

Different Heat Shock Application Effect on Gynogenetic Production of Zebrafish (*Danio rerio*)

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Received date: January 24, 2017; Accepted date: April 19, 2017; Published date: April 25, 2017

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Abstract

The present study aimed to obtain gynogenetic zebrafish. For this purpose, zebrafish spermatozoa exposed to UV irradiation to make it haploid gynogenetic fish and heat shock was applied to haploid zygote in order to obtain gynogenetic diploid fish.

Temperature, which is an important factor of the production, is taken into consideration in this study. In this respect, this study compared the results of 41.4°C and 41°C heat-shock applications and found that 12nd-24th-48th-72nd hour survival rate was maximum at 41.4°C ($P < 0.05$). When considered from hatching rate (72th-78th hour) view at 41.4°C was $17.3 \pm 3\%$ in gynogenetic diploid group and heat-shock application at 41°C survival rate was $14 \pm 2\%$ in gynogenetic diploid group and there is no survivor in haploid group, was observed ($P < 0.05$).

The result of the karyotype analysis in haploid gynogenetic embryos, ruptured chromosome fragments was identified. Also in karyotype analysis of diploid gynogenetic embryos, $2n=50$ chromosomes was identified. On 3rd day after fertilization, total body length of the haploid gynogenetic fish was 39.6% shorter and body thickness was 33% thicker than the diploid gynogenetic group. Our gynogenetic fish producing method and the different heat-shock applications improved survival rate of gynogenetic fish.

Keywords: Zebrafish; *Danio rerio*; Gynogenesis; Heat-shock

Introduction

The spermatozoa, of which genetic material has been destroyed by using Gamma (γ), X or Ultraviolet (UV) rays in order to eliminate the male genetic material, is used in fertilization of the egg cell during the gynogenesis; one of the chromosome manipulation techniques [1-5]. Various shock applications including heat / cold shock, hydrostatic pressure and chemical agents (Colchicine, Cytochalasin B and N_2O , etc.) have been used in the study in order to convert these haploid embryos, fertilized with spermatozoa and untreated, to diploid embryos [4,6-10].

The first gynogenesis study was conducted on trout (*Onchorhynchus mykiss*) by Opperman [11]. After this study, gynogenesis studies were conducted on weatherfish (*Misgurnus fossilis* L.) by Neifach [12], on *Gadus morhua* by Van Eenennaam et al. [13], on goldfish (*Carasius auratus*) by Paschos et al. [14] and on *Acipenser transmontanus* by Ottera et al. [15], respectively with various shock applications.

The zebrafish (*Danio rerio*) mentioned in this study is used as a live model in aquaculture and human diseases due to the facts that its embryo has the characteristics of in vitro development, its eggs have the transparent quality, its mutants can be examined morphologically and also it has high fecundity (100-300 eggs) and it can be manipulated easily [16-23]. Its embryonic development is highly rapid

at 26.5°C-28°C and the hatching of the eggs will begin 48-72 hours after the fertilization [24].

The method of gynogenesis is used for production of the generations with female genetic materials, easily conduction of genetic analysis of characters in living creatures and information gathering relevant to the issues such as nutrition and disease resistance when accurate clones are produced [25-29]. This study was conducted for biotechnology and molecular genetic studies. The problems encountered in here have been created in order to attain more comfortable solutions as mentioned above.

Materials and Methods

Care & feeding of fish

A male (7-8 months old) and a female (15-16 months old) zebrafish (*Danio rerio*), supplied from an importing company and brought to biotechnology laboratory of Istanbul University Faculty of Fisheries Department of Aquaculture, have been used in the study. The fish have been fed with *Artemia sp.*, frozen tubifex, dried *Daphnia sp* and fishmeal powder and the photoperiod has been applied to them (14-10 h, light-dark).

Mature fish selection and anesthesia treatment

A slim line, male zebrafish with yellowish colour and also a female fish with bloated appearance and silver colour on its abdomen have

been used as the mature fish. Than an anesthesia procedure has been applied by taking the mature zebrafish in a solution (Tricaine 0.2 mg/ml) for sucking eggs and sperm.

