JFMSE, 30(5), pp. 1519~1527, 2018.
수산해양교육연구, 제30권 제5호, 통권95호, 2018.
www.ksfme.or.kr
https://doi.org/10.13000/JFMSE.2018.10.30.5.1519

Occurance of Amitosis-like Nuclear Division in Erythrocytes of Induced Triploid Far Eastern Catfish, *Silurus asotus* and Marine Medaka, *Oryzias dancena*

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메기, *Silurus asotus*와 해산송사리, *Oryzias dancena*의 유도 3배체에서의 비정형 적혈구 출현

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Abstract

The differences of erythrocyte nucleus between diploid and induced triploid in Far Eastern catfish, *Silurus asotus* and marine medaka, *Oryzias dancena* were observed. The types of atypical cell were determined three types in induced triploid Far Eastern catfish; asymmetric division, irregular-shaped and absence of nucleus. The types of atypical cell were determined three types in induced triploid marine medaka; snippety nucleus, sickle-shaped and absence of nucleus. In Far Eastern catfish and marine medaka, the numbers of atypical cells in induced triploid were higher than those of diploid ($P < 0.05$). The absence of nucleus in diploid and induced triploid were shown in Far Eastern catfish and marine medaka, and this type in diploid samples was the lowest among the other types in both species ($P < 0.05$). Occurrence frequency of nucleus's absence and total number of atypical cells in induced triploid Far Eastern catfish was higher than that in induced triploid marine medaka.

Key words : Amitosis, Erythrocyte, Far eastern catfish, Induced triploid, Marine medaka

Ⅰ. Introduction

The aquaculture production of Far Eastern catfish, *Silurus asotus* in Korea was 4,194 tons at 2010, and has been increasing gradually since then (Lim et al., 2012b). Far eastern catfish has better taste than the channel catfish that is increasingly popluar in the spotlight to consumers (Yu et al., 2009). Marine medaka, *Oryzias dancena* is gaining attention as an experimental animal in the aquaculture. This fish is a truly euryhaline teleost, with a great capacity for hypo- and hyper-osmoregulation (Cho et al., 2010). Marine medaka's is used as an experimental model because

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※ This work was supported by a grant from the project titled 'Development and commercialization of high density low temperature plasma based seawater sterilization pulifiction system' funded by the Ministry of Oceans and Fisheries, Korea.

of its short generation cycle and large possibility of breeding in the laboratory (Kim et al., 2009; Park et al., 2011, 2012). So, Far Eastern catfish and marine medaka are optimum animals both in aquaculture and in experiment.

The triploidy induction of fishes have been achieved from a lot of studies. Among them, marine medaka was most used in the experiment to study polyploidy (Park et al., 2016a, 2016b, 2018; Lim et al., 2012b). Also, triploid induction of Far Eastern catfish served to study the freshwater fish and triplody research (Kim et al., 2001; Lim et al., 2017). The main purpose the triploid induction is that sterile energy is focus on the growth not on reproduction (Benfey, 1999; Park et al., 2006). And induced triploid can get a heavier weight, good meat quality than diploid (Swarup, 1959; Benfey, 1999; Park et al., 2006). In the haemotological analysis, induced triploid was 1.5 times larger than diploid in erythrocyte size. But giantism was not observed (Swarup, 1959; Seol et al., 2008). Also, induced triploid's erythrocytes were larger than those of diploid, whereas induced triploid's erythrocyte numbers were fewer than those of diploid (Seol et al., 2008). As mentioned by Seol et al. (2008), erythrocyte's nucleus observation is very important to distinguish diploid from induced triploid.

Atypical or divided erythrocyte nuclei are commonly shown in the induced triploid fish such as induced triploid rainbow trout, *Oncorhynchus mykiss* (Han et al., 2007). However, Dorafshan et al. (2008) suggested that atypical erythrocytes in the induced triploid Caspian salmon, *Salmo trutta* were far behind the mechanism to cope with stress in fish, and induced triploid was less tolerant of stress than diploids, as in a nuclear pattern in induced triploid brook trout, *Salvelinus fontinalis* reflected some kind of functional depression or mobilization of granulocytes (Wlasow et al., 2004). The atypical cells are regarded as a cytological marker for induced triploid (Liu et al., 2003).

So far, studies of amitotic erythrocytes have shown the problem in respiration and erythrocyte activity (Ueno, 1984). Therefore, through the observation of erythrocyte nucleus experiment in this study, the differences between diploid and induced triploid in Far Eastern catfish and marine medaka. have been defined, respectively.

Ⅱ. Materials and methods

Marine medaka, *Oryzias dancena* and Far Eastern catfish, *Silurus asotus* were reared for this experiment at the Fishery Genetics and Breeding Sciences Laboratory of the Korea Maritime and Ocean University in Busan, Korea. Experimental fish where in these places and reared in an aquarium maintained at a temperature of 23±1.5°C. Marine medaka was fed altemia twice a day, and Far Eastern catfish was fed 2% of feed of its average body weight feed during the experimental period twice a day.

