Optimum Conditions of UV Ray Irradiation for Genetic Inactivation of Loach Eggs^{*1}

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Optimum conditions of ultraviolet (UV) ray irradiation for genetic inactivation of eggs were examined to develop simple and easy techniques to induce androgenesis in the loach, *Misgurnus anguillicaudatus*, since ionizing radiation such as gamma- or X-ray, which has been used generally for fish androgenesis, gives technical problems of safety and the need for special facilities. In the first and second experiments, unfertilized eggs were exposed to UV rays from two sources, germicidal lamps affixed to the ceiling and below, at dosages from 1875 to 15000 erg/mm². While in the third experiment, eggs being shaken in a micro mixer were irradiated with UV rays from ceiling lamps at dosages between 4200 and 21000 erg/mm². Most embryos developing after fertilization with normal spermatozoa showed abnormal morphology and a haploid number of chromosomes ($n=25$) in all the experiments, indicating successful induction of androgenesis. Occurrence of some aneuploids and diploids suggests the necessity of further improvement for homogeneity of treatment.

Androgenetic development (all paternal inheritance) in fish can be triggered by fertilizing gamma- or X-ray irradiated eggs with normal spermatozoa, but the resultant embryos show inviable abnormality due to haploidy.¹⁻⁵⁾ Viable diploid androgenomes were produced in salmonids by doubling the paternal chromosome set through suppression of the first cleavage using hydrostatic pressure treatment⁶⁻⁸⁾ or by inseminating genetically inactivated eggs with diploid spermatozoa from induced tetraploid males.⁹⁾ The applicable potential of androgenesis involves the rapid establishment of an inbred line, sex control using anticipated super male (YY) in the male heterogametic species, production of a nucleo-cytoplasmic hybrid between different species, and the recovery of valuable strains or species from cryopreserved spermatozoa. However, much less work has been done to develop techniques for inducing androgenetic fish, because the inactivation of egg nucleus in fish with ionizing radiation such as gamma- or X-rays gives technical difficulty including problems of safety as well as facilities for managing the radioactive source. On the contrary, ultraviolet (UV) rays are used routinely and widely to inactivate spermatozoa for easy induction of gynogenesis in various species of fish,¹⁰⁻¹²⁾ after the successful development of efficient

methods for irradiation to overcome the weak penetrating power of UV rays.^{13,14)} A few attempts at UV inactivation of eggs have been reported in fancy carp *Cyprinus carpio*^{15,16)} but the optimum conditions of UV irradiation were inconclusive because of the low and unstable occurrence of androgenomes. In the present study, we examine the optimum conditions of UV ray irradiation of eggs in order to develop a simple and safe method for inducing androgenetic development in the loach *Misgurnus anguillicaudatus*.

Materials and Methods

Loach Specimens

Specimens were obtained from the Misakagawa River, Hiroshima Prefecture, and from a dealer in Mie Prefecture. Ploidy of these specimens was determined as diploidy, on the basis of erythrocyte measurements.¹⁷⁾ Ripe eggs were hand-stripped 10-15 h after injection with human chorionic gonadotropin (HCG). Spermatozoa were diluted to a concentration of 10^{-2} with M/7.5 Ringer's solution adjusted to pH 7.8. Each androgenetic experiment was made using eggs from one female and mixed spermatozoa from four males.

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UV Irradiation of Eggs

UV ray irradiation was performed in a box (approximately 700 mm high, 480 mm wide, and 350 mm deep) using germicidal lamps GL-15W (Toshiba Co. Ltd), commercially available, affixed to both the ceiling and the bottom of the box. In the first and second experiment, eggs were placed on a plastic dish (50 mm diameter and 10 mm deep) with a sheet of almost transparent cellophane film at the bottom. The dish containing about 200 to 460 egg specimens suspended in about 1.5 ml of Ringer's solution was covered with the cellophane film and then was set up on the stand, installed exactly between the ceiling-lamps and the bottom-lamps (350 mm distant from each UV source). The lamp was warmed up for stabilization before starting irradiation. Each dish was exposed to UV rays from both sides (ceiling and bottom) for varying durations of irradiation at 125 erg/mm²/s from 15 to 120 s. The final UV dosages against egg specimens were 1875, 3750, 7500, 11250, and 15000 erg/mm² for the first experiment and 3750, 7500, and 11250 erg/mm² for the second experiment.

In the third experiment, a different method of UV irradiation was used. A petri dish (90 mm diameter and 10 mm deep) containing egg specimens and 10 ml of Ringer's solution was placed on a micro mixer (Taiyo Co. Ltd.) which made

the treatment homogeneous by shaking. The distance between the ceiling-lamps and the specimens was 420 mm. Each dish was exposed to UV rays from the two lamps affixed to the ceiling of the box for varying durations of irradiation at 70 erg/mm²/s from 60 to 300 s, giving final UV dosages of 4200, 8400, 12600, 16800 and 21000 erg/mm².

Fertilization and Rearing of Embryos

After UV irradiation, eggs were mixed with normal spermatozoa and then immersed in freshwater. Fertilized eggs of each batch were kept and reared in freshwater in a container (270 mm long, 200 mm wide and 40 mm deep). The percentage of eggs which hatched and that of deformed fry were recorded. Typical fry from the control and the irradiated batch were photographed.