Gamete collection

The testicular tissues (two-piece) of male zebrafish obtained by dissection method have been transferred to 50 µl Hank's solution (+4°C) and the testicles have been smashed with the help of forceps [30]. They have been protected at +4°C in this solution in order to immobilize the spermatozoa without losing viability till the phase of in vitro fertilization [31,32]. The sperm motility has been identified by light microscopy and then the sperm has been diluted with an activation solution (9% NaCl) in 1:10 ratio at 22°C-23°C. In the gynogenesis study, motility of spermatozoa over 90% has been used in fertilization.

The eggs have been collected from female zebrafish by stripping to a dried Petri dish by means of massage method. During the fertilization period, at least 100 eggs have been used in each of haploid gynogen, diploid gynogen and control groups and the sperm/egg ratio to be performed has been 0.5 ml/number. The care and incubation of the fertilized eggs and larvae of the zebrafish in the oven have been performed as reported by Westerfield et al. [24,33] in order to prevent the risk of contamination.

Gynogenesis application

The sperm taken from 4 male zebrafish (sperm+Hank's solution) has been taken on ice by performing pooling procedure and transferred to a watch glass for the gynogenesis application. The watch glass with ice has been placed in UV cross-linker device (UVP TL-2000) so as to be 28 cms away from the UV lamp and (254 nm) UV has been applied for 2 minutes. Thirteen minutes later from using the UV-treated sperm in the fertilization process, the heat shock treatment (41°C or 41.4°C) has been conducted to the fertilized egg for two minutes.

Morphological analysis of gynogenetic zebrafish

The morphological developments of control, haploid and diploid gynogen groups from fertilization day to fifth day have been examined by means of shooting their photos with a stereo (Olympus SZ-PT) and invert microscope (Olympus CK40-F200) that has a camera attachment. Shock results at 41.4°C have been used in monitoring the embryo development of each group.

Karyotype analysis

Dechorionate: The chorion has been removed by the help of a fine-tipped forceps in 24 hours after the fertilization and the embryo of zebrafish has been transferred to Petri box by using a sterile Pasteur pipette.

Karyotype analysis: The embryo of zebrafish has been transferred into (4 mg/ml) freshly made colchicines solution after the dechoriation process and incubated at 28.5°C for 90 minutes in the dark. After that, the embryos has been rinsed at room temperature (~21°C) and transferred into 1.1% Sodium citrate (C₆H₅Na₃O₇). The yolk of the embryo has been punctured and a timer has started. After the dissection of the whole yolk, the Petri dish has been taken on the ice and hold there for 8 minutes. At the end of this time, the embryos have been transferred in 3:1 mixture of methanol/ acetic acid and hold

there for 20 minutes then, after this process methanol/acetic acid fixative has been added to the same degree in the solution and the embryos have been stored in a freezer overnight [24].

Spreading of chromosome: The embryos have been picked up one by one with forceps and blotted until dried. The dried embryos have been transferred on the watch glass and 2-3 drops of 50% acetic acid have been added on it and the pressing process with forceps has lasted for one minute. The suspended cells have been liquidized for two times in a 50 µl wiretrol micro capillary and droplets of the cell suspension have been dropped on a slide, pre warmed to 50°C, and the liquid has been pulled back to the wiretrol quickly. ~6 droplets have been dropped per slide. The slides have been left at 50°C for about 10 minutes to completely dry, then stained in Giemsa for 20 minutes and after that rinsed twice with H₂O. The slides have been left at room temperature to dry and the samples covered with entellan have been observed by light microscopy [24].

Osmotic pressure measurement

20 µl samples taken from Hank's solution are used in this study and measured in a micro osmometer with F FISKE (Fiske-210 brand name). 20 µl supernatant has been taken by centrifuging the sperm conserved in Hank's solution for 5 minutes at 12,000 rpm and its osmotic pressure has been measured in micro osmometer device.

Results

Results of osmotic pressure measurement

The osmotic pressures of Hank's solution and sperm cells measured in Fiske brand micro osmometer device are mOsm/kg and 306 mOsm/kg, respectively.

Results of embryo survival rates

Time dependent death rates of haploid gynogen, diploid gynogen and control group embryos are shown at Table 1. According to these data, the death rates in diploid gynogen group were observed as 80 ± 3% at 41.4°C and 85 ± 2% at 41°C (P<0.05).

