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Sperm fertilization capacity of Caspian salmon, *Salmo trutta caspius* under ultraviolet irradiation

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ABSTRACT
The optimum dose of ultraviolet (UV) irradiation to produce gynogenetic Caspian salmon, *Salmo trutta caspius*, with emphasis on the "Hertwig effect" and photoreactivation (PR) were investigated. The sperm of Caspian salmon was irradiated with UV at 2010 ± 200 μw.cm^{-2} in different times including 0, 1, 3, 5, 8, 10, 15, 20, 25, 35 and 45 min and was allowed to fertilize normal ova; the fertilization, eyed and hatching rates were calculated to assess the performance. Using the irradiated sperm decreased the fertilization, eyed as well as hatching rates and the so-called "Hertwig effect" was observed, with the time-dependent decrease in the hatching rates at 0$^+$ to 3 min irradiation, but better hatching rates were observed at more prolonged irradiation times. The best hatching rate was achieved at 25 min of UV irradiation; after that the survival rates rapidly declined to near zero. For PR studies, the semen was irradiated with UV (5, 30 and 120s) 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. UV irradiation as low dose as 5s, significantly decreased the hatching rate (P <0.05). Semen and/or eggs illumination with visible light could not improve the survival rates (P >0.05). So, based on the results of this study, it was impossible to detect any PR mechanism in Caspian salmon.

***Keywords**: Caspian Salmon, Salmo trutta caspius, Hertwig effect, Photoreactivation, Ultraviolet irradiation*

INTRODUCTION

Caspian salmon, *Salmo trutta caspius* is one of the nine subspecies of brown trout, *Salmo trutta* in the world (Quillet et al., 1992). This subspecies attains the greatest size, weight and growth rate of all brown trout, lives in the Caspian Sea but spawns in the rivers joining in to the Sea (Kalbassi et al., 2006; Pasha Zanousi et al., 2013). They are very rare but can be found mainly in the southwest part of the Caspian Sea, Iranian waters, where they used to be heavily fished for commercial purpose (Kiabi et al., 1999; Shiranghi et al., 2011; Rahbar et al., 2011). Some brown trout subspecies especially sea trout, *Salmo trutta trutta* show valuable potential for aquaculture (Krieg et al., 1991; Quillet et al., 1992) with special emphasis on the use of triploid and gynogenetic populations (Hulata, 2001). More recently, Caspian salmon has attracted interest for aquaculture especially in cages in Iran (Pasha Zanousi et al., 2013). Because of some advantages, culture of all-female population of this subspecies may be preferable. There are various ways to produce all-female population in fish such as hormonal treatment by estrogens (Donaldson, 1996; Piferrer, 2001), crossing the neomale (XX male) by normal female

(Devlin & Nagahama, 2002) and gynogenesis (Komen & Thorgaard, 2007). Gynogenesis is a chromosome set manipulation technique consisting of the generation of the progenies whose chromosome are exclusively inherited from the mother (Chourrout, 1982). It is a valuable tool to understand sex determination systems and produce inbreed line, clone fish and monosex (all-female) population in different fish species (Colburn et al., 2009; Fopp-Bayat, 2010; Zhang et al., 2011; Whitehead et al., 2012).

A critical point of gynogenesis induction is application of appropriate ray or chemical dose to achieve the complete DNA sperm inactivation while maintaining the capacity to trigger the embryonic development (Komen & Thorgaard, 2007). When semen is irradiated in different alternatives of dose or time and fertilize the normal ova, some fluctuations in fertilization, eyed and/or hatching rates are observed which called "Hertwig effect" (Chourrout, 1982). The Hertwig effect is a paradoxical phenomenon found in various fish and amphibian species, in which high doses of X, γ or ultraviolet (UV) rays for sperm irradiation seem to be more efficient in fertilizing normal ova than sperm irradiated at lower doses (Don & Avtalion, 1993).

Using UV as a sperm inactivation 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 its performance has more safety than ionizing rays. The disadvantage of this method is "photoreactivation (PR) effect" in which the chromatin of sperm that inactivate by UV may become active again when sperm and/or egg are exposed to visible light (Ijiri & Egami, 1980; Cleaver, 2003).

