2011 2nd International Conference on Agricultural and Animal Science
IPCBEE vol.22 (2011) © (2011) IACSIT Press, Singapore

Development of Cryopreservation Technique from Fresh Sperm Baseline Information of African Catfish (*Clarias gariepinus*)

Wan Khadijah Wan Embong[1 +], Noor Azlina Kamaruding[2] and Ramli Bin Abdullah[1]

[1]Animal Biotechnology-Embryo Laboratory (ABEL), Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, MALAYSIA.

[2]Department of Biological Sciences, Faculty of Science and Technology, Universiti Malaysia Terengganu, 21030 Kuala Terengganu, MALAYSIA.

Abstract. The objectives of this study were: (a) To evaluate fresh sperm motility according to individual body weight (BW) groups of catfish, and (b) To develop sperm freezing technique with an attempt to optimise the equilibration and vapor exposure factors. Two experiments were carried out in this study. In experiment 1, the effect of individual body weight on fresh sperm motility was evaluated in order to form the standard baseline information before sperm freezing procedures proper were carried out. In addition, experiment 2 was done to evaluate effect of combination factors of equilibration duration, vapor temperature and vapor exposure duration on post-thawed cryopreserved sperm motility rate. Methodology of freezing technique is outlined as follows: the straws containing the sperm were placed in refrigerator at 4^{o}C with three equilibration durations (120, 140 or 160 min) after which exposed to liquid nitrogen vapor at three vapor temperatures (-80, -90 or -100^{o}C) with three vapor exposure durations (5, 10 or 15 min). Finally, the straws were directly plunged into liquid nitrogen vapor at -196^{o}C. The frozen sperm were thawed at 30^{o}C for 30 s to evaluate the motility rate using the Automated Semen Analyzer-IVOS (Hamilton Thorne, USA). Result from experiment 1 revealed that large BW of African catfish gave the highest total motility (82.40±4.59%) followed by medium BW (51.64±9.82%) and small BW (40.40±12.16%), whereby small BW fish were significantly different in total motility compared with the other two groups. In experiment 2, the highest values of total motility and progressive motility were obtained from combination factors of 120 min equilibration duration, -100^{o}C vapor temperature and 15 min vapor exposure duration (87.44 ± 2.07% and 28.22 ± 2.16%, respectively). It can be concluded large body weight group obtained the highest total motility and progressive motility. We also successfully developed the freezing technique in combination factors of 120 min equilibration duration, -100 ^{o}C vapor temperature and 15 min exposure vapor duration with 28% was progressively motile out of 87% total motility.

Keywords: Sperm motility, Fresh sperm, Body weight, Equilibration, Vapor exposure

1. Introduction

Fresh sperm motility assessment is a useful step to indicate the success of cryopreservation technique as well as giving the baseline information on the fecundity of African catfish. A considerable amount of literature has been published on factors affecting sperm quality in fish such as rearing photoperiod and temperature, nutrition, water and food contamination, stress, age of broodstocks, breeding season, diseases of broodstocks, hormonal induction and spermiation [1],[2],[3]. Age of broodstocks could be apparently linked with their body weight and plays a major role in sperm maturation period. Until now, there is no study related to effect of body weight on sperm motility characteristics of African catfish.

To date, studies investigate the key parameters of sperm samples (e.g. ionic composition, osmolality [4], development of appropriate activation media, immobilization solutions, cryoprotective agents, equilibration time, cooling rates, sperm packaging unit, semen:extender ratio, storage vessel and thawing rates [5],[6],[7]

[+]Corresponding author. Tel: +60379674374
E-mail address: wkhadi@um.edu.my

have demonstrated a significant achievement in many freshwater fish species. These parameters are invariably different among and within fish species and consideration of interaction factors should be assessed in order to develop successful freezing protocol. Protocols of sperm cryopreservation can vary because of species-specific differences in sperm size, shape, and biochemical characteristics [8]. Development of sperm cryopreservation protocols for African catfish in the present study was no simple task due to the seminal composition of this fish which consists of high lipid content. Therefore, the choice of extender is important to ensure easy solubilisation and absorption into the sperm cells. Freshwater Fish Ringer Extender (FRE) is a diluent used in this study and was a preliminary attempt in our laboratory for sperm cryopreservation of African catfish.

