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Effects of Storage Periods on Viability, Hatchability, Survival and Motility of Cryopreserved C. Gariepinus Semen

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***Abstract*— The present study investigated the effect of storage periods (0, 24, 48, 72, and 96h) on fish semen cryopreserved with Mounibs Extender (500mg of potassium bicarbonate, 100mg of reduced glutathione and 2135mg of sucrose in 50ml of distilled water) and mixed with cyoprotectant (5% Dimethyl sulphide DMSO). The quality of semen (motility and duration) and performance (fertility, hatchability percentages and fry survival) were compared with control (unpreserved fresh semen not subjected to storage). The fertility rate was significantly higher among the stored semen at 24hrs (68.5%), 48hrs (64.7%), 72hrs (59.8%) and 96hrs (59.1%) than fresh semen (56.6%). Hatchability rate showed the highest performance at 24hrs storage period (67.9%) followed by 48hrs (64.2%) than unpreserved semen (57.9%) which did not differ with 72hrs (59.3%) and 96hrs (58.9%). Fry survival was significantly higher in preserved stored semen 24hrs (99.4%), 48hrs (99.1%), 72hrs (98.6%) and 96hrs (98.5%) than control (97.4%). Motility duration was significantly higher P<0.05) in control (50s) compared to stored semen at 24 (48s), 48 (47.6s), 72 (46.8s), and 96 hours (46.5s) but differences did not exist (P>0.05) in motility rate between control and stored semen. Cryopreserved semen at 24 and 48 hours storage periods gave better sperm viability, fertility, and hatchability and survival rate than fresh semen and should be adopted in fish hatcheries in the tropics, to prevent semen deterioration, ensure its quality and availability for artificial insemination.**

***Index Terms*— fish semen, fertility, hatchability.**

I. INTRODUCTION

Preservation of fish semen for a short duration is generally useful from the commercial point of view and facilitates various hatchery operations. Fish semen could be preserved by storage in diluted or undiluted form in which semen may be incorporated with or without extenders. Undiluted sperm stored at low temperature has been reported to cause a reduction in fertilization capacity. Storage of diluted semen with extenders provides better control, compared to undiluted storage. Extender solutions can be mixed with semen to increase the volume of the semen samples and to prevent its deterioration during shipment for insemination and other research purposes. The act of doing this under a relatively low temperature of about 4°C - 169°C is termed cryopreservation.

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Due to the fact that fish seed is important for a sustainable agriculture, there is a need for all round availability of fish seed (fingerlings). The high demand for catfish fingerlings increasing agriculture production has stimulated the need for the artificial propagation of fish seed (FAO, 2007). Sperm cells under cryopreservation are subjected to oxidative stress resulting from lipid peroxidation, which can lead to reduced sperm viability and fertility (Donghue and Donoghue, 1997). Although semen contain antioxidants that counteract the damaging effects of lipid peroxidation and prevent excessive peroxide formation (Lewis et al., 1997), the endogenous antioxidative capacity of semen may be insufficient during storage (Maxwell and Salamon, 1993). In vitro studies suggested that the addition of some antioxidants to semen extenders could improve the motility and survival of spermatozoa (Sanchez-Partida et al., 1997; Krzyosiak et al., 2000; Bilodeau et al., 2002).

Previous studies have indicated the use of various diluents to preserve fish semen without reducing fertilizing capacity (Rasowo et al., 2007; Wachirachaikam et al., 2009; Kovacs and Urbanyi, 2010; Agarwal, 2011). Results of previous workers showed that sperm motility, motility duration, viability and fertilizing capacity vary widely due to differences in species variation, collecting method and storage conditions which have affected the success in fish sperm dilution (Maxwell and Salamon, 1993; Agarwal, 2011)). Reported study on suitable temperature and extender chemicals for the preservation of semen vitality and performance of C.gariepinus is somewhat scarce. The aim of the present study was to determine the effects of different storage periods of cryopreserved catfish semen on the: Sperm viability, Sperm fertility, Hatchability percentage of fertilized eggs, survival percentage of the hatchlings.