Shock treatment temperature (°C)	Control		Diploid Gynogen		Haploid Gynogen	
	Survive	Death	Survive	Death	Survive	Death
41.4°C	75 ± 5	20 ± 5	17.3 ± 3	80 ± 3	0.5 ± 0.5	99 ± 1
41.0°C	80 ± 6	15 ± 5	14 ± 2	85 ± 2	0	100

Table 1: Survival and death ratio (%) of shock treatment applied embryo.

Time dependent death rates in heat shock implementation at 41.4°C are shown at Table 2. Accordingly, it is observed that the death rates of diploid gynogen group are higher than those of control group at time between 12th-24th hours and 24th-48th hours after the fertilization. In haploid gynogen group, the deaths occurred at the rate of 60.32% ± 13.46% and 53.03% ± 15.8% at time between 12th-24th hours and, 24th-48th hours, respectively. Moreover, no survival is observed in haploid group at 75th hour.

Experimet group	Control	Diploid gynogen	Haploid gynogen
1. hour	5.1 ± 6.8	18.89 ± 14.44	36.24 ± 21.02
Between 1-12 hours	7.3 ± 5.95	27.54 ± 8.05	39.16 ± 12.76
Between12-24 hours	8.7 ± 6.35	41.28 ± 5.12	60.32 ± 13.46
Between 24-48 hours	8.75 ± 7.03	48.89 ± 21.22	53.03 ± 15.80
Between 48-72 hours	0 ± 0	28,35 ± 20.83	90 ± 8.6

Table 2: Percentage of deaths result of shock treatment at 41.4°C for each experimental groups (%).

Time dependent death rates in heat shock implementation at 41°C are shown at Table 3. Accordingly, it is observed that the death rates of haploid gynogen are much more than those of diploid gynogen of which death rates are much more than those of control group. And furthermore, no survival is observed in haploid group.

Experimet group	Control	Diploid gynogen	Haploid gynogen
1. hour	4.9 ± 4.6	20.49 ± 16.24	31.84 ± 22.72
Between 1-12 hours	6.2 ± 4.75	27.54 ± 10.25	43.46 ± 14.36
Between12-24 hours	8.1 ± 4.15	43.78 ± 5.82	71.52 ± 15.16
Between 24-48 hours	8.25 ± 7.03	54.89 ± 21.72	73.03 ± 15.80
Between 48-72 hours	0 ± 0	30.35 ± 27.83	100

Table 3: Percentage of deaths result of shock treatment at 41°C for each experimental groups (%).

Experiment Groups	Morphological Development Stages			
	12nd. hour	24th hour	48th hour	72nd hour
Control	Bud stage	Prim-6	Long tail structure	Mouth development
Haploid Gynogen	%75 epiboli	18 somit	Prim-22	Pectoral fin stage
Diploid Gynogen	Bud stage	Prim-6	Beginning of Pectoral fin stage	Mouth development

Table 4: Comparison of embryonic development of control and gynogen groups.

Morphology screening of embryo development

At 12th hour after the fertilization; normal embryo development was observed in diploid gynogen (41.4°C shock applied) and control group (bud-stage), however, haploid embryos showed slower development than them, that is; in 8-hour stage (75% epiboly). At 24th hour after the fertilization; haploit gynogen embryo was observed at 18th hour stage (18-somit), on the other hand, diploid gynogen and control group was observed at 24th hour stage (prim-6). At 48th hour after the fertilization; haploit gynogen embryo was observed at 36th hour stage (prim-22), diploid gynogen embryo was observed at 42nd hour stage (beginning of pectoral fin) and control group was observed at 48th hour stage (long tail stage). At 72nd hour after the fertilization; haploit gynogen embryo was observed at 60th hour stage (pectoral fin), and diploid gynogen and control group were observed at 72nd hour stage (mouth development) (Table 4). On the basis of these data, the morphological development of haploid embryos is the latest and the morphological developments of diploid gynogen group and control group are found as similar. It is observed that, embryogenesis in haploid gynogen group comes up several phases from diploid gynogen group (Table 4). When embryo development of haploid gynogen group is compared with the other groups (control and diploid gynogen), it is seen that the phases of embryo development do not have normal development stage and the development stages differ from each other (Table 4). Furthermore, it is determined that the Haploid gynogen fry has body disorder which means it has shorter body length and also curvature of spine while the development of diploid gynogen fry has normal shape (Figure 1).