The triploid induction of Far Eastern catfish was carried out according to the method of Kim et al. (2001). Mature females of Far Eastern catfish were induced to spawn using a single intraperitoneal (IP) injection of 1000 IU of human chorionic gonadotropin (hCG, Sigma, USA) per kg body weight (BW). Sperm were obtained by cutting the surgically removed testes of males that had been given an IP injection of hCG at 500 IU/kg BW. Eggs were fertilized with sperm diluted in saline solution using the wet method. Five minutes after fertilization, the eggs were rinsed rapidly to remove excess sperm and were immediately subjected to

cold-shock treatment (4°C) for 60 min to prevent the extrusion of the second polar body. Untreated fertilized eggs were used as diploid controls.

The triploid of marine medaka is induced by the cold shock treatment (4°C) of fertilized eggs 2 min after fertilization for 45 min (Ko, 2013; Park et al., 2016a, 2016b, 2018). The induced triploid genotype was induced by all the thermal shock regimes by tests. The induced triploid of each species was confirmed with chromosome analyses, erythrocyte measurements, and flowcytometric analyses by using flowcytometry (PA-II, Partec, Germany).

There are two way to take blood samples of marine medaka. First, cutting the couda part of each sample then extracting the blood. Second, inserting the heparin coated syringe into the each sample's heart then extracting the blood. Blood of Far Eastern catfish was extracted by using syringe coated heparin at cauda part of each sample. To observe the erythrocytes of triploid in marine medaka and Far Eastern catfish, whole bloods of induced triploid fish were diluted 1:10 with phosphate-buffered saline (PBS: 0.8% NaCl, 0.02% KCl, 0.02% KH_2PO_4, 0.115% Na_2HPO_4) and a drop of cell suspension was placed in the centre of a slide glass, which was then covered with a coverslip and it was observed under an optical microscope (Axiostar plus, Carl Zeiss, Germany).

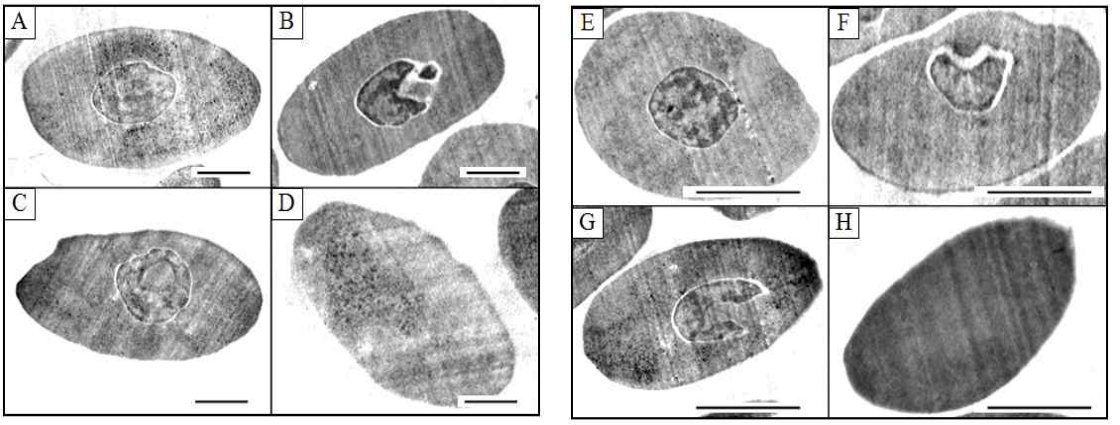
The amitotic erythrocytes were determined by transmission electron micrograph. For transmission electron microscopy, blood samples of diploid and induced triploid in experiment were pre-fixed in a cold 2.5% glutaraldehyde solution (pH 7.5) for 2 hrs. The erythrocytes of each group were centrifuged 1,000 rpm at 10 mins, and were pre-fixed for 2 hrs at 4°C in 2.5% glutaraldehyde solution buffered by 0.1 M phosphate buffer solution (PBS, pH 7.2). After washing with PBS for 10 min, the samples were post-fixed in 1% osmium tetroxide (OsO_4) for 2 hrs at 4°C. Samples were rewashed with PBS, then serially dehydrated with ethanol, and embedded in Epon 812.

Sections (0.5 μm thick) were cut using an ultramicrotome (LKB, Nova, Sweden) and then stained with toluidine blue to determine the investigation area. The sections were double-stained with uranylacetate and lead citrate solution and then examined by using a transmission electron microscope (JEM 1200 E-X II, 60-80 kv, JEOL, Tokyo, Japan). After processing, amitotic erythrocytes of diploid and induced triploid were observed and were counted with counter.