2. Methodology

2.1. Maintenance of Fish Broodstocks

Adult male African catfish, *Clarias gariepinus*, broodstocks that were healthy and sexually mature aged from 1 to 2 years old with body weight in a range of 1 to 2 kg were chosen for the experimental purposes. Routine management of fish includes periodical exchange the water with fresh clean water to ensure easy absorption of oxygen and disease-preventive measure. The tap water was dechlorinated before being supplied to the fish. The broodstocks were hand-fed with "commercial finisher layer mash" twice a day, *ad libitum* and daily monitored.

2.2. Induction of Spermatogenesis

Body weight of selected catfish was weighed to quantify the dosage of hormone per body weight for each individual fish. Ovaprim (0.5 ml/kg body weight; Syndel, Vancouver, Canada) was injected intramuscularly into the dorsal muscle of catfish. Prior to this procedure, the head of the catfish was covered by a wet towel in order to keep it quiet and calm during injection. Most of the fish kept still if their eyes were covered. After receiving the hormone treatment, these males were isolated for overnight in a separate tank to avoid aggressive interaction with other males and to maximise care during the experimental period.

2.3. Experimental Designs

2.3.1. Experiment 1: Effect of Individual Body Weight on Fresh Sperm Motility Characteristics in African Catfish

A total of 15 individual fish was weighed to get an actual body weight. The actual body weight was used to categorise three respective sizes, namely small (<1.0 kg), medium (1.0 -1.5 kg) and large (>1.5 kg). The testis of the sacrificed male African catfish was dissected out from the body cavity and the testis was cleaned with tap water to rinse the blood. Then, the testis was gently perforated with needle to collect the milt. Precaution during perforated of testis has to take into account to avoid the needle pierce into the capillary. The milt collected was diluted with diluents in a ratio of 1:10 to facilitate analysis of sperm motility. Without dilution, the analysis of sperm using IVOS was difficult because the sperm was too concentrated. Total motility and progressive motility were used for assessing survivability rate of pre-freezing semen using an automated semen analyzer (IVOS; Hamilton-Thorne, USA).

2.3.2. Experiment 2: Effects of Combination Factors of Equilibration Duration, Vapor Temperature and Exposure Vapor duration on Post-Thawed Cryopreserved Sperm of African Catfish

Semen was collected from testis of sacrificed catfish, diluted with Fish-Ringer Extender consisting of NaCl (0.75 g), KCl (0.10 g), $CaCl_2$ (0.016 g), $MgSO_4$ (0.023 g), NaH_2PO_4 (0.041 g) and Glucose (0.10 g) per 100 ml, in a ratio of 1:10 using 0.5 ml or 0.25 ml French straws. The straws containing the diluted semen were subjected to freezing process. This research involved a 3 x 3 x 3 factorial experiment consisting of 3 equilibration durations (120, 140 or 160 minutes), 3 vapor temperatures (-80, -90 or -100^{o}C) and 3 vapor exposure durations (5, 10 or 15 minutes). The molarity of cryoprotectant was fixed at 10% DMSO as described by [9]. Each of the combination treatments was replicated 3 times with 5 observations per replicate. Total motility and progressive motility were used for assessing survivability rate of post-thawed cryopreserved sperm using an automated semen analyzer (IVOS; Hamilton-Thorne, USA).

2.4. Statistical Analysis

All the data were subjected to analysis of variance (ANOVA), followed by comparison of means using Duncan's multiple range test (DMRT). Data analysis was performed by SPSS (Statistical Package for Social Sciences) for windows, version 12.0. The data was presented as mean±SEM which significant at ($P<0.05$).

