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Full Paper

Effects of extender type, sperm volume, cryoprotectant concentration, cryopreservation and time duration on motility, survival and fertilisation rates of Mekong giant catfish sperm

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Abstract: The objectives of this study are to evaluate the effects of some basic factors, namely extender type, sperm volume, cryoprotectant concentration, cryopreservation and storage time, on the quality of Mekong giant catfish (MGC) sperm. The following results are obtained from conducted experiments. The sperm kept in Hanks balanced salt solution (HBSS) extender consistently produced good results in terms of motility. The highest motility grade (4.0) was observed after 12 hours of examination and still a very satisfactory grade (3.3) was observed after 48 hours. The percentage of live cells of the sperm kept in HBSS was also highest (45.3%). The optimal amount of cryoprotectant (DMSO) prior to cryopreservation was 8%, which gave the best motility grade (4.0) up to the first 72 hours of observation while at 120 hours the motility grade was 3.3. The fertilisation rate of MGC fresh sperm in HBSS (2 ml) and 1 gram eggs was 47.1% while that of cryopreserved sperm under the same conditions was 36.2%. When crossed with *P. hypophthalmus*, the fertilisation rates of a 2-week- and a 1-year-cryopreserved sperm sample were 36.2% and 30.9% respectively.

Keywords: *Pangasianodon gigas*, Mekong giant catfish, cryopreservation, cryoprotectant, sperm motility, fertilisation rate

INTRODUCTION

At present, the aquaculture industry has become a highly important sector, especially in Asia and the Mekong River region. Catfish of the families Pangasiidae and Clariidae are commercially produced throughout the region. Indigenous species include the Chao Praya catfish (*Pangasius saitwonsei*), Pla Poa (*Pangasius berguti*), Pla Swai (*Pangasius hypophthamus*) and the Mekong giant

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catfish (MGC; *Pangasinodon gigas*), which is considered to be the world's largest freshwater fish. The MGC is of considerable popularity in the Indochina region and its population has declined precipitously in recent years in the Mekong River (Figure 1). It is also listed as an endangered species in the Convention on International Trade in Endangered Species (CITES) [1].

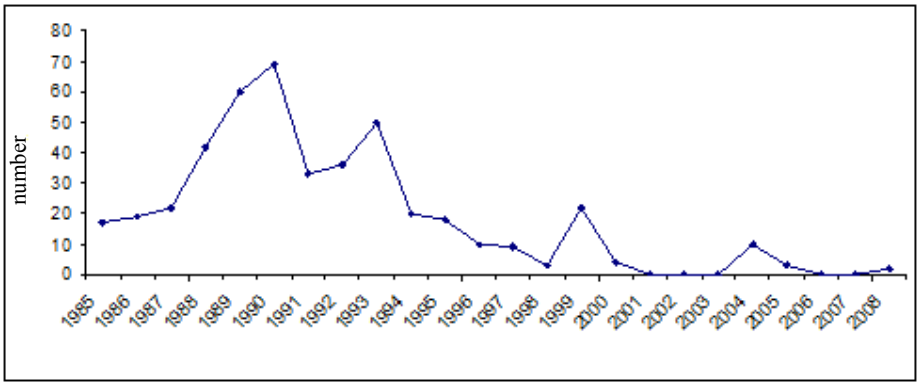

Figure 1. Annual catch of the Mekong giant catfish from the Mekong River [3].

The artificially bred MGC fingerlings have been produced from the brood stock in the Mekong River since 1983 and kept in earthen ponds in 2001-2002 by the Department of Fisheries, Maejo University. The MGC fingerlings have a fast growth in earthen ponds; they can grow to 25 kg in 3-4 years. As MGC is tasty and has a big market demand, current commercial aquaculture has been developed for both MGC and its hybrid (*P. gigas* x *P. hypophthamus*). The demand of the MGC fingerlings is about 500,000 per year [2].