Using the SPSS statistics package (SPSS 12.0, SPSS Inc., Chicago, IL, USA), one-way analysis of variance (ANOVA) were carried out to test for statistical significance ($P < 0.05$) between diploid and induced triploid fish. Multiple comparisons were performed using Duncan's multiple range tests (Duncan, 1955).

Ⅲ. Results

Three different types of atypical cells were determined in induced triploid Far Eastern catfish, *Silurus asotus*: asymmetric division, irregular-shaped, and absence of nucleus [Fig. 1-A, B, C and D]. Asymmetric division type has a nucleus of erythrocyte divided asymmetrically [Fig. 1-B]. Irregular-shaped type has an irregularly formed nucleus, and there are various irregular-shaped nuclei [Fig. 1-C]. Absence of nucleus type means no nucleus observed in erythrocyte [Fig. 1-D]. The atypical cell samples of induced triploid Far eastern catfish are shown in <Table 1>. The number of



[Fig. 1] Transmission electron micrograph of amitotic nucleus in erythrocyte of induced triploid Far Eastern catfish, *Silurus asotus* (A, B, C and D) and marine medaka, *Oryzias dancena* (E, F, G and H). A: normal; B: asymmetric division of nucleus; C: irregular-shaped; D: absence for nucleus; E: normal; F: snippety nucleus; G: sickle-shaped; H: absence for nucleus. Scale bars are 5 μm.

<Table 1> The quantity of different types of abnormal nucleus among 100 erythrocytes of induced triploid Far Eastern catfish, *Silurus asotus**

Samples	Abnormal types of erythrocyte's nucleus						Total number of atypical cells	
	Asymmetric division		Irregular-shaped		Absence of nucleus			
	2n	3n	2n	3n	2n	3n	2n	3n
1	0	5	0	6	0	7	3	6
2	1	6	1	7	0	6	5	5
3	0	4	0	5	0	5	3	6
4	2	8	0	8	1	8	6	6
5	0	10	2	9	0	9	5	8
6	0	5	0	10	0	6	3	7
7	1	7	1	6	2	5	7	5
8	2	6	0	8	0	6	5	5
9	0	8	0	9	1	10	4	10
10	0	9	0	10	0	6	3	6
Average	0.6^{a}	6.8^{b}	0.4^{a}	7.8^{b}	0.4^{a}	6.8^{b}	1.4^{a}	21.4^{b}
Standard devivation	0.84	1.93	0.70	1.75	0.71	1.69	1.43	4.48

*According to Wang et al. (2010). Means in rows with the different superscript letter are significantly different ($P < 0.05$).

cells of asymmetric division in the induced triploid is higher than that in diploid. In addition, The number of cells of the irregular-shaped nucleus or in the absence of nucleus in induced triploid is higher than that in diploid. The first result of number of cells for asymmetric division in induced

triploid was 11.3 times higher than that in diploid, and the number of cells for absence of nucleus in induced triploid was 17.0 times higher than that in diploid ($P < 0.05$). The number of irregular-shaped cells in induced triploid was 19.5 times higher than in diploid, which was the highest among the three types ($P < 0.05$). As a result, the number of atypical cells in induced triploid Far Eastern catfish was approximately 15 fold higher compared to that of diploid ($P < 0.05$). The difference in cell number among the three types was not significant in diploid and in induced triploid, respectively ($P > 0.05$).

The following three types of atypical cells were determined in induced triploid marine medaka, *Oryzias dancena*: snippety nucleus, sickle-shaped, and absence of nucleus [Figs. 1-E, F, G and H]. Snippety nucleus type has smaller size of atypical nucleus in erythrocyte than that of normal nucleus [Fig. 1-F]. Sickle-shaped type has the shape of a nucleus that is bent as in sickle and the curvatures of nuclei are shown to be various [Fig. 1-G]. Absence of nucleus type has no nucleus found in erythrocyte [Fig. 1-H]. <Table 2> shows the comparison of atypical cell numbers between the diploid and induced triploid marine medaka.

