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Research article

Induction of Gynogenetic Diploids the Tropical Oyster, *Crassostrea belcheri* 1873 (Ostreids: Ostreoidea) from Southern Thailand.

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Abstract: The induction of diploid gynogenesis in the Tropical oyster, *Crassostrea belcheri* 1873, was carried out through two main experiments: 1) the destruction of spermatozoa DNA using ultraviolet (UV) irradiation, and 2) the induction of gynogenetic diploids. UV light source was placed 30 cm over the sperm for various durations, including 0 (control), 30, 60, 90, and 120 seconds. The study revealed that the highest survival rate was observed in trochophore at 90 minutes (42.5 ± 2.50%) compared to the normal control *C. belcheri* ($p>0.05$). Based on cytogenetic study, haploid (n=10), diploid (2n=20), and aneuploid were observed in all trials, while only diploid (2n=20) could be seen in the control group. Diploid gynogenesis was induced using 100 μmol 6-DMAP for 10 minutes. The study found that the survival rate and developmental stages in both trochophore and D-shape were gradually decreasing as the duration of UV rays increased. The highest survival rate of trochophore in the control group was 75.20 ± 4.84%, while survival rates in other experimental groups ranged from 52.22 ± 4.82% to 59.33 ± 13.57%. However, regarding the survival rate of D-shape larvae, their survival rates were relatively low across all trials. They displayed abnormal embryo development, distorted shape, unnatural swimming, and were smaller than normal (40-50 μm). Moreover, haploid (n=10), diploid (2n=20), triploid (3n=30), tetraploid (4n=40), and aneuploid were observed in all trials, while only diploid (2n=20) could be discovered in the control group. However, gynogenesis in this tropical oyster is still limited at this time. Therefore, suitable conditions for inducing gynogenesis in oysters should be further developed.

Keywords: Gynogenesis; *Crassostrea belcheri*; UV-irradiated sperm; 6-DMAP; UV rays

1. Introduction

Diploid gynogenesis is a biotechnological method of manipulating chromosome sets in marine animals. This production strategy has an advantage over conventional methods as it only produces genetically identical females by destroying DNA in the spermatozoa using the UV irradiation

method [1, 2]. Research on artificial gynogenesis with ultraviolet light-irradiated sperm in the Pacific oyster, *Crassostrea gigas*, has been conducted to study its induction and survival rates [2]. It was found that some aquatic organisms such as *Haliotis discus* hannai, *C. gigas*, *Mulinia lateralis*, *Mytilus edulis*, and *M. galloprovincialis* have higher growth and survival rates in females, which is attributed to their high market demand compared to males [3]. The sexual transformation from male to female can be observed in bivalves and gastropods such as *H. discus* hannai, *C.gigas*, *M. lateralis*, *M. edulis*, *M. galloprovincialis*, and *Chlamys farreri* [4]. It is noteworthy that male *C. belcheri* can become female after its first breeding, and this sex change can occur once or multiple times during a breeding season depending on environmental factors such as temperature and food availability [5].

Some research demonstrated that mature females weigh more than males, indicating a faster growth rate in females [6]. Males also have a higher mortality rate than females, which could be related to changes in the sea [7]. It has been reported that diploid gynogenesis can be achieved in mollusk species such as *Haliotis diversicolor*, *H. discus* hannai, *C. farreri*, *M. edulis*, *M. galloprovincialis*, and *M. lateralis* through cold shock treatment, cytochalasin B, and 6-dimethylaminopurine [8-13]. However, the application of this technique in the economically important *C. belcheri* species in Thailand have not yet been reported. Therefore, this study aimed to apply diploid gynogenesis to this oyster species to promote its production and the aquaculture industry in the country. The successful implementation of this technique will contribute to the production of genetically identical female shellfish.