Cryopreservation is a valuable technique for artificial reproduction and genetic improvement of endangered species. The application of sperm cryopreservation to the MGC species (*P. gigas*) greatly contributes to its conservation and propagation, thereby avoiding its possible extinction in the Mekong River. The technique has also made possible the production of hybrids with striped catfish (*P. hypophthalmus*) in the event that the *P. gigas* females are not available, as well as the improvement of a hybrid of *P. hypophthalmus* [3]. This technique also makes it possible to formulate breeding programs to provide a constant supply of fingerlings for commercial purpose. However, the growth performance of the hybrid has not been better than that of the MGC [3].

The chemical composition of the extender media for cryopreserving spermatozoa varies greatly and simpler extenders contain only two or three substances with NaCl, KCl and $CaCl_2$ as the commonly used salts in fish spermatozoa cryopreservation studies [4]. With proper osmolality and an optimum percentage of cryoprotectant during the cryopreservation stage, a satisfactory level of post-thaw fertilisation can be achieved [5-6]. An optimum rate of decrease in temperature prior to storage at the freezing temperature has been established. With 9% DMSO mixed with the extender, the MGC sperm cryopreservation with a temperature decreasing rate of $12^{o}C\ min^{-1}$ has a fertilisation rate of 66% compared to 74.0% by control sample of undiluted sperm [3]. Since the availability of MGC sperm and eggs can be limited, the lowest volume possible of sperm is used to fertilise more oocytes with a satisfactory fertilisation rate. A prior study [7] achieved a fertilisation rate of 31% in common carp

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(*Cyprinus carpio*) from 1-ml volume of extended cryopreserved sperm (sperm: extender = 1: 4) and one gram eggs while a yield of 83% was obtained from fresh sperm.

This study aims to find optimum conditions for pre-freezing cold storage of sperm, identify the type of extender giving the maximum duration of sperm storage, and study factors influencing the fertilisation rate of MGC cryopreserved sperm.

MATERIALS AND METHODS

Brood Stock Preparation

Prior to the collection of sperm, 2 sexually mature male specimens of MGC weighing approximately 25 kg were subjected to two weeks of conditioning and fed with individually formulated pellet feed fortified with 30% crude protein (at 5% of their body weight per day). The MGC sperm were first stripped to check for the presence of milt and then the fish were injected with a gonadotropin-releasing hormone analogue (Suprefact®, Hoechst AG, Germany) and domperidone (Olic Limited., Thailand) at a dosage of 10 µg/kg and 5 mg/kg body weight respectively. After 8 hours after hormone injection, the MGC were stripped of milt. The collected milt was then kept at 15°C.

Effects of Extenders on Sperm Motility

The collected sperm samples kept at 15°C were treated with 4 different extenders, viz. 0.8% NaCl, Hanks balanced salt solution (HBSS), Hanks balanced salt solution–calcium free (HBSS-CF) and Kurokura. The sperm-to-extender ratio was 1:6 (v/v) and the volumes of extended sperm samples prepared were 0.5, 1.0, 2.0 and 4.0 ml. The chemical composition of each extender is shown in Table 1. The osmolality of each extender was determined using an osmometer (Osmomat 030, Gonotec, Germany).

The motility grades of all treatments were then obtained after 12, 24, 48 and 72 hours of storage by adding a drop of water to 0.1 ml of each sperm sample for motility activation and then estimating the motile sperm activity in a forward direction under the microscope at 40x magnification and interpreting the results following the grading guide for MGC sperm motility rate as shown in Table 2. A control was performed using fresh sperm.

Effects of Cryoprotective Agent

The effects of cryoprotectant (DMSO) on sperm were studied. Samples from the collected sperm were prepared and mixed with HBSS extender at a ratio of 1:6 v/v (sperm: extender). Different amounts of DMSO (5, 8 and 10 %) were then added and the mixture kept at 15°C. The motility grade was then examined after 12, 24, 48, 72, 96 and 120 hours.