The number of cells for snippety nucleus in induced triploid was 7.1 times higher than that in diploid ($P < 0.05$). The number of sickle-shaped cells in induced triploid was 12.3 times higher than that in diploid and the number of absence of nucleus cells in induced triploid was 12.8 times higher than in diploid, which was the highest among the three types ($P < 0.05$). As a result, the number of atypical cells in induced triploid marine medaka was 10 fold higher compared to that of

<Table 2> The quantity of different types of abnormal nucleus among 100 erythrocytes of induced triploid marine medaka, *Oryzias dancena**

Samples	Abnormal types of erythrocyte's nucleus						Total number of atypical cells	
	Snippety nucleus		Sickle-shaped		Absence of nucleus			
	2n	3n	2n	3n	2n	3n	2n	3n
1	0	5	0	5	0	5	3	15
2	0	4	0	5	1	4	4	13
3	0	6	0	5	0	6	3	17
4	2	4	0	4	0	6	5	14
5	2	4	1	4	0	6	5	14
6	0	4	1	6	1	4	5	14
7	1	5	0	5	2	5	5	15
8	1	6	0	5	0	4	4	15
9	0	7	2	4	0	5	5	16
10	1	5	0	6	0	6	4	17
Average	0.7^a	5.0^b	0.4^a	4.9^b	0.4^a	5.1^b	1.5^a	15.0^b
Standard deviation	0.82	1.05	0.70	0.74	0.71	0.88	1.08	1.33

*According to Wang et al. (2010). Means in rows with the different superscript letter are significantly different ($P < 0.05$).

diploid ($P < 0.05$). The difference in cell number among the three types was not significance in induced triploid ($P > 0.05$).

In the shape of blood of induced triploid, the types of atypical cell in Far Eastern catfish were shown with the shape of a nucleus that was abnormally transformed, and the nuclei of marine medaka had transformed shape and size. In both species, the number of atypical cells in induced triploid was higher than that of diploid. The absence of nucleus in diploid and induced triploid is shown in Far Eastern catfish and marine medaka, and this type in diploid samples was lowest among the three types in both species ($P < 0.05$). The frequency of nucleus absence occurring in induced triploid Far Eastern catfish was higher than that in induced triploid marine medaka. However, the total number of atypical cells in induced triploid marine medaka was lower than that in the induced triploid Far Eastern catfish.

Ⅳ. Discussion

In this experiment, occurrence of atypical cells by induction of triploid was observed in Far Eastern catfish, *Silurus asotu*, and marine medaka, *Oryzias dancena*. It is generally believed that erythrocytes in fish are terminally differentiated and can no longer undergo any further division. The peripheral blood of fish, however, possesses the erythroblasts which have the ability to undergo mitosis (Wang et al., 2010). As the cells with multiple nuclei are in the process of mitosis, chromatin condensation should take place in cells and the electron density of the nuclei should increase.

The other events in the process of mitosis would also occur such as pairing of homologous chromosomes, which could be observed histologically. In animals, amitosis can occur in the epithelium, connective tissue, muscle and liver (Shi et al., 2000). But in amphibians, amitosis occurs throughout the erythrocytes (Hu et al., 2005; Wang et al., 2010).

The type of irregular-shaped nucleus in induced triploid Far Eastern catfish was the highest in all groups of this experiment. This type of nuclear division was also found in other polyploid fish species (Zhou et al., 2002). Although some researchers have reported that multiple atypical cells seldom appeared in diploids and tetraploids (Gao et al., 2007), others showed that the percentage of atypical erythrocytes increased with the rise of ploidy level (Han et al., 2007).

Some cell nuclei in the induced triploid fish were constricted at the middle or towards one side, which resulted in nuclear membrane invagination. A long period of evolution has allowed the tension of the membrane to adapt to the volume of the diploid nucleus, which could lead the division of polyploid nuclei to recover their inherent ploidy (Wang et al., 2010). For example, the hybrid of the pentaploid crucian carp, *Carassius carassius* and blunt snout bream, *Megalobrama amblycephala* showed triple nucleated erythrocytes (Liu et al., 2007).

Structural changes in induced triploid erythrocytes can disrupt some of their functions such as the ability of gas transportation (Johari et al., 2008).

Because of the entirely the same nutritional and environmental conditions in Johari et al. (2008), it was hypothesized that the increase of abnormalities in induced triploid erythrocytes could not be due to these conditions. These changes have also been reported in induced triploid brook trout, *Salvelinus*

fontinalis taken by hydrostatic pressure shock, but the shock technique cannot make these abnormalities (Wlasow et al., 2004). This phenomenon should have direct relationship to the increased ploidy level.

On the other hand, red blood cells with divided nuclei have been observed in coho salmon, *Oncorhynchus kisutch*, due to the lack of folic acid in its diet (Smith, 1968). The presence of folic acid is essential for the normal formation of red blood cells. The lack of this vitamin in fish can result in the decrease of total number of erythrocytes. Therefore, the occurrence of these abnormalities in induced triploid Far Eastern catfish and marine medaka could be due to disruption in the function of gene replicates related to folic acid synthesis or metabolism in DNA molecule (Smith, 1968; Johari et al., 2008). Folic acid content in the used feed was not investigated in this experiment. Consequently, future investigationare are sure to examine the relationship of these abnormalities related to folic acid content in the used feed for triploid induction.

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• Received : 19 February, 2018
• Revised : 28 March, 2018
• Accepted : 17 July, 2018