3. Results and Discussion

Large BW of African catfish gave the highest total motility (82.40±4.59%) followed by medium BW (51.64±9.82%) and small BW (40.40±12.16%), whereby small BW fish were significantly different in total motility compared with the other two groups. Progressive motility values for small, medium and large BW of fish were 8.20±3.65%, 14.00±4.29% and 17.40±3.36%, respectively ($P>0.05$) (Table 1).

Table 1: Assessment of fresh sperm motility of African catfish in different body weight groups

	Body size (BW, kg)		
	Small (< 1.0) N=5	Medium (1.0-1.5) N=14	Large (>1.5) N=5
Total motility (%)	40.40±12.16[a]	51.64±9.82[ab]	82.40±4.59[b]
Progressive motility (%)	8.20±3.65[a]	14.00±4.29[a]	17.40±3.36[a]

N* = Number of fish.
[ab]Means with different superscripts within a row were significantly different ($P<0.05$).

Table 2 shows total motility and progressive motility of post-thawed cryopreserved sperm of African catfish. The highest values of total motility and progressive motility were obtained from combination factors of 120 min equilibration duration, -100°C vapor temperature and 15 min vapor exposure duration (87.44 ± 2.07% and 28.22 ± 2.16%, respectively). Combination of 140 min, -100°C and 10 min showed the lowest values of total motility (59.27 ± 8.00%), but the lowest value of progressive motility was observed at combination of 160 min, -90°C and 10 min (12.20 ± 2.24%).

Fish Ringer Extender (FRE) found to be suitable diluent for African catfish based on this study, as it maintains inactivity of sperm when semen is diluted before freezing due to stabilization of physicochemical properties [10]. Extenders have been developed using saline- and sugar-based diluents [11],[12]. Exposure to cryoprotectant prior to freezing is another important parameter in cryopreservation of sperm from many species, and effect will vary depending on the cryoprotectant, duration of exposure and concentration [13]. Increased exposure to cryoprotectants can improve the cryoprotective effect, but can also result in increased toxicity to the sperm cells [14],[15]. Besides advantages, cryoprotectants have disadvantage as it can induce protein denaturation at higher temperature and cause cellular toxicity at cellular systems. It has long been recognized that exposure to cryoprotectants can cause damage to the cells and tissues during equilibration prior to freezing due to their toxicity [16]. The apparent toxicity of cryoprotectant is dependent on type and concentration of cryoprotectant, the equilibration duration and the temperature during loading [17]. Several cryoprotectants have been reported to be toxic on sperm and embryos of the fish. For instance, sodium citrate is known to be harmful to the structural and integrity of some fish sperm [18], while propylene glycol and ethylene glycol are reported to be more toxic than DMSO in oyster embryos [19]. However, high levels of DMSO can be toxic to fish sperm compared with other commonly used cryoprotectants such as methanol, ethanol and glycerol [20]. Several methods of freezing can be performed such as programmable temperature changes or simple immersion in liquid nitrogen vapor above the surface of liquid nitrogen [21]. For African catfish, we applied two steps freezing procedure by allowing reduction of temperature slowly with time. As a result, we found the sperm motility rate fluctuates with combination of equilibration and vapor exposure factors due to multiple steps and their interactions, some errors occur at each step can accumulate and lead to considerable losses of viable cells. Thus, careful attention should be given to the numerous details at each step, and care should be taken to reduce or eliminate sources of uncontrolled variation [22].

Table 2: Total motility and progressive motility (mean ± S.E.M.) of post-thawed cryopreserved sperm of African catfish (*Clarias gariepinus*) using 10% DMSO in FRE extender for combination of equilibration duration, vapor temperature and vapor exposure duration.

