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Cryopreservation of Sperm in Red Tilapia (*Oreochromis niloticus*)

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ABSTRACT

Cryopreservation of fish sperm is a valuable method for restoration of endangered species as well as a technique for manipulation of reproduction for genetic improvement in fish. The objective of this study was to determine the effect of equilibration time, vapour temperature and exposure time on post-thawed sperm motility characteristics in red tilapia. Semen was collected from matured male, diluted using TCAYE extender in French straws (0.25 ml) and stored in liquid nitrogen tank. This research involved a 3 x 4 x 4 factorial experiment consisting of 3 equilibration times (30, 45 or 60 minutes), 4 vapour temperatures (-70, -80, -90 or -100°C) and 4 exposure times (5, 7, 9 or 10 minutes). Sperm movement and velocity distribution after frozen-thawed were evaluated using Automated Semen Analyzer (IVOS, Hamilton-Thorne). The highest percent motility was obtained significantly ($P \leq 0.05$) when red tilapia fish sperm were equilibrated for 60 minutes (63.2±1.9%), vapourized at -80°C (61.2±2.1%) and exposed for 10 minutes (57.6±2.0%). The results from this study on red tilapia fish sperm diluted with TCAYE extender suggested that the optimal percent motilities of sperm could be obtained from combination of 60 minutes equilibration time, -80°C of vapour temperature and 10 minutes of exposure time.

Keywords: Cryopreservation, red tilapia, sperm motility, equilibration, liquid nitrogen vapour.

INTRODUCTION

Since the past few decades, sperm cryopreservation has been developed and widely applied in various species of animals including human. In the fishes, sperm cryopreservation has been used as a method to conserve the endangered species, artificial reproduction and genetic improvement. Sperm cryopreservation has been studied with different protocols in more than 200 fish species, among them are salmon, catfish and carp (Leung and Jamieson, 1991; Tiersch, 2000). Cryopreservation of sperm from genetically superior brood fish can preserve genetic resources.

During the past few years, cryobiological studies focusing on the adaptation of cooling rates to biophysical properties of sperm, changes of sperm packaging systems as well as the accurate and consistent freezing of large numbers of samples have led to the improvement of cryopreservation protocols (Roca *et al.*, 2006). The objective of cryopreservation is to minimize the formation of damaging intracellular ice crystals. In frozen-thawed sperm, the reduction in fertilizing capacity and in motility characteristics have been largely attributed to the alteration of membrane structure and function during the prosesses of cooling, freezing and thawing (Parks and Graham, 1992).

In this study, cryopreservation of red tilapia sperm was carried out in an attempt to develop the suitable technique of cryopreservation. To date, poor success rate and little information on the sperm cryopreservation in fish have been reported in the literature. Cryoinjury during freezing has been the major cause for the ineffective fish cryopreservation method. Therefore, the aim of this study was an attempt to develop protocol for red tilapia fish sperm cryopreservation with a special reference to equilibration time, vapour temperature and exposure time. Red tilapia has been used as a sample because of its extremely rapid growth, tolerance to crowding and resistance to poor water quality.

Generally, the sperm of most fish with external fertilization (such as red tilapia) are motile after release into the aquatic environment, and the motility persist less than 1-2 min (Billard and Cosson, 1992). Adjustment of the various parameters such as equilibration time, vapour temperature and exposure time should minimize the cellular injuries. To enable cryopreservation of fish sperm to become an efficient and routine technique much more detailed information is required. The technical aspects of freezing and thawing sperm and preparing cryopreservation media have been refined over the years. Various methods of cryopreservation have been evaluated for their effects on sperm motility (Taylor *et al.*, 1982; Ragni *et al.*, 1990; Verheyen *et al.*, 1993). However, there is no standard method for cryopreservation that optimize motility recovery. The optimum rate of temperature drop during freezing remains controversial (Ragni *et al.*, 1990; Verheyen *et al.*, 1993; Leffler and Ntlers, 1996).

