Optimization of UV irradiation for production of gynogenetic rainbow trout, *Oncorhynchus mykiss*: emphasising Hertwig effect and photoreactivation

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Abstract: The optimum dose of UV irradiation to produce gynogenesis rainbow trout, *Oncorhynchus mykiss* with emphasis of Hertwing effect and photoreactivation (PR) was investigated. For this purpose, the sperm of rainbow trout were irradiated with UV at 2010 ± 200 $\mu w.cm^{-2}$ intensity in different alternatives of 0, 1, 3, 5, 8, 10, 15, 20, 25, 35 and 45 minutes and allowed to fertilize normal ova; the fertilization, eyed and hatching rate were calculated to assess the performance. Using the irradiated sperm decreased the fertilization, eyed as well as hatching rates and the

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so-called "Hertwig effect" was observed, with the time-dependent decrease in hatching rate at 0^+ to 10 min of irradiation, but a better hatching rate at greater time of irradiation was observed. The highest hatching rate was observed in 20 min of UV irradiation; after that the survival rate decreased rapidly. Interestingly, irradiation even up to 45 min could not eradicate sperm fertility and a hatching rate above zero was observed at this treatment. For PR studies the semen was irradiated with UV for 5, 30 and 120 seconds and untreated semen (0s) was used as control. Irradiated semen and/or fertilized eggs by treated semen were exposed to visible light (60 W) at a distance of 30 cm for 10 min; the eyed and hatching rates were measured. The results showed that UV irradiation in as low as 5s could cause deleterious effect on semen chromation and decrease the eyed and hatching rates ($P<0.05$). Illumination of semen and\or eggs with visible light resulted in PR in rainbow trout when sperm was irradiated by UV at 120s.

We could conclude that the best UV irradiation time for production of gynogenesis rainbow trout was 20 min and UV irradiation, fertilization and egg hardening must be done far from visible light to eliminate the PR mechanism.

Keywords: Gynogenesis, Hertwig effect, *Oncorhynchus mykiss*, Photoreactivation, Rainbow trout, Ultraviolet irradiation

Introduction

Being the sole representative of cold-water fish species that is commercially cultivated in Iran, rainbow trout, *Oncorhynchus mykiss*, production has steadily increased at 38% annually from 835 MT in 1992 to 23138 MT in 2002 (FAO, 2005), and expected to reach 60 thousands MT by 2010 (www.shilat.com).

Culture of all female diploid or triploid population of rainbow trout is preferred because of some advantages (Thorgaard, 1983 ; Donaldson, 1996 ; Hulata, 2001). There are various ways to produce all female population in fish such as hormonal treatment by estrogens (Donaldson, 1996 ; Piferrer *et al.*, 2004), crossing the neomale (XX male) by normal female (Thorgaard, 1983 ; Devlin & Nagahama, 2002) and gynogenesis (Hulata, 2001; Devlin & Nagahama, 2002 ; Piferrer *et al.*, 2004).

Gynogenesis is a short cut approach to obtain all female population in fish. Gynogenesis is a chromosome set manipulation technique consisting of the generation of the progenies whose chromosome are exclusively inherited from the mother (Chourrout, 1982 ; Thorgaard, 1983).

A critical point of gynogenesis induction is the application of appropriate ray or chemical dose to achieve the complete inactivation of sperm DNA while maintaining the capacity to trigger the embryonic development (Piferrer *et al.*, 2004). When semen irradiate at different alternatives of dose or time and fertilize the normal ova, some fluctuations in fertilization, eyed and/or hatching rates are observed which is called "Hertwig effect" (Chourrout, 1982).

The Hertwig effect is a paradoxical phenomenon found in various fish and amphibian species, in which irradiated sperm with high doses of X or γ or ultraviolet rays seem to be more efficient in fertilizing normal ova than sperm irradiated with lower doses (Don & Avtalion, 1993). This phenomenon is explained to occur through the partial inactivation of sperm chromatin at the lower doses, resulting in the development of embryos in an aneuploid chromosomal condition and the complete inactivation of the sperm chromatin at higher doses with maternal haploid chromosomes fully participating in the embryo development (Valcarcel *et al.*, 1994). The highest dose of radiation destroys completely the physiological conditions and/or morphological aspects of sperm, the embryo viability drop to near zero (Piferrer *et al.*, 2004).

