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Induction of gynogenetic diploids and cytological studies in the zhikong scallop, *Chlamys farreri*

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Abstract – The induction of gynogenetic diploids during meiosis II in the scallop *Chlamys farreri* was attempted using the treatments of cytochalasin B (0.5 μg ml^{-1}; CB) and 6-dimethylaminopurine (60 μg ml^{-1}; 6-DMAP). The effects of CB and 6-DMAP treatments on meiosis and fertilization of eggs were also examined. Haploid gynogenesis was induced by sperm which were ultraviolet-irradiated for 30 s at an intensity of 2561 μW cm^{-2} s^{-1}. CB and 6-DMAP treatments were highly effective in suppressing meiosis II, yielding 19% and 28% gynogenetic diploids, respectively. Compared with the CB treatment, the 6-DMAP treatment produced more expected D-shaped larvae. Cytological observations revealed that 6-DMAP inhibited pronuclear movements and the segregation of chromosomes, resulting in the formation of one big diploid female pronucleus with strong fluorescence, while CB inhibited cytokinesis, resulting in two female pronuclei formations. The UV-irradiated sperm nucleus developed into a male pronucleus, but did not participate in karyokinesis at mitotic anaphase, suggesting that gynogenetic diploids were induced in the scallop.

Key words: Gynogenetic / Cytology / Bivalve / *Chlamys farreri*

Résumé – Induction de diploïdes gynogénétiques et études cytologiques du pétoncle *Chlamys farreri*. L'induction de diploïdes gynogénétiques a été effectuée durant la seconde division méiotique (méiose II), chez le pétoncle *Chlamys farreri,* par traitement à la cytochalasine B (0,5 μg ml^{-1} ; CB) et à la 6-diméthylaminopurine (60 μg ml^{-1} ; 6-DMAP). Les effets des traitements à la CB et à la 6-DMAP ont aussi été examinés sur la méiose et la fertilisation des œufs. La gynogenèse haploïde est induite par du sperme irradié aux ultra-violets pendant 30 s, à une intensité de 2561 μW cm^{-2} s^{-1}. Les traitements à la CB et 6-DMAP ont été très efficaces en supprimant la méiose II, rapportant 19 % et 28 % de diploïdes gynogénétiques, respectivement. Le traitement à la 6-DMAP, comparé à celui de CB, produit davantage de larves-D. Des observations cytologiques révèlent que la 6-DMAP inhibe les mouvements nucléaires et la ségrégation des chromosomes, ayant pour résultat la formation d'un seul pronucléus femelle diploïde avec une forte fluorescence, tandis que la CB inhibe la cytocinétique, ayant pour résultat la formation de 2 pronucléus femelles. Le noyau des spermatozoïdes irradiés aux UV se développe dans un pronucléus mâle, mais n'a pas participé à l'anaphase mitotique de la caryogenèse, ce qui laisse penser que des diploïdes gynogénétiques sont induits chez ces pétoncles.

1 Introduction

Chromosome engineering has been considered one of the genetic tools that could improve mollusk production. The last decade has seen considerable progress in the development of chromosome set manipulation techniques in mollusks which includes both the production of polyploidy and of gynogenesis (Beaumont and Fairbrother 1991). The induction of gynogens represents a valuable addition to classical genetic methods. The production of gynogenetic diploids offers the potential applications of the techniques in genetic research as well as a part of breeding technologies for genetic improvement including gene mapping and study of genetic sex-determination (Avtalion and Don 1990; Guo and Allen 1994) and gene-centromere recombination (Arai et al. 1991). Moreover, gynogenesis may be critical to the rapid production of inbred lines, mono-sexual broods, or clones (Naruse et al. 1985; Tabata and Gorie 1988; Taniguchi et al. 1988; Kobayashi et al. 1994; Li et al. 2000a). Viable gynogenetic diploids have been successfully produced in many species of fish and amphibians (Fujino 1989). Induced gynogenesis requires the fertilization of eggs activated with genetically inactivated spermatozoa and subsequent diploidization of the maternal chromosome set, through inhibiting either the polar body extrusion or the first cleavage. Chemical, thermal, and hydrostatic pressure treatments have all been shown to

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be effective to some degree in preventing the polar body extrusion and the first mitotic division without interfering with DNA replication (Fujino 1989; Guo et al. 1993; Li et al. 1999).