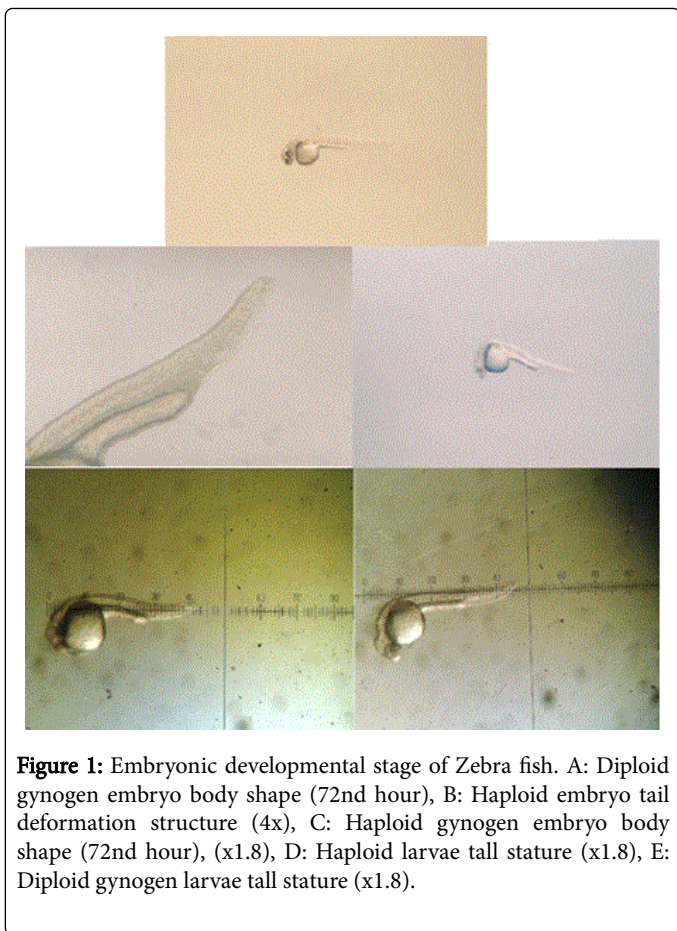


Figure 1: Embryonic developmental stage of Zebra fish. A: Diploid gynogen embryo body shape (72nd hour), B: Haploid embryo tail deformation structure (4x), C: Haploid gynogen embryo body shape (72nd hour), (x1.8), D: Haploid larvae tall stature (x1.8), E: Diploid gynogen larvae tall stature (x1.8).

Data of karyotype analysis

The results of the karyotype analysis of embryos of the experimental groups (control, haploid and diploid gynogen) at 24th hour after the fertilization, is shown at Figure 2. In haploid gynogenetic embryos, the ruptured chromosome fragments were identified at Figure 2C. Moreover, the number of the chromosomes was determined as $2n=50$ in the karyotype analysis of diploid gynogenetic and control group embryos (Figure 2A and 2B).

