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Induction of meiotic gynogenesis in Indian catfish *Heteropneustes fossilis* (Bloch) using irradiated sperm of *H. fossilis* and *Clarias batrachus* (Linn.)

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ABSTRACT

Gynogenesis was induced in Indian catfish (*Heteropneustes fossilis*) to produce all female population. The irradiated spermatozoa from the two species *viz*.,, *H. fossilis* and *Clarias batrachus* were successful in inducing parthenogenetic development and resulted in the production of viable gynogens. The irradiation of sperm was accomplished by exposing them to ultraviolet light (260 nm) for a duration of 60 sec. Both cold shock and heat shock treatments were given at 5 min post-fertilization. The effects of cold shock given for diploidization at 2 and 4°C for a duration of 15 and 30 min and heat shock at 38 and 41°C for 1, 2 and 3 min resulted in diploid gynogens. Cold shock at a temperature of 2 °C was found to be better than that of 4 °C and the duration of 15 min was better than 30 min. Heat shock at 41°C for 1 min was found to be better than 38 °C . The karyological examination of gynogens showed 2n=56 number of chromosomes. The survival of gynogens produced from the irradiated sperm of *H. fossilis* and *C. batrachus* sperm after 60 days of rearing was found to be 4.12 and 2.78% respectively.

Keywords: Chromosome number, Gynogenesis, *Heteropneustes fossilis*, Shock treatment, Survival rate

Introduction

Gynogenesis is the development of an egg triggered by genetically inactivated sperm to obtain viable gynogens which requires restoration of diploidy by the retention of the second polar body. Ultraviolet (UV) treatment of sperm has been used successfully in a number of gynogenetic studies (Stanley, 1976; Streisinger *et al*., 1981; Thorgaard, 1983; Karal Marx and Sukumaran, 2007; Karal Marx, 2011). UV light damages chromosomes mainly by inducing dimers. Hussain and Mazid (2001) has induced meiotic gynogenesis in *H. fossilis* by applying cold shock to the eggs that were fertilized with UV-irradiated sperm. The aim of the present study was to compare the effectiveness of cold shock and heat shock given 5 min after fertilization to duplicate the haploid number of chromosomes in *H. fossilis* embryos. Irradiated milt of *H. fossilis* and *C. batrachus* were tried to induce gynogenesis in *H. fossilis*. Use of heterologous sperm in assuring the production of viable gynogens were reported in many previous studies (Chourrout, 1982; Na-Nakorn *et al*., 1993; Varadi *et al*., 1999).

Materials and methods

Mature male and female *H. fossilis* (120-200 g body weight) with gravid gonads were purchased from local fish market and acclimatized in the laboratory for 4 days. The fishes were given antibiotic treatment (tetracycline @ 500 mg l^{-1}) for three days. Then the fishes were transferred to circular cement tanks (2 m dia; 1 m height; water depth of 50 cm). Male and female fishes were maintained separately in different tanks under natural photoperiod. Mature male and female specimens were identified on the basis of genital papilla and distension of the abdomen. The males have well-defined pointed papilla and reddish vent while the females have blunt genital papilla, reddish vent and bulging abdomen during the breeding season (Khan, 2008). The males of *C. batrachus* also possessed similar features and were easily identified. Partial water exchange was done once in a week with complete water exchange once in a month. The brood fishes were fed *ad libitum* on formulated pellet feed, twice a day.

Experimental fish were fed from a suspended tray . The females were injected with the hormone, Ovatide (Hemmo Pharma, Mumbai, India) intramuscularly @ 1.5 ml kg^{-1} body weight just below the dorsal fin and above the lateral line during late evening. After 10-13 h of injection, the female fishes were checked for the release of eggs and the male fishes were dissected out and the testis were removed . The milt was preserved in Hanks Balanced Salt Solution (Himedia, Mumbai, India). Motility was checked by adding a drop of water to the milt under microscope. The sperm was diluted 1:10 (100 µl: 900 µl) in Hanks Balanced Salt Solution. Five millilitre of diluted

sperm was taken in a petridish and exposed to UV irradiation as described by Karal Marx (2011). Irradiation was done in a simple UV chamber with 4 nos. of 15 W germicidal lamps (Philips, Holland). Two lamps were fixed on the top and two lamps on the bottom at a distance of 25 cm from the centre. The petridish with the milt suspension was placed in the middle of the chamber so that complete irradiation can be achieved across the chamber.