Various irradiation doses and times were used for different species (Table 1) and the Hertwig effect has been reported in various fish species by means of ionizing radiation (X & γ rays) in rainbow trout (Chourrout, 1980), chum salmon, *O. keta* (Onozato, 1982) and UV in rainbow trout (Goryczko *et al.*, 1991), catfish, *Rhamdia sapo* (Valcarcel *et al.*, 1994) and turbot, *Scophthalmus maximus* (Piferrer *et al.*, 2004).

Using UV as a sperm inactivator has received a great attention recently,

because of the fact that no super mandatory chromosome fragment in the embryo cells will occur if the treatment is completed (Goryczko *et al.*, 1991) and UV performance has more safety than ionizing rays. The disadvantage of this method is "photoreactivation (PR) effect" in which the inactivated chromatin of sperm may become active again when sperm and/or egg are exposed to visible light (Ijiri & Egami, 1980 ; Cleaver, 2003).

It has been established that UV produces cyclobutane type dimmers between adjacent pyrimidines on the same DNA strand. These dimmers can be split in situ by visible light. The existence of such a repair mechanism has been described extensively by Sancar (2000) and Cleaver (2003). Some studies have shown PR in Japanese medaka, *Oryzaias latipes* (Ijiri & Egami, 1980 ; Armstrong *et al.*, 2002) and African clawed frog, *Xenopus laevis* (Legerski *et al.*, 1987), although Valcarcel *et al.* (1994) failed to find this phenomenon in the catfish.

The objectives of this study were to determine the appropriate time of UV irradiation on fertilization capacity of rainbow trout sperm, the occurrence of the Hertwig effect to find out the optimum UV time for producing gynogenetic progenies and the PR mechanism in the semen as well as eggs of rainbow trout.

Materials and Methods

Specimens of rainbow trout (6 females and 6 males) were selected from broodstocks in Shahid Bahonar Salmonid Hatchery Centre, Kelardasht, Mazandaran, Iran.

The experiments were performed in three replicates by using 3 donor couples. Sperm irradiation was carried out at a distance of 5 cm, by means of two 15-watt germicidal tube (TUV1W/G 15 T8 UV lamp, Philips) located in a box. Irradiation intensity of one tube was estimated about 1005 $\mu w.cm^{-2}$ at 5 cm distance in 254 nm wave length (Iranian Atomic Energy Organization), so we expected the total energy received by sperm was 2010±200 $\mu w.cm^{-2}$. Irradiation doses were

adjusted by changing the exposure time as following: 1, 3, 5, 8, 10, 15, 20, 25, 35 and 45 minutes. To assay the time effect between the first and final insemination, there were two controls, initial (IC) and final (FC), using similar pattern of insemination without irradiation.

High quality semen (above 90% activity) with a typical concentration of 3-4×10^9 spermatozoa/ml was diluted 1:10 (v/v) in 20 mM Tris, 50 mM Glycine, 0.6% NaCl, 0.2 % KCl, pH = 9 (Chourrout, 1982). UV irradiation performed on aliquots of diluted semen (0.6 mm thickness) including 0.5 ml of milt + 4.5 ml of extender (Plati *et al.*, 1997). They were placed in a 10 cm diameter petridish on ice and were mixed by using a magnet stirrer (2c/s) during irradiation.

Oocyte was then added at a ratio of about 105 oocyte per 2.5 ml of diluted milt. Fertilization was done by adding 0.9 % NaCl, 0.01 M Tris, 0.02 M Glycine, pH = 9 as an activator media (Chourrout, 1982) and evaluation of viability rate was carried out at fertilization, complete eyed and hatching stages.

For PR studies, sperm were subjected to irradiation for 5, 30, 120 seconds, and none irradiated sperm as control. Irradiated/control milt was divided into two separate groups (2.5 ml) and put in the petridishes of 15 cm in diameter, one subjected to darkness for 10 min while the other was illuminated of a distance of 30 cm using 60 watt room lamp for 10 min.

Immediately after 10 min exposure to darkness or visible light, each group was used to inseminate 4 groups of ova at the rate of about 0.6 ml of diluted milt per 70 ova. After 10 min., eggs were washed by incubator water of 8°C, and subjected to darkness or visible light for 10 min. as above in two replicates (Table 3). Incubation period was done in California trays and routine care was subjected to all groups until hatch.

Statistical analysis

Statistical analysis of results was performed by one way ANOVA and Tukey HSD and Duncan multiple range test at minimum significance of $P<0.05$.

Results

Determination of embryo survival rate as a function of UV time examined at three developmental stages from fertilization to hatching (Fig. 1) showed that survival at hatching stage was the most sensitive marker of the effect of irradiation, though it was observed at the eyed stage as well.