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Table 1. Composition of sperm extenders

	0.8%NaCl (mM)	HBSS (mM)	HBSS-CF (mM)	Kurokura (mM)
NaCl	136.89	136.89	136.89	61.60
KCl	-	5.37	5.37	-
$CaCl_2H_2O$	-	1.24	-	1.71
$MgCl_2$	-	-	-	0.84
$MgSO_4.7H_2O$	-	0.81	0.81	-
$Na_2HPO_4.7H_2O$	-	0.45	0.45	-
KH_2PO_4	-	0.44	0.44	-
$NaHCO_3$	-	4.17	4.17	2.38
Glucose	-	5.55	5.55	-
Osmotic pressure (mosm/kg)	270	233	258	297

Table 2. Grading guide for examining motility rate of MGC sperm [2]

Motility grade	Motility (%)	Period movement after activation (sec)
0	0	0
1	1-25	10
2	25-50	20
3	50-75	30
4	75-100	40

Cryopreservation Method

Immediately after milting, the sperm sample was subjected to a low temperature (15^{o}C) and was diluted with the prepared extenders (0.8%NaCl, HBSS, HBSS-CF and Kurokura). Samples of the extended sperm were then placed in cryovials at different volumes (0.5, 1.0, 2.0 and 4.0 ml). All samples were placed in a cryochamber and plunged into the cryobath with liquid nitrogen at 10-cm depth. A computer program (Cryogenesis V5, Australia) was then run to attain a constant cooling rate of 10^{o}C per minute until the target temperature of -80^{o}C was achieved. The samples were then kept in a liquid nitrogen tank.

Live Sperm Cells Determination

The two-week-cryopreserved sperm samples in the four extenders were thawed in warm water (40^{o}C) for one minute and stained with eosin-negrosin dye (Merck) for a determination of the percentage of both living and dead sperm. A small drop each of an extended sperm sample and eosin-negrosin dye solution (0.2 g eosin, 1 g nigrosin, 0.3 g sodium citrate dehydrate and 20 ml distilled water) were placed on the slide, mixed together and distributed evenly. The stain was dried and the

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numbers of dead and living sperm were examined under the microscope (100x). The live sperm that did not absorb the dye appeared somewhat clear while the dead sperm absorbed the dye and appeared pink or purple in colour. Forty sperm cells, living and dead, from five different points were separately counted.

Fertilisation Test

Fertilisation experiments were performed to compare the fertilisation rate of frozen sperm and fresh sperm. In the first experiment, artificial fertilisation was performed by placing 1 or 2 ml of HBSS-extended fresh MGC sperm (sperm-to-extender ratio = 1:6 v/v) and eggs (1 gram) in a Petri dish and mixing for 2 minutes using a rooster's tail feather and 50 ml of distilled water was then added. The mixture was placed in a small fine net enclosure (6"x6"x6"). Aeration was provided in a nursery concrete pond. After approximately 24 hours, the fertilisation rate was determined by observing the fertilised eggs (somite stage) under a dissecting microscope.

In the second experiment, the above procedure was repeated with one-year-cryopreserved MGC sperm in place of fresh sperm. In addition, 2 ml of the HBSS-extended cryopreserved MGC sperm were used to fertilise one gram of *P. hypophthalmus* eggs. A control was performed using fresh sperm.

In the third experiment, different volumes (0.5, 1.0, 2.0 and 4.0 ml) of two-week-cryopreserved MGC sperm samples in different extenders (0.8%NaCl, HBSS, HBSS-CF and Kurokura) with the same sperm-to-extender ratio as above were used to fertilise one gram of *P. hypopthalmus* eggs. A control was performed using fresh sperm.

Statistical Analysis

Every experiment was performed in triplicate. Data of living sperm cells (%) and fertilisation rate (%) were normalised by arcsine transformation. One-way analysis of variance (ANOVA) was used to analyse significant differences between treatments ($p<0.05$). Duncan's multiple range test was used to compare the means of significant difference of each treatment using SPSS software.