Exposure of UV irradiation was carried out for different durations 30, 60, 90, 120, 150, 180, 210, 240, 270 and 300 sec and the duration of motility was recorded. Motility of the sperm was tested before (control) and after irradiation by microscopic examination. Arbitrary scoring system modified from Kurokura (1979) was used to assess the sperm motility. The irradiation was carried out in darkness in order to avoid genetic photo-reactivation of sperm (Kaastrup and Horlyck, 1987).

The sperms with high motility after UV irradiation was studied and used for activating the eggs. The optimum duration was found to be 60 sec. The sperm suspension was irradiated for 60 sec prior to fertilization. The females were stripped gently by pressing the abdomen and eggs were collected in a white enamel tray. The non-irradiated milt sample was used as control. Water was added for the activation of the sperm. Ten milliliter of the sperm suspension of *H. fossilis* prior to UV irradiation was added gently to a batch of eggs. Each batch of eggs consisted of 600 numbers. The eggs were mixed gently with the sperm suspension using a feather and tap water was added for the activation of the sperm. These eggs were maintained in separate rectangular plastic troughs with 5 l of water. Similarly, 10 ml each of the sperm suspension of *H. fossilis* and *C. batrachus* after UV irradiation was added to another batch which consisted of about 3000±200 numbers of eggs and *in vitro* fertilization was carried out.

A batch of 600 numbers of fertilized eggs with the UV-irradiated sperm suspension was taken and the fertilization rate was calculated. The portion of eggs retained without giving any shock treatment served as haploids. Two types of shock treatments (cold shock and heat shock) were administered for diploidization in which the haploid set of chromosomes get doubled with another set of chromosomes from the second polar body. For the induction of meiotic gynogenesis, shock treatment was given to all the batches of eggs 5 min after fertilization.

Batches of 600 numbers fertilized eggs were placed in a netting basket (filter) in the container such that the eggs were dipped in the water between the ice cubes. Cold shock was given at 2 °C and 4 °C with a duration of 15 and 30 min at each temperature. Heat shock was given at 38 °C and 41 °C for a duration of 1, 2 and 3 min. A batch of 600 eggs were placed in a netting basket (filter) using a spatula and suspended in water bath for a duration of 1, 2 and 3 min. After shock treatment, the fertilized eggs in the netting basket (filter) were taken out from the water bath and were released into rectangular troughs containing 5 l of water.

The different groups of eggs were maintained in separate troughs with 5 l of water for hatching at 27±1 °C. The eggs maintained in different groups hatched out at 24 h post-fertilization. Deformed larvae were observed in the shock treated and haploid groups. The larvae rely on the yolk sac for the first three days. From the third day onwards, the larvae were fed with *Artemia* nauplii *ad libitum* (*Artemia* cysts O.S.I. Pro 80, Ocean Star International, Inc. Snow Ville, USA). After seven days, *Artemia* nauplii was partially replaced with zooplankton collected from fish culture ponds followed by powdered pellet feed after 15 days. Water was exchanged twice daily and aeration was also provided in the larval rearing tanks. The survival and growth for different groups were calculated. The technique of Kligerman and Bloom (1977) with simple modifications was followed for chromosome preparation.

Results

The effect of UV irradiation on the motility of sperm was studied for *H. fossilis* and *C. batrachus*. More than 70% motility was found when the UV exposure duration was 60 sec. The average motility score for *H. fossilis* was found to be 78 sec and for *C. batrachus* was 64 sec when they were exposed to UV irradiation for 60 sec. As the shocks were given 5 min post-fertilization, the offsprings produced were meiotic gynogens. The study showed that the hatching rates were high in eggs when minimal period of shock treatment was given. The eggs fertilized with irradiated sperm of *H. fossilis* after cold shock treatment showed better hatching rate than that of heat shock (Table 1 and 2). There was no significant difference ($p>0.05$) between the two temperatures tried for cold shock (2 °C and 4 °C). Cold shock at 2 °C for 15 min showed a hatching rate of 12.82% followed by 12.50% for 30 min.

