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Cryopreservation of Sperm in Common Carp (*Cyprinus Carpio*) and Its Strain Amur Carp (*Cyprinus Carpio Haematopterus*): Sperm Motility and Hatching Success of Embryos

Adita Sharma[1*], R. N. Ram[1], Shiv Prasad[2]

[1]Department of Fisheries Resource Management, College of Fisheries, G. B. Pant Univ. of Agri. & Tech, Pantnagar

[2]Department of Veterinary Gynecology and Obstetrics, College of Vet. Sci., G. B. Pant Univ. of Agri. & Tech, Pantnagar

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ABSTRACT

The present study was conducted for comparing the performance of cryopreserved sperm of Common carp (*Cyprinus carpio*) with its Amur strain (*Cyprinus carpio haematopterus*) in monsoon season (MS) and post monsoon season (PMS). The parameters used for milt quality analysis are sperm motility percentage and motility duration. Fertilization rate, hatching rate and embryonic development were also recorded in monsoon and post monsoon season. Results of this experiment are indicative of successful cryopreservation of common carp and Amur carp sperms either by using TRIS+DMSO+Glucose or RPMI+DMSO or PBS+DMSO. In Amur carp comparison of three diluent combinations, maximum motility, fertilization rate and hatching rate were obtained with TRIS+DMSO+Glucose in both the seasons. In case of common carp, the higher percentages of viability and fertilization rate were observed in PBS+ DMSO during MS and PMS. On the premise of this test, it can be concluded that TRIS+DMSO+Glucose is the best suited combination for freezing Amur carp sperm. On the other hand, PBS+DMSO is quality combination for freezing of common carp sperm. The overall result of the present study revealed that in comparison between Amur carp and common carp, best embryonic development were found in Amur carp indicating that Amur carp performed well than common carp. It indicating that reproduction capacity of Amur carp is higher than common carp. So, if Amur carp is cultured on large scale, a high profit will be obtained which will augment fish production. In this way food security may be ensured across the globe by reducing food crisis.

1. Introduction

Common carp is the most important cultured fish species in India, consisting 80–85% of the average annual yield. The fish has acclimatized to a wide range of environmental circumstances and habitats and consequently is a preferred choice in world-wide cultivation purposes (Sultana *et al*., 2010). It has significant contribution in inland fish production. To keep the production cost minimum, hatchery owners in India maintain limited number of broods to minimize effective breeding numbers (Lip *et al*., 2013). The Amur wild carp is an ancient form that originated from the Asian carp centre (Amur-China type of wild carp, *Cyprinus carpio haematopterus*) and spread to the water bodies of Western Asia. However, cryopreservation of its sperm is not used in the hatchery practice in spite of its advantages. The currently existing methods either produce unsatisfactory fertilization and hatching results (Babiak *et al*., 1997) or they describe the fertilization of minute volumes of eggs: 200–800 eggs altogether (Linhart *et al*., 2000). They allow the utilization of cryopreservation in frozen gene banks but they are impractical for the hatchery practice as it involves the fertilization of large volumes of eggs at the same time.

*Corresponding author: sharmaadita1989@gmail.com

Moreover, only Dimethyl-sulfoxide (DMSO) has been extensively studied as a cryoprotectant for common carp sperm. Therefore, one of our objectives was to investigate the effect of three very simple basic extenders and two cryoprotectants on the motility and fertilizing ability of common carp and Amur carp sperm in two different seasons *i.e.* Monsoon and Post- Monsoon season.

2. Materials and Methods

For the present study, sexually mature males of common carp and Amur carp (2-2.5 kg) of body weight were chosen randomly from College of Fisheries. Males were examined to determine the sexual maturity by the presence of milt in the genital papilla after a light pressure of the abdomen. Only sperm samples showing more than 40% motility were used for cryopreservation. Diluents for the milt consisted of an extender plus a cryoprotectant in the v/v ratio 9 parts extender: 1 part cryoprotectant. The milt: extender was maintained at a ratio of 1:4. Carp brood stocks will also be maintained in indoor tanks supplied with a continuous flow of tap water. TRIS, RPMI and PBS were used as diluents and DMSO and glucose were taken as cryoprotectant. First we performed the dose approximation on common and amur carp for standardization of diluents concentration in second week of august (mid breeding season). Twelve combinations of extender and cryoprotectant were prepared. These combinations were Milt+ (TRIS + DMSO), Milt+ (TRIS + Glucose), Milt+(TRIS+DMSO+Glucose), Milt+(TRIS), Milt+(RPMI 1640+DMSO), Milt+(RPMI 1640+Glucose), Milt +(RPMI1640+DMSO+Glucose), Milt+(RPMI 1640), Milt +(PBS+DMSO), Milt+(PBS+Glucose), Milt+(PBS+ DMSO +Glucose) and Milt+(PBS). Out of twelve combinations only three group were selected that were TRIS+ DMSO+Glucose, PBS+DMSO and RPMI+DMSO. So, only these groups were used for further study.

