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Full Length Research Paper

Potentials of short term and long term cryopreserved sperm of the African giant catfish (*Clarias gariepinus* Burchell, 1822*)* for aquaculture

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To service the growing demand for male African giant catfish (*Clarias gariepinus*) broodstock for aquaculture in Nigeria, and to conserve valuable genetic resources, we improved both short-term (in deep freezer at -35°C) and long-term cryopreservation (in liquid nitrogen at -296°C) of catfish sperm. Catfish sperm cryopreserved at -35°C using three different types of cryodiluents composed of 10% methanol, glycerol or dimethylsulphoxide (DMSO) with 15% non-permeating cryoprotectant (skim milk) and 75% phosphate-buffered saline (PBS) as extender was evaluated at day 1 to 28. Sperm motilities on thawing for the two cryodiluents that contained glycerol and DMSO were the same ($p > 0.05$) while those for the cryodiluent that contained methanol were significantly lower ($p < 0.05$). Sperm cryopreserved in DMSO and glycerol provided hatching at least 50% of catfish eggs. Liquid nitrogen (LN_2) was with the same 3 cryoprotectants and one new extender based on glucose and skim milk (Ginzburg fish Ringer, GFR). Two thawing procedures were tried: 1) In the first trial, one set of sperm was frozen with different cryodiluents with extenders in the freezer at -10°C and 2) Stored for up to 8 months in LN_2. Sperm with up to eight months cryopreservation from the first trial when thawed at room temperature at 27°C for 15 min gave 0 - 6.25% fertilization rate (FR). The sperm stored for two months in the second trial when rapidly thawed at 35°C for 5 min gave better results. The FR of 94% was achieved with a cryodiluent of DMSO with 5% glucose solution and PBS when the cryopreserved sperm was thawed at 35°C. Also, DMSO in combination with PBS resulted in a higher FR than either glycerol or methanol. However, sperm cryopreserved with glycerol had the lowest motility rate in the 2 trials ($p < 0.05$). The use of cryopreserved sperm did not significantly affect ($p < 0.05$) the survivability of the fry. The use of cryopreserved sperm of the African catfish for fry production will be especially beneficial in Nigeria where reliable sperm supply is a major bottleneck in the catfish aquaculture industry.

Key words: Cryopreservation, sperm motility, *Clarias gariepinus*, African giant catfish.

INTRODUCTION

Freshwater aquaculture (fish farms) in Nigeria presently covers an estimated 60,000 ha of the country producing some 25,000 to 30,000 metric tons of fish per year. Freshwater aquaculture currently contributes to 5% of Nigeria's fish consumption; Nigeria is still one of the largest importers of fish in the developing world, importing more than 600,000 tons annually (FAOTCP/ NGR:http:www.fao.org/documents/). Freshwater aquaculture

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Abbreviations: DMSO, Dimethyl sulfoxide; **LN_2,** liquid nitrogen; **PBS,** phosphate buffered saline.

production must be improved to supply an unsatisfied demand for fish from Nigeria's population of 154 million people (UN, 2009).

In Nigeria, the most common aquaculture fish include catfish (*Clarias gariepinus* and *Heterobranchus*, sp.), tilapia *(Oreochromis* sp.) and carp (*Cyprinus carpio)*. Catfish can have a market value of two to three times that of other fish and they can be artificially propagated to meet the supply of fingerlings. A major impediment to catfish aquaculture is the shortage of reliable males for breeding. This has been very difficult because a lot of fish broodstock are wasted during natural and artificial induced spawning of fish breeding. The shortage of male broodstock can be overcome through cost effective, reliable and secure storage of frozen sperm. Loss of genetic variation on both wild (Kirpichnikov, 1981) and farmed (Hallerman et al., 1986) populations of fish is a major problem. Loss of genetic variation makes improvement of farmed strains of catfish more difficult or impossible, and may result in poor growth, survival, reproduction, and susceptibility to disease. Sperm storage can also provide a means to maintain the genetic variation of catfish. Consequently, the development of the best methods for the short-term and long-term storage of catfish sperm would enable economic growth in fresh water catfish aquaculture and reduce Nigeria's reliance on imported fish.

