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Effect of extender composition on sperm cryopreservation of Asian catfish *Heteropneustes fossilis* (Bloch) and *Clarias batrachus* (Linnaeus)

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Abstract

Air breathing catfish species *Heteropneustes fossilis* (Bloch) and *Clarias batrachus* (Linn.) are important table fish and fetch high market price. Cryopreservation of spermatozoa can be a useful tool in captive seed production for domestication and aquaculture of these catfish species. The objective of the present study was to identify optimum extender composition for sperm cryopreservation of the two species, *H. fossilis* and *C. batrachus*. Four extender compositions Hank's Balanced Salt Solution (HBSS), Modified Hank's Balanced Salt Solution (M-HBSS), Modified Hank's Balanced Salt Solution with hen's egg yolk (M-HBSS with EY) and European catfish were evaluated for cryopreservation of catfish sperm and 10 % Dimethyle Sulphoxide (DMSO) was used as a cryoprotectant. The pooled milt exhibiting 70-80% motile sperm was used for cryopreservation experiment. After storage for 48 hrs at -196ºC, the milt was thawed and evaluated for fertility test. The percentage of hatching was used as a parameter for the comparative evaluation of different extender composition. In *H. fossilis* extender M-HBSS indicated highest hatching rate (49.06%), followed by HBSS (42.76%), M-HBSS with EY (37.46%) and European catfish (29.47%). The hatching success with extender M-HBSS did not differ significantly ($P > 0.05$) from the control group (51%). In *C. batrachus* extender HBSS exhibited highest hatching (62.1 %), followed by M-HBSS with EY (51.6%), European Catfish (46.3%) and M-HBSS (40.9%). The hatching rate in control was 90% in *C. batrachus*. The results indicated that the two species differ in the protocol for sperm cryopreservation. The paper presents successful cryopreservation of sperm with the production of viable hatchlings of *H. fossilis* and *C. batrachus* for the first time. The protocol reported in the study can be used for scaling up of seed production of these two catfish species.

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Introduction

Asian catfishes *Heteropneustes fossilis* (Singhi) and *Clarias batrachus*, commonly known as (Magur) are commercially important air breathing fishes of Southeast Asian countries especially in the Indian subcontinent. These catfishes are economically important and prioritized species for aquaculture and fetch a good price. Cryopreservation is a technique that allows preservation of biological material at a very low temperature, particularly to keep the cells viable. Fish sperm cryopreservation technology has wide application as a powerful *ex-situ* conservation and aquaculture tool to alleviate milt related problem.

Fish sperm cryopreservation protocols are experimented for more than 200 finfish species and have been found to be species specific with respect to the protocol parameters (Rana, 1995). Some of the catfish species for which successful sperm cryopreservation has been reported include *Silurus glanis* (L) (Marian and Krasznai, 1987), Channel catfish *Ictalurus punctatus* (Guest et al., 1976), *Pangasius sutchi* (Withler, 1995), *P.hypophthalmus* (Samorn and Bart, 2003), *P.larnauduei* (Samorn and Bart, 2006). In catfish species, sperm cryopreservation can help to overcome inadequate availability of milt, which is obtained through sacrificing male brood fish. Limited success on the cryopreservation of *H. fossilis* and *C. batrachus* sperm has been reported based on development of embryo up to a few stages of cell division and motility of post thawed preserved sperm (Lakra & Krishna, 1997; Padhi and Mandal, 1995).

However, production of hatchlings is necessary to demonstrate an effective sperm cryopreservation protocol. The present work evaluates the effect of different extender composition through production of viable hatchling to obtain suitable extender composition for sperm cryopreservation for both *H. fossilis* and *C. batrachus*.

Materials and Methods

Live *H. fossilis* and *C. batrachus* specimens were collected from local markets at Lucknow, nearly five months prior to breeding season (June to August). Fishes were maintained in 500 l capacity FRP tank for acclimatization and fed with chopped fish and pelleted prawn feed at 3-5 % of body weight.

Both the fishes are seasonal breeders and developed secondary sexual character during breeding season (June to August). Matured female fish having round and bulging abdomen with reddish coloured bottom and round shaped genital papilla, whereas male having conical elongated genital papilla with a pointed reddish tip. Mature male and female fishes were kept in separate tanks 3-4 days before the experiment. For collection of gametes the fishes were primed by intramuscular Ovaprim (Syndel Laboratory, Canada) injection at 0.6 ml $^{-1}$ Kg body weight (female) and at 0.3 ml^{-1} Kg body weight

(male). After injection male and female fishes were kept in separate tank with showering of water.

The fishes were incubated at 28°C for 15 hrs following the injection. As the males do not readily ooze milt, testes were collected surgically. After removing the extra fat tissue and blood, testes were cut into small pieces and macerated in mortar and pestle with 1: 3.5 ratio of 0.9% NaCl solution (sperm: NaCl) and passed through bolting silk (0.2mm). The pooled milt that exhibited more than 70-80 % motility was cryopreserved in 0.25 cc French medium straws.

Four extenders Hank's Balanced Salt Solution (HBSS, Samorn and Bart 2003), Modified HBSS (M-H BSS, Steven et al. 2006), Modified HBSS with hen's egg yolk (M-HBSS with EY) and European catfish (Linhart et al. 1987) were tested in this experiment with different combination of composition for both the species (Table.1). Sperm diluents were mixed with extender and cryoprotectant in the ratio of 1:3.5:0.5 (sperm: extender: cryoprotectant). DMSO was used as a cryoprotectant @10% v/v final concentration. The sperm suspension diluted with extender and cryoprotectant was filled in 0.25cc french straws and equilibrated over ice for 10 min. followed by liquid nitrogen (LN_2) vapour exposure by horizontal freezing for 10 min and finally plunging in LN_2.