Freshly collected milt mixed with different diluents were discharged into polyvinyl straws. The straws were immediately sealed from open end, wiped and immediately transferred into biological freezer. The biological freezer holding the straws after allowing varying equilibration period were frozen by exposing them to liquid nitrogen vapour at about the surface of liquid nitrogen for 2-5 minutes, after which the straws were immersed and frozen at -196°C. Fertility of preserved milt was tested using evoluted eggs stripped from hypothesized females. Eggs were collected from selected spawners by dry stripping. Frozen milt was thawed by swirling straws in tap water (30°C) and sperm tested for its fertilizing ability were applied immediately to fresh ova in a 250 ml beaker. Milt and ova were mixed by stirring with a feather. Tap water was added immediately after the milt was added. The ova were poured in large-sized beakers and kept under the running tap. Sample of ova were withdrawn at intervals and examined under a low power microscope.

3. Statistical Analysis

All values were showed as mean±S.D. The effects of different extenders, cryoprotectants and their combinations on post thaw motility parameters in different seasons were analyzed using two factor ANOVA of MSTAT followed by Duncan's Multiple Range test at 5% level of significance. Student's t-test was applied to compare the fertilization and hatching rate of carp for different diluents in two seasons. All statistical examinations were performed at $P < 0.05$ considered significant.

4. Results and Discussion

4.1 Effect of diluents on frozen semen

Various factors which affect the post thaw motility of the spermatozoa of common carp and Amur carp during cryopreservation were studied. In addition to the viability researches through the post thaw motility of spermatozoa, the fertilization and hatching rate obtained with cryopreserved spermatozoa for the two carp were also evaluated. Impact of different concentrations of extenders and cryoprotectants on post thaw motility percentage of spermatozoa of common carp and Amur carp in monsoon season (MS) and post-monsoon season (PMS) are given in table 1 and 2. Among the different seasons and different diluents maximum motility (87.68±1.6%) was obtained in Amur carp in MS and 80.36±0.6% in PMS. In case of common carp, motility percentage was 73.14±1.2% in MS and 60.67±0.2% in PMS.
In case of Amur carp, longest period of motility of sperm frozen with Tris+Glucose+DMSO was 104.67±0.9 seconds in MS and 98.02±0.5 seconds in PMS, while in common carp, maximum motility period with PBS+DMSO in MS and PMS (84.98±0.6 seconds and 87.12±0.8 seconds respectively). These values were substantially different from that of sperm frozen with other diluents ($p<0.05$). In case of Amur carp, total sperm count recorded was maximum in Tris+Glucose+DMSO 55.68±1.9x10^9/ml (maximum motile sperm =53.79±1.8 x10^9/ml) in MS and 50.68±0.2x10^9/ml (maximum motile sperm 48.96±0.5x10^9/ml) in PMS, PBS+DMSO shows maximum non-motile sperm *i.e.* 2.71±0.3x10^9/ml in MS. Tris+Glucose+DMSO shows maximum non-motile sperm *i.e.* 1.72±0.3x10^9/ml in PMS.

Table 1. Post thaw sperm motility percentage, motility duration, total sperm count, motile and non-motile sperm, fertilization and hatching percentage in response to different combinations of extenders and cryoprotectants of common carp and Amur carp in monsoon seasons.

S. No.	Extenders with cryoprotectants	monsoon season													
		common carp							Amur carp						
		Motility percentage (%)	Motility duration (Seconds)	Total sperm count ($x10^9$/ml)	Motile sperm ($x10^9$/ml)	Non-motile sperm ($x10^9$/ml)	Fertilization Rate (%)	Hatching Rate (%)	Motility percentage (%)	Motility duration (Sec)	Total sperm count ($x10^9$/ml)	Motile sperm ($x10^9$/ml)	Non-motile sperm ($x10^9$/ml)	Fertilization Rate (%)	Hatching Rate (%)
1	TRIS + DMSO + Glucose (1:5:5)	62.66 ±0.8	80.78 ±0.8	25.66 ±0.8	23.84 ±0.7	1.82 ±0.1	54.26 ±1.0	51.52 ±0.6	87.68 ±1.6	104.67 ±0.9	55.68 ±1.9	53.79 ±1.8	1.89 ±0.1	78.15 ±0.8	69.15 ±0.8
2	RPMI1640+ DMSO (1:10)	55.28 ±0.9	77.89 ±0.7	27.46 ±0.7	25.12 ±0.8	2.34 ±0.1	50.48 ±1.2	47.69 ±0.6	83.16 ±0.8	98.76 ±0.9	50.86 ±0.2	49.87 ±0.8	0.99 ±0.6	71.68 ±0.9	65.89 ±0.2
3	PBS + DMSO (1:10)	73.14 ±1.2	84.98 ±0.6	25.98 ±0.2	24.11 ±0.9	1.87 ±0.6	66.57 ±0.9	62.69 ±0.8	80.67 ±0.7	97.86 ±0.8	48.72 ±0.9	46.01 ±0.6	2.71 ±0.3	70.63 ±0.2	63.56 ±0.3

Table 2. Post thaw sperm motility percentage, motility duration, total sperm count, motile and non-motile sperm, fertilization and hatching percentage in response to different combinations of extenders and cryoprotectants of common carp and Amur carp in post-monsoon seasons.

