Preliminary investigations on the cryopreservation of *Clarias gariepinus* (Clariidae: pisces) sperm

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Abstract

Semen of the sharptooth catfish *Clarias gariepinus* was diluted with combinations of different extenders and cryoprotective agents to evaluate their suitability as cryodiluents. An effective freezing rate and thawing temperature of the semen were established. Spermatozoa were successfully stored in liquid nitrogen for periods exceeding sixty days, with satisfactory survival rates and high sperm motility.

Introduction

The establishment of aquaculture to supplement the dwindling protein resources of the world has necessitated the development of large scale artificial spawning techniques of a number of fish species. Problems encountered in the spawning of fish used for production, were the selection of breeding stock with emphasis on qualities such as low temperature tolerance, disease resistance and growth rate. In South Africa where a number of endemic freshwater fish species like the Border barb (*Barbus trevelyani*), Fiery redfin (*Barbus phlegethon*), Treur River barb (*Barbus treurensis*) and the Drakensberg minnow (*Oreodaimon quathlambae*) are endangered (Skelton, 1977), it became necessary to investigate the large-scale spawning of indigenous fish species in order to restock river systems. Moreover, the lack of synchronization in male and female gonadal maturation has further hampered the artificial propagation of fish. A recent development to assist in the controlled reproduction of fish is the cryopreservation of sperm and ova. The cryopreservation of gametes can be of practical value, for it allows banking of semen which could be used in the manipulation of spawning programmes (Ginzburg, 1972; Harvey, 1982).

In the case of livestock, artificial insemination has been made possible by the successful development of sperm cryopreservation techniques. Attempts in recent years to transfer this technology to the preservation of fish sperm showed difficulties, because techniques developed for mammalian sperm were not compatable with the physiological peculiarities of fish semen (Harvey, 1982).

Fish spermatozoa can be refrigerated successfully at 0-4 °C for several days (Ginzburg, 1972). Survival of fish sperm can also be extended at above zero temperatures by providing adequate air space above the semen sample or by storing semen under oxygen (Truscott *et al.*, 1968; Billard, 1981).

Since the first experiments on the long-term preservation of fish spermatozoa at sub-zero temperatures by Hoyle and Idler (1968) and Graybill and Horton (1969), much work has been done with variable success rates on the cryogenic preservation of fish semen, especially *Oreochromis mossambicus*, *Salmo gairdneri* and *Mugil cephalus* (Scott and Baynes, 1980; Harvey, 1983; Stoss and Holtz, 1983, Chao *et al.*, 1975; Chao, 1982). In these experiments various cryodiluents were employed with the simplest formulations still being the most successful. Semen of *M. cephalus* was preserved for one year in liquid nitrogen after which a fertility success of 2,7 % was obtained (Chao *et al.*, 1975). Chao (1982) improved the techniques and achieved a 90 % motility and a fertility rate exceeding 28 % after one year. Investigations by Mounib (1978) yielded a 30 % motility of *Salmo salar* sperm after thirteen months. Up to date, no attempt has been made to cryopreserve indiginous freshwater fish spermatozoa in South Africa.

The aim of the present investigation was to develop a suitable extender and cryoprotective agent for the long-term preservation of sharptooth catfish *Clarias gariepinus* sperm, which at present is being evaluated for its aquacultural potential in Southern Africa.

Materials and methods

Mature male sharptooth catfish were obtained from the Roodeplaat Dam near Pretoria, Transvaal during October 1983 and acclimatized for three weeks at 23 ± 1 °C in four separate holding tanks. Sperm production was induced by the administration of chorionic gonadotropins (Choragon) and alcohol preserved catfish pituitary extract (PGE) as described by Schoonbee *et al.* (1980). Semen was obtained by dissecting out and squeezing the testes. Five different combinations of extenders and cryoprotective agents were then evaluated for their suitability as cryodiluents.

TABLE 1
CRYOPROTECTIVE AGENTS AND EXTENDERS USED IN CRYOPRESERVATION EXPERIMENTS WITH *C. GARIEPINUS* SEMEN

Code	Cryodiluent		Reference
	Cryoprotectant	Extender	
A	5 % Methanol	15 % Milk 80 % Fish saline	
B	5 % Glycerol	5 % Glucose	Chao *et al.* (1975) Chao (1982)
C	12,5 % DMSO	Reduced glutathione 2 g/ℓ $KHCO_3$ 10 g/ℓ Sucrose 42 g/ℓ	Mounib (1978)
D	12,5 % Glycerol	Same as C	Mounib (1978)
E	5 % DMSO	15 % Milk 80 % Fish saline	Chao *et al.* (1975)