Chromosome Preparation

About 18 h after fertilization, randomly selected developing eggs were dechorionized by treating with 2% trypsin dissolved in Ringer's solution (pH 9.0) for 10–15 min, and then treated with 0.5% colchicine for 2.5 h. They were treated with 0.075 mol KCl for 25 min and fixed with 3 ethanol:1 acetic acid mixture. Chromosome slides were prepared according to the procedure proposed by Yamazaki *et al.*¹⁸⁾

Table 1. Rates of hatched and deformed fry and ploidy status of embryos developing after UV irradiation of eggs

Exp. no.	UV dosage (erg/mm ²)	No. of eggs used	Hatched fry (%)	Deformed fry (%)	Ploidy			
					Total* ²	2n* ³	Aneuploid* ⁴	n* ⁵
1	0* ¹	282	36.9	1.0	—	—	—	—
	1875	203	44.8	84.6	4	0	0	4
	3750	226	35.0	94.9	11	0	2	9
	7500	278	39.6	90.9	7	0	3	4
	11250	270	23.7	98.3	8	0	2	6
	15000	225	7.1	94.3	9	0	1	8
2	0	183	66.7	0	5	5	0	0
	3750	221	47.1	94.3	12	0	1	11
	7500	265	23.0	96.3	11	0	0	11
	11250	464	16.6	100	6	0	1	5
3	0	141	31.2	0	4	4	0	0
	4200	301	24.3	93.0	6	0	1	5
	8400	187	33.7	92.0	7	0	0	7
	12600	386	17.9	97.2	10	2	1	7
	16800	159	3.8	100	3	0	1	2
	21000	263	22.1	100	7	2	0	5

*¹, non-irradiated control; *², total number of embryos examined; *³, diploid (2n=50); *⁴, near haploid with maternally derived chromosome(s) and/or chromosome fragment(s); *⁵, haploid (n=25).

Results

The hatching rates of the irradiated batches tended to decrease, depending on the increase of UV dosage on specimens in all the experiments, and were markedly lower than that of the control in the batches irradiated at above 11250 erg/mm² in the first and second experiment and at above 12600 erg/mm² in the third experiment (Table 1). More than 90% of hatched fry showed deformity in all batches even with 3750 erg/mm² irradiation (Table 1). The appearance of the deformed fry (Fig. 1) was similar to that of haploid embryos described by Suzuki *et al.*¹⁰⁾

Results of chromosome counts showed that most embryos from UV irradiated eggs were haploidy with 25 chromosomes, while all control embryos examined had diploidy with 50 chromosomes (Table 1, Fig. 2a, b). However, aneuploidy with supernumerary and/or fragmented chromosome (s), which are probably residues of

the maternal nucleus, were observed in some specimens from a low dose to a high dose of UV irradiation (Fig. 2c). No diploid embryos were detected in the first or second experiments but some in the third, in which the irradiating procedure was different from the former two experiments.

Fig. 1. External appearance of normal fry (a) and deformed fry (b), developing from the control and the UV irradiated batch, respectively.

Fig. 2. Metaphase spreads of normal diploid with 50 chromosomes (a), androgenetic haploid with 25 chromosomes (b), and aneuploid with supernumerary chromosome fragments of possibly maternal origin (c). Arrow indicates chromosome fragment.

Discussion

Abnormal embryos developing from UV irradiated eggs are proved to be primarily haploidy, on the basis of chromosome observations. The results demonstrate that the two simple procedures of UV irradiation used in the present study effectively inactivate genetic materials of the egg nucleus in order to induce androgenetic development. However, we did not succeed in eliminating the occurrence of aneuploidy, including possible residues of maternal chromosome. Occasional diploids, appearing exclusively in the third experiment, may be escapers developing from eggs which were shaded completely or partly by other eggs in the course of UV exposure. Although the possibility of spontaneous cleavage suppression and dispermy cannot be ruled out, further improvement of homogeneity of treatment should be needed.

The weak penetrating power of UV rays is the major reason why UV irradiation has not been used in inactivating the eggs of fish. One exception is preliminary work done by J. Idomoto and K. Ueno,* who reported UV-induced haploid androgenesis in the bitterling *Rhodeus ocellatus* in which species the orientation of the animal pole is quite suitable for irradiation because of the pear-like shape of the eggs. Although their technique for UV-based androgenesis is specific to the bitterling species, our methods using UV irradiation from both sides, or egg-shaking during exposure, can be useful in most fish species which have spherical eggs. In fancy carp *Cyprinus carpio* it was reported that androgenetic progeny of a yellowish color were obtained when UV irradiated eggs of wild type carp were fertilized with normal spermatozoa of the fancy carp and then heat shocked (40°C, 2 min duration, 50–60 min after fertilization).^{15,16)} However, these works seemed to be inconclusive regarding UV irradiating conditions for genetic inactivation, because the hatching rates were extremely low and a large number of normally fertilized carp (wild type) appeared together with a small number of haploid and diploid progeny of a yellowish color. The occurrence of escapers from UV irradiation was also noticed in our experiments, but we succeeded in producing more than 90% of androgenetic haploidy even after 3750 erg/mm² irradiation.

Diploidization of androgenomes is the next

step for the application of androgenesis in aquaculture. Suppression of the first cleavage for duplication of paternal chromosomes is now underway in our laboratory.

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