S. No.	Extenders with cryoprotectants	Post-monsoon season													
		common carp							Amur carp						
		Motility percentage (%)	Motility duration (Seconds)	Total sperm count ($x10^9$/ml)	Motile sperm ($x10^9$/ml)	Non-motile sperm ($x10^9$/ml)	Fertilization Rate (%)	Hatching Rate (%)	Motility percentage (%)	Motility duration (Sec)	Total sperm count ($x10^9$/ml)	Motile sperm ($x10^9$/ml)	Non-motile sperm ($x10^9$/ml)	Fertilization Rate (%)	Hatching Rate (%)
1	TRIS + DMSO + Glucose (1:5:5)	55.98 ±0.8	82.98 ±0.7	21.08 ±0.8	19.35 ±1.2	1.73 ±0.4	43.42 ±0.6	36.51 ±0.9	80.36 ±0.6	98.02 ±0.5	50.68 ±0.2	48.96 ±0.5	1.72 ±0.3	74.82 ±0.8	68.49 ±0.2
2	RPMI1640+ DMSO (1:10)	50.79 ±0.9	80.28 ±0.8	18.66 ±0.9	17.11 ±0.8	1.55 ±0.1	40.62 ±0.5	31.06 ±0.8	75.71 ±0.7	95.11 ±0.5	45.89 ±1.2	44.89 ±0.8	1.00 ±1.4	69.66 ±0.2	65.12 ±0.8
3	PBS + DMSO (1:10)	60.67 ±0.2	87.12 ±0.8	24.89 ±1.8	22.86 ±0.1	2.03 ±1.7	52.89 ±0.3	42.68 ±0.9	70.82 ±0.5	90.89 ±0.2	41.10 ±0.5	40.01 ±0.1	1.09 ±0.4	61.89 ±0.8	56.02 ±0.9

Total sperm count was minimum in PBS+DMSO 48.72±0.9x10^9/ml (minimal motile sperm=46.01±0.6x10^9/ml) in MS and 41.10±0.5x10^9/ml (minimum motile sperm=40.01±0.1x10^9/ml motile sperm) in PMS. Minimum non-motile sperm was 0.99±0.6x10^9/ml in RPMI+DMSO (MS) and 1.00±0.4x10^9/ml (PMS). The distinction amongst diluents was significant ($p<0.05$). In case of common carp, total sperm count recorded was maximum in RPMI+DMSO 27.46±0.7x10^9/ml and maximum motile sperm =25.12 ±0.8x10^9/ml in MS. PBS+DMSO 24.89±1.8x10^9/ml (maximum motile sperm 22.86±0.1x10^9/ml) in PMS, RPMI+DMSO shows maximum non-motile sperm *i.e.* 2.34±0.1x10^9/ml in MS and PBS+DMSO shows maximum non-motile sperm *i.e.* 2.03±1.7x10^9/ml in PMS. Total sperm count was minimum in Tris+DMSO+Glucose 25.66±0.8x10^9/ml (minimum motile sperm=23.84±0.7x 10^9 /ml) in MS and in RPMI+DMSO is 18.66±0.9x10^9/ml (minimum motile sperm=17.11±0.8x10^9/ml motile sperm) in PMS. Minimum non-motile sperm was 1.82±0.1x10^9/ml in PMS.

Minimum non-motile sperm was 1.82±0.1x10^9/ml in Tris+DMSO+Glucose (MS) and RPMI+DMSO shows 1.55±0.1x10^9/ml (PMS). The differences among diluents and among species were significant ($p<0.05$). The maximum motility period of frozen Amur carp and common carp sperm were 104.67±0.9 seconds and 84.98±0.7 seconds (MS) and 98.02±0.5 seconds and 87.12±0.8 seconds (PMS) respectively. Longest motility period observed was 104 seconds and after that all sperm were found immotile. The motility of carp spermatozoa ranged from 80 to 110 seconds in different species and maximum duration of spermatozoa motility in cyprinids is up to 120 seconds (Suzuki, 1959). Fish sperm concentration, which is a significant parameter in hatchery reproduction management, is highly variable and this depends on species, individual, fish size and season (Glogowski *et al*., 2002). The total sperm count, motile sperm and non-motile sperm has direct correlation in fishes (Routray *et al*., 2006). The maximum value of sperm count was 55.68±1.9x10^9/ml (MS) and 50.68±0.2x10^9/ml (PMS) in Amur carp and 27.46±0.7x10^9/ml (MS) and 24.89±1.8x 10^9/ml (PMS) in common carp. In other Cyprinids (Indian major carps), the sperm count between 2.6x10^{10}/ml to 3.5x10^{10}/ml in six species of carps and reported wide variation within the spermatocrit and sperm count in all the six carp species (Verma *et al*., 2009). They attributed this is due to the spermatozoa size and species-specific nature of carps. In the present study, the sperm count values are similar than other cyprinids and this might an improvement in fertility rate and hatching rate of this species.