Cryopreservation is the process of storing biological materials at below freezing temperatures using cryodiluents to protect the biological materials from damage during the freezing and subsequent thawing cycles (www.genpro. mag.com, www.viacellince.com). Since 1970, there have been many of publications demonstrating the feasibility of cryopreserving fish spermatozoa. The majority of publications and protocols relate to three groups of fish of aquacultural importance: the salmonids, tilapia and carp (Scott and Bayries, 1983; McAndrew et al., 1980). Extenders are required for diluting fish milt prior to cryopreservation. Also, both permeating and non-permeating cryoprotectants have been used to prevent cryoinjuries. Extenders are required for diluting fish milt prior to cryopreservation and are generally designed to be compatible with the physiochemical composition of the fish seminal plasma. They are used to maintain the spermatozoa in an immotile state until required. There are two different types of cryoprotectants used for cryopreservation of fish spermatozoa namely: permeating and non-permeating cryoprotectants. The two types of cryoprotectants are to be mixed at a varying combination to provide proper cryoprotection (Stoss, 1983). Examples of permeating cryprotectants are ethylene glycol, glycerol, dimethyl sulfoxide (DMSO), methanol, dimethyl acetamide etc. The efficacy of cryoprotectants will depend on the pre-freezing toxicity of compounds to spermatozoa concentration, equilibration period and the extender used (Harvey, 1983). Examples of non-permeating cryoprotectants are skim milk, egg yolk and protein which serve as extracellular cryoprotectants. Chao et al. (1987) recommended 15% of skim milk as non-permeating cryoprotectant for the cryopreservation of fish spermatozoa in extender-cryoprotectant combinations.

This study is therefore designed to investigate the possibility of cryopreservation of catfish sperm under short and long-term conditions in a deep freezer at -35°C and at -196°C in liquid Nitrogen respectively and evaluate its effects on its motility and ability to fertilize normal eggs.

MATERIALS AND METHODS

Experimental site, selection of broodstock and cryopreservative preparation

The experiments were conducted at the Biotechnology and Wet Laboratories of the Department of Animal Sciences, Obafemi Awolowo University (OAU), Ile-Ife. Mature broodstock were obtained from reputable fish farms in Ibadan. The broodstock were transported to Ile-Ife in a large plastic container filled up to half of its capacity with water. The males were selected for maturity based on reddish or pinkish and pointed vascularized papilla and of age 7 ± 2 months while the female broodstock were chosen on the basis of distended swollen abdomen, and extrusion of eggs after a gentle pressure was applied on the abdomen towards the genital papilla.

The two different types of extenders were tested before they were used to prepare cryodiluents. The extenders were phosphate-buffered saline (PBS) formulated as Hank's balanced saline solution and 0.9% sodium chloride solution (w/v) (normal saline solution). Only PBS formed homogeneous mixture with cryoprotectants and then provided the extender for further trials. PBS in combinations of 75% extender - 10% cryoprotectant (v/v) were combined with, 15% skim milk (DANO). Ten percent of either of the three different types of permeating cryoprotectants was used: methanol (BDH), glycerol (BDH) and dimethylsulphoxide (DMSO, SIGMA and ALDRICH Co., USA) initially designated as E-CPA A, E-CPA B and E-CPA C, respectively (Oyeleye and Omitogun, 2007). Skim milk was used for its potential to protect cells against freezing shock and formation of ice crystals at the extra cellular level.

Sperm collection and cryopreservation, cryopreserved sperm motility assessment

Milt cannot be stripped out of the male catfish because the testes are located in the dorsal region of the abdomen. The vertebral column must be broken and testicle surgically extracted and carefully cleaned, removing the main blood vessels (Figure 1). The milt was obtained by careful laceration of the testis to release the sperm using a new razor blade (Adewumi, 2004; Lamai, 1996).

The fresh milt was observed under the microscope using high power objective (40X) and the motility was assessed and counted before they were cryopreserved. The good, agile and highly motile sperm were cryopreserved (Billard et al., 1996). The milt was mixed with E-CPA at the ratio 1:1 (v/v); the motility was assessed under high power microscope (40X) and stored in 2 ml cryotubes. The cryotubes were labeled for each type of E-CPA combination used.

Figure 1. Extraction of testes from male broodstock.

The cryotubes were gently shaken to allow milt to properly mix with E-CPA, then arranged in a larger container and placed in the refrigerator for 15 min to reduce their temperature to 4°C before fast freezing in the freezer. A deep freezer capable of freezing to -35°C (IGNIS) capacity was used.

In the thawing process, the container with cryotubes were first transferred into refrigerator for 25 to 30 min to allow the milt to thaw from -35 to 4°C inside the refrigerator. Thereafter, they were transferred at room temperature and allowed to stand from 4 - 5 min before the motility was assessed, but in the case of using cryopreserved sperm for fertilization fast thawing is advisable. Motile sperm were observed and counted. Sperm counts were determined after dilution using hemacytometer. The numbers of motile sperm were recorded at various periods of cryopreservation namely: day 1 (24 h), 2, 3, 4, 5, 6 and 7, week 2 and 4. The percentage of motile sperm was calculated after Hafez (1985).