The objectives of this study were to determine the appropriate duration of UV irradiation on fertilization capacity of Caspian salmon sperm, the occurrence of the Hertwig effect to find out the optimum UV exposure time for producing gynogenetic progenies and to occur the PR mechanism in the semen as well as eggs of Caspian salmon. The results of this study may be useful as a suitable tool to optimize gynogenesis induction in Caspian salmon.

MATERIALS AND METHODS

Specimens of Caspian salmon (6 females and 6 males) were selected from wild broodstocks in the Shahid Bahonar Salmonid Hatchery Centre, Kelardasht, Mazandaran, Iran. The broodfish were captured during the spawning migration in the Cheshme Kileh River, Tonekabon, Mazandaran and transported to the hatchery for artificial breeding.

The experiments were performed in three replicates using 3 donor couples. Sperm irradiation was carried out at a distance of 5 cm, by means of two 15-watt germicidal tubes (TUV1W/G 15 T8 UV lamp, Philips) located in a box. According to Atomic Energy Organization of Iran (AEOI) assay, irradiation intensity of one tube was about 1005 μwcm^{-2} at 5 cm distance in 254 nm wave lengths, so the total energy received by sperm was expected to be about 2010 ± 200 μwcm^{-2}. Irradiation doses were adjusted by changing the exposure times as follow: 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 9.6×10^9 $spermatozoa.ml^{-1}$ 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 milt + 4.5 ml extender (Plati et al., 1997). They were placed in a 10 cm diameter Petri dish on ice and were mixed by a magnet stirrer (2 $c.s^{-1}$) during irradiation.

Ova were then added at a ratio of about 75 oocytes 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, 120s, and none irradiated sperm as control. Irradiated / control milt was divided into two separate groups (2.5 ml each) and put in a Petri dish, 15 cm in diameter, subjected to darkness (D) or visible light (L) at a distance of 30 cm using 60 watts regular lamp (Yazd, Iran), 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 about 74 ova. After 10 min., eggs were washed by incubator water at 8°C and were subjected to darkness (D) or visible light (L) for 10 min as above in two replicates. So, four different groups were created as L.L., L.D. D.L. and D.D. where L. and D. describing visible light and darkness respectively. In each treatment, the first symbol is linked to milt treatment and the second one shows fertilized egg treatment. For example, L.D. represents the group that milt is subjected to visible light before fertilising ova (L), and then the eggs are subjected to darkness (D). Incubation period was done in California trays and routine care was subjected to all groups until hatch.

Sperm fertilization capacities after UV irradiation were analysed based on the fertilization success, survival until eyed and hatching stages. For each parameter at first, normality was checked using Kolmogorov-Smirnov test. Then the mean value of each parameter at every irradiation time plus two control groups, initial and final controls were analysed using One Way ANOVA at 5 % significant level followed by Tukey's HSD. Before performing it, the data for each pair of fish were analysed as above. For PR studies, the normality test was done as above. The mean value of the survival rates at each stages (fertilization, eyed and hatching) at every irradiation time was compared as defined above.

RESULTS

There were no differences in hatching rates either among individuals from different couples or between IC and FC, indicating the similar quality of gametes between different broodfish that could remain viable throughout the experiment (Fig. 1; Table 1). Determination of embryo survival rate as a function of UV time exposure at three developmental stages from fertilization to hatching (Fig. 1) showed that survival rate at hatching stage was the most sensitive marker of the effect of irradiation, though it was observed at the eyed stage as well.

The highest fertilization rate (84.7 %) was observed for IC groups while the lowest one has been measured when the milt irradiated for 15 min (Fig. 1). The only significant differences were found between IC and the milt irradiated at 15 min ($P < 0.05$). There were no significant differences among other treatments ($P > 0.05$). UV irradiation of sperm affected the survival rates in eyed and hatching stages much more than in fertilization rate (Fig. 1). The only 1 min UV irradiation time could decrease the eyed and hatching rates extensively to 47.5 and 16.7 %, respectively. Survival rate in eyed stage decreased up to 10 min UV irradiation and then started to improve gradually and reached maximum, 54.3 %, at 20 min (Fig. 1).