3.2 Effects of frozen sperm on fertilization and hatching capacity

Fertilization rate and hatching rate of Amur carp eggs ranged between 70.63±0.2 to 78.15±0.8% and 63.56±0.3 to 69.15± 0.8% in MS, whereas during the PMS these rates were lower 61.89±0.8 to 74.82±0.8% and 56.02±0.9 to 68.49±0.2% respectively. Eggs of MS, Amur carp showed significantly higher percent of fertilization rate and hatching rate ($P<0.05$) than PMS eggs. Common carp showed the same trend with the fertilization and hatching of 50.48±1.2 to 66.57±0.9% and 47.69±0.6 to 62.69±0.7% were determined in MS and 40.62±0.5 to 52.89±0.3% and 31.06±0.8 to 42.68±0.9% in PMS respectively. Among the different diluents, maximum motility, fertilization rate and hatching rate were obtained in Amur carp with Tris+DMSO+Glucose in both the seasons. In case of common carp, the higher percentage of viability and fertility rate were observed in PBS+ DMSO with respect to MS and PMS.

Long-time cryopreservation of milt will be successful after conducting in fertility trial with cryopreserved milt. From this fertility trial scientist can find the best extender and cryoprotectant combination for cryopreservation. In the present study, three extender mixtures (Tris, RPMI 1640 (SIGMA) culture medium in 0.9% NaCl solution and Phosphate buffer saline) with cryoprotectants (DMSO and Glucose) were used for milt cryopreservation of Amur carp and common carp. The fertilization rate of common carp ranged from 43.42±0.6% to 54.26±1.0% in Tris+ DMSO +Glucose, 40.62±0.5% to 50.48±1.2% in RPMI+DMSO and 52.89±0.3% to 66.57±0.9% in PBS+ DMSO. There is higher fertilization rate in PBS+ DMSO. The fertilization rate of Amur carp ranged from 74.82±0.8% to 78.15±0.8% in Tris+ DMSO+Glucose, 69.66±0.2% to 71.68±0.9% in RPMI+ DMSO and 61.89±0.8% to 70.63±0.2% in PBS+DMSO. There is higher fertilization rate in Tris+DMSO+Glucose combination. Akcay *et al.* (2004) found that fertilization rate was subjected to Student 't' test and it was observed that all extender combination significantly differed. But, fertilization rate using Tris+DMSO+Glucose and PBS+DMSO differed significantly from other groups. This extender combination slightly differed from other extender combinations in having glucose and DMSO. Simple sugar based extenders had been examined on common carp sperm (Babiak *et al*., 1997) however met with little success. Fructose and glucose have been suggested as additives of different extenders for the freezing of sperm of different cyprinid species (Kumar, 1988; Zhang and Liu, 1991). In the present study, also glucose and DMSO based diluent gave better result. Sugar extenders were

successfully used for the cryopreservation of sperm of numerous other fish species including African catfish (Urbanyi *et al*., 1999). In the present study, it is found that Tris+DMSO+Glucose and PBS+ DMSO were more efficient diluents group for Amur carp and common carp sperm respectively. The experimentation concludes that observations are indicative of successful cryopreservation of common carp and Amur carp sperm either by using Tris+DMSO+Glucose and RPMI+DMSO and PBS+ DMSO on these species. On the basis of this experiment, it is concluded that Tris+DMSO+Glucose is rated as best combination for freezing Amur carp sperm. Alternatively, PBS+DMSO are best combination for freezing of common carp sperm. With the current state of demand for Amur carp and common carp seed for aquaculture in India, hatcheries need statistics on the sperm biology of artificially induced broods to enhance the current practice. Present research represents the first successful cryopreservation of Amur carp sperm based totally on using glucose with the addition of DMSO. The usage of Tris diluent with DMSO and glucose as a cryoprotectant were an effective extender for freezing Amur carp and common carp sperm. Using Tris with DMSO and glucose for freezing Amur carp and common carp sperm in the liquid nitrogen vapor allowed utilizing practical and inexpensive protocol for captive breeding program.

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