Fertilization of ovulated eggs with cryopreserved sperm

The cryopreserved sperm after thawing at day 3, 7 and 14 were mixed with 5% glucose intravenous water at a ratio 1:1 separated in each Petri dish labeled with E-CPA A, E-CPA B, E-CPA C, respectively. For each of the Petri dish 1g (700 ± 100) of eggs were measured which was used for the fertilization. The cryopreserved sperm was allowed to fertilize the eggs and were stirred continuously with a soft brush placing in hatching plastic tanks (Figures 2 and 3). The colour of the fertilized eggs changed from light green to pale yellow or orange. After 24 h of fertilization, the fertility rate were assessed and recorded. Hatched eggs were counted.

Fertilized eggs were placed in the plastic aquaria measuring (50 x 35 x 30 cm). The aquaria were filled with clean filtered, UV-sterilized and well-oxygenated water with an aerator pump. The eggs were spread homogeneously in one single layer on the net placed on the wide plastic sieve, 2 - 3 stones were placed on the net and the plastic to press it down into the water, such that the eggs were continuously suspended in contact with the water. When the eggs are not fertilized, they start to swell and stick to the net ensuring separation of the hatched eggs from the dead eggs.

Hatching took place after about 48 h of fertilization. The temperature of the water was recorded at 22°C.

Preparation of the cryoprotectants for liquid nitrogen cryopreservation

Phosphate-buffered saline (PBS) also known as Hank's balanced saline solution, and Ginzburg fish ringer (GFR) were used as extenders with pH of 7.4 and 7.6, respectively. These extenders however were sterilized for 20 min at 15 lbs/inch^2 using a pressure cooker before being used to avoid any bacterial contamination and deterioration of the spermatozoa. The extenders were prepared as shown in Table 1.

In the first trial carried out, dimethylsulphoxide (DMSO), which had earlier been proven effective for cryopreservation under short-term conditions (Oyeleye and Omitogun, 2007), was evaluated with 5% glucose and 5% skim milk inclusion and at the same time the effect of glycerol was also tested but now under long-term cryopre-servation purpose in liquid Nitrogen.

In the second trial, 2 different extenders (that is, PBS and GFR) and cryoprotective agents earlier tested (that is, dimethylsulphoxide, DMSO; methanol and glycerol this time designated as D,G and M for easy labeling in liquid Nitrogen Dewar canisters) which are capable of crossing the plasma membrane (that is, molecular weight less than 400) as shown in Table 2 were tested each at a dilution ratio of 1:1 with Phosphate buffered saline, PBS but at a

Figure 2. Control (fresh) and thawed cryopreserved sperm were used respectively to fertilize 1 g each of fertile eggs for each type of sperm.

Figure 3. Fertilization and hatching of fertilized eggs were conducted in plastic containers covered with black polyethylene bags and illuminated with 200-W electric bulbs to elevate water temperature.

Table 1. Composition of the extenders tested for cryopreservation of catfish sperm in liquid nitrogen (LN_2).

Composition (g/1000 ml)	Phosphate- buffered saline (PBS)	Ginzburg fish ringer (GFR)
NaCl	8.0	7.0
KCl	0.02	0.28
$CaCl_2$	-	0.33
$NaHCO_3$	-	0.23
$NaHPO_4$	1.15	-
KH_2PO_4	0.20	-
Distilled water	Up to 1000 ml	Up to 1000 ml

Table 2. Effect of different cryoprotectants on the viability of African catfish sperm cryopreserved in liquids nitrogen.

Cryoprotectants[1]	Fertility	Hatchability	Motility
DP	6.25[b]	3.00[b]	51.00[c]
DGP	5.00[b]	2.75[b]	57.00[b]
GP	1.25[b]	0.00[b]	45.00[d]
DS	2.50[b]	0.00[b]	NA
C	45.00[a]	45.00[a]	71.00[a]
MP	NA	NA	48.00[cα]
DF	NA	NA	56.00[b]
DGF	NA	A	47.50[cα]
GF	NA	NA	46.50[cα]
MF	NA	NA	48.00[cα]
SEM[2]	4.55	3.57	2.06

[abcd]Means in the same column with different superscripts are significantly different at $p < 0.05$.44

[1]Cryoprotectants + extender.

DP = Dimethlsulphoxide (DMSO) + Phosphate buffered saline (PBS), DGP = DMSO + 5% glucose + PBS, GP = Glycerol + PBS, GS = Glycerol + PBS, DS = DMSO + 5% skim milk, MP = Methanol + PBS, DF = DMSO + Ginzburg fish ringer (GFR), DGF = DMSO + 5% glucose + GFR, GF = Glycerol + GFR, MF = Methanol + GFR, C = control, NA = Not available.