Although among marine bivalves many species are known to be economically important for aquaculture, the induction of gynogenetic diploids has been reported only for *Haliotis discus hannai* (Fujino et al. 1990; Li et al. 1999), *Crassostrea gigas* (Guo et al. 1993; Li et al. 2000b), *Mytilus edulis* (Fairbrother 1994), *Mytilus galloprovincialis* (Scarpa et al. 1994), and *Mulinia lateralis* (Guo and Allen 1994). However, efficient procedures for gynogenetic diploid induction have not still been established for bivalves as they have for fishes, and few efforts have been made on production of adult gynogenetic mollusks (Fujino et al. 1990; Guo et al. 1993).

The zhikong scallop, *Chlamys farreri* (Jones and Preston), is one of the most commercially important bivalves in northern China, with an annually production of over 500 000 tons in recent years. Numerous studies have been performed on propagation and cross breeding of the scallop, and artificial polyploid has been successfully produced (Lu and Wang 1992; Yang et al. 1999; Yang et al. 1997, 2000a, 2000b). However, gynogenetic diploids of the scallop have not been induced. In a previous study, we have already demonstrated that ultraviolet (UV) irradiation of sperm for 30 s at a UV intensity of 2561 $\mu W\,cm^{-2}\,s^{-1}$ was the optimum dose to achieve haploid gynogenesis in the scallop (Pan unpublished data). In this study, we attempted to produce gynogenetic diploids by blocking the release of the second polar body (PB II) with treatments of cytochalasin B and 6-dimethylaminopurine in *C. farreri*, and also observed the nuclear behaviors of CB-treated and 6-DMAP-treated eggs that had been inseminated with UV-irradiated sperms under a fluorescence microscope.

2 Materials and methods

2.1 Gamete collection

Mature cultured scallops, *C. farreri* (shell height, 6.8 ± 0.5 cm; shell length, 6.3 ± 0.5 cm), were collected locally in late March and early May from the coast of Weihai, Shandong, China. They were separated into males and females and kept in running seawater tanks until used. Eggs and sperms were obtained by artificially inducing spawning with the stimulations of dryness and raising water temperature from 16 to 20 °C. Discharged eggs were collected by suction and rinsed in filtered seawater several times. Suspensions of sperm and egg were prepared at concentrations of 1.0×10^7 sperm ml^{-1} and 2.0×10^4 egg ml^{-1} by dilution with filtered seawater and held at room temperature before use. All seawater was 1.2 μm filtered and maintained at room temperature (20 °C) for culture.

2.2 Ultraviolet irradiation of sperm

Two milliliters of sperm suspension was spread on a 9.0 cm diameter plastic Petri dish (Nunclon dish; Nalge Nunc Co, Denmark). The dish was placed on a recipro shaker (Guohua Inc., Changzhou, China) 15 cm below a 15W UV germicidal light (Toshiba GL15, 254 nm of UV wave length; Toshiba Inc. Tokyo, Japan) in which provided a UV intensity of 2561 $\mu W\,cm^{-2}\,s^{-1}$ as measured by a digital radiometer (Model 97503-00; Cole-Parmer Inc., France). The sperm suspension in Petri dish was exposed to the UV light for 30 s. During the UV irradiation, the sperm suspension was shaken at 50 cycles min^{-1}.

2.3 Induction of gynogenetic diploids by inhibiting meiosis II

Six milliliters of UV-irradiated sperm suspension were mixed with 30 ml of egg suspension, and the mixture was then divided into three groups. One group was the untreated gynogenetic haploid, while the other two groups were used for inducing gynogenetic diploids by inhibiting meiosis II (Meiotic-G2N) with treatments of 6-dimethylaminopurine (6-DMAP) or cytochalasin B (CB). For the 6-DMAP treatments, the zygotes were placed into 50 ml of seawater and exposed to 6-DMAP (60 $\mu g\,ml^{-1}$) at 30 min postinsemination for 15 min. After treatment, the zygotes were filtered through a 20 μm mesh, rinsed with seawater and transferred into normal seawater, and then were allowed to develop at 20 °C. For CB treatments, the zygotes were placed into 50 ml of seawater and exposed to CB (0.5 $\mu g\,ml^{-1}$ seawater containing 0.1% dimethylsulfoxide, DMSO) at 25 min postinsemination for 20 min. At the end of the 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 normal seawater for culture. During insemination and treatment, the water temperature was maintained at 20 °C. The procedures following the UV-irradiation of the sperm were performed in the absence of visible light to prevent the possible photoreactivation of the sperm's DNA (Ijiri and Egami 1980). The starting time of the 6-DMAP and CB treatments was based on cytological observation (Pan et al. 2004). CB and 6-DMAP concentrations were based on previous papers (Lu and Wang 1992; Yang et al. 1997; Yang et al. 1999).