MATERIALS AND METHODS

Collection of milt

Fresh water fish (red tilapia) was chosen for this project. All the donor males used were at age 5 to 6 months old (which reach their sexual maturity) and at weight 150 to 200g. Milt collection was done by gentle squeezing technique at the male fish abdomen. When the milt was given, pipette was used to collect the milt and it was transferred to 100ml capillary tubes. This technique should be done carefully to avoid any mixing of milt with the urine or waste matter which may reduce the sperm activation. Sometimes, training of fish squeezing is important, to avoid contamination.

Freezing and thawing

Sperm were processed immediately upon arrival to the laboratory to avoid its degradation in quality. After

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accumulation of the sperm, the sperm were diluted 1: 9, semen: extender (1 = 20 microlitre). Eppendorf tube was then shaken slowly to mix the extender and sperm thoroughly.

The diluted sperm was placed in a French straw (0.25ml), letting some air space in between. Each end of the straw was sealed with an electric sealer. Then these straws were arranged on a special rack for further processing. The rack of straw filled with sperm was placed into the freezer at 4^0C for different equilibration time (30, 45 or 60min). Next process reduced the sperm temperature to enable cryoprotectant to seep through the sperm cell.

This is a two stage process. The first stage was done by using nitrogen vapour for different exposure time (5, 7, 9 or 11min) and vapour temperature (-70, -80, -90 or -100 °C). The second stage was when the straw was completely submerge into the liquid nitrogen (-196 ^{0}C) for 10 minutes. The last stage was for storage, which all the sealed straws were placed in the liquid nitrogen tank for long-term sperm preservation.

The last process was done by putting the straws in water bath, at 30°C for 7 seconds. After that, the straws were wiped dry with tissue paper and both sealed end was cut to extract the sperm.

Analysis of sperm

Fresh sperm and post-thawed sperm were analyzed using "Automated Semen Analyzer-IVOS" to evaluate the sperm movement and velocity distribution.

Extender

The extender used was Tris Citric Acid Extender (TCAYE) which formulated in our laboratory as described by Asmad (2005).

Statistical analysis

All data were assessed using the software package SPSS (Statistical Package for Social Science). Mean and Standard Error of the Means (SE) for each traits were estimated. Data were assessed by one-way multifactorial analysis of variance (ANOVA), followed by Duncan's multiple range test.

RESULTS

Comparison of different equilibration times on sperm motility characteristics

Post-thawed sperm motility characteristics in red tilapia were estimated for different equilibration times (30, 45 or 60 min). There were significantly different of motility between 30 to 45 min and 45 to 60 min. (Table 1, Fig. 1).

Comparison of different vapour temperatures on sperm motility characteristics

Post-thawed sperm motility of red tilapia was estimated for different vapour temperatures (-70, -80, -90 or 100˚C). There were significantly different between -70 to -80 or -90˚C and -100 to -80 or -90˚C. (Table 2, Fig.2).

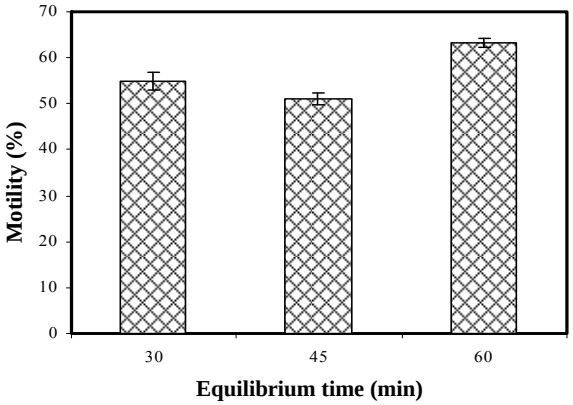

Figure (1): Mean ± SEM of motility at different equilibration times.

Table (1): Mean ± SEM of frozen-thawed sperm characteristics for different equilibration times.

