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Gynogenetic Tetraploid Larvae of the Pacific Oyster *Crassostrea gigas* Induced by Inhibition of the 1st and 2nd Meiotic Divisions

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Summary

The induction of gynogenetic tetraploids by the suppression of the meiotic division in the Pacific oyster *Crassostrea gigas* was attempted in three trials using the treatments of cytochalasin B (0.5 μg/mL ; CB). The effects of various durations of CB treatment on tetraploid induction frequency were examined. Haploid gynogenesis was induced by sperm which were UV-irradiated for 60 s at an intensity of 72 erg/mm^2/s. CB treatment was effective in inhibiting meiosis, and the optimum time for the suppression of both the 1st and 2nd polar bodies was from 10 to 45 min postinsemination, yielding 51.4% gynogenetic tetraploids. The results of this study suggest the possibility of producing gynogenetic tetraploid Pacific oysters.

Key words : Pacific oyster, *Crassostrea gigas*, gynogenetic tetraploid

Introduction

The first use of biotechnology in molluscan aquaculture was induced triploidy which has realized commercial success in the USA and is of great interest in most other parts of the world for their superior growth and improved meat quality (Allen and Guo, 1997). Currently, in the hatcheries triploid mollusks are produced by inhibiting the second polar body (PBII) with cytochalasin B (CB) (Beaumont and Fairbrother, 1991), or more recently with 6-dimethylaminopurine (6-DMAP) (Desrosiers et al., 1993). However, there are limitations to the use of these chemicals that include toxicity of the treatment reduced survival of the progeny, and often unpredictable results. All these problems are eliminated with the development of tetraploids, since all-triploids can be produced by mating tetraploids and diploids.

As an ideal approach to triploid production, tetraploid induction in mollusks has been widely investigated by several methods, including inhibition of the first polar body (PBI) in eggs from diploids (Stephens and Downing, 1988; Yang et al., 2000; Yang and Guo, 2004), blocking of both polar bodies in eggs from diploids (Scarpa et al., 1993; Peruzzi and Guo, 2002), mitosis I inhibition, and cell fusion (Guo et al., 1994). However, tetraploid embryos rarely survived beyond metamorphosis in mollusks. A different method of tetraploid induction was developed in the Pacific oyster *Crassostrea gigas*, when inhibiting PBI in eggs from triploids produced 2000 viable tetraploids (Guo and Allen, 1994). Although this method is reproducible in the pearl oyster *Pinctada martensii* (He et al., 2000), it is limited to species in which triploids produce significant numbers of eggs. Therefore, effective methods for direct induction of tetraploids from diploids are needed.

Gynogenesis is a form of development in which eggs are activated by sperm which do not contribute genetically to resulting embryos. In general, the induction of gynogenesis involves genetic inactivation of sperm by using radiation before fertilization, and diploidization of the female chromosomes by suppressing the first or second polar body, or the first mitotic division by thermal or hydrostatic pressure shock, or chemical treatments in order to produce viable diploid gynogenetic offspring. In mollusks, production of meiotic gynogenetic diploids by retention of the second polar body has been carried out successfully in *Haliotis discus hannai* (Fujino et al., 1990), *C. gigas* (Guo et al., 1993; Li et al., 2000a), *Mulinia lateralis* (Guo and Allen, 1994), *Mytilus edulis* (Fairbrother, 1994), *Mytilus galloprovincialis* (Scarpa et al., 1994) and *Chlamys farreri* (Pan et al., 2004). Theoretically only tetraploids can be produced by the suppression of both PBI and PBII after eggs are fertilized with genetically inactivated sperm. In the Pacific abalone *H. discus hannai*, tetraploid gynogenesis produced high percentage of tetraploid embryos, suggesting that this method is effective for tetraploidization (Okumura et al., 1996). In the present study, in order to search for a new candidate method for tetraploid induction in the Pacific oyster, we examined conditions necessary for the induction of gynogenetic tetraploids by UV-irradiated sperm and inhibition of the 1st and 2nd meiotic divisions with CB treatment.