Table 1. Composition of extenders used in cryopreservation of Heteropneustes fossilis and *Clarias batrachus* sperm.

	Extender			
Chemical	HBSS	M-HBSS	M-HBSS With EY	European catfish
NaCl	137mM	137mM	137mM	200.15mM
KCl	5036mM	5036m	5036m	-
$CaCl_2$	1.26mM	1.43mM	1.43mM	-
$NaHCO_3$	0.35mM	0.72mM	0.72mM	-
$MgSO_4$	0.41mM	0.41mM	0.41mM	-
NaH_2PO_4	0.34mM	0.68mM	0.68mM	-
Glucose	5.55mM	5.55mM	5.55mM	-
KH_2PO_4	0.44mM	0.44mM	0.44mM	-
Tris	-	-	-	333 mM
Egg yolk	-	-	2%	-

Eggs were collected from female fishes by stripping method. For this fishes were removed from water, their genital apertures wiped with a dry cloth and then eggs were collected in plastic basins. For fertility experiment, three replications of each extender as well as control were carried out. Good quality (100 μl) eggs were poured in glass petri plates using micropipette and fertilized with two days old cryopreserved sperm for the experiment. For thawing straws were taken out from LN_2 and put in water bath at 37 °C for 15 sec. and then sperm solution were mixed with eggs. Sperm were activated by adding of 50 μl of water. After 2-3 min of mixing the fertilized eggs were washed with well-oxygenated water. Post fertilization of eggs after two hrs, were transferred to a flow through water system for incubation. Hatching occurred after incubation of 20 hrs at 26°C.

All the experiments were carried out in three replicates. Statistical analysis of data was carried using SPSS 12.0 version software. Results were subjected to one-way analysis of variance (ANOVA) at the significance level $p \leq 0.05$ after data was verified for normal distribution of variance and Duncan's multiple range tests.

Results and Discussion

Fertility data indicated that the extender composition has significant affect on the performance of cryopreserved sperm (one way analysis of variance; $P<0.05$). In *H. fossilis* extender M-HBSS exhibited highest hatching percent (49.06±2.67 %), followed by HBSS (42.76±32.0 %), M-HBSS with EY (37.46±24.34 %) and European catfish (29.47±25.61%) respectively. The hatching value with cryopreserved sperm, in M-HBSS extender did not differ significantly ($P<0.05$) from the control value of 51±7.24 % (Fig.1.-a). In *C. batrachus* the extender HBSS exhibited maximum hatching value (62.09±9.74 %), followed by M-HBSS with EY (51.59 ±8.26%), European Catfish (46.31±14.24%) and M-HBSS (40.90±4.54%). The hatching success in *C. batrachus* was significantly lower than control value of 90±5 % ($P < 0.05$) (Fig.2.-b). In both the fish species, addition of egg yolk was not found to exhibit any improved performance of extenders. DMSO has been successfully used as cryoprotectant in another cultivable clarid fish, *Clarias gariepinus* Wayman & Tiersch (2000) and Horvath & Urbanyi (2000). Padhi and mandal (1995) reported sperm cryopreservation of these two species with three different cryodiluents with glycerol as cryoprotectant; however, the embryos did not develop beyond a few cell divisions. Nayak et al. (2006) achieved 10 % fertilization using extender BWW (Biggers et al 1971) and 8% DMSO as cryoprotectant. The present study demonstrated good rate of hatching success (>50%) in the two catfish species, *H. fossilis* and *C. batrachus* using cryopreserved sperm. The comparison of results revealed that optimum extender composition required for the sperm cryopreservation is different in the two species.

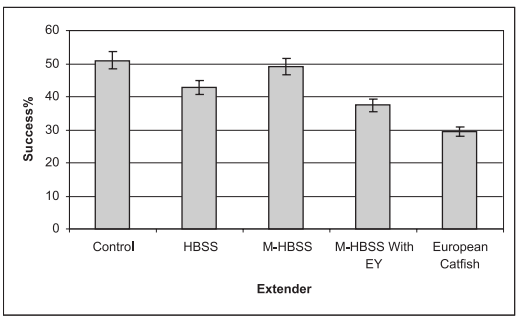

Figure 1. a. *Heteropneustes fossilis*

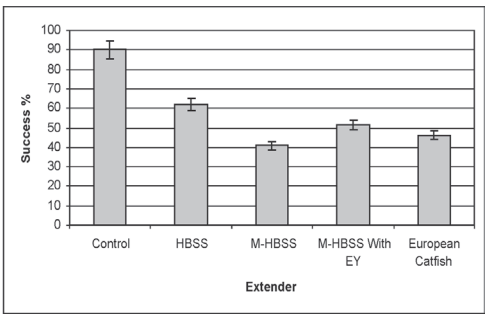

Figure 1. b. *Clarias batrachus*.

Figure 1. Hatching value (mean±sd) obtained with fresh sperm (control) and sperm cryopreserved with 4 different extender compositions a. Heteropneustes fossilis; b. Clarias batrachus

Conclusion

The present paper demonstrated the successful sperm cryopreservation of *Heteropneustes fossilis* and *Clarias batrachus* using M-HBSS and HBSS respectively with DMSO (10% v/v) as cryoprotectant with hatching success of 50 to 60 %.

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