Significant difference ($p<0.01$) was found among the two heat shock treatments. Heat shock at 41 °C for a minute recorded a hatching rate of 11.65 % while the hatching rate was 11.45 % for a period of 2 min (Table 3). The maximum survival of hatchlings was observed in the cold shock at 2 °C for 15 min duration and in heat shock at 41 °C for 1 min duration. The haploids showed a poor survival of 4.76% and subsequently all the haploids died. When compared to treated groups and haploids, maximum survival (86.72%) was recorded in the control groups. Induction of gynogenesis using irradiated sperm of *C. batrachus* by cold shock at 2 °C for a duration of 15

and 30 min resulted in 15.26% and 14.96% induction respectively (Table 3). Statistical analysis of the treatments revealed significant difference in survival ($p<0.01$) between the two temperatures. Heat shock given at 41 °C for a minute gave a hatching rate of 14.93% (Table 4) and shock at 38 °C for a minute resulted in hatching rate of 14.64%. Significant difference was found in the hatching rate between 38 °C and 41 °C. Maximum survival (60%) was observed till the feeding stage (72 h) in the cold shock treated groups at 2 °C for 15 min duration. Highest survival of 57.14% was recorded in the heat shock treated groups at 41°C for one minute duration.

The survival percentage of gynogens and control produced by fertilizing *H. fossilis* eggs with *H. fossilis* sperm is shown in Fig. 1. The survival percentage of control was found to be greater (86.72 %) than that of the gynogens. After a rearing period of 60 days, the survival declined to 4.12% for gynogens when compared to 51.78 % for the control group. The survival of the gynogens produced by the irradiated *C. batrachus* sperm was found to be 2.78% after 60 days of rearing (Fig. 2). Haploids showed poor survival of 3.33% with haploid syndrome and died within 24-72 h of hatching. Haploids showed different types of deformities such as small eyes, dwarf body size and bent

Table 1. Effect of cold shock on the induction of gynogenesis in *H. fossilis* eggs fertilized with irradiated sperm of *H. fossilis*

Offspring	Shock temperature (°C)	Shock duration (min.)	Fertilization (%)	Hatching (%)	Survival (%) (72 h)
Gynogenetic female	2	15	52.00	72.50	12.82
		30	51.33	60.00	12.29
	4	15	51.50	63.16	12.29
		30	50.67	57.43	11.51
Haploid	–	–	27.17	4.76	15.77
Control	–	–	92.20	86.72	92.25

Table 2. Effect of heat shock on the induction of gynogenesis in *H. fossilis* eggs fertilized with irradiated sperm of *H. fossilis*

Offspring	Shock temperature (°C)	Shock duration (min.)	Fertilization (%)	Hatching (%)	Survival (%) (72 h)
Gynogenetic female	38	1	48.83	11.95	63.89
		2	46.33	10.43	61.76
		3	43.67	9.51	54.17
	41	1	51.50	11.65	71.43
		2	49.50	11.45	68.97
		3	47.17	8.48	60.00
Haploid	–	–	27.17	15.77	4.76
Control	–	–	92.20	92.25	86.72

Table 3. Effect of cold shock on the induction of gynogenesis in *H. fossilis* eggs fertilized with irradiated sperm of *C. batrachus*

Offspring	Shock temperature (°C)	Shock duration (min.)	Fertilization (%)	Hatching (%)	Survival (%) (72 h)
Gynogenetic female	2	15	47.50	15.26	60.00
		30	47.00	14.96	45.86
	4	15	46.67	14.64	56.85
		30	46.63	14.32	42.67
Haploid	–	–	23.50	11.28	3.33
Hybrid	-	-	36.83	3.17	33.33