The extender is the solution of balanced salts that inhibits the activation of sperm. A long range of extender media have been used on different fish species but Mounib's extender has been recommended as suitable for carp and catfish (Agarwal *et al.*, 2009) and is prepared by dissolving 1000mg of $KHCO_3$ which serves as a buffer, 200mg of reduced glutathione to provide normal component of seminal fluid and 4270mg of Sucrose as source of energy in 100ml of distilled water, and enriched with Mannitol to protect against any toxic effect of DMSO frequently added as a cryoprotectant substance. Reported study on suitable temperature and extender chemicals for the preservation of semen vitality and performance of C.gariepinus is somewhat scarce. The objectives of this study was to determine the effects of different storage periods of cryopreserved catfish semen on the: Sperm viability, Sperm fertility, Hatchability percentage of fertilized eggs, Survival percentage of the hatchlings. The

African catfish (*Clarias gariepinus*) is widely cultured in Nigeria and it is one of the best aquaculture species under culture (FAO, 1994). The present study investigated the effect of storage periods (0, 24, 48, 72, and 96h) on C.gariepinus semen cryopreserved with Mounibs extender.

II. Materials and Methods

Research Area

This research was carried out at Prof. Nwamba Research and Teaching Farm, Mbaukwu Street along Old airport road in Emene, Enugu State latitude of 6030'E and Longitude of 6.5000N – 7.5000E, average daytime temperature 26^{o}C, night time temperature 20^{o}C, mean elevation 450m above sea level and an annual rainfall of 1800mm - 2100mm (NiMet, 2013).

Cryopreserving catfish semen

Diluent preparation (extender and cryoprotectant)

Diluents (a mixture of Extender and Cryoprotectant) was prepared on the first day of the research and stored in the refrigerator at 4^{0}C for 24hrs before use according to (Argwal et al 2007). Extender (mounibs) was prepared by dissolving 500mg of potassium bicarbonate, 100mg of reduced glutathione and 2135mg of sucrose in 50ml of distilled water, which is equivalent to dissolving 1000mg of potassium bicarbonate, 200mg of reduced glutathione and 4270mg of sucrose in 100ml of distilled water as proposed by Mounibs (1978). The various chemical components were repeatedly weighed with Ohaus Cooperation Chemical Balance to obtain the accurate weight, afterwards 50ml of distilled water was poured into a beaker of 100ml, then 500mg of potassium bicarbonate was added and stirred with spatula to dissolve properly, afterwards 2135mg of sucrose was added and stirred, followed by addition of 100mg of reduced glutathione. The solution was thoroughly stirred to obtain a homogenous mixture (Moczarski, 1977). Five (5%) of Dimethyl Sulphide (DMSO) was prepared and used as Cryoprotectant. 5% Dimethyl sulphide was prepared by adding 5ml of Dimethylsulphide into 95ml of distilled water. This was done using two beakers, one was used to obtain 95ml of distilled water while the other was used to collect 5ml of DMSO, which was gently introduced into 95ml of distilled water. It was stirred to obtain an even mixture. The extender (50ml) was gradually poured into the 100ml (1:2) Cryoprotectant, Afterwards, the mixture was thoroughly stirred with a spatula to obtain a homogenous mixture referred as the diluents (Agarwal, 2011). The prepared diluents were poured into a container having a lid and well tightened and kept in the refrigerator at temperature 4^{0}C for 24hrs (Chao et al., 1989). The male broodstock was weighed, cleaned with a towel before dissection was done with a new razor blade. Semen was extracted by dissecting the male broodstock from the abdomen towards the papilla to obtain the testis from which the semen was obtained by incising it followed by a collection with cryovials and refrigeration. Four beakers were washed and rinsed with saline solution, then using a 5ml syringe, 2ml of diluent was collected and poured into each of the four beakers containing 0.5ml semen to dilute at 1: 4 (Rana 1995)

Semen Storage and freezing.

Ampoules/vials method was used in storing semen for freezing (Mounibs, 1978). The diluted semen was stored with a syringe. This was done for the four beakers containing the diluted semen, therefore four syringes were used to store the semen for freezing. The volume of semen in each syringe was 2.5ml (i.e. 2ml of diluents and 0.5ml of catfish semen). After the storage, the semen was allowed to stay for equilibration before freezing. The semen was kept in the fridge at 40C for equilibration. The period of this equation is termed "equilibrium time. This is the time allowed to facilitate the penetration of Cryoprotectant into the cells for effective protection during freezing. (Grout and Morris, 1987). The semen was allowed to equilibrant for 45mins with the time of gradual addition of diluent to semen and storage taken into account. Equilibrium time was not longer than 45 – 60mins to maximize later recovery (Agarwal, 2007). The most appropriate equilibrium time is 45mins for DMSO (Sanni, 2008). The stored semen in cryovial (Syringe) was laid in the Freezer, before then the freezer was allowed to freeze up to -300C before the semen was kept. The semen was stored in the freezer at -30^{0}C for 4days but at the end of each day, each tube containing the cryopreserved semen was thawed (warned) and used for analysis and fertilization, to determine hatchability and survival.