A fourth group, normal diploid control, was also produced by fertilizing eggs with untreated sperm, and giving no treatment. The fertilization rate, abnormality rate of embryo and the development rate of D-shaped larvae were calculated at 2 h, 12 h and 32 h postinsemination, respectively. The experiment was repeated three times separately using gametes obtained from different individuals.

2.4 Ploidy determination

The ploidy of embryos was determined with chromosome preparations at 22 h postinsemination, following the procedures mentioned by Li et al. (2000c). 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 stored

in Carnoy's solution at −20 °C until used. The samples were dropped on warmed glass slides (50 °C), air-dried and stained for 30 min with 10% Giemsa solution diluted with phosphate buffer solution (pH 6.8). Chromosome numbers were counted from well-spread metaphase figures.

2.5 Cytological observation of nuclear behavior

For cytological examination, eggs were collected from each group at 5 min intervals up to 2 h after the addition of sperm, and fixed in 2% formalin in seawater at 4 °C. After rinsing three times with 0.1 M phosphate buffer containing 8% sucrose, the samples were stained with DAPI (4′, 6-diamidino-2-phenylindole dihydrochloride) following Li et al. (2000d). Cytological events in more than 100 DAPI-stained eggs per sample were observed under an Olympus fluorescence microscope BH-2.

3 Results

3.1 Induction of gynogenetic diploids

The effects of CB and 6-DMAP treatments on the rate of fertilization, abnormality rate of embryo and developmental rate of D-shaped larvae in various experimental groups are shown in Figure 1. The fertilization rate for the control group was 78%, but fell gradually to 62%, 57% and 44% in the haploid, CB-treated and 6-DMAP-treated G2N groups, respectively. Abnormality rate of embryo for the haploid group was 2%, but rose obviously to 20% and 8% in the CB-treated and 6-DMAP-treated G2N groups, respectively. On the other hand, the developmental rate of D-shaped larvae was 98% in the control group, but no survival to D-stage in haploid group. Viable D-shaped larvae occurred in both the CB-treated and 6-DMAP-treated G2N groups, and reached a developmental rate of 28% in the 6-DMAP-treated G2N group, which was considerably higher than that observed in the CB-treated G2N group (11%).

To ascertain whether or not the chromosomes were doubled in the larvae from the CB-treated and 6-DMAP-treated G2N groups, we counted chromosome numbers. Mitotic metaphase plates from larval cells are shown in Figure 2. Figure 3 presents the frequency distribution of chromosome numbers in larval cells from the control, haploid, CB-treated and 6-DMAP-treated G2N groups in the three experiments. The modal numbers of the control and haploid groups were 38 and 19 (Figs. 2a,b), respectively, which coincided with the expected numbers of the diploid and haploid chromosomes for *C. farreri* (Wang et al. 1990). Despite many cells showing the aneuploid number, the mode at $N = 38$ was verified in the CB-treated and 6-DMAP-treated G2N groups (Fig. 2c).

Since aneuploid cells were observed in all groups (Fig. 3), the ploidy was classified in haploids with a modal chromosome number of 19 and diploids with a modal chromosome number of 38. Table 1 shows the levels of haploids and diploids in the larval cells of various experimental groups. In the control and haploid groups, 51% and 54% of the trochophore larvae contained the expected ploidy levels. CB and 6-DMAP treatments

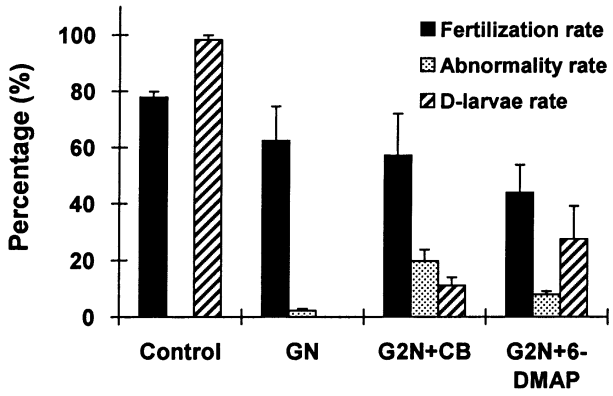

Fig. 1. The rate of fertilization, abnormality rate of embryo and developmental rate of D-shaped larvae in various experimental groups. CB, cytochalasin B; 6-DMAP, 6-dimethylaminopurine.