The fertilization rates varied between 60.3-82.7%, for 15 min UV irradiation time and initial control (IC) treatments respectively (Fig. 1). Only the lowest fertilization rate was significantly lower than IC ($P<0.05$), and no significant differences were found between other treatments ($P>0.05$).

UV irradiation of sperm affected the survival rates at eyed and hatching stages more than fertilization rate (Fig. 1). Just 1 min UV irradiation time could decrease the eyed and hatching rates extensively to 46.38 and 28.28%, respectively. Survival to eyed stage decreased till 8 min of UV irradiation and then started to improve gradually. The hatching rate also showed a similar pattern, although its decreasing trend continued till 10 min and then improved up to 27.20 % at 20 min as the maximum hatching rate in comparison to FC (60.37%). The results coincided with the highest survival rate (32.1%) of eyed stage (Fig. 1). Survival rate started to decrease again in both eyed and hatching stages over 20 min of UV irradiation, which was comparable with those at 8 min of irradiation. Hence it maybe concluded that 10 min of UV irradiation was a starting point for Hertwig effect to become evident (Fig. 1 and Table 2). Some hatching rate differences were detected between couples as well as IC and FC treatments, indicating different quality of brood stock gametes (Table 2).

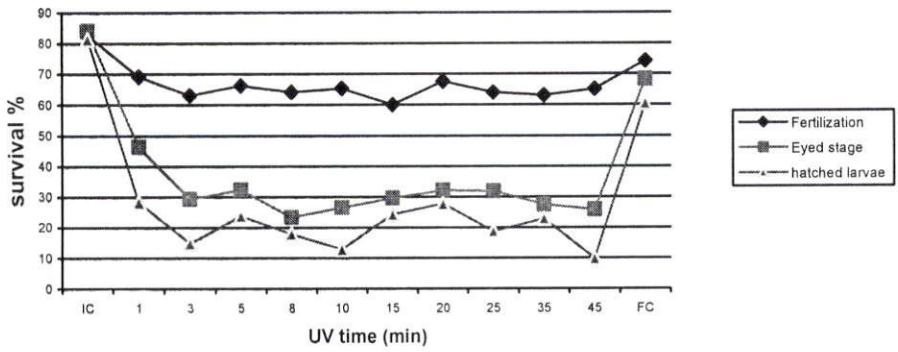

Figure 1: Mean survival rates of embryos at different UV-times used for irradiation of fertilizing sperm of rainbow trout. IC and FC are initial and final controls

Table 1: Method of sperm treatment using UV rays as reported by numerous authors

Author(s)	Irradiation time(min) or dose	Best irradiation time (min) or dose	Irradiation dose and unit given	Dilution rate	Species
Chourrout, 1982	0 - 6	> 4	MAZDA.TG1 5 W/G	1:4	*O. mykiss*
Chourrout, 1982	0 - 8	>4	MAZDA.TG1 5 W/G	1:4	*O. kisutch*
Chourrout, 1982	0 - 8	>5	MAZDA.TG1 5 W/G	1:4	*Salmo trutta*
Thopmson & Scott, 1984	1 - 30	Not mentioned	5 $mWcm^{-2}$	Not mentioned	*O. mykiss*

Table 1 continued:

Author(s)	Irradiation time(min) or dose	Best irradiation time (min) or dose	Irradiation dose and unit given	Dilution rate	Species
Goryzko *et al.*, 1991	5 - 10	>5	2075 μWcm^{-2}	1:10 & 1:40	*O. mykiss*
Don & Avtalion,1993	0.6 - 3.5	1.5	1800 $Jm^{-2}min^{-1}$	Not mentioned	*Oreochromis niloticus*
Valcarcel *et al.*, 1994	0 - 25	16	9.36×10^3 $erg.min^{-1}.mm^{-2}$	1:40	*Rhamdia sapo*
Plati & Thorgaard, 1997	3 - 20	15 - 20	1000 $erg.mm^{-2}$	1:10	*O. mykiss*
Azari Takami *et al.*, 2000	0 - 15	8	2887 μWcm^{-2}	1:4	*O. mykiss*
Piferrer *et al.*, 2004	300-100,000 $erg.mm^{-2}$	30,000 $erg.mm^{-2}$	-	1:10	*Scophthalmus maximus*

Table 2: Survival rate (%) of embryos at hatching stage after fertilization of oocyte with UV-irradiated sperm of rainbow trout.