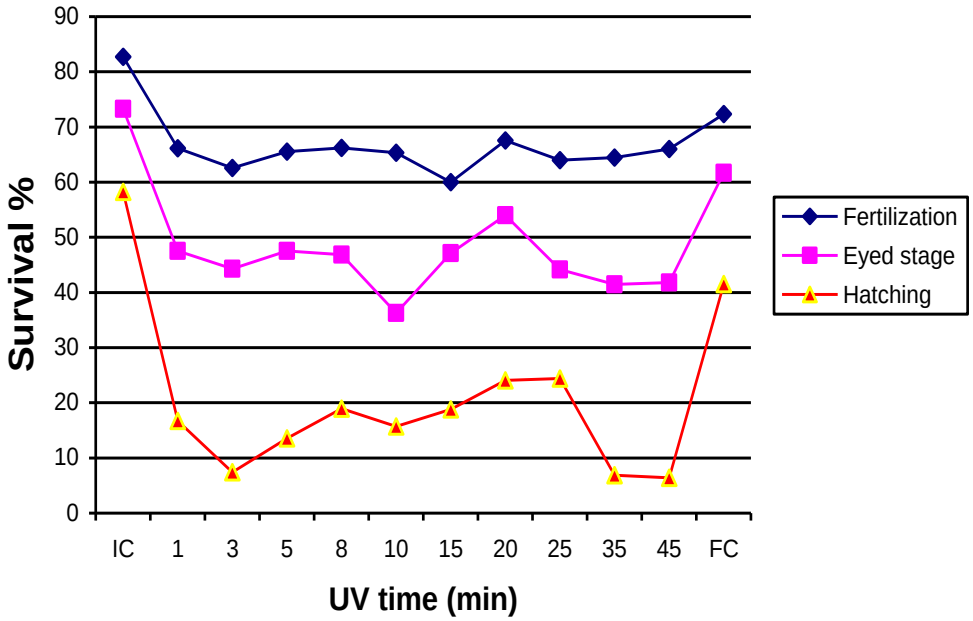

Fig.1. Mean survival rate of embryos at different UV-times used for irradiation of fertilizing sperm. IC and FC are initial and final control respectively. The hatching stages were the most sensitive marker showing obvious Hertwig effect.

Table 1. Mean ± S.D. of survival rate (%) of embryos at hatching stage after fertilization of oocyte with UV-irradiated sperm of Caspian salmon, *Salmo trutta caspius*.

	Experiments			**Mean**	**S.D.**
	1	2	3		
IC*	63.11	70.16	41.38	58.12	15.00
FC*	34.3	41.33	47.33	40.98	6.52
Time (min)					
1	24.86	9.28	16.02	18.05	9.47
3	7.99	7.95	6.33	7.42	0.94
5	11.77	5.93	22.90	13.53	8.62
8	28.17	9.20	19.40	18.92	9.49
10	17.30	8.1	27.90	17.77	9.90
15	21.23	16.20	-	18.75	3.55
20	27.62	29.7	14.95	24.09	7.98
25	25.25	25.6	22.3	24.38	1.81
35	14.59	4.71	1.3	6.88	6.09
45	8.00	5.84	5.4	6.41	1.39

*. Initial and final control.

The hatching rates showed similar fluctuation. Although the lowest hatching rate was achieved at 3 min UV irradiation and then started to increase gradually up to 24.3 % at 25 min as the maximum hatching rate in comparison to FC, 40.37 % (Fig. 1). Eyed and hatching survival rates started to decline again over 20 and 25 min UV irradiation respectively, were comparable with those at 10 and 3 min UV irradiation, respectively.

For PR studies, a 10 min time interval was chosen for treatment with visible light and only survival rate at eyed and hatching stages summarized here. As a general conclusion, UV time (5, 30 and 120s) did not have any adverse effect on average fertilization rate, but these treatments showed negative impact on the hatching rates. Just 5s exposure to UV irradiation was sufficient to significantly decrease the average hatching rates from 44.4 to 12.9 % (Table 2). At fertilization stage, only the lowest fertilization rate in L.L. portion of control group (0s UV irradiation) was significantly lower than that of the others (P <0.05). As illustrated in Table 2, there were no significant differences among portions (L.L. L.D. D.L. and D.D.), except for L.L. in the control, in each UV treated times (P <0.05). So, it was impossible to detect any PR mechanism in sperm or egg of Caspian salmon based on the explained condition.

