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Nuclei fluorescence microscopic observation on early embryonic development of mitogynogenetic diploid induced by hydrostatic pressure treatment in olive flounder (*Paralichthys olivaceus*)

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ABSTRACT

Sperm genetic material of olive flounder (*Paralichthys olivaceus*) was inactivated by ultraviolet irradiation. The nuclear phase changes during early embryonic development of diploid, haploid, and mitogynogenetic diploid induced by hydrostatic pressure treatment were observed under fluorescent microscope with 4′,6-diamidino-2-phenylindole staining. The parameters of hydrostatic pressure treatment were 600 kg/cm^2 for 6 minutes at prometaphase stage. The data showed that developmental timing sequence of diploid and haploid fertilized eggs was similar. The cell cycle was about 48 minutes, including interphase (about 21 minutes), prophase (about 3 minutes), prometaphase (about 6 minutes), metaphase (about 6 minutes), anaphase (around 9 minutes), and telophase (about 3 minutes). After entering the fertilized egg, ultraviolet-inactivated sperm formed a male pronucleus and became a dense chromatin body in the cytoplasm. Dense chromatin body did not participate in nuclear division and unchanged all the time. For hydrostatic pressure–treated embryos, the first nuclear division and cytokinesis after treatment proceeded normally after about 15 minutes recovery. During the second mitosis, having undergone interphase, prophase, and prometaphase stage, chromosomes began to slowly spread around and scattered in the cell but not entered into metaphase and anaphase. The second nuclear division and cytokinesis was inhibited. The occurrence frequency of developmentally delayed embryos also showed that the second cleavage of about 80% treated eggs was inhibited. The inhibition of the second cleavage resulted to chromosome set doubling. So chromosome set doubling for mitogynogenetic flounder diploid induced by hydrostatic pressure treatment, performed at prometaphase stage, was mainly due to inhibition of the second mitosis rather than the first one.

1. Introduction

Gynogenesis refers to the development of eggs activated by genetically inactivated sperm, whose genetic material does not contribute to the resulting embryo. Artificial induction of gynogenesis includes two types: meiogynogenesis and mitogynogenesis. In mitogynogenesis induction, chromosome set doubling was performed by blocking the first or second egg cleavage. Mitogynogens, theoretically homozygous at almost all loci, have many potential applications in genetic mapping, rapid establishment of pure lines, production of all-female stock, and accelerated

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elimination of recessive deleterious genes from aquaculture population [1]. Since the first article on mitogynogenesis in zebra fish (*Brachydanio rerio*) was published in 1981 [2], mitotic gynogenesis has been successfully applied to more than 20 freshwater fishes [3], including the zebrafish (*B rerio*) [4], medaka (*Oryzias latipes*) [5], paddlefish (*Polyodon spathula*) [6], goldfish (*Carassius auratus*) [7–9], amago salmon (*Oncorhynchus rhodurus*) [10], and rainbow trout (*Oncorhynchus mykiss*) [11]. However, mitogynogenesis techniques have been developed only in several marine species such as red sea bream (*Pagrus major*) [12], large yellow croaker (*Pseudosciaena crocea*) [13–16], half-smooth tongue sole (*Cynoglossus semilaevis*) [17,18], turbot (*Scophthalmus maximus*) [19], and European sea bass (*Dicentrarchus labrax*) [1,20].

Olive flounder (*Paralichthys olivaceus*) is one of the most economically important marine fish in China. With the improvement of farming technology, flounder has become the main aquaculture species in East Asia in recent years. Because the female flounder grows much faster and bigger than the male, artificial gynogenesis induction has great significance for improving aquaculture production and economic benefit [21]. Since the first article on mitogynogenesis in olive flounder was published in 1999 [22], some related research on mitogynogenesis induction in flounder has been reported. Zhuang et al. [23] and You et al. [24] had investigated the conditions for inducing mitogynogenesis by blocking cell division with hydrostatic pressure shock or cold shock in flounder, but the result was different. You et al. concluded that the optimal induction parameters of hydrostatic pressure treatment were 600 kg/cm^2 for 6 minutes at 85 minutes after fertilization (AF) when the developmental temperature was 15 $\pm$ 0.2 °C. But the result of Zhuang et al. showed that the optimal induction parameters of hydrostatic pressure treatment were 55 MPa for 6 minutes at 75 minutes AF when the developmental temperature was 15.5 $\pm$ 0.5 °C. The difference might result from different developmental temperature, egg, and semen quality, and so on.