2. Materials and Methods

In this study, Four females and one male of the mature *C. belcheri* (shell width 12.5 ± 0.2 cm; shell length 11.8 ± 0.3 cm.) were purchased from a farmer farm in Trang Province and were thoroughly washed to remove all dirt from the shell. The oysters were then transferred to a semi-closed recirculation system for cultivation. The oysters were fed with single-cell algae such as *Isochrysis galbana*, *Chaetoceros calcitrans*, and *Tetraselmis suecica* at a rate of 3% calculated by dry meat weight, as described by [14]. The maturity of the oysters was monitored routinely for up to 4 weeks. To examine the maturity of the shellfish, their gametes were collected and analyzed under a light microscope (Motic, Spain) following sampling and dissection, as described by [15].

2.1 Egg preparation

After inducing mature *C. belcheri* broodstock with cold shock at 19 °C to release their gametes, the suspension of eggs at a concentration of $1x10^5$ eggs/ml was filtered through a 70-90 μm pore size filter and transferred to a clean container with 25 psu seawater.

2.2 Seminal fluid preparation

The seminal fluid generated in seawater was filtered through a 20-30 μm pore size to eliminate grease and dirt before being stored in a clean, dry container. Next, the fluid was diluted with 0.85% NaCl at a ratio of 1:9 (fluid:0.85% NaCl). To prepare a sample for UV radiation, 1 mL of the diluted fluid was transferred to 15 petri dishes with a diameter of 8 cm each.

2.3 Exposure of the seminal fluid to UV light and gamete fusion (fertilization)

The petri dishes containing the diluted fluid were subsequently exposed to a 30-watt UV lamp at a distance of 30 cm for 0 (control), 30, 60, 90, and 120 seconds. After exposure, the diluted fluid from each petri dish was combined with eggs prepared on filtered cloth about 50-60 μm pore size for 15 to 30 minutes before diploid induction.

2.4 Induction of gynogenetic diploids

1. Diploid gynogenesis induction

At the designated time, 100 μmol of 6-DMAP was added to the sample on each plate and left to incubate for 10 minutes and 30 minutes post-insemination. This concentration and timing of 6-DMAP for treatment was based on that described in the previous research in this oyster [16].

2. Hatchery and nursery

To prevent the larvae from falling to the bottom of the container, they were nurtured in aerated seawater (30 psu) for 24 hours (D-shape stage) at an ambient temperature of about 27 °C following the diploid induction.

3. Data collection

One mL of rearing water was collected at 6-8 hours post-fertilization (trochophore larvae stage) to determine egg hatching and the percentage of haploid larvae. The survival rate and percentage of induced diploid gynogenesis were further examined by metaphase chromosome counting, as described by [17]. The experimental design used in this study was a completely randomized design (CRD) with three replicates.

2.5 Chromosome identification

The oyster larvae, including the trochophore and D-shape stages, were immersed in 0.05% colchicine for 90 minutes. After washing with seawater to remove the chemical, they were treated with 0.075 M KCl for 40 minutes, followed by a fixative for 5 minutes. Next, 15-20 drops of 50% acetic acid were added to 0.2 mL of the sample on a petri dish. After incubation for 20 minutes, the sample was placed on a slide, dried by heating, and stained with 10% Giemsa solution for 40 minutes. Finally, the sample was rinsed with water and examined under a light microscope to determine the metaphase chromosome count.

2.6 Data Analysis

The statistical analysis of all values was performed using a one-way analysis of variance (ANOVA) followed by the New Duncan Multiple Range Test. A p-value of less than 0.05 was considered statistically significant.

3. Results and Discussion

Gynogenesis induction in the tropical oyster *Crassostrea belcheri* (1873) involves two main steps: 1) UV irradiation to inactivate the sperm and 2) gynogenesis induction during the larval stage.

1) Inactivation of sperm chromosomes using UV-irradiated sperm

This experiment was conducted using a UV bulb at a distance of 30 cm, with irradiation periods of 0 (control), 30, 60, 90, and 120 minutes. Afterward, normal eggs were mixed with irradiated sperm. The highest survival rate of trochophore in the control group was found to be 42.58 ± 12.06%. In contrast, the survival rates in the other experimental groups ranged from 32.58 ± 6.66% to 42.50 ± 2.50%, which were not significantly different ($p > 0.05$), as displayed in Table 1.