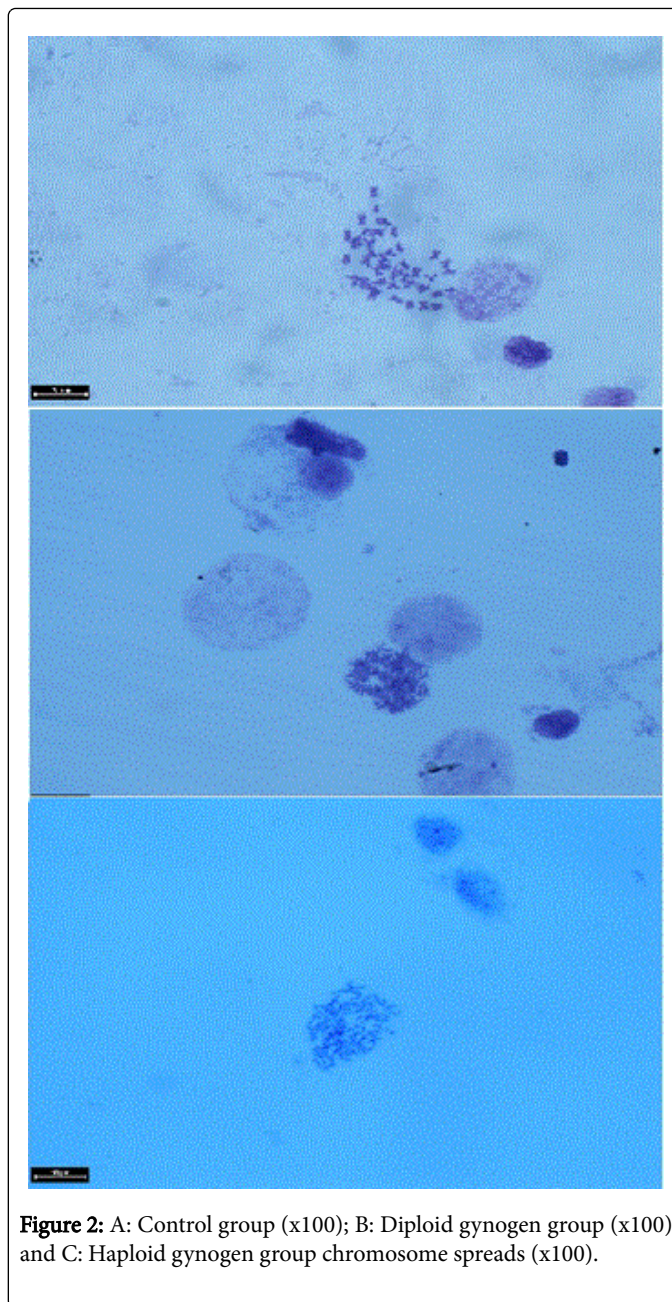


Figure 2: A: Control group (x100); B: Diploid gynogen group (x100) and C: Haploid gynogen group chromosome spreads (x100).

Discussion

The results of the heat shock implementation at 41.4°C used in this study show similarities with those of concerning the zebrafish [34,35]. It is determined that the survival rates of diploid gynogen embryos are higher at 41.4°C is used in this study- than at 41°C (Table 3).

The shocks implemented to get the diploid gynogen embryos have been done 13 minutes after the fertilization for 2 minutes, and this is the same implementation with that conducted by Walker and Streisinger. So; the results were similar.

10% haploid larvae were obtained as a result of the gynogenesis implementation on *Carasius auratus*, however all larvae were dead after this stage. The heat-shock implementation at 39.5°C for 2.5

minutes was conducted at 15, 20, 30, 35 and 45 minutes later than the fertilization in order to get diploid gynogen. It is determined that mitogynogenetic and mayo-gynogenetic larvae can be obtained with the hot shock implementations conducted at different intervals in the study [14]. The shock implementation was conducted 13 minutes after the fertilization and mayo-gynogenetic larvae were obtained in the study.

254 nm UV implementation from 4 cms away was used for 3-4 minutes to corrode spermatozoa genome in a study on *Cyprinus carpio* [36]. The spermatozoa of *Oreochromis niloticus*, of which genetic material was neutralized with an exposure to radiation, was used for the fertilization in a study carried out on *Betta splendens* and after the fertilization, gynogen fish was obtained by implementing pressure shock.

It is informed that 50% heterozygote gynogenetics fish can be obtained when the pressure shock is implemented 2.5 minutes after the fertilization for 6 minutes. However, the rate of success achieved has been 21% when the pressure shock is implemented 34 minutes after the fertilization for 5 minutes. According to the results; under these conditions, the leave time of 2nd polar body of *Betta splendens* eggs actualizes 2.5-8.5 minutes after the fertilization [26].

In a study of Schwark [35] performed on *Danio rerio*, the sperm was exposed to UV implementation from 27.5 cm distance for 2 minutes. All haploid larvae exposed to heat shock implementation (41.4°C) 13 minutes after the fertilization for 2 minutes were dead 2-3 days after running out of vitellus and their caudal length is 1/3 shorter when compared to those of diploid group [35].