Thawing and artificial fertilization with cryopreserved semen

The semen was thawed in a water bath at 57^{0}C for 10secs. But prior to the time of removal from the freezer, hot water was boiled to be used for the thawing(warming). The hot water was used for thawing at 57^{o}C for 10secs according to (Rana 1995). In several freshwater fish species, thawing is done at 30 – 80^{0}C in a water bath. The tube containing the cryopreserved semen were played in the water for 10secs. Afterwards, a dropper was used to collect a drop of the semen for Analysis under the microscope to determine the viability of the sperm cells which include the sperm swimming velocity (motility), motility percentage and motility duration.

Egg stripping

The female broodstock was held by two persons and gently pressed at the abdomen for the eggs to move freely out of the cloaca and were poured into 3 dry 100ml beakers.contained with12g of stripped eggs. Afterwards, the female broodstock was kept, and then fertilization commenced. After each egg collection with each beaker, the eggs were fertilized with 0.2ml of the cryopreserved semen. 0.2ml of semen was spread on the eggs, afterwards was gently stirred to ensure even fertilization. The same was done to the two breakers containing 12g of eggs. Afterwards, the three replicates of the fertilized eggs were incubated. The male broodstock initially brought out with the female broodstock, was dissected to extract fresh unpreserved semen for analysis. Fertilized eggs were incubated in a kakaban for 24h.

Viability, Fertility, Hatchability and Survival Evaluation.

The unpreserved semen,(0.0 h), 24hrs,48hrs,78hrs, and 96hrs cryopreserved semen were analyzed under the microscope of 10x100 magnification. Using a dropper, a drop of each semen was dropped on the microscope slide and activated with saline solution, this was done to determine if the sperm cells were still alive after being stored in the freezer at -30oC for 24hrs, 48hrs, 72hrs, and 96hrs. Also, their movement behavior was evaluated such as motility(swimming velocity), % motility and motility duration (Randall et al., 1971). 0.2ml of unpreserved, 24hrs, 48hrs, 72hrs and 96hrs (Scott et al., 1980).

Statistical analysis

Data were analyzed with SPSS package (Statistical Package for Social Sciences). The difference between each treatment and control was subjected to one-way analysis of variance (ANOVA) and differences in mean was separated with the Duncan multiple range test.

III. RESULTS

Table 1 showed that the storage periods of cryopreserved catfish semen had some effects on the sperm viability, fertility, hatchability and survival.

Table 1: Viability, Motility and Survival Rates of Cryopreserved Clarias gariepinus Semen

Parameters	storage period	Mean	Std. Error ±
fertility_rate	Fresh semen	56.6333[a]	.95277
	24.00	68.5000[d]	1.22882
	48.00	64.7000[c]	1.19304
	72.00	59.8000[b]	.36056
	96.00	59.1000[b]	.28868
	Total	61.7467	1.19151
hatchability_rate	Fresh semen	57.9667[a]	.52387
	24.00	67.9333[c]	1.16667
	48.00	64.2000[b]	1.30767
	72.00	59.3667[a]	.40961
	96.00	58.9667[a]	.52387
	Total	61.6867	1.06592
Motility rate	Fresh semen	92.3000[a]	.33456
	24.00	96.1000[a]	.57735
	48.00	96.9000[a]	.57735
	72.00	98.4000[a]	.57735
	96.00	99.2000[a]	.57735
	Total	95.8000	.46496
motility_duration	Fresh semen	50.0000[b]	.57735
	24.00	48.0000[a]	.57735
	48.00	47.6000[a]	.57735
	72.00	46.8000[a]	.57735
	96.00	46.5000[a]	.57735
	Total	47.7800	.39535
survival rate	Fresh semen	97.4000[a]	.40415
	24.00	99.4000[c]	.00000
	48.00	99.1333[c]	.03333
	72.00	98.5667[b]	.08819
	96.00	98.5000[b]	.20000
	Total	98.6000	.20000

Sperm viability

Result showed that the cryopreserved semen of 24hrs, 48hrs, 72hrs and 96hrs were still alive and exhibited lower motility duration, than unpreserved semen. It took lesser time (48.0sec, 47.6secs, 46.8secs, and 46.5secs) respectively to cover 1mm distance, while fresh semen took longer time (50.0secs) to cover 1mm. This is because the cryopreserved sperm cells had more energy in them, due to the sucrose content of the extender that supplied energy to the sperm cells during preservation. There were no significant (P>0.5) difference between the storage periods of the semen on the sperm viability but between cryopreserved and fresh semen. Therefore the storage periods does not have significant effect on the sperm viability.