Table 1. Survival rate of Trochophore after fertilized eggs with UV irradiated semen**.**

Periods (Second)	Survival rate (%)
Control	42.58 ± 12.06^{ns}
30	32.58 ± 6.66^{ns}
60	37.17 ± 9.57^{ns}
90	42.5 ± 2.50^{ns}
120	36.75 ± 2.82^{ns}

Note: ns= not significant difference (P>0.05)

Previous reports have documented the induction of diploid gynogenesis. For example, [10] investigated gynogenesis induction in the *C. farreri* using a UV intensity of 256 μW cm^{-2} s^{-1} for 30 seconds. They found that the egg development rate in the control group was 73.3%, but this rate gradually decreased to 33.5% after 30 seconds of exposure. After 60 seconds of irradiation, no development of oocytes was

observed. On the other hand, [2] reported that sperm DNA destruction in *C. gigas* could be achieved with 5-6 minutes of exposure at a UV intensity of 108 erg mm^{-2} min^{-1}. In contrast, [16] stated that the use of UV-irradiated sperm from *C. gigas* for 60 seconds at 72 erg $mm^{-2}s^{-1}$ could accomplish sperm DNA destruction to induce gynogenesis. Our study found that embryonic development occurred with an irradiation time of about 25 seconds, but the development rate of D-shape larvae reached 0% in this case. The highest survival rate of trochophore was observed after 90 seconds of UV exposure, which is consistent with the Hertwig effect caused by UV irradiation of sperm in *C. farreri* [10].

Numerous pieces of evidence have been reported on the destruction of genetic material in sperm. It has been postulated that the development of oocytes would decline with increasing duration of irradiation, resulting in a reduction of the breeding rate, such as the case with the Pacific abalone, *H. discus* hannai [18]. Furthermore, the destruction of sperm at various durations depends on several factors, including the density and quantity of sperm, the intensity of the UV light source, and the aquatic animal species being studied.

The destruction of sperm DNA was examined using a chromosome counting technique with simple dyeing. The results showed that the number of chromosomes ranged from 10 to 20, characterized as haploids, aneuploids, and diploids. (Fig 1.)