Equilibration duration (min)	Vapor temperature (^{o}C)	Vapor exposure duration (min)	N*	Total motility (%)	Progressive motility (%)
120	-80	5	15	74.00 ± 4.69^{abc}	20.00 ± 2.59^{abc}
		10	15	62.73 ± 6.95^{ab}	12.80 ± 2.49^{a}
		15	13	59.92 ± 9.11^{a}	15.77 ± 3.63^{a}
	-90	5	13	71.38 ± 9.76^{abc}	15.62 ± 2.54^{a}
		10	15	80.80 ± 4.54^{abc}	19.93 ± 2.73^{abc}
		15	15	72.60 ± 7.45^{abc}	19.93 ± 2.71^{abc}
	-100	5	20	84.00 ± 2.86^{bc}	25.95 ± 2.36^{bc}
		10	20	80.95 ± 3.71^{abc}	25.95 ± 2.74^{bc}
		15	18	87.44 ± 2.07^{c}	28.22 ± 2.16^{c}
140	-80	5	7	62.86 ± 7.74^{ab}	14.00 ± 3.51^{a}
		10	6	59.67 ± 13.56^{a}	16.00 ± 6.54^{a}
		15	7	78.29 ± 7.51^{abc}	19.57 ± 3.72^{abc}
	-90	5	15	73.73 ± 5.17^{abc}	18.73 ± 2.83^{ab}
		10	15	68.00 ± 5.89^{abc}	16.13 ± 2.35^{a}
		15	18	76.33 ± 4.59^{abc}	20.00 ± 2.18^{abc}
	-100	5	11	61.27 ± 8.10^{a}	13.27 ± 3.21^{a}
		10	15	59.27 ± 8.00^{a}	13.53 ± 2.87^{a}
		15	10	60.80 ± 9.43^{a}	13.80 ± 3.21^{a}
160	-80	5	14	76.07 ± 5.57^{abc}	15.64 ± 2.67^{a}
		10	12	78.75 ± 6.04^{abc}	20.08 ± 2.74^{abc}
		15	12	73.75 ± 4.76^{abc}	16.08 ± 2.82^{a}
	-90	5	13	74.15 ± 6.40^{abc}	16.08 ± 1.95^{a}
		10	15	60.20 ± 7.12^{a}	12.20 ± 2.24^{a}
		15	19	73.21 ± 4.50^{abc}	14.05 ± 2.23^{a}
	-100	5	20	71.45 ± 6.69^{abc}	18.60 ± 2.66^{ab}
		10	19	80.00 ± 3.65^{abc}	20.16 ± 2.04^{abc}
		15	20	73.75 ± 4.86^{abc}	17.80 ± 2.00^{ab}

N* = Total number of observations (straws).
^{abc}Means with different superscripts within a column were significantly different (P<0.05).

4. Acknowledgements

We would like to express our appreciation and gratitude to ABEL members and staff of Institute of Biological Sciences Mini Farm (Livestock), who helped directly or indirectly in this project. This project was financially supported by PPP Research Grant (P0060 / 2008A), University of Malaya.

5. References

[1] J. Bobe, and C. Labbe. Egg and sperm quality in fish. *General and Comparative Endocrinology*. 2009, 1-14.

[2] S. M. H. Alavi, M. Rodina, T. Policar, and O. Linhart. Relationship between semen characteristics and body size in *Barbus barbus* L. (Teleostei : Cyprinidae) and effects of ions and osmolality on sperm motility. *Comparative Biology and Physiology*. 2009,153: 430-437.

[3] D. E. Kime, K.J.W. Van Look, B.G. McAllister, G. Huyskens, E. Rurangwa, and F. Ollevier. Computer-assisted sperm analysis (CASA) as a tool for monitoring sperm quality in fish. *Comparative Biochemistry and Physiology*. 2001, 130: 425– 433.

[4] S. M. H. Alavi, and J. Cosson. Sperm motility in fishes. (II) Effects of ions and osmolality: a review. *Cell Biology International.* 2006, 30: 1–14.