	Replicates of treatments			Average	S.D.
	1	2	3		
IC*	86.21	66.30	91.68	81.39	13.35
FC*	57.40	44.07	79.64	60.37	17.8
UV irradiation time (min)					
1	16.65	30.2	22.8	28.18	6.83
3	20.60	5.50**	18.51	14.87	8.18
5	23.77	18.78	28.63	23.7	4.95
8	-	11.1	24.29	17.69	9.32
10	18.75	9.25	13.44	12.81	4.76
15	22.06	20.80	28.87	24.28	4.66
20	30.86	20.47	30.29	27.20	5.77
25	5.33**	14.1	36.7	18.71	16.18
35	38.82	11.00	26.45	22.42	19.33
45	11.1	10.4	7.55	9.68	1.88

*** Initial and final control**

**** Significant differences between replicates at the same time.**

For PR studies, a 10 min time interval was chosen for treatment with visible light and only survival to eyed and hatching stages summarized here. In a general conclusion, UV time (5, 30 and 120 seconds) did not have any adverse effect on average fertilization rates, but showed the greatest impact on survival to hatching stages. Just 5s of UV irradiation was sufficient to significantly decrease the survival rate up to eyed and hatching stages (Table 3). At eyed and hatching stages there were no significant differences in all L.L. L.D. D.L. and D.D. in each UV time exposure except those of 120s (Table 3).

In groups that received 120s of UV irradiation, the highest survival rates were observed in L.L. and L.D. portions in both eyed and hatching stages (Table 3). Interestingly, in both stages, the lowest survival rate was observed in D.D.

portion ($P<0.05$). In other UV treated time (5 and 30s), the lowest eyed and hatching rates were also achieved in D.D. portion (Table 3), although no significant differences among potions (L.L., D.L., L.D., D.D.) were observed ($P>0.05$); we failed to detect any PR effect at these periods. Light treatment of semen and/or eggs could, therefore, reverse the deleterious effect of UV on embryo viability only at 120s of irradiation time.

Table 3: Survival rates (%) of embryos obtained after treatment with visible light of semen and/or eggs of rainbow trout at different stages of development

Treatment / Stage	UV time (s) semen	egg		0 (control)	5	30	120
Eyed	Vis. Light	Vis. Light	L.L.	73.33 [ab]	37.80	34.92	48.10 [a]
	Vis. Light	Darkness	L.D.	86.46 [a]	34.87	41.86	43.78 [ab]
	Darkness	Vis. Light	D.L.	82.90 [a]	29.84	34.61	31.18 [b]
	Darkness	Darkness	D.D.	63.12 [b]	42.32	32.54	13.39 [c]
	Mean			**76.45**	**36.21**	**35.98**	**34.11**
Hatching	Vis. Light	Vis. Light	L.L.	60.23	33.91	30.02	44.00 [a]
	Vis. Light	Darkness	L.D.	73.38	33.92	33.92	40.88 [ab]
	Darkness	Vis. Light	D.L.	61.21	26.71	26.71	22.36 [b]
	Darkness	Darkness	D.D.	60.11	35.02	21.65	10.89 [c]
	Mean			**63.73**	**27.68**	**28.07**	**29.53**

*** At eyed or hatching stage group assigned with similar letter don't show any significant differences in each UV time treatment**

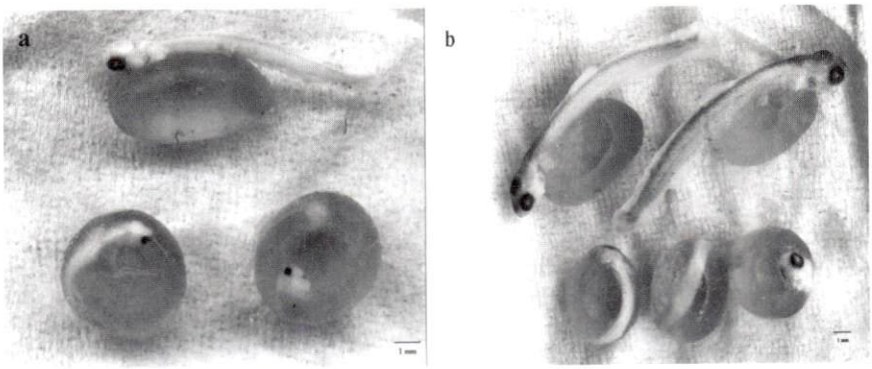

Figure 2: Embryos (eyed and hatched) resulting from fertilization with and without irradiated sperm of rainbow trout
(a) irradiated for 10 min b) 0 min (control) Bar = 1 mm

Discussion

When irradiated semen of fish is used for fertilization, the occurrence of malformed embryos becomes very frequent. Even though the assessment of this effect is difficult at early developmental stages, the normally developed embryos are readily distinguished from affected ones at eyed and hatching stages (Fig. 2 a, b). Such an abnormality, called "haploid syndrome" was observed in the catfish, *R. sapo* (Valcarcel *et al.*, 1994) and turbot, *S. maximus* (Piferrer *et al.*, 2004). Haploid syndrome is caused because of decreasing in chromosome number or penetrating destroyed chromatin of sperm into oocyte and embryo formation (Piferrer *et al.*, 2004).