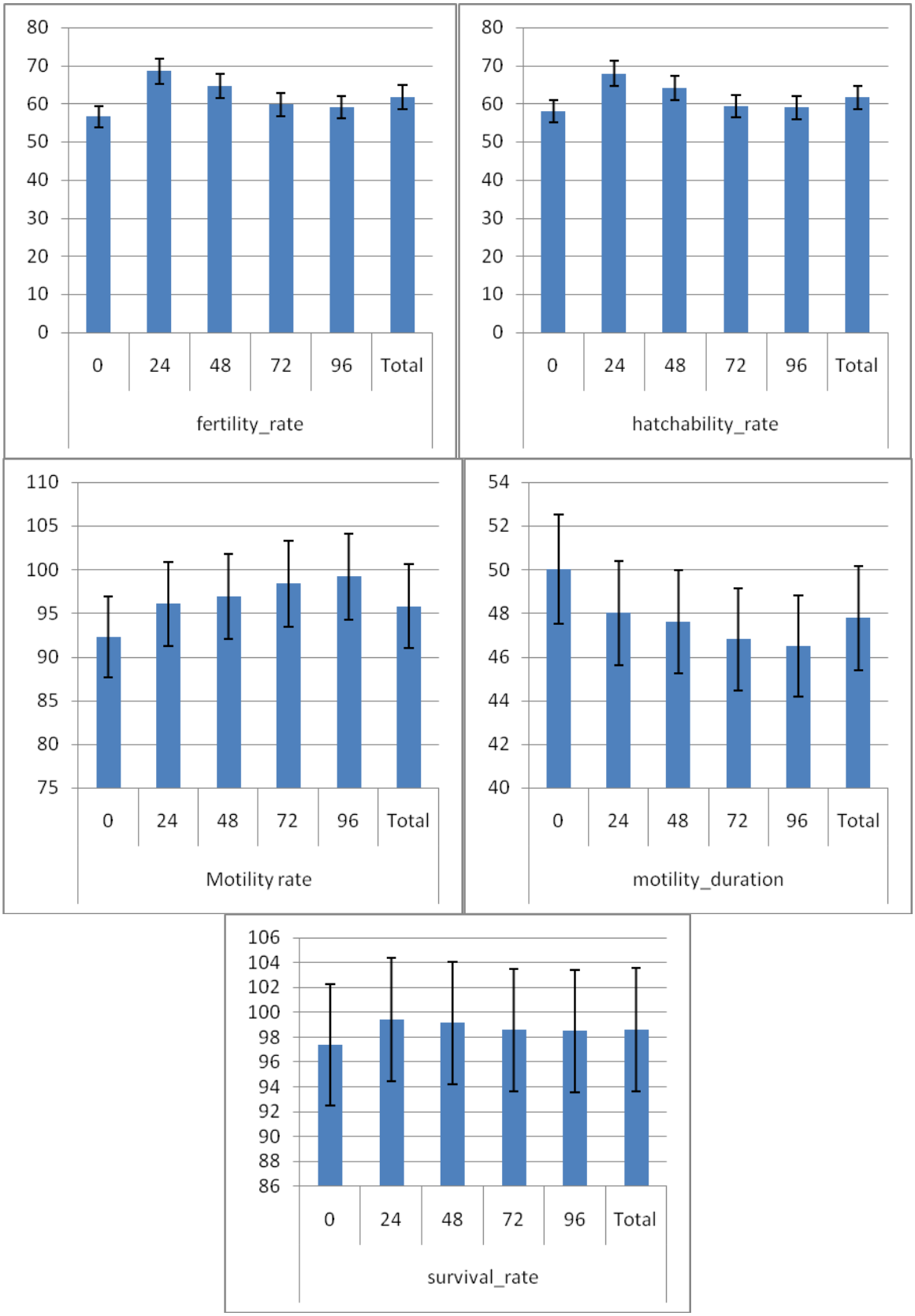

Figure 1: reproductive qualities of C. gariepinus semen cryopreserved with Mounibs extender over a period of 96h