The occurrence of malformed embryos was very frequent in the groups treated with different doses of UV irradiation. These malformations were usually observed at hatching stages and afterward, however, this phenomenon was also observed in the eyed eggs (Fig. 2 a, b, c).

DISCUSSION

The results of the present study showed that treatment of semen with UV light had the greatest effect on hatching rates. This may be because aneuploid zygote can either start the embryonic development or may develop to eyed stage, while in the advanced stage such as hatching; the zygote cannot survive due to abnormalities. Similar results were obtained by Valcarcel et al. (1994) on catfish, *Ramdia sapo* and rainbow trout, *Oncorhynchus mykiss* (Dorafshan et al., 2006). Initially, the minimum survival rate at hatching stage was at 3 min irradiation, then gradually increased and reached maximum at 25 min, thereafter decreased rapidly to below 10 %. Similar pattern was observed at eyed stage with an exception that the lowest survival rate was achieved at 10 min UV irradiation (Fig. 1).

Table 2. Survival rates of embryos obtained after treatment with visible light (L) or darkness (D) of semen and/or eggs at hatching stage in Caspian salmon, *Salmo trutta caspius.*

Stage \ Treatment	Semen	Egg	UV time (s)	0 (control)	5	30	120
Fertilization	L.	L.	L.L.	48.78 [a*]	70.12	71.26	62.94
	L.	D.	L.D.	64.35 [b]	71.53	61.30	64.98
	D.	L.	D.L.	66.69 [b]	62.30	66.01	67.17
	D.	D.	D.D.	66.23 [b]	63.70	63.21	65.97
		Mean		**61.51**	**66.91**	**65.44**	**65.26**
Eyed	L.	L.	L.L.	44.80	44.22	42.60	39.87
	L.	D.	L.D.	54.11	53.35	38.92	44.51
	D.	L.	D.L.	58.21	48.23	33.32	47.88
	D.	D.	D.D.	55.57	52.60	35.92	38.30
		Mean		**53.17**	**49.6**	**37.69**	**42.66**
Hatching	L.	L.	L.L.	30.68	10.9	16.4	16.9
	L.	D.	L.D.	44.03	8.01	12.7	15.1
	D.	L.	D.L.	56.37	17.88	21.62	15.2
	D.	D.	D.D.	47.2	15.2	16.09	9.9
		Mean		**44.45**	**12.99**	**16.70**	**14.26**

*There were no significant differences at each UV time of irradiation (P >0.05). Only fertilization rate in the control group showed significant differences.

Fig. 2. Caspian salmon, *Salmo trutta caspius* embryos (eyed and hatched) resulting from fertilization with or without irradiated sperm at different times. (a) 3 min, aneuploid (b) 25 min, haploid and (c) 0 min (control), diploid. Malformation is obvious at eyed and hatching stage (a) in comparison to control (c). Bar = 1 mm.

Various irradiation doses and/or times were used for different species to achieve the best gynogenetic results (Table 3) and the Hertwig effect has been reported in various fish species by means of ionizing radiation (X and γ rays) in rainbow trout *Oncorhynchus mykiss* (Chourrout, 1980), chum salmon *Oncorhynchus keta* (Onozato, 1982) and non-ionizing rays such as UV in coho salmon *Oncorhynchus kisutch* and brown trout *Salmo trutta* (Chourrout, 1982), tilapia *Oreochromis niloticus* (Don & Avtalion, 1993), rainbow trout (Goryczko et al., 1991; Dorafshan et al., 2006), catfish *Rhamdia sapo* (Valcarcel et al., 1994), turbot *Scophthalmus maximus* (Piferrer et al., 2004) and southern flounder *Paralichthys lethostigma* (Luckenbach et al., 2004).