Table 4. Effect of heat shock on the induction of gynogenesis in *H. fossilis* eggs fertilized with irradiated sperm of *C. batrachus*

Offspring	Shock temperature (°C)	Shock duration (min.)	Fertilization (%)	Hatching (%)	Survival (%) (72 h)
Gynogenetic female	38	1	46.67	14.64	53.85
		2	45.33	14.04	45.45
		3	44.17	13.77	40.00
	41	1	47.33	14.93	57.14
		2	46.50	14.30	50.00
		3	45.17	14.06	45.45
Haploid	–	–	23.50	11.28	3.33
Hybrid	-	-	36.83	3.17	33.33

body shape. These haploids were unable to swim. The mean length of the larvae from the different groups such as hybrid, gynogens and control is shown in Fig. 3. The control and gynogens possessed 2n=56 (Fig. 4a and b) chromosomes and hybrids possessed 2n=53 (Fig. 4c).

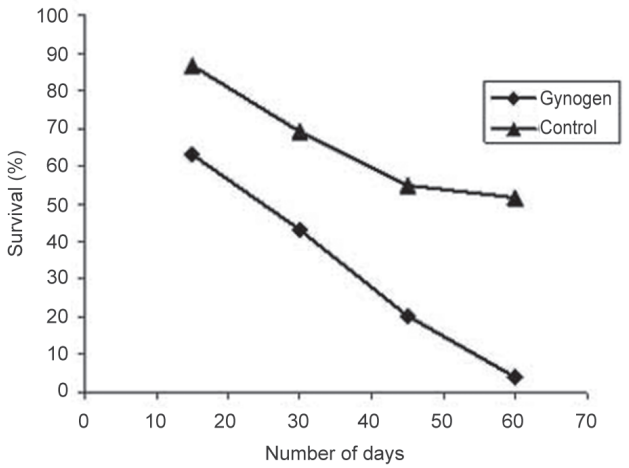

Fig. 1. Survival rate of gynogen and control group embryos obtained from *H. fossilis* eggs after fertilization with irradiated sperm of *H. fossilis*

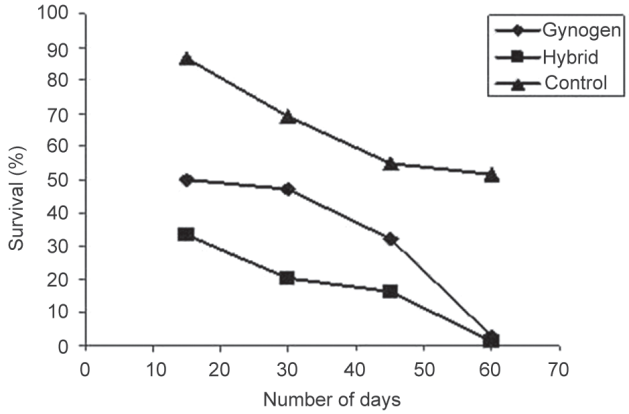

Fig. 2. Survival rate of gynogen, hybrid and control group embryos obtained from *H. fossilis* eggs after fertilization with irradiated sperm of *C. batrachus*

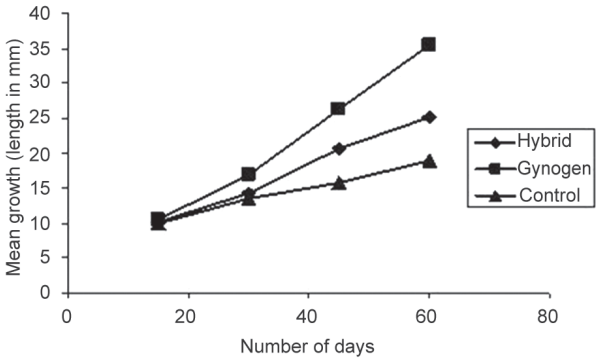

Fig. 3. Mean growth of different groups of *H. fossilis* larvae during a period of 60 days