Materials and Methods

Gametes

Mature cultured Pacific oysters (shell height, 10.8 ± 0.7 cm; shell length, 5.6 ± 0.5 cm) were collected in late July of 2002 at Onagawa Bay, Miyagi Prefecture, Japan. In the Bay the oysters spawn between August and September (Li et al., 2000b). The eggs and sperms were obtained by mincing the ovary and testis, and then suspended in seawater. Suspensions of sperm and egg were prepared at

concentrations of 1.0×10^7 sperm/mL and 2.0×10^4 egg/mL by dilution with seawater. All seawater used was filtered using membrance cartridge filters whose pore size is 1 μm.

UV irradiation of sperm

Two milliliters of sperm suspension was spread on a 9.0 cm diameter plastic dish (Nunclon dish; Nalge Nunc Co., Denmark). The dish was placed on a recipro shaker (TAITEC Inc., Japan) 15 cm below a 15 W UV germicidal light (TOSHIBA GL15, 254 nm) which provided a UV intensity of 72 erg/mm^2/s as measured by a digital radiometer (DRC-100X; Spectronics Inc., USA). The sperm was treated with UV light for 60 s. During UV irradiation, the sperm suspension was shaken at 40 cycles/min.

Induction of gynogenetic tetraploids

Eight milliliters of UV-irradiated sperm suspension was mixed with 40 mL of egg suspension, and the mixture was then divided into four batches. One was a gynogenetic haploid group in which the eggs were inseminated with UV-irradiated sperm but not exposed to CB, the others were gynogenetic tetraploid (G4N) groups in which the eggs were inseminated with UV-irradiated sperm and followed by CB treatment.

For CB treatment, the three batches of eggs were placed into 50 mL of seawater and exposed to CB (0.5 μg/mL seawater containing 0.1% dimethylsulfoxide, DMSO) at 10 min postinsemination for three durations: 30 min (abbreviation: G4N-30 group), 35 min (abbreviation: G4N-35 group), and 40 min (abbreviation: G4N-40 group), respectively. The timing of the CB treatments was based on that described in the previous paper (Li et al., 2000c) and cytological observation. After treatment, the zygotes were filtered through a 20 μm mesh, rinsed with seawater, and resuspended in seawater with 0.1% DMSO to remove the residual CB. Thirty minutes later, the zygotes were transferred into 5,000 mL of seawater for culture. During insemination and CB treatment, the water temperature was maintained at 23°C.

Rates of the fertilization and development of D-shaped larvae were calculated by counting the cleaved zygotes and the D-shaped larvae at 2 and 24 h after insemination, respectively. The experiment was repeated three times separately using gametes obtained from different individuals.

Ploidy determination

The ploidy of embryos was determined with chromosome preparations at 10 h postinsemination. Samples of the larvae (trochophores) were collected from each group, placed in 0.1% colchicine for 2 h, subjected to a hypotonic treatment with 0.075 M KCl solution for 30 min, and fixed by Carnoy's solution (methanol:

acetic acid, 3 : 1, v/v). During fixation the solution was changed several times, and the samples were finally stored in 50% acetic acid. The samples were dropped on warmed glass slides, air-dried and stained with 2% Giemsa solution diluted with phosphate buffer (pH 6.8). Chromosome numbers were counted from well-spread metaphase figures.

Statistical analysis

Data were expressed as mean ± standard deviation of three replicate trials and were compared using one-way analysis of variance (ANOVA). When the F-ratio for the ANOVA was statistically significant ($P<0.05$), these values were further analyzed by Tukey's test of multiple comparison. All statistical analyses were carried out using the Statistical Package for the Social Sciences (SPSS) program (Chicago, IL, USA).

Results

Fertilization and development

The effects of UV irradiation of sperm and CB treatments on the rates of fertilization and development of D-shaped larvae are shown in Table 1. The fertilization rate for the control was 99.7%, but fell to 92.5% in the haploid group, and 83.6–88.1% in the CB-treated G4N groups. The developmental rate of D-shaped larvae was 97.0% in the control group, however no D-shaped larva was obtained in the haploid group. Viable D-shaped larvae occurred in all three CB-treated G4N groups, and reached a development rate of 3.0%, 1.2%, and 0.8% in the G4N-30, G4N-35, and G4N-40 groups.

