurensis* can be obtained from November to next April (Lee *et al.*, in preparation). The spawning periods of echinoids utilizable for bioassays from Korean coasts are known as March–April for *Hemicentrotus pulcherrimus*, June–August for *Anthocidaris crassispina*, June–September for *Strongylocentrotus nudus*, and September–November for *Strongylocentrotus intermedius* (Shin and Rho, 1996; NFRDI, personal communication). Therefore, it can be possible to perform sperm bioassays at any time of a year owing to the addition of *A. amurensis* to the list of bioassay organisms.

CONCLUSIONS

We determined several conditions for sperm bioassay with *A. amurensis*. The conditions determined as optimal are as follows. (1) The fertilization time is 60 min. (2) The sperm to egg ratio should be >3,000. (3) The sperm should be used within 3 hr after dilution in seawater. (4) The exposure time is 20 min. (5) The salinity of sample should be within 26~38 psu. Since the sensitivity is intermediate among marine organisms commonly used in aquatic bioassays, *A. amurensis* can be applied to the quality assessments of marine environments.

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REFERENCES

ASTM. Standard guide for conducting static acute toxicity tests with echinoid embryos. E1563-95, American Society for Testing and Materials, Philadelphia, PA. 1995; 19 pp.

Byrne M, Morrice MG and Wolf B. Introduction of the northern Pacific asteroid *Asterias amurensis* to Tasmania: reproduction and current distribution. Mar. Biol. 1997; 127: 673-685.

Carr RS, Long ER, Windom HL, Chapman DC, Thursby G, Sloan G and Wolfe DA. Sediment quality assessment studies of Tampa Bay, Florida. Environ. Toxicol. Chem. 1996; 15(7): 1218-1231.

Chia FS and Walker CW. Chapter 5. Echinodermata: Asteroidea. *In*: Giese AC, Pearse JS and Pearse VB (eds). Reproduction of Marine Invertebrates. Volume VI. Echinoderms and Lophophorates. Boxwood Press. Pacific Grove, CA. 1991; 301-353.

Dinnel PA, Link JM and Stober QJ. Improved methodology for a sea urchin sperm cell bioassay for marine waters. Arch. Environ. Contam. Toxicol. 1987; 16: 23-32.

Ghirardini AV, Birkemeyer T, Novelli AA, Delaney E, Pavoni B and Ghetti PF. An integrated approach to sediment quality assessment: the Venetian lagoon as a case study. Aquat. Ecosyst. Health Manage. 1999; 2(4): 435-447.

Hamilton MA, Russo RC and Thurston RV. Trimmed Spearman-Karber method for estimating median lethal concentrations in toxicity bioassays. Environ. Sci. Technol. 1977; 11: 714-719.

Hatanaka M and Kosaka M. Biological studies on the population of the starfish, *Asterias amurensis*, in Sendai Bay. Tohoku J. Agric. Res. 1959; 9: 159-178.

His E, Heyvang I, Geffard O and De Montaudouin X. A comparison between oyster (*Crassostrea gigas*) and sea urchin (*Paracentrotus lividus*) larval bioassays for toxicological studies. Water Res. 1999; 33(7): 1706-1718.

Lee CH. A study on the sea urchin (*Strongylocentrotus nudus*) bioassay: Sperm and fertilized egg tests. Ph. D. Thesis, Seoul National University. 2000; 185 pp. (in Korean)

Lee CH, Choi JW and Chung EY. Effects of temperature on the development of a starfish, *Asterias amurensis* Lütken (Echinodermata). (in preparation).

Lee CH, Choi JW and Ryu TK. Gametogenic cycle of a starfish *Asterias amurensis* Lütken from Geoje Island, southern coast of Korea. (in preparation).

Nacci D, Jackim E and Walsh R. Comparative evaluation of three rapid marine toxicity tests: Sea urchin early embryo growth test, sea urchin sperm cell toxicity test and Microtox. Environ. Toxicol. Chem. 1986; 5: 521-525.

OECD. Detailed review paper on aquatic testing methods for pesticides and industrial chemicals. OECD series on testing and assessment, number 11. ENV/MC/CHEM (98)19/PART1. 1998; 260 pp.

Ramachandran S, Patel TR and Colbo MH. Effect of copper and cadmium on three Malaysian tropical estuarine invertebrate larvae. Ecotoxicol. Environ. Saf. 1997; 36(2): 183-188.

Rand GM, Wells PG and McCarty LS. Chapter 1. Introduction to aquatic toxicology. *In*: Rand GM (ed.). Fundamentals of Aquatic Toxicology. Effects, Environmental Fates, and Risk Assessment. 1995; 3-67.

Rayburn JR and Fisher WS. Developmental toxicity of copper chloride, methylene chloride, and 6-aminonicotinamide to embryos of the grass shrimp *Palaemonetes pugio*. Environ. Toxicol. Chem. 1999; 18(5): 950-957.

Riveros A, Zúñiga M, Larrain A and Becerra J. Relationships between fertilization of the southeastern Pacific sea urchin *Arbacia spatuligera* and environmental variables in polluted coastal waters. Mar. Ecol. Prog. Ser. 1996; 134(1-3): 159-169.

Shin S and Rho BJ. Illustrated Encyclopedia of Fauna and Flora of Korea. Vol. 36. Echinodermata. Ministry of Education. 1996; 780 pp.

Sokal RR and Rholf FJ. Biometry. 2nd Ed. WH Freeman and Company, San Francisco, CA. 1981.

Strathmann MG. Reproduction and development of marine invertebrates of the northern Pacific coast: Data and methods for the study of eggs, embryos and larvae. University of Washington Press. Seattle and London. 1987; 670 pp.

USEPA. Biomonitoring for control of toxicity in effluent discharges to the marine environment. EPA/625/8-89/015. 1989; 58 pp.

USEPA. Technical support document for water quality-based toxics control. EPA/505/2-90/001. 1991; 145 pp.

USEPA. Short-term methods for estimating the chronic toxicity of effluents and receiving water to marine and estuarine organisms. 2nd Ed. EPA/600/4-91/003. 1994; 300-340.

USEPA. Short-term methods for estimating the chronic toxicity of effluents and receiving waters to west coast marine and estuarine organisms. EPA/600/R-95/136. 1995; 389-465.

Won NI. Sea urchin bioassay using Korean purple sea urchin *Strongylocentrotus nudus*: Standardization of well plate method. MS Thesis, Seoul National University. 2000; 103 pp. (in Korean)

Wong PPK, Chu LM and Wong CK. Study of toxicity and bioaccumulation of copper in the silver sea bream *Sparus sarba*. Environ. Int. 1999; 25(4): 417-422.

Yang JS. Jeong JY, Kang JW and Choi MS. Toxicity tests of seawater on the developmental stages of sea urchin, *Hemicentrotus pulcherrimus*, in Ulsan Bay. "The Sea" J. Korean Soc. Oceanogr. 1996; 1(1): 13-19.

Yu CM. Evaluation of offshore water quality by bioassay using the gametes and embryos of sea urchins. "The Sea" J. Korean Soc. Oceanogr. 1998; 3(3): 170-174. (in Korean)

Zar JH. Biostatistical analysis. 2nd Ed. Prentice-Hall International, Inc. Englewood Cliffs, NJ. 1984; 718 pp.

Zúñiga M, Roa M and Larrain A. Sperm cell bioassays with the sea urchin *Arbacia spatuligera* on samples from two polluted Chilean coastal sites. Mar. Pollut. Bull. 1995; 30(5): 313-319.