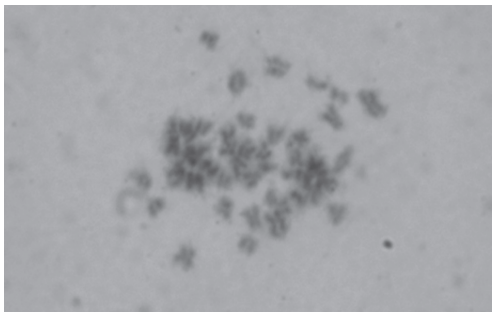

Fig. 4 (a). Metaphase chromosome spreads of *H. fossilis* (control) (2n=56, 100X)

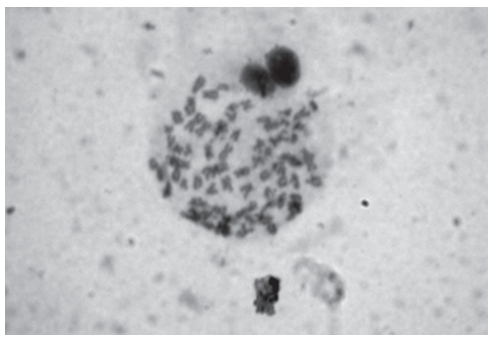

Fig. 4 (b). Metaphase chromosome spread of *H. fossilis* (gynogenetic) (2n=56,100X)

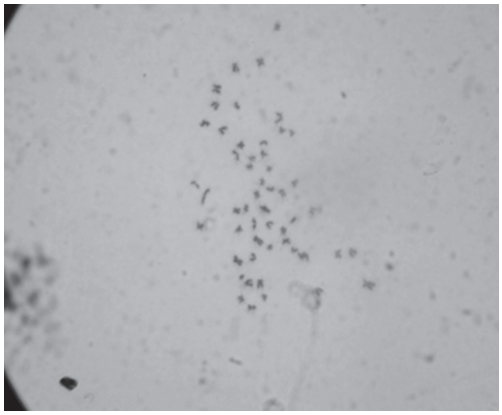

Fig. 4.(c). Metaphase chromosome spreads of hybrid of *H. fossilis* x *C. batrachus* (2n=53, 40X)

Discussion

The sperm of *H. fossilis* recorded good motility followed by the sperm of *C. batrachus*. From the present study, the optimum duration for irradiation was fixed as 60 sec. Since irradiation was done from top and bottom, irradiation of spermatozoa for a short duration of 60 sec was found effective. Horstgen-Schwark (1993) reported slightly longer irradiation duration of 2 min for zebra fish (*Brachydanio rerio*). Varadi *et al*. (1999) carried out irradiation of *Clarias gariepinus* milt in ultra-violet light for 2 to 3 min. Similar to the present study, Karal Marx and Sukumaran (2007) observed irradiation dosage with high motility for 60 sec in *C. gariepinus* spermatozoa.

Hussain and Mazid (2001) used cold shock of 2-6 °C for 10 and 15 min for the induction of gynogenesis in *H. fossilis*. They reported that the treatment at 2 °C for 10 min resulted in a survival of 59-90 % for 15 min and 36-76 % up to a period of three days. Also cold shock at 4 °C for 10 min gave a meager survival of 0-1 % and for 15 min gave 15-28 % survival. In the present study, cold shock at 2 °C for 15 min resulted in a survival of 72.50% and for 30 min resulted in 60 % survival. Cold shock at 4 °C for 15 min and 30 min gave survival rates of 63.16 and 57.43% respectively. Cold shock at 2 °C has given similar results as that of Hussain and Mazid (2001). But the results of cold shock at 4 °C is in contrast to the survival obtained by Hussain and Mazid (2001). In the present study, heat shock at 38 °C for 1, 2 and 3 min resulted in survival of 63.89, 61.76 and 54.17% respectively. Also heat shock at 41 °C for 1, 2 and 3 min gave 71.43, 68.97 and 60 % survival respectively. This indicates the adverse effects of increased temperature as well as duration of exposure on the survival rate.