[2]Standard error of mean.

dilution ratio of 1:10 with Ginzburg fish ringer, GFR. Furthermore, the effects of addition of 5% glucose and skim milk designated as g and s (a non-permeating cryoprotective agent) to DMSO on the cryopreserved semen were also evaluated. The tubes used for storing the cryopreserved semen were labeled as CPA-D_1 (10% DMSO, 90% PBS), CPA-D_2 (10% DMSO, 90% GFR), CPA-G_1 (10% Glycerol, 90% PBS), CPA-G_2 (10% Glycerol, 90% GFR), CPA-M_1 (10% Methanol, 90% PBS), CPA-M_2 (10% DMSO, 90% GFR), CPA-D_1+5%g (10% DMSO, 90% PBS, 5% Glucose), and CPA-D_2 + 5% g (10% Methanol, 90% GFR, 5% Glucose), CPA-D_1+5%s (10% DMSO, 90% PBS, 5% Skim milk).

Extraction of sperm

Gravid males weighing 700 ± 100 g were selected and kept in different 50 l tanks for about 18 h through the night before the actual day of experiment and thereafter were made unconscious by breaking their backbone. Testes were removed by dissection using a sterilized scissors and lacerated with a needle; meanwhile semen was gently squeezed out and collected in a sterilized Petri dish (Viveiros et al., 2000). However, this process was carried out in a disinfected environment to avoid bacterial contamination which is a commonly encountered problem and even inaccurate estimation of motility (Jenkins, 2000). Sterilized instruments and aseptic techniques for collection of sperm within extenders were incorporated to reduce the possibilities of bacterial contamination of sperm samples.

Cryopreservation of the semen

In this section, 2 trials were conducted. In the first trial using PBS as the extender, the volume of the extracted spermatozoa was measured with a 5.0 ml syringe (DISCARDM , NIG.) and diluted with the extender on a ratio 1:1 (v/v) and thereafter mixed evenly with the different cryoprotective agents at a ratio of 1:1 final concentration for fertility and hatchability evaluation.

In the second trial now using Ginzburg fish Ringer and phosphate-buffered saline solutions as the extenders, the same design was followed except that the fresh spermatozoa was directly

mixed with the cryoprotective agents now at a 1:10 (v/v) in the case of GFR for semen motility evaluation.

In both trials, the resulting semen in each experiment after thorough mixing was then dispensed into labeled 1 ml cryotubes while a 2-step freezing protocol of first freezing on the chilled water blocks at -10°C for 30 min before the final transfer into the liquid nitrogen was used to preserve the semen.

Induced spawning of the female broodstock

The readiness of the female broodstock to be used for fertilization was tested by holding it in a head-up vertical position during which the eggs begin to run freely from the genital pore (Richter and Van den Hurk, 1982). The selected broodstock were kept separately in different tanks without feeding them after they were injected with 0.35 ml Ovaprim (SYNDEL, Canada) per kg live weight (Oyeleye and Omitogun, 2007) and then left for 12 h as a post ovulatory maturation period (Richter and Van den Hurk, 1982) to ensure high hatching rates and low proportion of deformed larvae (Hogendoorn and Vismans, 1980). The first free running eggs obtained at a slight pressing of the induced female brood stock were then collected for fertilization (Legendre and Oteme, 1995).

Evaluation of the tested parameters

Motility evaluation

The motility of the spermatozoa after the addition of cryoprotective agents was evaluated for each trial before freezing on the day of preservation and thereafter every time the cryopreserved spermatozoa was evaluated for fertility and hatchability. This is done by diluting a drop of the post-thawed semen either with PBS or GFR to match the extender used both at a ratio of 1:200 from which 1 drop was put on the hemacytometer and viewed under 40X magnification power of a compound microscope. The figure arrived which is converted to the total number of spermatozoa per ml by multiplying it by the dilution factor (200) and 10^4 (SIGMA, 1994) as follows: Total no of spermatozoa per ml = Average nnumber of counted spermatozoa $\cdot$ 200 $\cdot 10^4$ of the cryopreserved semen.

Fertility and hatchability evaluation

In the first trial, the cryopreserved semen was thawed at a room temperature of 27°C for 15 min while it was thawed at a temperature of 35°C for 5 min in the second trial. In both cases, sodium chloride and glucose infusion B.P. (UNIDEXAL , Nig.) was added at a ratio 1:1 v/v in the case of those preserved with phosphate buffered saline as their extenders but no activating solution was added to those preserved with GFR as the extenders.