Ploidy levels of larvae

To know whether the chromosomes were doubled in the larvae from the

Table 1. Rates of the fertilization and development of D-shaped larvae of Crassostrea gigas from the control, haploid and G4N groups in the three experiments. G4N, gynogenetic tetraploid ; CB, cytochalasin B. Each value represents the mean ± SD (n = 3). Values in a column with different superscript letters are significantly different (P < 0.05).

Groups	UV irradiation of sperm	Duration of CB treatment	Fertilization (%)	D-shaped larvae (%)
Control	Unexposed	Untreated	99.7 ± 0.5^{a}	97.0 ± 2.4^{a}
Haploid	Exposed	Untreated	$92.5 \pm 6.1^{a,b}$	0.0 ± 0.0^{b}
G4N-30	Exposed	30 min	$88.1 \pm 11.6^{a,b}$	3.0 ± 2.3^{b}
G4N-35	Exposed	35 min	$86.1 \pm 11.0^{a,b}$	1.2 ± 0.6^{b}
G4N-40	Exposed	40 min	83.6 ± 10.3^{b}	0.8 ± 0.7^{b}

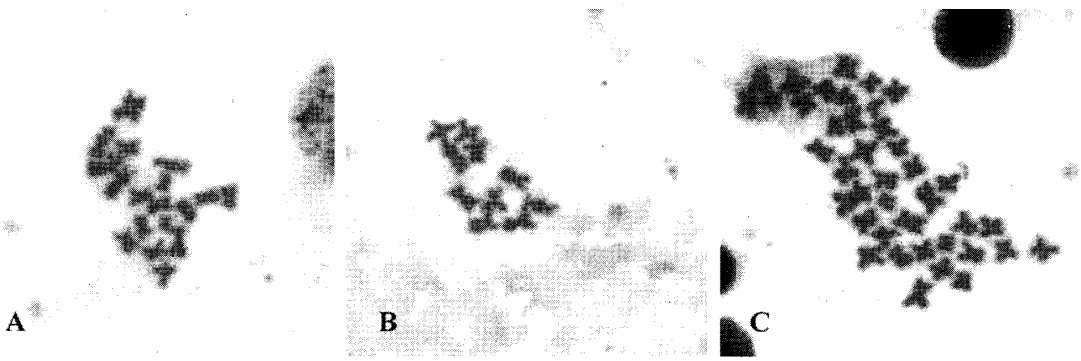

FIG. 1. Mitotic metaphase plates from the larval cells of *Crassostrea gigas*. (A) A diploid cell with 20 chromosomes from the control group. (B) A haploid cell showing 10 chromosomes from the haploid group. (C) A tetraploid cell with 40 chromosomes from the G4N-35 group. Scale bar = 10 μm.