Sperm fertility

Figure 1 showed that cryopreserved semen of 24hrs, 48hrs, 72hrs and 96hrs, had greater fertility capacity than fresh semen. It was seen from the result that 24hrs cryopreserved semen exhibited the highest fertility (68.5%) followed by 48hrs cryopreserved semen with 64.7% fertility, then 72hrs cryopreserved semen with 59.8%. and 96hrs cryopreserved semen with 59.1%, while fresh semen exhibited 56.6%. There were significant difference ($p<0.5$) between the storage periods. There were slight decline in fertility as storage period progresses, this is due to the oxidative stress resulting from lipid peroxidisation which could lead to reduced fertility.

Hatchability

Result indicated that there were significant difference ($P < 0.5$) in the hatchability of eggs fertilized in 24hrs and 48hrs cryopreserved semen while there was no difference existed

between 72hrs, 96h and fresh semen. There were significant difference between 24hrs and 48hrs cryopreserved semen with 67.9% and 64.2% respectively while 72hrs, 96hrs and fresh semen had 59.3%, 58.9% and 57.9% respectively. This implies that hatchability is best for 24hrs and 48hrs cryopreserved semen.

IV. Survival

Result showed that fries of cryopreserved semen survived more than the fries of fresh semen with significant difference . Also there were significant difference ($p<0.5$) between the storage periods of cryopreserved semen on the survival rate of the fries. Fries of 24hrs cryopreserved semen had higher survival rate (99.4%), followed by fries of 48hrs cryopreserved semen with 99.1%, fries of 72hrs exhibited 98.5% survival rate, 96hrs gave 98.5% while unpreserved semen gave 91.4% survival rate, indicative that fries survival was best at 24hrs and 48hrs of cryopreservation.

TABLE 2 : WATER QUALITY PARAMETERS

Storage periods	pH.	DO mg/L	^{0}C
Fresh semen.	7.00	6.0	25
24hrs storage	7.10	6.1	24
48hrs storage	7.00	6.1	23
72hrs storage	7.10	6.0	24
96hrs storage	7.00	6.1	23

4. Discussion

Cryopreservation of fish semen of a commercially important freshwater species was developed in order to make all availability of viable and fertile semen for healthy seed production in the hatchery, for conservation and propagation strategies and for selective crossbreeding programmes. The success of semen cryopreservation technique lies in the proper collection of semen from healthy donor fish, development and use of suitable extenders to prevent depletion of sperm energy reserve and to maintain sperm in quiescent condition but alive. Using appropriate cryoprotectants in the right concentration to reduce cryo-injury from cold and thermal shock, freezing and thawing procedure to minimize sperm damage. Fertilization success of gametes and subsequent development of early life stage are the reliable measures of cryopreservation success. It has been seen from this study that cryopreserved semen gave better sperm viability,fertility, hatchability and survival rate. This agrees with the research conducted by (Kovas and Ubanyi, 2010) who observed from several trials of an experiment that cryopreserved semen resulted in significantly higher fertilization and hatchability percentage than freshly extracted semen.

The present study corrobated the report of Rana (1995) which indicated that storage periods of cryopreserved semen with mounibs extender did not reduce the viability of the sperm cells probably owing to the fact that the dimethyl sulphide gives the best cryoprotection effect on sperm cells (Rana, 1995). Hence sperm cells could be stored for long periods without losing its viability. Cryopreservation at 24hrs exhibited the highest fertility, hatchability and survival which agree with Maxwell and Salamon (1993). Farmers are advised to use cryopreserved semen for artificial fertilization since it performs better than fresh semen. Most preferably they should use cryopreserved semen at 24hrs storage. This research showed that the longer storage of gave depreciated values compared with short duration which improved parameter results such as the sperm viability, fertility, hatchability and survival (Kovas and Urbani, 2010).

V. Conclusion

.The fertility/viability test of cryopreserved semen should be performed by artificial fertilization experiments and incubation and hatching of fertilized eggs in the hatchery system to evaluate the success of the cryopreserved technique. Cryopreservation was duration dependent and showed best performance in 24hrs storage, followed by 48hrs, 72hrs and 96hrs respectively. Cryopreserved semen at 24 and 48 hours storage periods gave better sperm viability, fertility, and hatchability and survival rate than fresh semen and should be adopted in fish hatcheries in the tropics, to prevent semen deterioration, ensure its quality and availability for artificial insemination.

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