After stripping of the induced female broodstock, 1.0 g of eggs (numbering 700 ± 100) (Oyeleye and Omitogun, 2007) was mixed with the semen in the two experiments. The mixture was stirred for 2 - 5 min to allow contact between eggs and the spermatozoa (Hogendoorn and Vismans, 1980; Legendre, 1986). The resulting non-sticky eggs were then transferred to the hatching troughs (50 x 35 x 30 cm^3; l x b x h) containing well aerated water inside which plastic buckets containing 1 mm mosquito nets were suspended. The hatching troughs were completely covered on all sides with black polythene bag and placed under 200-Watt bulbs meanwhile the top were screened from insect contamination of eggs by covering the troughs with perforated lids that would allow enough air into those troughs.

The development time during incubation was temperature-dependent but usually fell between 27 - 28.5°C during this experiment. Once hatching occurred after 24 h, the new hatchlings simply swim to the bottom of the hatching troughs while the egg envelopes remain adhered to the mosquito net. The larvae were then simply separated from the unfertilized eggs and eggshells by simply lifting the basket and the net out of the hatching trough.

The percentage fertility and hatchability were determined after 12 h of fertilization by identifying the fertilized eggs as those with embryonic discs and eye spots on one side of the eggs while the dead ones were being counted.
Thus,

$$\% \text{ Fertility} = \frac{\text{Total number of eggs fertilized} - \text{Total number of unfertilized eggs}}{\text{Total number of eggs fertilized}} \cdot 100$$

and

$$\% \text{ Hatchability} = \frac{\text{Total number of fertile eggs} - \text{Total number of unhatched fertile eggs}}{\text{Total number of fertile eggs}} \cdot 100$$

Post-hatching survivability evaluation

The newly hatched larvae of all the treatments considered and that of the control were allowed to live on the remains of their yolk sacs for the first 4 days (Heicht et al., 1988) after hatching out of the eggs and were then carefully removed from the hatching troughs into other different containers where they were fed *Artemia* (INVE AQUACULTURE , USA) on a regular basis for about 2 times per day while observing any irregularity in their activities in terms of feeding, movement inside water, and at the same time taking note of dead fry which were then immediately removed to avoid contamination of water. This was done for a period of 1 month using the larvae that resulted from fertilization of normal eggs with fresh sperm (the control experiment) and also for the larvae that resulted from the fertilization of normal eggs with the cryopreserved semen each time fertility and hatchability trial were carried out. The post-hatching survivability was then evaluated as follows:

$$\% \text{ Survivability} = \frac{\text{Total number of hatched larvae} - \text{Total number of dead larvae}}{\text{Total no of hatched larvae}}$$

The control experiment

The control for both trials was set up by using fresh semen obtained from the lacerated testes using sterilized materials but now activated with sodium chloride and glucose infusion B.P. (UNIDEXAL , Nig.) on a ratio 1:1 v/v and subsequently used to fertilize 1.0 g normal eggs from the same batch of eggs used any time fertility and hatchability were evaluated for cryopreserved sperm during the course of this work.

The motility of the post-thawed cryopreserved sperm was evaluated after dilution with the extender on a ratio of 1:200 using hema-cytometer as stated for the cryopreserved sperm.

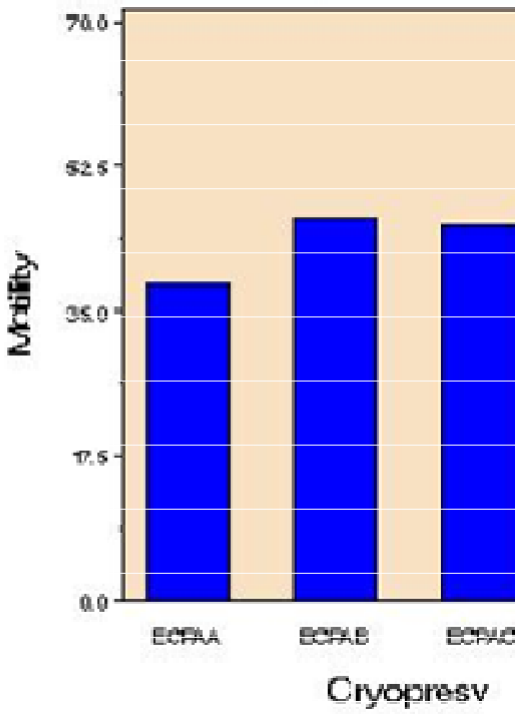

Figure 4. Effect of cryopreservatives on sperm motility after 28 days. E-CPAA, E-CPAB, E-CPAC: The cryopreserved sperm after thawing at day 3, 7 and 14, respectively, were mixed with 5% glucose intravenous water at a ratio 1:1.

Statistical analysis

The data collected were analyzed using analysis of variance to find a level of significance at $p < 0.05$. The number of motile sperm counted per square of hemacytometer and percentage of motile sperm obtained in 28 days of cryopreservation were subjected to Duncan's multiple range test to evaluate effects of type of cryopreservatives and period of cryopreservation on sperm motility.