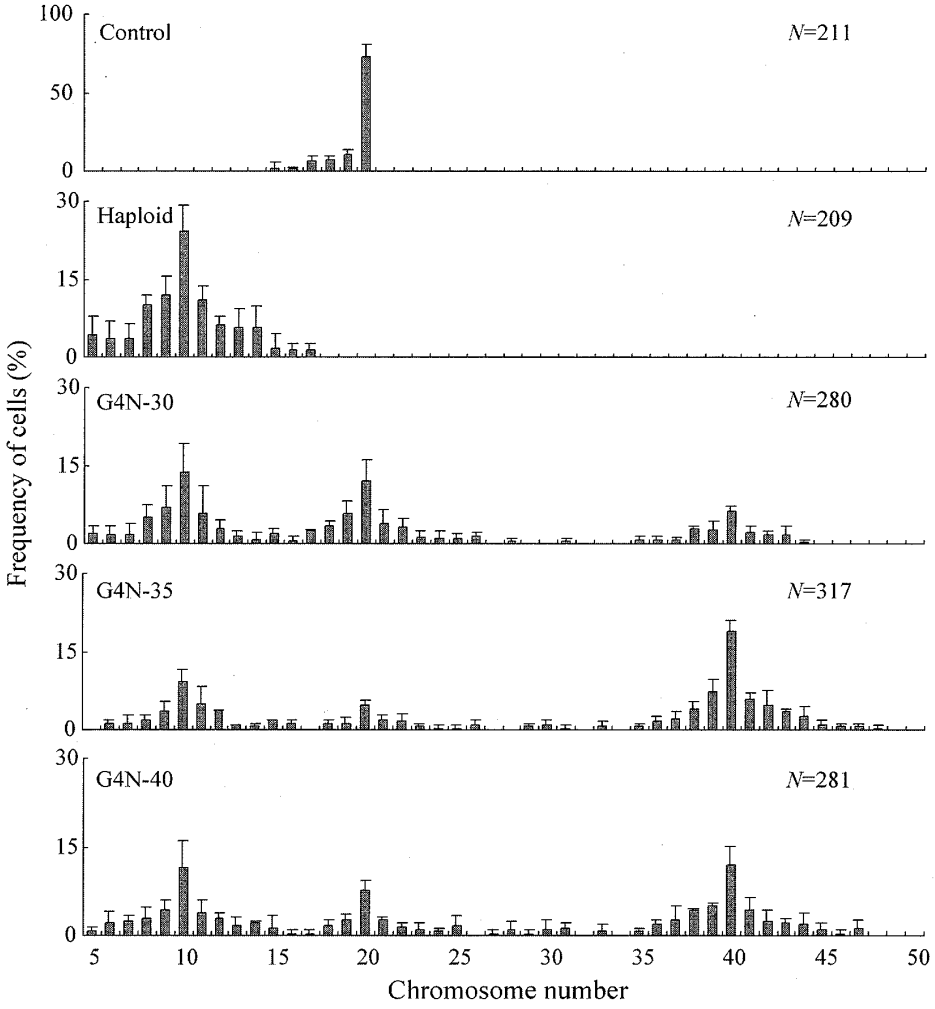

FIG. 2. Frequency distribution of chromosomes numbers in larval cells of *C. gigas* from the control, haploid, and CB-treated gynogenetic tetraploid groups in the three experiments. *N*, total number of cells observed. Each value represents the mean $\pm$ SD ($n = 3$).

Tabel 2. Levels of haploids, diploids, triploids, tetraploids and pentaploids in various groups. Each value represents the mean ± SD (n = 3). Values in a column with different superscript letters are significantly different (P < 0.05).

Groups	Haploid (%)	Diploid (%)	Triploid (%)	Tetraploid (%)	Pentaploid (%)
Control	0.0 ± 0.0^{a}	100.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
Haploid	95.5 ± 4.7^{b}	4.5 ± 4.9^{b}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
G4N-30	42.0 ± 19.4^{c}	35.2 ± 17.2^{c}	3.2 ± 2.3^{b}	19.6 ± 3.3^{b}	0.0 ± 0.0^{a}
G4N-35	27.5 ± 9.8^{c}	14.8 ± 2.3^{c}	3.8 ± 3.4^{b}	51.4 ± 9.2^{c}	2.5 ± 1.5^{b}
G4N-40	34.8 ± 9.7^{c}	19.6 ± 4.1^{c}	6.0 ± 3.1^{b}	37.1 ± 10.6^{c}	2.6 ± 2.4^{b}

CB-treated G4N groups, we counted chromosome numbers. Mitotic metaphase plates from the larval cells are shown in Figure 1. Figure 2 presents the frequency distribution of chromosome numbers in larval cells from the control, haploid, and CB-treated G4N groups. The modal numbers of the control and haploid groups were 20 and 10 (Fig. 1A, B), respectively, the diploid and haploid numbers for *C. gigas* (Ahmed and Sparks, 1967). Despite numbers of cells showing the aneuploid number, the mode at $N = 40$ was verified in the G4N groups in which the CB treatment was performed for 30, 35, and 40 min, respectively (Fig. 1C). Cells showing a diploid number were also observed in the three G4N groups. Compared with those in the G4N-30 and G4N-40 groups, the mode in the G4N-35 group was more apparent.

Since many aneuploids were observed in the various groups, we classified the cell ploidies as: haploid, 5–14 chromosome; diploid, 15–24 chromosomes; triploid, 25–34 chromosomes; tetraploid, 35–44; and pentaploid, 45–54 chromosomes. Table 2 shows the percentages of ploidy levels in the larval cells from various experimental groups. In the control and haploid groups, 100% and 95.5% of the trochophore larvae contained the expected ploidy levels. CB treatment was effective for tetraploidization of the chromosome set, and resulted in 19.6%, 51.4%, and 37.1% tetraploids in the G4N-30, G4N-35, and G4N-40 groups, respectively.