effectively suppressed PB II extrusion, resulting in 19% and 28% diploids in the G2N groups. Compared with CB treatments, 6-DMAP treatment was more effective for diploidization of the chromosome set and resulted in higher diploid rate.

3.2 Cytological observation

In the CB-treated G2N group, maternal and paternal chromosomes of eggs formed two female pronuclei and one male pronucleus resulting from retention of PB II at 60 min postinsemination (Fig. 4(1)). At metaphase of the first mitosis, the chromatin of the male pronucleus condensed, but did not transform into chromosomes (Fig. 4(2)). Concomitant with the movement of the maternally derived chromosomes towards two spindle poles at mitotic anaphase, the sperm nucleus was left between the two groups of chromosomes (Fig. 4(3)). At the completion of cytokinesis, the sperm nucleus was seen on the first cleavage furrow as two partitioned parts (Fig. 4(4)).

In the 6-DMAP-treated G2N group, most zygotes had a female pronucleus with greater fluorescence compared to the male pronucleus at 50 min postinsemination (Fig. 5(1)). At metaphase and anaphase of the first mitosis and the 2-cell stage, sperm nuclei in the zygotes of the 6-DMAP-treated G2N group behaved in the same manner as those in the CB-treated G2N group (Figs. 5(2–4)).

4 Discussion

The results obtained in this study demonstrated that although CB and 6-DMAP treatments reduced the development rate of D-larvae, they were highly effective in inhibiting PB II extrusion, and successfully produced gynogenetic diploids. Moreover, 6-DMAP was more efficient than CB. D-shaped larvae, which did not occur in the haploid group but were observed in the CB-treated and 6-DMAP-treated G2N group, were suggested to be gynogenetic diploids. Viable gynogenetic diploid has been induced in the other mollusks including *C. gigas*, *M. edulis*, *M. galloprovincialis* and *H. discus hannai* by inhibiting meiosis II with CB or thermal shock treatment (Guo et al. 1993; Fairbrother 1994; Scarpa et al. 1994;

Table 1. Levels of haploids and diploids of larval cells in various groups.

Groups	Haploid (%)	Diploid (%)
1. Eggs + sperm (control)	0 ± 0*	51 ± 0
2. Eggs + UVsperm (haploid)	54 ± 8	3 ± 6
3. Eggs + UVsperm + CB (G2N)	21 ± 5	19 ± 7
4. Eggs + UVsperm + 6-DMAP (G2N)	29 ± 4	28 ± 4

* Each value represents the mean ± SD (n = 3).

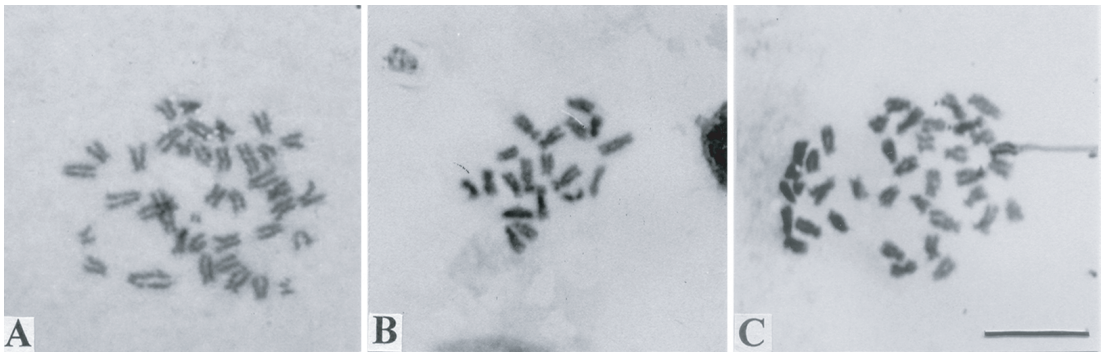

Fig. 2. Mitotic metaphase plates from the larval cells of *Chlamys farreri*. (A) A diploid cell with 38 chromosomes from the control group. (B) A haploid cell with 19 chromosomes from the haploid group. (C) A diploid cell showing 38 chromosomes from the gynogenetic diploid group treated with CB. Scale bar = 10 μm.