The data collected on motility, fertility and hatchability in the first trial were subjected to 2- way analysis of variance (ANOVA) at a significant level of $p < 0.05$ while those data collected in the second trial including that of survivability was analyzed using Chart Wizard of Microsoft Excel (2003).

The following figures show the cryopreservation materials and the various stages of cryopreservation carried out in the course of this study are as follows:

RESULTS AND DISCUSSION

Effect of cryopreservatives on sperm motility

The two extenders recommended by Kwantong and Amrit (2000) were experimented on (phosphate-buffered saline and 0.9% NaCl solution). Both were good activators of sperm motility during fertilization, however, it was observed that PBS formed a mixture that was more homogenous with the cryoprotectants than did 0.9% NaCl solution. This was the reason for using PBS in preparing three different types of cryopreservatives for this experiment. The sperm motility of the fresh sperm was found to be significantly different from that of cryopreserved sperm ($p < 0.05$). Glycerol (B) had the mean value which was not significantly different from that of DMSO (C) but significantly different from methanol. The spermatozoa frozen in E-CPA A (methanol) had the lowest mean value (38.20) (Figure 4).

There was a sharp decline in motility percentage after 24 h (93 to 46%). There was a gradual decline from day 1 to day 4, and between days 4 and 5 there was a decrease in motility percentage at a higher rate. From day 5 to 7, there was a gradual decrease while from day 7 to day 28 the decrease in motility percentage was at a very slow rate.

Cryopreserved sperm in a -35°C deep freezer

The 3, 7 and 14- day old cryopreserved sperm were able

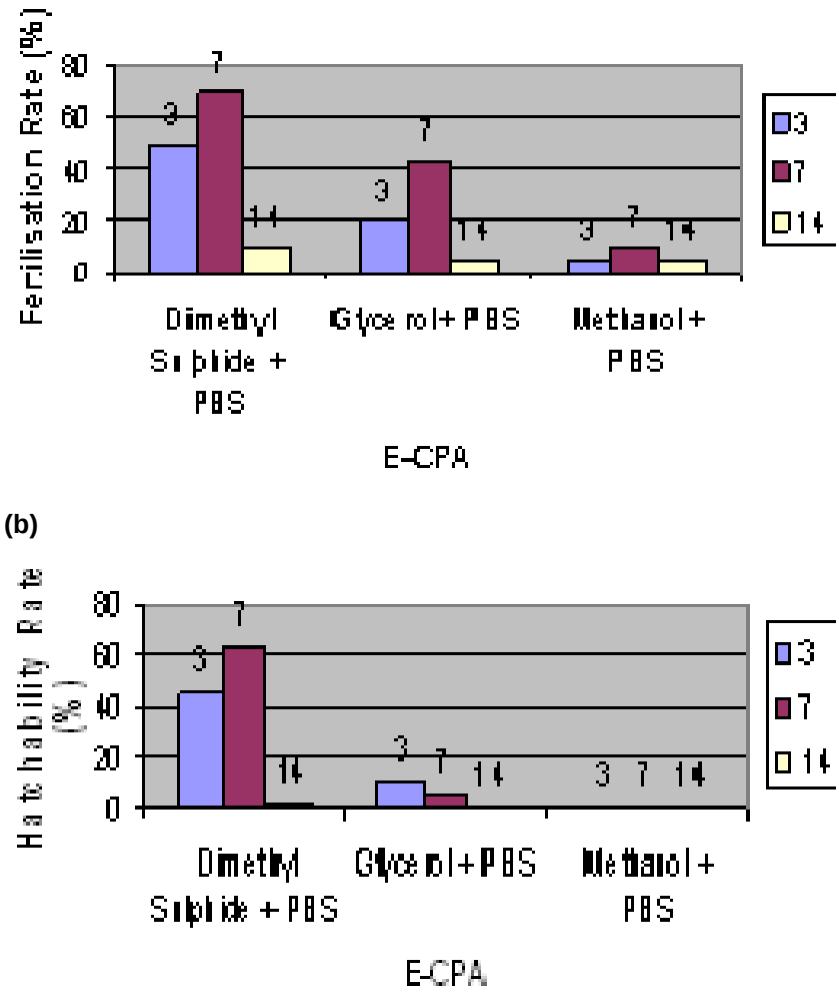

Figure 5. (a) Fertilization rate using 3-, 7-, 14-day old cryopreserved sperm in deep freezer and hatchability of fertilized eggs. (b) DMSO was proven to be the most efficient cryopreservative. E-CPAA: The cryopreserved sperm after thawing at day 3 and mixed with 5% glucose intravenous water at a ratio 1:1.

to fertilize the ovulated eggs ($p < 0.05$), however fertilization and hatchability rates were comparatively very low using 14-day old cryopreserved sperm (Figures 5a and b). Significantly, sperm cryopreserved in DMSO proved more efficient glycerol and methanol as hatchability was significantly higher ($p < 0.05$).