Conclusions

The gynogenetic fish producing method and the different heat-shock applications mentioned in this study enhanced the survival rate of gynogenetic fish. Some important stages are in gynogenetic production which is UV irradiation, short-term storage of sperm, heat shock time and duration, karyotype analysis and morphological screening were applied.

In our study, time dependent death rates of diploid gynogen embryos in heat shock implementation at 41.4°C at times between 12th-24th hours and 24th-48th hours were observed as 41.28% ± 5.12% and 48.89% ± 21.22% respectively, such as the same those in the studies of Walker et al. [34,35] on *Danio rerio*. In haploid gynogen group, deaths occurred at the rate of 60.32% ± 13.46% at times between 12th-24th hours and 24th-48th hours were observed as 60.32% ± 13.46% and 53.03% ± 15%, respectively. Furthermore, survived haploid larvae were observed on the second day after the hatch of larvae from the eggs. Time dependent death rates of gynogen diploid embryos in heat shock implementation at 41°C used in our study were observed as 43.78% ± 5.82% and 54.89% ± 21.72% at times between 12th-24th hours and 24th-48th, respectively. In haploid gynogen group, the rate of death occurred was observed as 71.52% ± 15.16% and 73.03% ± 15.80% at times between 12th-24th hours and 24th-48th, respectively. Moreover, there is no larvae were survived in between 48th-72nd hours (Table 2 and 3).

In the study, the sperm was exposed to UV (254 nm) from 28 cms away for 2 minutes and the survival rate of diploid embryos was higher (17% ± 3%) in 41.4°C hot shock implementation when compared to that in 41°C (14% ± 2%).

In screening the morphological development of haploid and diploid embryos; assembly of cell cycle, axis, epiboly at 12nd hour; general body shape, eyes, notocord and death cells at time between 24th-30th hour; hearth beat, blood cells, pectoral fins and newly death cells at time between 48th-54th and swimming movement and embryo death at time between 72nd -78th hour were observed [2]. In this study; bud stages were observed in control and diploid gynogen groups at 12nd hour after the fertilization but, this stage is observed in haploid group at 12-18th hour after the fertilization. Delay in the development stages, shortness in body length and death cells were observed more in haploid embryos than gynogen embryos at time between 24th -30th hour. In haploid embryos, heart beats are slower than those of gynogen embryos (heart beats observed at 36-42nd) at 48-54th hour. In this case, deformation in the embryonic development was caused by the fact that circulatory system was not developed enough at time between 48th-54th hour as it is pointed out in study [37,38].

In the diploid embryos; the developments of eye, pigment cells, notochord and otic placod were observed as normal. Hatching from the egg, normal swimming movement and body shape with normal evolution were observed in the investigation on diploid gynogen and control group at time between 72nd-78th hours. Deformation of tail, body shape and, slow blood flow were observed in haploid gynogen. In conjunction with these deformations, they died 1 or 2 days after hatching from the eggs.

On the 3rd day after the fertilization, total body length of the haploid gynogenetic fish (1277 µm) was 39.6% shorter and its body thickness was 33% thicker than those of the diploid gynogenetic group (2553 µm).

In this study, ruptured chromosome fragments were identified as a result of the karyotype analysis in haploid gynogenetic embryos. Furthermore, the number chromosomes were detected as 2n=50 in the karyotype analysis of diploid gynogenetic embryos. All these results share similarities with those of found in study [35].

This study forms the basis of gynogenesis which will be held later and also the method of further biotechnological studies. The study also puts forward the points to be considered in the gynogenesis application. The data obtained in this study will contribute to the gynogenesis applications to be conducted on the fish with high commercial value.

Authors Contributions

AE was consulted in the study. RCO carried out the animal experiments and data analysis, and drafted the manuscript. RCO and AE designed the study and revised the manuscript together. RCO and AE participated in the animal trial. RCO made the data collection and analyses. All authors read and confirmed the final manuscript.

Acknowledgment

The research is a part of master thesis with 10850 project number which was supported by Istanbul University Scientific Research Project Agency.

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