RESULTS AND DISCUSSION

Motility of MGC Sperm in Different Extenders

In evaluating the potential of some extenders for use in MGC sperm cold storage, it was observed that the highest motility grade (4.0) was found at all volumes of HBSS that were examined after 12 hours of refrigeration, and a satisfactory motility grade (3.3) was observed up to 48 hours of refrigeration (Figure 2). This was followed by that obtained with HBSS-CF (3.3-4.0) and Kurokura (3.0-4.0) in that order, where satisfactory motility grades were observed up to 24 hours of refrigeration. The motility grade results in 0.8%NaCl and undiluted sperm were the least satisfactory (3.0-4.0 and 2.7-4.0 respectively for the first 24 hours). From the results in Figure 2(c-d), sperm with extenders have more satisfactory motility grades than fresh sperm within 48 hours. Sperm with motility grade of 0.5-1 cannot be used for artificial fertilisation. Thus, the storage time for fresh sperm and sperm with extenders in the refrigerator (15^{o}C) seems to be a maximum of 48 hours.

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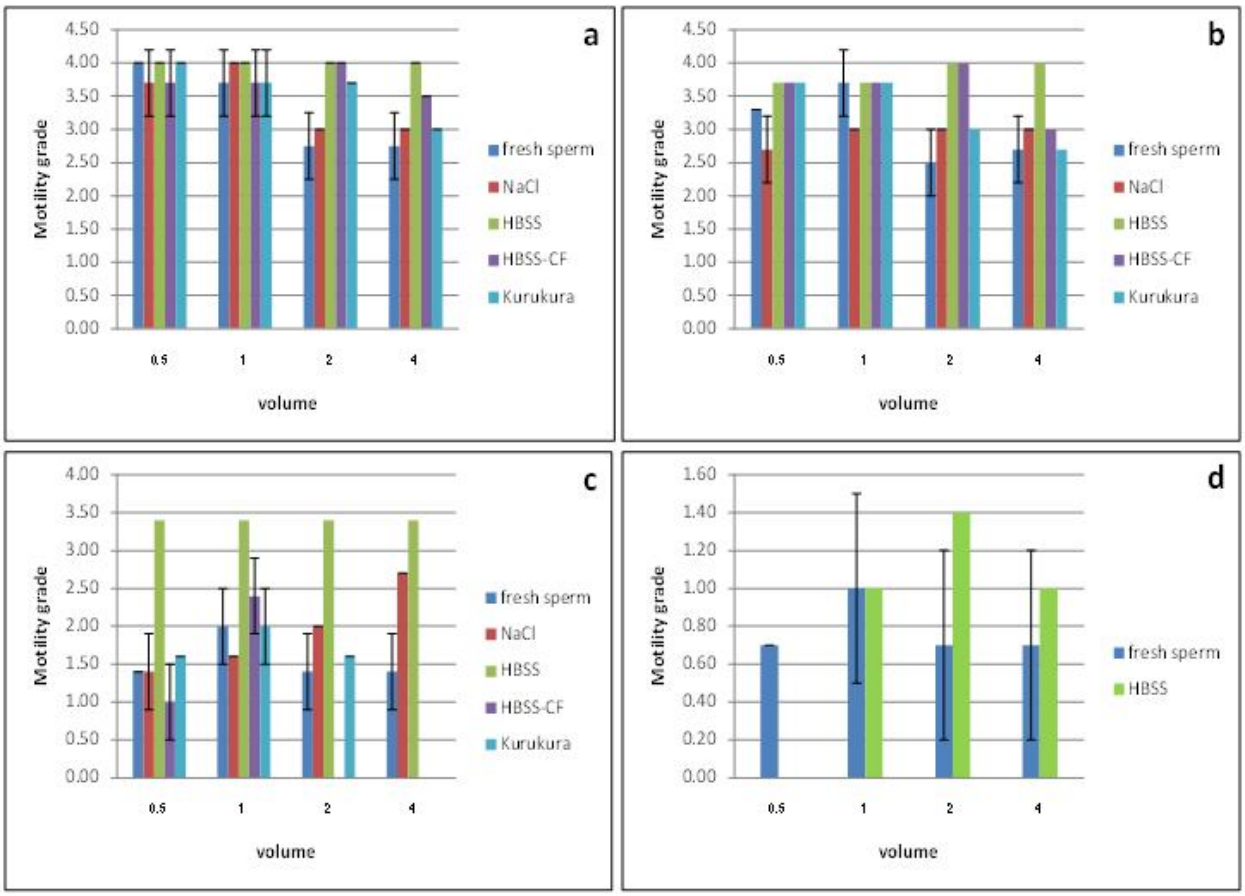