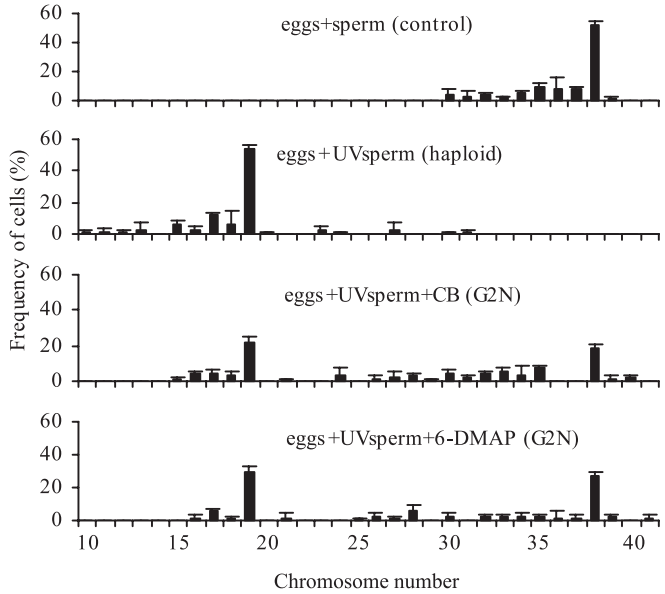

Fig. 3. Frequency distribution of chromosome numbers in larval cells of *C. farreri* from the control (eggs + sperm), haploid (eggs + UV sperm), CB-treated and 6-DMAP-treated G2N groups. Each value represents the mean ± SD (n = 3).

Fujino et al. 1990). 6-DMAP was first used in the induction of molluscan gynogenetic diploid, although it has been extensively used in molluscan polyploid production (Gérard et al. 1994; Cai and Beaumont 1996; Zhang et al. 1998; Tian et al. 1999). Compared with CB, 6-DMAP is considered safer and less harmful to embryos (Desrosiers et al. 1993). This might explain the fact that the efficiency of gynogenetic induction was higher for 6-DMAP-treated group than for CB-treated group. The optimum condition for PB II inhibition is CB treatment at 20 °C for 20 min starting at 25 min postinsemination, and 6-DMAP treatment at 20 °C for 15 min starting at 30 min postinsemination, respectively. The difference of the treatment condition between CB and 6-DMAP may be due to the different mechanisms of CB and 6-DMAP for inhibiting meiosis II.

CB, an inhibitor of microfilament assembly, is found to be especially effective in preventing polar body formation (Longo 1972; Stanley et al. 1981). In this study, CB-treated zygotes formed two female pronuclei and a male pronucleus at anaphase of meiosis II; this process was also observed in other bivalves (Longo 1972; Komaru et al. 1990; Komaru and Wada 1991; Li et al. 2000b), which indicates that CB is able to suppress cytokinesis but not the segregation of chromosomes. In contrast, 6-DMAP-treated zygotes formed a more fluorescent female pronucleus than the male pronucleus; these findings were basically identical to the observations of Yang et al. (1999) for triploid induction. Since the segregation of chromosomes was not observed in the zygotes at anaphase of meiosis II, the female pronucleus seems to possess one chromosome set which should be extruded from the zygotes as PB II, and become diploid. It is suggested that 6-DMAP inhibits chromosome segregation and pronuclear movements (Dufresne et al. 1991; Rime et al. 1989; Szollosi et al. 1991). Although the process of female pronuclear formation is different in CB-treated and 6-DMAP-treated zygotes, chromosome doubling induced by the suppression of PB II formation was satisfactorily achieved with CB and 6-DMAP in the scallop.