Equilibration time (min)	Sperm movement (%)		Velocity distribution (%)			
	Motility	Progressive	Rapid	Medium	Slow	Static
30	54.97±1.86^a	21.25±0.99^a	25.03±1.17^a	5.84±0.20^a	24.05±1.07^a	45.04±1.86^a
45	50.99±1.23^a	20.99±0.76^a	23.80±0.84^a	6.04±0.23^a	21.37±0.72^a	48.58±1.23^b
60	63.22±0.93^b	24.41±1.08^b	29.02±1.27^b	5.73±0.18^a	28.52±1.16^b	36.78±1.88^b

$^{a, b}$ means with different superscripts in a column were significantly different (P<0.05).

Table (2): Mean ± SEM of frozen-thawed sperm characteristics for different vapour temperatures.

Vapour temperature (ºC)	Sperm movement (%)		Velocity distribution (%)			
	Motility	Progressive	Rapid	Medium	Slow	Static
-70	50.99±1.59^a	19.79±0.89^a	22.99±1.02^a	6.17±0.20	21.85±0.88^a	49.02±1.59
-80	61.24±2.14^b	26.74±1.33^c	31.04±1.53^c	6.24±0.40	24.37±1.33^b	38.24±2.13
-90	59.36±1.51^b	23.22±0.92^b	26.77±1.03^b	5.65±0.22	26.98±0.99^c	40.28±1.49
-100	47.43±2.21^a	17.79±1.06^a	20.79±1.25^a	5.61±0.27	21.02±1.23^a	52.58±2.21

$^{a, b, c}$ means with different superscripts in a column were significantly different (P<0.05)

Comparison of different exposure times on sperm motility characteristics

Post-thawed sperm motility of red tilapia was estimated for different exposure times (5, 7, 9 or 10 min). There were significantly different of motility between 7 to 5 and 10 min. (Table 3, Fig. 3).

DISCUSSION

The mature *Oreochromis niloticus* (red Tilapia) males have been used in this study. A previous study showed that equilibration times of 10-20 min were most commonly used for fish semen (Billard and Zhang, 2001). In this study, the sperm motility of *Oreochromis niloticus* showed the highest motility when equilibrated with 60 min equilibration time (63.22±0.93%). There were significantly different between 60 to 30 or 45 minutes, but no significant different between 30 to 45 min equilibration time. Progressive movement of sperm was shown to have higher percentage when equilibrated under 60min equilibration time. For velocity distribution optimal percentage of rapid movement was also found at 60 min equilibration time.

Temperature changes affect membrane integrity as do water and cryopretectant movement through the membrane, which leads to cell volume and osmolarity changes: these factors result in damages to the different cell compartments (Morshedi *et al.*, 1995). Sperm from *Oreochromis niloticus* showed the highest motility with vapour temperature of -80 or -90˚C (61.24±2.14 or 59.36±1.51% respectively). There were significantly differences between -70 to -80 or -90˚C, and -100 to -80 or -90˚C, but no significant difference between -70 to -100˚C and -80 to -90˚C vapour temperature. From this study there was lower percentage of motility with too low (-70˚C) or too high (-100˚C) vapour temperature. The optimal vapour temperature was found to be between -80 and -90˚C. Progressive movement of sperm was high with vapour temperature of -80 and -90˚C. For velocity distribution optimal percentage of rapid movement was found to be at -80˚C vapour temperature (31.04±1.53%). For fish sperm, optimal reported cooling rates vary from 5 to 45˚C/min for cooling from 5 to -80˚C, but some species show highest post-thawed motility with a combination of different cooling rates (Rana and Gilmour, 1996; Sansone *et al.*, 2002). Exposure to cryoprotectant prior to freezing is an important factor in cryopreservation of sperm in many species. The effects are varied depending on the cryoprotectant, time of exposure, and concentration (Morris, 1981). Exposure time for liquid nitrogen also one of the factors determined in this experiment, the results showed that 5 and 10 min (56.67±1.54 and 57.61±1.96% respectively) exposure time gave optimal motility percentage compared to 7 and 9 min (50.78±1.88 and 54.13±2.17%, respectively). There were significantly differences in motility between 7 to 5 or 10 min. Progressive movements of sperm have