Figure 2. Motility grades of MGC fresh and frozen sperm kept at 15°C after 12 hrs (a), 24 hrs (b), 48 hrs (c) and 72 hrs (d) in different extenders

Previous studies have established the need for appropriate diluents for cool storage of sperm [5, 8]. Moreover, storage of sperm in diluents with optimal constituents prolongs their lives compared to undiluted sperm. Morisawa and Suzuki [9] determined the factors whereby the motility of teleost spermatozoa is initiated, i.e. the decrease or increase in osmolality of the environment. The composition of the diluent as well as its osmolality also has a significant influence on the success of storage. Mongkonpanya et al. [3] concluded that a suitable extender should have an osmolality level near the range of that of fresh sperm. The finding seems to agree with the data in this study, i.e. the HBSS osmolality value of 233 mOsmo/kg was almost the same as that of the fresh sperm (206 mOsmo/kg), but quite different from those of NaCl and Kurokura (270 and 297 mOsmo/kg respectively).

Since HBSS extender at all volumes (0.5, 1, 2 and 4 ml) gave the best result from the first part of the study (Figure 3), it was used to check the motility grade of MGC sperm in different levels of DMSO (a cryoprotectant usually used in sperm cryopreservation).

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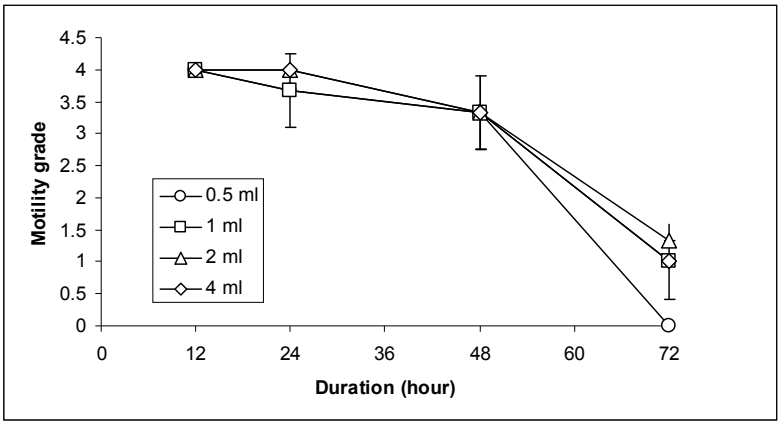

Figure 3. Sperm motility at different duration and volumes in HBSS

Effects of DMSO on Post-thaw Sperm Motility

After 12 hours of refrigeration, the sperm motility grade was 4.0 in all levels of DMSO. Mongkonpunya et al. [6] observed similar patterns when they added DMSO (5 and 9%) to a diluent (bicarbonate buffer) to diluted MGC sperm. They concluded that the use of DMSO at higher percentages than 10% could be toxic to the MGC sperm but that DMSO at 5% was not as effective as 9%. In this study, the sperm extended with 8% DMSO exhibits the highest motility grade of 4 for up to 72 hours of refrigeration. It then declines to 3.7 and 3.3 at 96 and 120 hours respectively (Figure 4). This finding agrees with a study on the cryopreservation of sperm of red snapper, which indicated that the DMSO cryoprotectant of 10% yields the highest post-thaw motility [11].