Considering the effect of type of cryopreservative used on sperm motility, DMSO and glycerol were shown to preserve the motility best. DMSO was found to be the most effective cryopreservative because it preserved sperm motility for the longest period of time. This result correlates with what was found in the literature. Methanol, although had the least performance, still preserved sperm motility for 28 days at appreciable percentage range of 42 - 25%. It was observed that both the freezing process and cryopreservative reagents decreased sperm motility after each period of cryopreservation. It could be deduced that cryopreserved sperm still needs to be activated after thawing in order for them to fertilize the eggs since there is a direct relationship between motility and fertility rates (Hafez, 1985).

Effect of cryopreservation in liquid nitrogen on fertilization and hatchability rate

In the first trial in which the liquid nitrogen-cryopreserved sperm were thawed at 27°C for 15 min, dimethyl-sulphoxide in combination with phosphate buffered saline (PBS) represented as DP gave the highest fertility rate

but was not significantly different ($p > 0.05$) from the other cryoprotectants. However, in the second trial, dimethyl-sulphoxide with the addition of 5% glucose in combination with PBS represented as GDP gave the highest fertility rate ($p < 0.05$) where the sperm was thawed at 35°C for 5 min. This may be explained by the extracellular protection offered by the glucose. In both the two cases, glycerol in combination with phosphate buffered saline represented as GP gave the lowest rate as shown in Table 2.

Since fertility closely relates to hatchability, the same trend was observed with the fresh sperm having the highest hatchability rate and was significantly different ($p < 0.05$) from the cryoprotectants tested in the first trial. However, no significant difference was observed among the different cryoprotectants tested in the two trials. The differences in fertility and hatchability between the control and the cryoprotectants tested may be due to the mild damage done to the spermatozoa during the process of lacerating the testes to extract semen. Also this may be due to intracellular vitrification- a commonly occurring problem in the process of freezing the sperm prior to cryopreservation in liquid nitrogen.

Effects of different cryoprotectants on the motility rate

In the motility evaluation, sperms cryopreserved with DGP gave the highest motility value but was not significantly different from that of the DF. Followed by this was the DP, MP, MF, DGF while those cryopreserved with GP, GF gave the lowest value ($p < 0.05$). The result was in conformity with that obtained under the short-term cryopreservation of the African catfish sperm by Omitogun et al. (2006).

Effect of the use of liquid nitrogen on the survivability of the cryopreserved sperm

The use of liquid nitrogen does not have adverse effect on the survivability of *C. gariepinus* larvae produced from the cryopreserved sperm (Table 2). However, those cryopreserved sperm thawed at 35°C for 5 min gave the best result to fertilize normal eggs.

This present study proves that the sperm of African catfish can be preserved in some cryodiluents at -20°C in a refrigerator, or in a deep freezer at -35°C or at -196°C in LN_2. This will reduce sperm wastage and increase the use of genetic variation for maintaining and breeding better fish strains. The ability to store sperm for days means that broodstock do not have to be kept on the farm, and spare sperm may be saved from wastage. With storage the sperm from good quality male broodstock can be used for fertilizing many females in different locations, and be kept for decades in LN_2.

Conclusion

Both the short-term and long-term storage of African catfish (*C. gariepinus*) sperm is practical, with the best techniques providing high levels of recovery of motility and fertility. Sperm to be cryopreserved should be frozen for 15 min on chilled water blocks before a final transfer into liquid nitrogen and then should be thawed rapidly at 35°C for 5 min. Moreover, it was observed from this study that the sperm to be cryopreserved could be contaminated by microorganisms. Hence, all activities during the process of cryopreservation should be conducted in a sterile environment. In addition, slow thawing of the cryo-preserved sperm at room temperature did not give a high percentage of fertility and hatchability, hence a higher temperature (35°C for 5 min) is recommended for rapid thawing of the cryopreserved sperm in liquid nitrogen to optimize sperm viability. In conclusion, dimethyl-sulphoxide (DMSO) gave the highest viability value among the cryoprotectants tested and can be further increased when 5% glucose solution is incorporated.

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REFERENCES

Adewumi AA (2004). The Effect of heating time of soyabean for broodstock nutrition on the reproductive performance of *Clarias gariepinus* Burchell, 1822. Ph.D. Thesis, Department of Zoology, Obafemi Awolowo University (OAU), p. 8.

Billard R, Cosson Y, Crim LW, Suquet M (1996). Sperm physiology and quality In: Broodstock management, egg and larval quality, Blackwell Ltd, Osney Mead, Oxford, pp. 25-52.