Researchers had noted that spindles were disorganized by physical shocks and concluded that such treatments suppressed the cell division in the cell cycle concerned because of the inactivated or disorganized spindle, and resulted in chromosome set doubling. It was speculated that induction of mitogynogenesis inactivated or disassembled spindles, thereby blocking the anaphase movement of chromosomes and duplicating the nucleus without the first cell division. Zhang and Onozato [25] investigated the behaviors of spindles and nuclei of tetraploid induced by hydrostatic pressure or heat shock treatment at metaphase of the first cell cycle in the rainbow trout (*O mykiss*). The result showed that either heat shock or hydrostatic pressure shock did not inhibit the first cleavage because of the regeneration of the bipolar spindle during the first cell cycle, but the second cleavage was blocked because of the monopolar spindle forming during the second cell cycle. Zhu et al. [26,27] had investigated the mechanism on chromosome set doubling of mitogynogenetic diploids induction through a series of dynamic behaviors of microtubules in gynogenetically activated eggs by antitubulin immunofluorescence microscopy in olive flounder (*P olivaceus*). The result was consistent with that of Zhang and Onozato. It showed that the chromosome set doubling of mitogynogenetic diploid induced by either hydrostatic pressure or cold shock around prometaphase of the first cell cycle was due to the second cleavage inhibition by forming a monopolar spindle during the second cell cycle. But the related research on fluorescence microscopic observation of nuclear phase in early developmental embryo of mitogynogenetic diploid has not reported until now.

Our study aimed at clarifying the mechanism on chromosome set doubling in mitogynogenetic diploid induction through observation of the dynamic behaviors of chromosome so as to improve induction efficiency. We investigated in detail the behaviors of nuclei in haploid, normal diploid, and mitogynogenetic diploid fertilized eggs by a fluorescent microscopic labeling technique. Mitogynogenetic diploid was induced by hydrostatic pressure treatment around prometaphase of the first cell cycle. In the article presented here, occurrence frequency of developmentally delayed embryos was used to estimate mitogynogenetic diploidization rates.

2. Materials and methods

2.1. Eggs, milt collection, and sperm inactivation

Male and female olive flounder (aged approximately 3–4 years) was obtained from Shenghang Aquafarm, Weihai city, Shandong Province of China, for use in this study. Semen from two to three males was stripped by gently pressing the male flounders' abdomen, collected using glass Pasteur pipettes, and stored on ice until use. Ovulated eggs were stripped from three to four females, pooled in a 600-mL glass beaker, and stored in dark until use. The quality of the eggs was examined by external appearance observed under a stereoscope, and the motility of spermatozoa was checked under a light microscope. Approximately 100 mL of good-quality ovulated eggs from one female and spermatozoa from one male flounder were selected to use.

Before treatment, 2 mL of good-quality semen was diluted 50 times with Ringer's solution (0.75% NaCl, 0.04% $CaCl_2$, and 0.02% KCl in distilled water, pH 7.0) at 0 °C to 2 °C (which did not activate sperm motility) and then divided into four aliquots in precooled glass Petri dishes (diameter, 13 cm). Sperm were irradiated under ultraviolet (UV) light (wavelength, 254 nm) for 180 seconds, and the total irradiation dose was 3770 ergs/mm^2 (verified with a UV dosimeter VLX-3W sensor; Cole–Parmer Instrument Company, Chicago, IL, USA).

2.2. Fertilization and hydrostatic pressure treatment

Embryonic development often speeds up with rising temperature [28,29]. The start timing for hydrostatic pressure treatment was different with different developmental temperature. So the start timing for hydrostatic pressure treatment should be adjusted according to developmental temperature. According to our previous study [27], the result of Zhuang et al. [23] and You et al. [24], we had conducted a series of start timing experiments