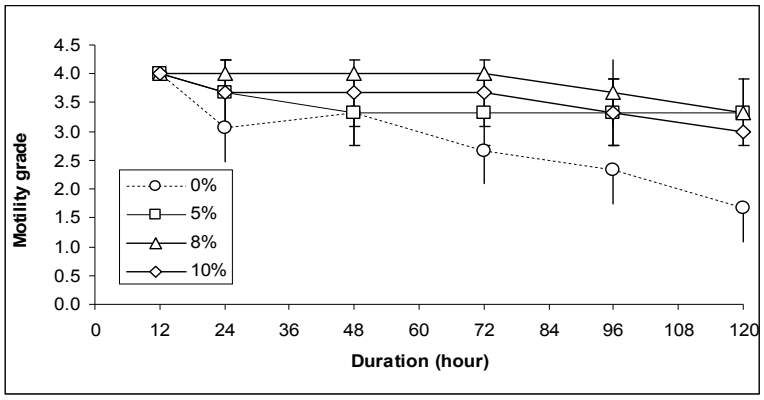

Figure 4. Motility grade of MGC sperm kept at 15°C in HBSS with different percentages of DMSO

Survival of Sperm Cells

The highest percentage of live sperm was observed in HBSS-extended sperm (45.3%), which is statistically comparable with those in Kurokura (29.3%) and HBSS-CF (28.9%), but significantly different (p<0.05) from that in 0.8%NaCl-extended sperm (22.4%) (Table 3).

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Table 3. Percentage of live cells in cryopreserved MGC sperm in different extenders

Extender	Viability (%)
0.8%NaCl	22.4 ± 4.8^{a}
HBSS	45.3 ± 6.0^{b}
HBSS-CF	28.9 ± 4.7^{ab}
Kurokura	29.3 ± 4.3^{ab}

Note: Data are expressed as mean±S.E. (from 3 replicates).
Means that do not share the same superscript are statistically significant ($p<0.05$).

Fertilisation Efficiency of MGC Sperm

The results of fertilisation rate of MGC sperm in HBSS extender are shown in Table 4. This result suggests that the higher the sperm volume of MGC is, the greater the fertilisation yield is achievable. From Table 4, an extended sperm volume of at least 2 ml is needed to achieve a satisfactory fertilisation. The decrease in sperm quality and consequent reduction in fertilisability of cryopreserved sperm was also observed in *Cyprinus carpio* [12] and *Misgurnus anguillicaudatus* [13]. However, Ding et al. [14] showed that the fertilisation rate and hatching rate of Mandarin fish (*Siniperca chuatsi*) by sperm cryopreserved for 1 week or even 1 year in liquid nitrogen were similar to those obtained when fresh sperm was used. Thus, this cryopreservation technique should be further improved to get a better fertilisation rate for MGC sperm.

Table 4. Fertilisation rate of MGC sperm in HBSS with 1 g MGC eggs

MGC sperm	Volume (ml)	
	1	2
Fresh sperm (control)	30.3 ± 8.9^{Ba}	47.1 ± 5.1^{Bb}
One-year-cryopreserved sperm	16.8 ± 0.3^{Aa}	36.2 ± 9.9^{Ab}

Note: Data are expressed as mean±S.E. (from 3 replicates).
Means that do not share the same superscript in the same column (capital letters) and row (small letters) are statistically significant ($p<0.05$).