Discussion

In this study, fertilization was reduced in the haploid and G4N groups compared with the control group, suggesting that UV irradiation affected the mobility of sperm and decreased their ability to activate eggs. In fact, in a previous study we observed the clear destruction of the sperm acrosome and flagellum in UV-irradiated oyster sperm (Li et al., 2000d). For the haploid group, though aneuploid cells were also observed, the mode at $N = 10$ was verified,

indicating that haploid gynogenesis of the Pacific oyster was successfully achieved by the dose of UV exposure used here. In the Pacific oyster, Guo et al. (1993) reported that the threshold dosage for genetic inactivation of sperm was 5-6 min at a UV intensity of 108 erg/mm^2/s. The UV intensity and exposure time were obviously higher than those of the present study, suggesting that the optimum dose of UV irradiation depends on the density and volume of sperm suspension and UV intensity.

Inactivation of sperm chromosomes by UV irradiation is suggested to be a continuous process (Guo et al, 1993). Aneuploids shown here have also been reported in the previous gynogenetic induction of mollusks (Fujino et al., 1990 ; Fairbrother, 1994 ; Scarpa et al., 1994 ; Pan et al., 2004). In this study, 4.5% of cells which showed the chromosome counts of 15-17 and were classified as "diploids" were seen in the haploid groups. As the development of eggs in the haploid groups terminated before reaching the D-shaped stage, the cells were considered to be aneuploids but not normal diploids.

The results obtained in this study demonstrated that CB treatment is highly effective in inhibiting the extrusion of both polar bodies, and successfully produced 19.6-51.4% gynogenetic tetraploids in the various G4N groups. Moreover, CB treatment for 35 and 40 min has been found to induce more expected ploids ($P<0.05$). In mollusks, the production of gynogenetic tetraploids from inhibition of both polar bodies is possible. Mature eggs of marine mollusks are arrested at either prophase I or metaphase I. Before fertilization, eggs of the oysters are actually tetraploids and, theoretically, tetraploids should be produced after fertilization with genetically inactivated sperm and successful inhibition of PBI and PBII. The induction of gynogenetic tetraploids by blocking both polar bodies has been reported in the Pacific abalone *H. discus hannai* (Okumura et al., 1996).

In addition to the gynogenetic tetraploids, many diploids and a small number of triploids were also seen in the G4N groups. Since the early development of Pacific oyster eggs shows asynchronicity (Li et al., 2000c), the diploids were considered as resulting from zygotes in which either PBI or PBII was suppressed, while the occurrence of triploids might result from failure of genetic inactivation of sperm or chromosome loss of tetraploids during the development of embryos (Peruzzi and Guo, 2002). D-shaped larvae did not appear in the haploid group but were observed in the CB-treated G4N groups, and were suggested to be gynogenetic tetraploids or diploids.

In this study, the development rate of gynogenetic tetraploid D-larvae was considerably low compared with that of normal diploids. This result is consistent with the previous studies on gynogenetic diploid production in many species of marine mollusks (Fujino et al., 1990 ; Fairbrother, 1994 ; Scarpa et al., 1994 ; Pan et al., 2004), and has been reported also in gynogenetic tetraploid induction of

Pacific abalone (Okumura et al., 1996). The plausible causes for the low viability of gynogenetic tetraploids are considered to be related to the expression of lethal recessive genes, developmental dysfunction caused by an incorrect nucleus to cytoplasm ratio, or genetic damage resulting from UV irradiation of sperm (Scarpa et al., 1993; Guo et al., 1993). The probability that the CB treatment itself was responsible for the inviability is very small since triploidy is easily induced in mollusks with similar treatments (Scarpa et al., 1994; Barber et al., 1992; Beaumont and Kelly, 1989).

Although the gynogenetic tetraploid larvae yielded in this study were not further cultured after D-shaped larvae stage, it is not known whether they could survive beyond metamorphosis and to adulthood, the results given here indicate the possibility of producing tetraploids by using UV-irradiated sperm and the inhibition of both polar bodies in the Pacific oyster.

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