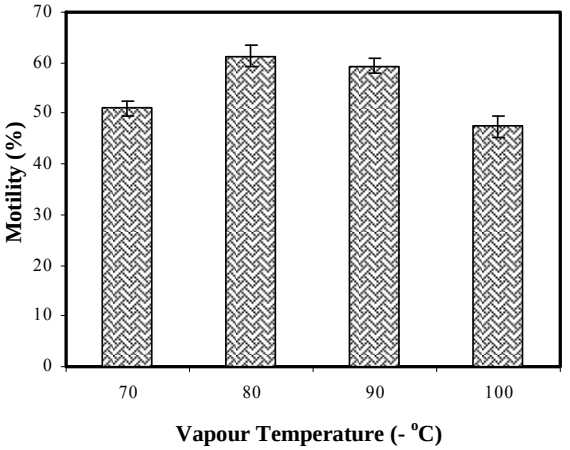

Figure (2): Mean ± SEM of motility at different vapour temperature.

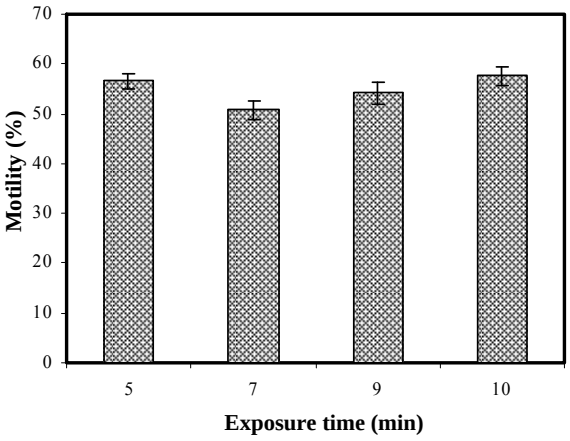

Figure (3): Mean ± SEM of motility at different exposure time.

Table (3): Mean ± SEM of frozen-thawed sperm characteristics for different exposure times.

Exposure time (min)	Sperm movement (%)		Velocity distribution (%)			
	Motility	Progressive	Rapid	Medium	Slow	Static
5	56.67±1.54^{b}	24.26±0.97^{b}	27.57±1.07^{b}	5.74±0.21^{a}	23.34±0.93^{ab}	43.32±1.54^{ab}
7	50.78±1.88^{a}	20.27±1.02^{a}	23.41±1.16^{a}	5.81±0.35^{a}	22.00±1.03^{a}	48.36±1.88^{b}
9	54.13±2.17^{ab}	20.13±1.09^{a}	23.71±1.28^{a}	5.73±0.25^{a}	24.75±1.33^{ab}	45.87±2.17^{ab}
10	57.61±1.96^{b}	21.35±1.11^{ab}	25.45±1.35^{ab}	6.48±0.23^{a}	25.64±1.18^{b}	42.38±1.96^{a}

$^{a, b}$ means with different superscripts in a column were significantly different (P<0.05).

shown that there were high percentages of motility with 5 min exposure time (24.26±0.97%). For velocity distribution optimal percentage of rapid movement also was found to be at 5 min exposure time (27.57±1.07%). Sperm are not adapted to survive cryopreservation, and therefore have variable responses to cooling and rewarming rates depending both on individual male and species (Watson, 1990; Holt, 2000; Thurston *et al.*, 2002).

The results in this study have demonstrated that 60 minutes equilibration time, -80˚C vapour temperature and 5 minutes exposure time gave the highest motility for sperm cryopreservation in red tilapia. These results have shown that vapour temperature is a critical factor compared to equilibration time and exposure time that subsequently affect sperm cryopreservation in red tilapia.

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