Chao NH, Chdo WC, Liu KC, Liao IC (1987). The properties of tilapia sperm and its cryopreservation. J. Fish Biol. 30: 107-118.

Hafez ESE (1985). Preservation and cryopreservation of gametes and embryo techniques for improving reproductive efficiency, In: E.S.E. Hafez (ed.). Reproduction in Farm Animals. Lea & Fabiger, USA, pp. 571-600.

Hallerman EM, Dunham R, Smitherman RO (1986). Selection or drift-isozyme allele frequency changes among channel catfish selected for rapid fish growth. Trans. Am. Fish Soc. 115: 60-68.

Harvey B (1983). Cryopreservation of *Sarotherodon mossambicus* spermatozoa. Aquaculture, 32: 313-320.

Hogendoorn H, Vismans MM (1980). Controlled propagation of the African catfish, *Clarias gariepinus* (C&V), II: Artificial reproduction. Aquaculture, 21: 39-53.

Heicth T, Uys W, Briitz PJ (1988). The culture of sharptooth catfish,

Clarias gariepinus in Southern Africa. South Afr. Nat. Sci. Prog. Rep. 153: p.133.

Jenkins JA (2000). Infectious diseases and quality assurance considerations for the transfer of cryopreserved fish gamates. In: Cryopreservation in Aquatic species. TR Tiersch, PM Mazik (Eds.). World Aquacult. Soc. 28: 282-288.

Kirpichnikov VS (1981). Genetic bases of fish selection. Springer-Verlag Berlin. p. 410.

Kwantong S, Amrit NB (2000). Effect of cryopreservation, extender and freezing rate on fertilization rate of frozen stripped catfish: *Pangasius hypothalamus* sperm. http: //www.blackwell-synergy.com /link /dio /10.1046/j.1365-2109. 2003. 00897.x/abs /:jresswind.

Lamai SL (1996). Successful handstripping of hatchery breeding reared male African catfish *Clarias gariepinus.* Proceedings of the 13th Annual Conference of the Fisheries society of Nigeria, New Bussa 1999, pp. 159-162.

Legendre M (1986). Seasonal changes in sexual maturity and fecundity, and HCG-induced breeding of the catfish, *Heterobranchus longifilis* Val. (Clariidae) reared in Ebrien lagoon (Ivory Coast). Aquaculture, 55: 201-213.

Legendre M, Oteme ZJ (1995). Effect of varying latency period on the quality and quantity of ova after HCG-induced ovulation in the African catfish, *Heterobranchus longifilis* (Teleostei: Clariidae). Aquat. Living Resour. 8: 309-316.

McAndrew BJ, Rana KJ, Penman OJ (1980). Conservation and preservation of genetic variation in aquatic organisms in recent advances in aquaculture (Muir JF, Robert R Eds.). Blackwell Scientific Publication, Oxford, UK, pp. 295-336.

Omitogun OG, Oyeleye OO, Betiku CO, Ojiokpota C, Aladele S, Sarumi MB (2006). Potentials of short-term cryopreserved sperm of the giant African catfish (*Clarias gariepinus* Burchell, 1982) for aquaculture in Nigeria. In: Olakojo SA, Ogunbodede BA, Akande SR Eds.). Proceedings of the 31st Annual Conference of the Genetics Society of Nigeria, NACGRAB, Moor Plantation, Ibadan. November 6-9, 2006. pp. 141-146.

Oyeleye OO, Omitogun OG (2007). Evaluation of motility of the short-term cryopreserved sperm of African giant catfish (*Clarias gariepinus*). Ife J. Agric. 22(1): 11-16

Richter CJJ, van den Hurk R (1982). Effects of 11-desoxycortisosterone-acetate and carp pituitary suspension follicule maturation in the ovaries of the African catfish, *Clarias lazera* (C.& V.). Aquaculture, 29: 53-66.

Scott AA, Bayries SM (1983). A review of the biological handling and storage of salmonid spermatozoa. J. Fish Biol. 17: 707-739.

SIGMA (1994). Cell counting and cell viability. Saint Louis, MO 63178 USA, pp. 1634-1635.

Stoss JT (1983). Short-term storage and cryopreservation of milt from Atlantic salmon and sea trout. Aquaculture, 30: 229-236.

UN (2009). Population Division of the Department of Economic and Social Affairs of the United Nations Secretariat (2009). World Population Prospects: The 2008 Revision. Highlights. New York: United Nations.

Viveiros AT, So N, Komen J (2000). Sperm cryopreservation of African catfish, *Clarias gariepinus*: cryoprotectants, freezing rates and sperm: egg dilution ratio. Therionology, Vol. 54, Num. 9.