For hybrid artificial breeding in different extenders, the best fertilisation rate (89.7%) of MGC sperm with *P. hypophthalmus* eggs was observed with 2 ml of the control (fresh sperm) in HBSS. This was followed by the 2-week-cryopreserved sperm in HBSS at 2 ml (36.2%), while at 0.5 ml volume no fertilisation was observed (Figure 5). An unsatisfactory rate was obtained at 1.0 ml while at 2-ml volume, fertilisation rate was highest among all treatments including fresh sperm. In a similar study involving interspecific fertilisation, a fertilisation rate of 26-45% from MGC sperm and *P. hypophthalmus* oocytes was obtained [3].

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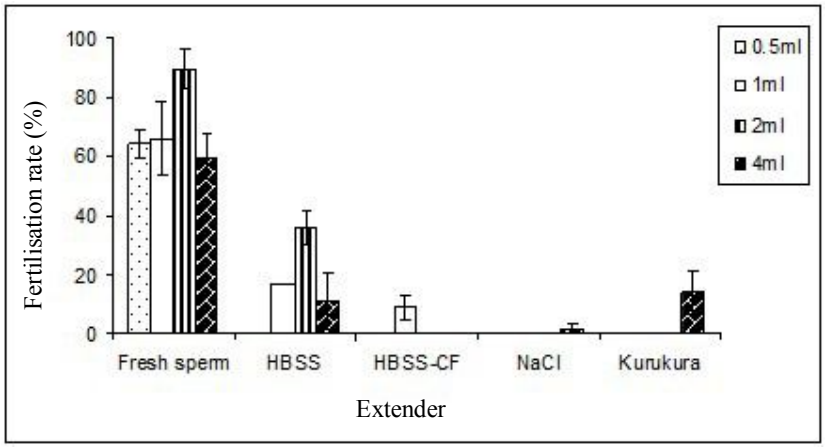

Figure 5. Fertilisation rate of fresh and cryopreserved MGC sperm in different extenders of different volumes with *P. hypophthalmus* eggs

The fertilisation rate of 30.9% by one-year-old cryopreserved MGC sperm and fresh *P. hypophthalmus* eggs proved to be satisfactory (Table 5). This finding confirms all previous experiments involving the suitability of HBSS extender for MGC sperm cryopreservation. Theoretically, extended sperm can be kept for many years and according to the findings of this study, cryopreserving for a period of one year can be considered suitable for achieving a reasonable rate of fertilisation. The availability of cryopreserved MGC sperm can now be increased since the viability of preserved sperm can be maintained for a considerably long period. More consideration should be given to the mass application of the cryopreservation process for endangered species. However, cryo-preservation technique still needs to be improved in a further study to increase the fertilisation rate. Moreover, the proper ratio of cryopreserved sperms and eggs will also be evaluated in the further study.

Table 5. Fertilisation rate of 1-year-cryopreserved MGC sperm in HBSS and *P. hypophthalmus* eggs

Sperm	Fertilisation rate (%)
Fresh sperm (control)	76.6 ± 3.5^{b}
Cryopreserved sperm	30.9 ± 4.1^{a}

Note: Data expressed as mean±S.E. (from 3 replicates)
Means which do not share the same letter superscript are statistically significant ($p<0.05$).

CONCLUSIONS

The motility, survival and fertilisation rates of Mekong giant catfish sperm were found to depend on a number of factors, i.e. type of extender, sperm volume, cryoprotectant concentration, cryopreservation and storage time. HBSS appeared to be the best extender for the fish sperm which, when present in 1:6 (sperm:extender) ratio, gave the highest motility grades (3.3-4.0) and the highest fertilisation rates at 2-ml volume. The percentage of live cells of the sperm kept in HBSS was also

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highest (45.3%).A cryoprotectant (DMSO) concentration of 8% of the extended sperm was found to be optimum. The highest fertilisation rate of MGC fresh sperm and MGC eggs was 47.1% while that of one-year-cryopreserved sperm under the same conditions was 36.2%. Sperm cryopreserved for 2 weeks and 1 year gave a satisfactory fertilisation rates of 36.2 and 30.9% respectively with *P. hypophthalmus* eggs.

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