In the present study, cold shock at 2 °C for 15 min proved to have higher survival than that of heat shock. When the irradiated milt from *H. fossilis* itself was used, it gave better results. Cold shock at 2 °C for 15 min gave the highest fertilization rate of 52 % and hatching rate of 12.82% whereas the fertilization rate was 51.50% and hatching rate 11.65 % for shock at 41 °C for a duration of one minute. This is in agreement to the results of Karal Marx and Sukumaran (2007) who observed that heat shock (39±1 °C) for short duration of a minute was better than two minutes for diploidization in *C. gariepinus*. They also reported that cold shock (3±1 °C) for a duration of 30 min gave better results than 60 min shock for the same species.

When the irradiated (heterologous) milt from *C. batrachus* was used, the results showed the effect of cold shock and heat shock on the fertilized eggs of *H. fossilis* for diploidization. Cold shock at 2 °C for 15 min gave a fertilization rate of 47.50 % and a hatching rate of 15.26 % for the gynogens. Heat shock at 41 °C for 1 min gave a fertilization rate of 47.33 % and hatching rate of 14.93%. Hence cold shock was found to be more effective than heat shock. Significant difference ($p<0.01$) was found between the different temperatures for cold shock. Cold shock at a temperature of 2 °C for 15 min was found to give better results. Karal Marx and Sukumaran (2007) induced meiotic gynogenesis in *C. gariepinus* for 5 min after fertilization. In the present study, the shocks were given 5 min post-activation for diploidization which is in agreement with the shock given in the gynogenesis of *C. gariepinus* by Volckaert *et al*. (1994) and they reported that in general, the most efficient moment to induce retention of the second polar body (Meiosis II) was 3 to 5 min post-activation.

In the present study, the eggs fertilized with *C. batrachus* milt which were not irradiated resulted in the production of hybrids. The hybrids of *H. fossilis* and *C. batrachus* hatched out and showed good survival. This is in contrast to Nam (2001) who reported that the hybrids (female catfish *Silurus asotus* x male mud loach *Misgurnus mizolepis*) did not hatch although the embryonic development of hybrid proceeded until late gastrula stage.

Gynogens produced from the irradiated milt of *H. fossilis* showed a survival of 54.17 to 72.50% up to 72 h. The control group showed a survival of 86.72%. The gynogens produced from irradiated *C. batrachus* milt showed a survival of 42.67 to 60%. The survival rates of gynogens produced with *H. fossilis* male after 60 days showed a survival of 4.12% and those produced with *C. batrachus* male showed a survival of 2.78%. Karal Marx and Sukumaran (2007) reported a survival rate of 9% among the gynogens of *C. gariepinus* after a rearing period of 4 weeks. The eggs fertilized with irradiated milt without diploidization resulted in haploids. Haploids in *H. fossilis* survived hatching stage with hatching rate of 10%. In the present study, haploid embryos showed thick bodies in particular with poorly developed tails and usually with small

under developed eyes. Peter *et al.* (1980) reported that the haploid embryos of *C. carpio* were non-viable. This result is in agreement with the present study in which the haploids hatched out, died in 3 days.

In the present study, the eggs which were fertilized with non-irradiated milt of *C. batrachus* resulted in the production of hybrids. Padhi *et al.* (1995) reported that the survival of hybrids produced (female *H. fossilis* x male *C. batrachus*) got reduced due to high rate of mortality of the hatchlings. Kushwaha *et al*. (2002) studied the karyotype of *H. fossilis* and the diploid chromosome number was found to be between 56 to 58. Similar to this karyological study, the present study also revealed that gynogens had two sets of chromosomes (2n=56). Padhi *et al*. (1995) recorded that the modal diploid chromosome number of hybrid was found to be 2n=53, the average of the chromosomal number in two parental species. Similarly, the chromosomal number of the hybrid (*H. fossilis* x *C. batrachus*) in this study was also found to be 53. For the production of 100% gynogens, the irradiated milt of *C. batrachus* with cold shock at 2 °C for 15 min can be used. Since the females of *H. fossilis* grow faster and larger in size than the males, production of gynogens of this species gains aquaculture importance.

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