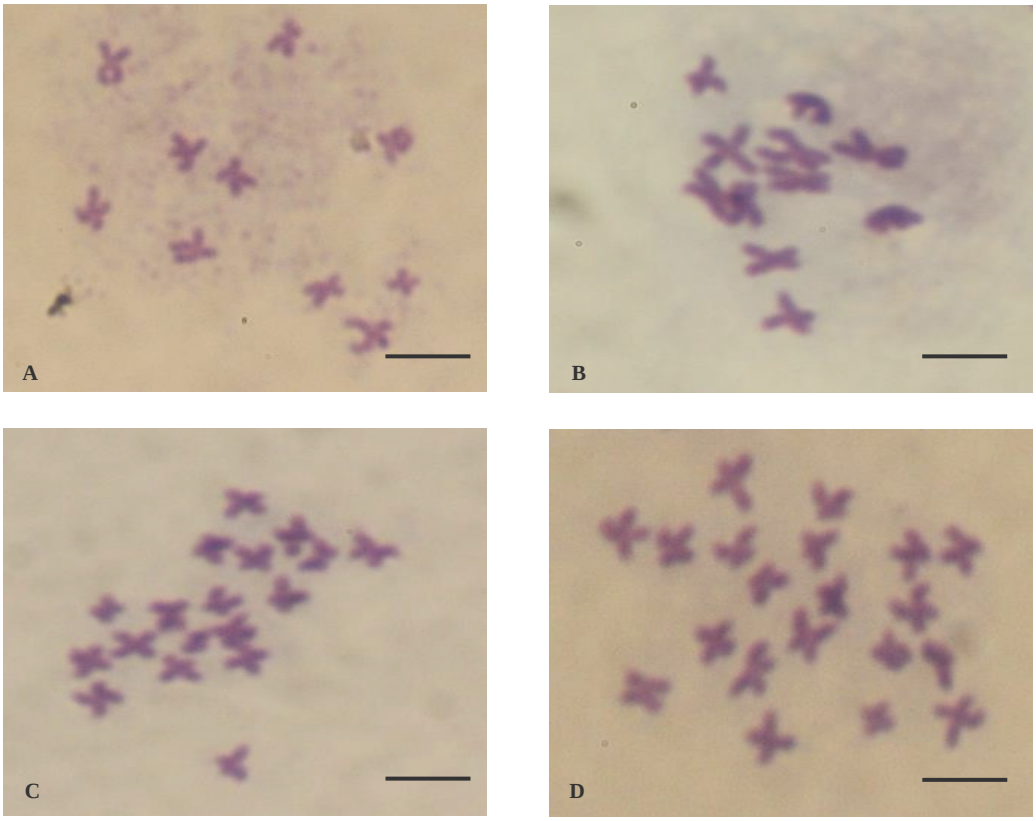

Figure 1. Metaphase chromosome plates of Trochophore after fertilized eggs with UV irradiated semem: haploid=10 (A), aneuploid 11-18 (B-C) and diploid=20 (D) (scale bars=5 μm).

2) Induction of gynogenetic diploids of larval stage

As mentioned above, the results of the first phase indicated that the genetic material of *C.belcheri* sperm was destroyed, and then it was used to fertilize a normal egg. After that, the sample was treated with 100 μM of 6-DMAP for 10 minutes, 30 minutes after fertilization, resulting in the successful production of artificial polyploid in the tropical oyster (*C. belcheri*) [16]. In this study, both trochophore and D-shape larvae were observed, and the survival rate for trochophore in the control group was 75.20 ± 4.84%, but it gradually decreased to 52.22 ± 4.82 - 59.33 ± 13.57% in the 6-DMAP-treated group (Table 2).

Table 2. Survival rate of gynogenetic diploid Trochophore and D-shape stages.

Periods (Second)	Survival rate (%)	
	Trochophore	D-shape
Control	75.20 ± 4.84^{a}	23.03 ± 6.05^{a}
30	59.33 ± 13.57^{ab}	7.87 ± 2.66^{b}
60	52.22 ± 4.82^{b}	4.67 ± 1.15^{bc}
90	52.66 ± 6.42^{b}	0^{c}
120	55.33 ± 10.47^{b}	0^{c}

Means (± SD) in the same column with different superscripts show significant differences ($p<0.05$)

This result is consistent with a previous study by Pan et al. [10], who stated that the survival rate of the trochophore stage in the control group was 56.3%, gradually falling to 31.3% with 20 seconds of UV intensity. The study also showed that the survival rate of D-shape larvae decreased continually and reached 0% with 15 seconds of UV intensity. The low survival rate could be attributed to the longer duration of UV exposure. [19] conducted a study on gynogenetic diploid induction in *C. gigas* using Cytochalasin B (CB) at a concentration of 0.5 μg/ml, which was found to effectively inhibit cell division during metaphase II when combined with caffeine at a concentration of 10 mM and an incubation temperature of 32°C. The study showed that the highest production of gynogenetic diploid larvae was achieved at 74.6% and 63.7%, with additional production of triploid larvae at 72.7% and 68.1%. However, the D-shape larval survival rate in gynogenetic diploid induction was relatively low compared to triploid and diploid larvae [19]. Previous research on gynogenetic diploid induction in other bivalves, such as *M. edulis*, demonstrated that the highest haploid results were achieved in 15 minutes [11]. *M. galloprovincialis* was operated for 2 minutes at 62 erg/mm2 per second [12], and *H. discus* hannai was also studied [9, 20]. However, these studies showed that low production of gynogenetic diploid induction might be due to polar body inhibition in the meiosis II process or damage from UV-irradiated genetic material.

This study found that the survival rate of larvae in the D-shape stage was low after 30 and 60 seconds of exposure to UV-irradiated sperm, whereas no larvae developed to the D-shape stage after 90 and 120 seconds of exposure. In addition, upon examining the overall condition of the larvae that were able to develop up to the D-shape stage, it was discovered that they exhibited a relatively impaired shape, with deformities and smaller sizes of approximately 40-50 μm in comparison to the normal D-shape stage. Some larvae were also found to be non-motile. All surviving larvae were investigated for chromosomal characteristics using conventional staining to quantify the number of chromosomes discovered during gynogenesis induction. It was discovered that haploid (1N), diploid (2N), triploid (3N), and tetraploid (4N) chromosomes could be identified (Fig 2). Additionally, chromosomal characteristics of various aneuploid types were also present. [10] conducted a study to examine the effect of destroying genetic material and inducing diploid gynogenesis on the normal developmental stage of *C. farreri*. The study found that the normal diploid number of chromosomes was 2n=38. However, after 15 seconds of sperm DNA destruction, the number of chromosomes observed was 32, with some being in the form of aneuploidy. Furthermore, 19 haploid chromosomes were discovered within 30 seconds.