[5] T. R. Tiersch. Introduction. In: T.R. Tiersch et al (eds.). Cryopreservation in Aquatic Species, World Aquaculture Society, Baton Rouge, LA, 2000, pp. xix–xxvi.

[6] A. P. Scott, and S. M. Baynes. A review of the biology, handling and storage of salmonid spermatozoa. *Journal of Fish Biology*. 1980, 17: 707–739.

[7] M. Suquet, C. Dreanno, C. Fauvel, J. Cosson, and R. Billard, Cryopreservation of sperm in marine fish. *Aquaculture Resources*. 2000, 31: 231–243.

[8] H. Yang, and T.R. Tiersch. Current status of sperm cryopreservation in biomedical research fish models: zebrafish, medaka, and xiphophorus. *Comparative Biochemistry and Physiology*, Part C. 2009, 149: 224-232

[9] N. Basavaraja, and S.N. Hedge. Cryopreservation of the endangered mahseer (*Tor khudree*) sperm: Effect of extender composition, cryoprotectants, dilution ratio and storage period on post-thaw viability. *Cryobiology*. 2004, 49: 149-156.

[10] H. Ohta, and T. Izawa. Diluent for cold storage of the Japanese eel (*Anguilla japonica*) spermatozoa. *Aquaculture*. 1996, 142:107–118.

[11] Z. Yao, L. Crim, G.F. Richardson, and E.J. Emerson. Cryopreservation, motility and ultra structure of sperm from the ocean pout *Macrozoarces americanus* L., an internally fertilizing marine teleost. In: F.W. Goetz et al (eds.), Proceedings of the Fifth International Symposium on Reproductive Physiology of Fish. Austin, TX. 1995, p. 149.

[12] B. Urbanyi, A. Horvath, Z. Varga, L. Horvath, I. Magyary, and F. Radics. Effect of extenders on sperm cryopreservation of African catfish. *Clarias gariepinus* (Burchell, 1822). *Aquaculture Resources*. 1999, 30:145–151.

[13] G. J. Morris. Cryopreservation: an introduction to cryopreservation in culture collections. Cambridge (England): Institute of Terrstrial Ecology, 1981.

[14] B. G. M. Jamieson. Fish evolution and systematics from sperm. Cambridge University Press. Cambridge. 1991, pp. 319.

[15] J. M. Christensen, and T.R. Tiersch. Cryopreservation of channel catfish sperm: effect of cryoprotectant, straw size and extender formulation. *Theriogenology*. 1996, 47: 639-645.

[16] G. M. Fahy. The relevance of cryoprotectant toxicity to cryobiology. *Cryobiology*. 1986, 23: 1-13.

[17] N. H. Chao, and I.C. Liao. Cryopreservation of finfish and shellfish gametes and embryos. *Aquaculture*. 2001, 197: 161-189.

[18] J. C. Gwo, and C.R. Arnold. Cryopreservation of Atlantic croaker sperm: evaluation of morphological changes. *Journal of Experimental Zoology*. 1992, 264: 444-453.

[19] N. H. Chao, C.P. Chiang, H.W. Hsu, C.T. Tsai, and T.T. Lin. Toxicity tolerance of oyster embryos to selected cryoprotectants. *Aquatic Living Resources*. 1994, 7: 99–104.

[20] F. P. Simione. Cryopreservation manual. Nalge Nunc International Corporation. New York. 1998, pp. 8.

[21] H. Irawan, V. Vuthiphandchai and S. Nimrat. The effect of extenders, cryoprotectants and cryopreservation methods on common carp (*Cyprinus carpio*) sperm. *Animal Reproduction Science*. 2010, 122: 236-243.

[22] Leibo, S. P. Sources of variation in cryopreservation. In: T.R. Tiersch et al (eds.). Cryopreservation in aquatic Species. World Aquaculture Society, Baton Rouge, Louisiana. 2000, pp. 75–83.