Our result showed that treatment of semen with UV light at only 1 and 3 min resulted in a great decrease in survival rate at hatching stage (Fig 1). Although this similar pattern was observed at eyed stage, the greatest decrease occurred at hatching stage. This may be due to the fact that aneuploid zygote can start the

embryonic development and may be developed to eyed stage, but in the advanced stage like hatching can not survive due to abnormalities (Valcarcel *et al.*, 1994).

Initially, the minimum survival rate at hatching stage was at 10 min irradiation, then gradually increased and reached maximum at 20 min, after which the survival rate decreased rapidly to below 10%. Similar pattern was observed at eyed stage with an exception that the lowest survival rate was achieved at 8 min of UV irradiation, although they were very close (Fig. 1).

This phenomenon was described as Hertwig effect in several species like coho salmon, *O. kisutch* and brown trout, *S. trutta* (Chourrout, 1982), tilapia, *Oreochromis niloticus* (Don & Avtalion, 1993), catfish, *R. sapo* (Valcarcel *et al.*, 1994), rainbow trout, *O. mykiss* (Chourrout, 1982 ; Azari Takami *et al.*, 2000) and turbot, *S. maximus* (Piferrer *et al.*, 2004).

The Hertwig effect can express itself on 3 stages: (1) initially, when the UV irradiation is used at lower dose (time), the survival rates decrease rapidly because of partial inactivation of semen chromatin, resulting in aneuploid embryos that have a very low survival capability. In this study, it coincided with 0^+ to 8-10 min of irradiation. The maximum aneulploid larvae, the minimum survival rate are the starting point of Hertwig effect (8-10 min in this study). (2) by increasing of irradiation dose (time), the complete inactivation of semen chromatin occurs which produces the haploid embryos and shows the better survival rate than aneuploid ones; by increasing the haploid larvae, the survival rate increases gradually and the maximum survival rate is achieved, which in our experiment was coincided with 20 min of UV irradiation; (3) irradiation above the maximum survival rate (20 min of UV irradiation) brings about a rapid decrease in survival rate which is expected because of damages in the physiological (Piferrer *et al.*, 2004) and/or morphological (Don & Avtalion, 1993) aspects of semen.

The range of times (0.25-30 min) were described by different researchers for starting the Hertwig effect or complete inactivation of semen chromatin (Table 1), Such differences in time may be originated due to variations in the semen

quality, motility and spermatocrite, fish species as well as experimental conditions such as kind of irradiation rays (X or γ or UV), irradiation dose, dilution rate, semen thickness and egg quality (Chourrout, 1982; Plati *et al.*, 1997).

The first reports on reversal of UV damage by illumination with visible light were reported in microorganisms (bacteria) in 1940s and 1950s (Cleaver, 2003). Later research showed that among vertebrate only mammals (Placentaria) are deficient in PR activity due to the absence of light-dependence repair enzyme (Valcarcel *et al.*, 1994 ; Cleaver, 2003), and other vertebrates such as fish, amphibians and birds contain large amount of PR enzyme and can show PR activity (Natarjam *et al.*, 1980 ; Valcarcel *et al.*, 1994).

In our experiment, we could detect the PR activity only in 120s of UV irradiation time, in which all portions of semen and/or eggs treated with visible light showed a better survival rate than D.D. ($P<0.05$); the best survival rates (eyed and hatching) were resulted from L.L. portion in which both semen and fertilized eggs were treated with visible light for 10 min (Table 3).

Although in general agreement, the sperm do not contain the PR enzyme and so the L.D. treatment can not improve the survival rate (Valcarcel *et al.*, 1994), we could detect the clear occurrence of PR in L.D. portion (at 120s of UV treatment) of rainbow trout semen. This may show that PR enzyme is active in semen as well as eggs of rainbow trout.

In conclusion the best time of UV irradiation at 2010±200 $\mu w.cm^{-2}$ intensity for producing gynogenesis in rainbow trout was 20 min and visible light illumination (10 min post fertilization for 10 min) to semen and/or eggs could reverse the deleterious effect of UV.

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