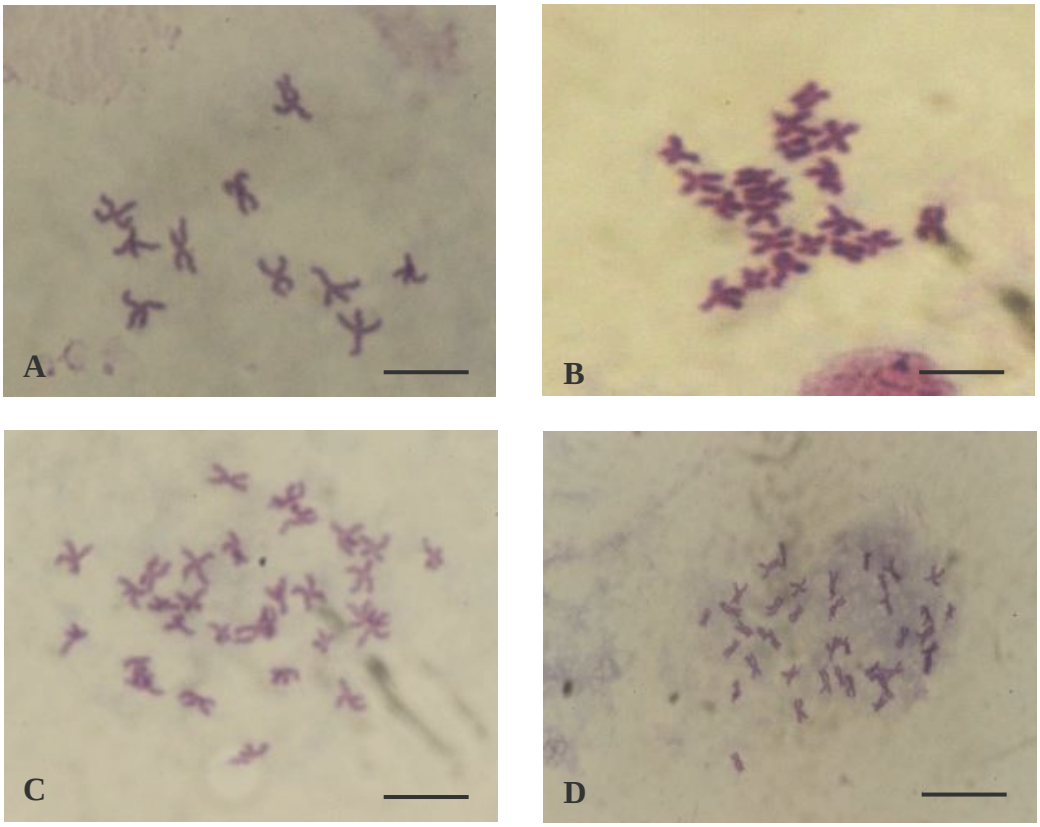

Figure 2. Metaphase chromosome of gynogenetic diploid *C.belcheri*: haploid =10 (A), diploid=20 (B), triploid =30 (C), and tetraploid=40 (D) (scale bars=5 μm).

4. Conclusions

The control group had the highest trochophore survival rate (42.58 ± 12.06%), which was not significantly different from the 90-second UV exposure group. Additionally, chromosomal characteristics were determined using the conventional staining method, and both 10 and 20 chromosomes and various aneuploid types were observed. The control group had the highest rate of trochophores, indicating diploid gynogenesis (75.20 ± 4.84%). However, it should be noted that a relatively low survival rate of the D-shaped stage was observed, which may be due to increased stress during development.

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