However, Chakraborty et al. (2006) could not find any Hertwig effect during sperm irradiation of Sarpunti, *Puntius sarana*. The Hertwig effect usually can express itself in 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 3 min of irradiation. The maximum aneulploid larvae, the minimum survival rate are the starting point of Hertwig effect (3 min in this study); (2) by increasing the 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 25 min UV irradiation; (3) Finally, irradiation above the maximum survival rate (25 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.

Different ranges of irradiation time or intensity were described by numerous researchers for starting the Hertwig effect or complete inactivation of semen chromatin (Table 3). Such differences in time or dose 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 works 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 (Cleaver, 2003), and other vertebrates such as fish, amphibians and birds contain large amount of PR enzyme and can show PR activity (Natarjan et al., 1980; Valcarcel et al., 1994).

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 Cleaver (2003).

PR mechanism has been previously reported in Japanese medaka *Oryzias latipes* (Ijiri and Egami, 1980; Armstrong *et al.*, 2002), rainbow trout (Dorafshan et al., 2006) as well as some amphibians like African clawed frog *Xenopus laevis* (Legerski et al., 1987), although Valcarcel et al. (1994) failed to detect PR in catfish, *R. sapo*. Usually, to reduce the risk of PR during gynogenesis induction, irradiation usually is conducted in the dark (e.g. Zhang et al., 2011; Whitehead et al., 2012). In our experiment, there were no significant differences in survival rates in different UV treated portions (L.L., L.D., D.L. and D.D.) ($P > 0.05$). In general agreement, the fish eggs contain large amount of PR enzyme (Valcarcel et al., 1994) and so the D.L. and L.L. portions must show better survival rates than others, but we failed to detect any PR activity in Caspian salmon. Different factors such as illumination starting point and exposing duration (Ijiri & Eghami, 1980) as well as both visible and UV intensities and spectra can affect PR mechanism (Bohrerova & Linden, 2007). Another explanation could be structure and colour intensity of eggs in the wild Caspian salmon. The eggs have a large diameter (about 5 mm) with very intense orange colour which could affect visible light penetration into the eggs that is essential for any PR phenomena.

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, c). Such an abnormality, generally called "haploid syndrome" was observed in different fish species such as Southern flounder *Paralichthys lethostigma* (Luckenbach et al., 2004), rainbow trout (Dorafshan et al., 2006), Atlantic halibut *Hippoglossus hippoglossus* (Tvedt et al., 2006), grass carp *Ctenopharyngodon idellus* (Zhang et al., 2011) and Atlantic cod *Gadus murphua* (Ghigliotti et al., 2011). Haploid syndrome is caused because of decreasing in chromosome number or penetrating destroyed chromatin of sperm into oocyte and embryo formation.

In conclusion, the results of this study showed that UV irradiation on Caspian salmon milt can reduce the fertilization capacity of the male gamete which showed the greatest effects on the survival to eyed and hatching stages. The best time of UV irradiation at 2010 ± 200 $\mu w.cm^{-2}$ intensity for producing gynogenetic progeny of Caspian salmon was 20 - 25 min and visible light illumination (10 min post fertilization for 10 min) to semen and/or eggs could not reverse the UV effect.