On the other hand, after its incorporation into the egg cytoplasm, the UV-irradiated sperm nucleus successively developed into a male pronucleus, showing the behavior of a normal sperm nucleus. However, at mitotic prophase, the male pronucleus formed no chromosomes, unlike the female pronucleus. Instead, the male pronucleus became a dense chromatin body

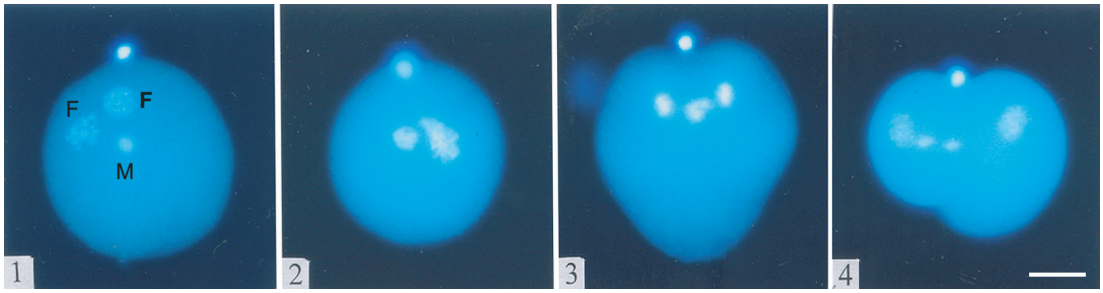

Fig. 4. Nuclear behavior of *C. farreri* eggs in the CB-treated G2N group. (1) Two female pronuclei (F) and one male pronucleus (M) at 60 min postinsemination (pi). (2) Metaphase of the first cleavage (80 min pi). (3) Anaphase of the first cleavage (95 min pi). (4) 2-cell (105 min pi). Scale bar = 20 μm.

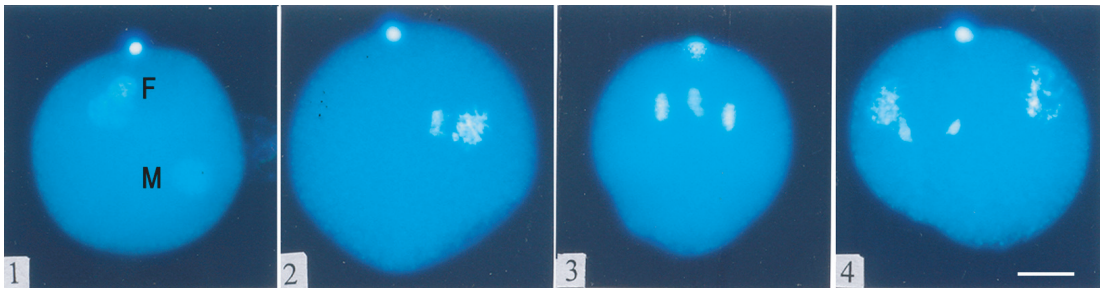

Fig. 5. Nuclear behavior of *C. farreri* eggs in the 6-DMAP-treated G2N group. (1) One large female pronuclei (F) and one male pronucleus (M) (50 min pi). (2) Metaphase of the first cleavage (80 min pi). (3) Anaphase of the first cleavage (100 min pi). (4) 2-cell (120 min pi). Scale bar = 20 μm.

which did not participate in karyokinesis. These results are fundamentally identical to cytological observations of gynogenetic haploid scallop zygotes (Pan et al. 2004), indicating that CB and 6-DMAP treatments did not affect the behavior of the UV-irradiated sperm nucleus during meiosis and cleavage.

In this study, the development rate of gynogenetic diploid D-larvae was obviously low compared with that of normal diploids (Fig. 1). This result is similar to what has been reported for *C. gigas* (Guo et al. 1993; Li et al. 2000b), *M. edulis* (Fairbrother 1994), *M. galloprovincialis* (Scarpa et al. 1994) and *H. discus hannai* (Fujino et al. 1990; Li et al. 1999). The plausible causes for the low viability of gynogenetic diploids are considered to be the homozygosity of recessive lethal genes resulting from the inhibition of meiosis II, genetic damage resulting from UV irradiation of sperm, and/or side effects of chemical treatments.

The cytological results shown here indicate that the UV-irradiated sperm nucleus, which did not participate in the first karyokinesis, was still recognized in the cytoplasm at the 2-cell stage. It remains unknown whether or not UV-irradiated sperm nuclei affect the development of zygotes after the 2-cell stage. Further studies on the fate of paternal nuclei derived from UV-irradiated sperm are needed to elucidate the mechanisms of gynogenetic diploid induction, and to improve the yield of gynogenetic individuals.

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