Table 3. 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	Sperm donor, (common or scientific name)
Chourrout, 1982	0-6	>4	MAZDA.TG15 W/G	1:4	Rainbow trout
Chourrout, 1982	0-8	>4	MAZDA.TG15 W/G	1:4	*Oncorhynchus kisutch*
Chourrout, 1982	0-8	>5	MAZDA.TG15 W/G	1:4	*Salmo trutta*
Thopmson and Scott, 1984	1-30	Not mentioned	5 mW.cm^{-2}	Not mentioned	Rainbow trout
Goryczko et al., 1991	5-10	>5	2075 µW.cm^{-2}	1:10 and 1:40	Rainbow trout
Don and Avtalion, 1993	0.6-3.5	1.5	1800 J.m^{-2}min^{-1}	Not mentioned	*Oreochromis niloticus*
Valcarcel et al., 1994	0-25	16	9.3×10^{3} erg.min^{-1}.mm^{-2}	1:40	*Rhamdia sapo*
Plati et al. 1997	3-20	15-20	1000 erg.mm^{-2}	1:10	Rainbow trout
Azari Takami et al., 2000	0-15	8	2887 µW.cm^{-2}	1:4	Rainbow trout
Piferrer et al., 2004	300-100,000 erg.mm^{-2}	30,000 erg.mm^{-2}	-	1:10	*Scophthalmus maximus*
Luckenbach et al., 2004	0-190 J/cm2	70 J.cm2	-	1:20	*Paralichthys lethostigma*
Dorafshan et al., 2006	0-45	20	2010 µW.cm^{-2}	1:10	Rainbow trout
Tvedt et al., 2006	0-1382 mJ.cm^{-2}	65 mJ.cm^{-2}	-	1:80	*Hippoglossus hippoglossus*
Fopp-Bayat et al., 2007	45-60-70 s	60-70 s	Philips 15 W	1:10	Bester*
Colburn et al., 2009	-	70 mJ.cm^{-2}	-	1:10	*Centropristis striata*
Fopp-Bayat, 2010	-	288.75 J.m^{-2}	-	-	Hybrid Siberian sturgeon×Russian sturgeon
Ghigliotti et al., 2011	-	3689 µW.cm^{-2}	-	1:40	*Gadus morhua*
Zhang et al., 2011	20-25	3000-3600 mJ.cm^{-2}	Quartz UV lamp (ZSZ20D)	1:4	Hybrid red crucian carp × common carp**

* *Huso huso* × *Acipencer ruthenus.*
** *Carassius auratus* × *Cyprinus carpio.*

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اثر پرتودهی با اشعه فرابنفش بر قابلیت بارورکنندگی اسپرم ماهی آزاد دریای خزر *Salmo trutta caspius*

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چکیده

دوز بهینه پرتو فرابنفش به منظور القای ماده‌زایی در ماهی آزاد دریای خزر با تاکید بر اثر هرتویگ و فعال‌سازی نوری مورد ارزیابی قرار گرفت. به این منظور اسپرم ماهی آزاد دریای خزر با استفاده از پرتو فرابنفش در شدت $\mu W.cm^{-2}$ ۲۰۱۰±۲۰۰ برای مدت ۰، ۳، ۵، ۸، ۱۰، ۱۵، ۲۰، ۲۵، ۳۵ و ۴۵ دقیقه پرتودهی و سپس برای تلقیح تخمک‌های معمولی مورد استفاده قرار گرفت. نتایج نشان داد که استفاده از اسپرم پرتودهی شده منجر به کاهش معنی‌دار درصد لقاح، چشم زدگی و تفریخ با روندی که اصطلاحا اثر هرتویگ نامیده می شود، می گردد. در این میان میزان تفریخ ارتباط مشخصی را با دوره پرتودهی نشان داد. به طوری که با افزایش زمان پرتودهی تا ۳ دقیقه میزان تفریخ کاهش و سپس با افزایش طول دوره پرتودهی تا ۲۵ دقیقه، افزایش یافت. پس از آن روند کاهشی مجددا نمایان شد تا در زمان ۴۵ دقیقه پرتودهی، میزان تفریخ به حدود صفر کاهش یافت. جهت بررسی فعال‌سازی نوری، اسپرم به مدت ۵، ۳۰ و ۱۲۰ ثانیه در معرض تابش فرابنفش قرار گرفت. اسپرم پرتو ندیده به عنوان گروه شاهد در نظر گرفته شد. اسپرم یا تخم بارور شده با آن در معرض نور مرئی (۶۰ وات) در فاصله ۳۰ سانتی‌متری به مدت ۱۰ دقیقه قرار گرفت و درصد چشم‌زدگی و تفریخ محاسبه شد. پرتودهی حتی در زمان کوتاه ۵ ثانیه نیز قادر به کاهش معنی دار میزان تفریخ بود ($p<0.05$). پرتودهی تخم یا اسپرم با نور مرئی قادر به بهبود بازماندگی نبود ($p>0.05$)، لذا در این تحقیق امکان شناسایی فرایند فعال سازی نوری در ماهی آزاد دریای خزر فراهم نشد.

* مولف مسئول