TABLE 2
PROCEDURES FOLLOWED IN CRYOPRESERVATION OF *C. GARIEPINUS* SPERM AND THE EXPERIMENTAL RESULTS OBTAINED

Exp.	Before freezing			Cryo-protectant	Ex-tender (see Table 1)	Tech-nique	Equili-bration time (min)	Freez-ing rate	Thaw temp. (0 °C)	Storage Time	After thawing			Dilution ratio
	Moti-lity time (s)	Moti-lity grade	% Moti-lity								Motility grade	Moti-lity %	Moti-lity time (s)	
1	116	+++++	60	Methanol	A	Straw	20	X	20	24 hours	–	0	0	1:1 to 1:10
2	120	+++++	50	Glyc.	B	Straw	20	Y	20	24 hours	–	0	0	1:1
3	114	+++++	30	DMSO	C	Straw	20	Y	20	24 hours	–	0	0	1:3
4	118	+++++	30	Glyc.	D	Straw	20	Y	20	24 hours	–	0	0	1:3
5	120	+++++	50	Glyc.	B	Straw	20	X	20	24 hours	–	0	0	1:1
6	114	+++++	30	DMSO	C	Straw	20	X	20	24 hours	–	0	0	1:3
7	118	+++++	30	Glyc.	D	Straw	20	X	20	24 hours	–	0	0	1:3
8	120	+++++	50	Glyc.	B	Vial	20	Y	20	24 hours	–	0	0	1:1
9	114	+++++	30	DMSO	C	Vial	20	Y	20	24 hours	–	0	0	1:3
10	118	+++++	30	Glyc.	D	Vial	20	Y	20	24 hours	–	0	0	1:3
11	120	+++++	50	Glyc.	B	Vial	20	X	20	24 hours	+++	1	0	1:1
12	114	+++++	30	DMSO	C	Vial	20	X	20	24 hours	–	1	0	1:3
13	118	+++++	30	Glyc.	D	Vial	20	X	20	24 hours	–	0	0	1:3
14	100	+++++	80	Glyc.	B	Vial	60	Z_1	25	24 hours	+++++	40	76	1:1
15	100	+++++	80	Glyc.	B	Vial	60	Z_2	25	24 hours	++++	30	74	1:1
16	100	+++++	80	Glyc.	B	Vial	60	Z_3	25	24 hours	++++	10	68	1:1
17	100	+++++	80	Glyc.	B	Vial	60	Z_4	25	24 hours	++++	10	56	1:1
18	100	+++++	80	Glyc.	B	Vial	60	Z_5	25	24 hours	++++	20	68	1:1
19	100	+++++	80	Glyc.	B	Vial	60	Z_1	7	24 hours	++++	30	74	1:1
20	100	+++++	80	Glyc.	B	Vial	60	Z_2	7	24 hours	++++	20	72	1:1
21	100	+++++	80	Glyc.	B	Vial	60	Z_3	7	24 hours	+++	2	35	1:1
22	100	+++++	80	Glyc.	B	Vial	60	Z_4	7	24 hours	++++	4	52	1:1
23	100	+++++	80	Glyc.	B	Vial	60	Z_5	7	24 hours	++++	10	64	1:1
24	95	+++++	80	Glyc.	B	Vial	0	Z_1	25	30 days	+++++	20	61	1:1
25	84	+++++	80	DMSO	E	Vial	0	Z_1	25	30 days	+++++	15	57	1:1
26	110	+++++	70	Methanol	A	Vial	0	Z_1	25	30 days	++++	2	47	1:1
27	95	+++++	80	Glyc.	B	Vial	0	Z_1	25	30 days	+++++	15	52	1:1
28	84	+++++	80	DMSO	E	Vial	0	Z_1	25	30 days	++++	10	44	1:1
29	110	+++++	70	Methanol	A	Vial	0	Z_1	25	30 days	++++	1	35	1:1
30	95	+++++	80	Glyc.	B	Vial	120	Z_1	25	30 days	+++++	20	64	1:1
31	84	+++++	80	DMSO	E	Vial	120	Z_1	25	30 days	++++	10	51	1:1
32	110	+++++	70	Methanol	A	Vial	120	Z_1	25	30 days	++++	5	47	1:1
33	95	+++++	80	Glyc.	B	Vial	0	Z_1	25	60 days	++++	20	66	1:1
34	84	+++++	80	DMSO	E	Vial	0	Z_1	25	60 days	++++	20	68	1:1
35	110	+++++	70	Methanol	A	Vial	0	Z_1	25	60 days	++++	4	54	1:1

Glyc. – Glycerol; DMSO – Dimethylsulphoxide;
Vial – Bio-Freeze Vial; Straw – polyethelene straw

Various dilution ratios as well as seven freezing rates, three cryoprotective agents and two thawing temperatures were investigated (Table 2). Different rates of freezing were obtained by introducing the semen samples into nitrogen vapour (– 80 °C), liquid nitrogen (– 196 °C) and iso-propanol cooled down with dry ice (+ 20 to – 75 °C). Freezing rate X (Table 2) was achieved by first leaving the samples in nitrogen vapour for two minutes followed by rapid freezing in liquid nitrogen. A fast freezing rate Y, as described by Mounib (1978), was employed by submerging the diluted semen sample directly into liquid nitrogen. Freezing rates Z_1-Z_5 presented in Figures 1-5, were obtained by adding known quantities of dry ice to iso-propanol.

The effect of pressure release on thawing and freezing was investigated by incorporating a pressure release mechanism to the cap of the bio-vials containing the semen.

Only good quality semen with high sperm counts and motility was used in the cryopreservation experiments. Total motility, motility grade and percentage motile spermatozoa were determined before freezing, after 24 h, 30 days and 60 days. Motility was evaluated microscopically by employing the following scale: +++++ very strong; ++++ strong; +++ moderate; ++ weak and + very weak. Observations were subsequently made on the suitability of Costar bio-vials and polyethelene straws in semen preservation (Chao, 1982).

Results

The experimental procedures followed and the results obtained in the cryopreservation of *C. gariepinus* sperm are presented in Table 2. Sperm counts varied from 0,75 to as high as 9,8 million/mm³. After preservation periods of 30 to 60 days, 1,96 million sperm/mm³ survived.

Survival of *C. gariepinus* spermatozoa which was evaluated according to motility, showed glycerol to be an effective cryoprotective agent. The most suitable cryodiluent (B) is shown in Table 1. This cryodiluent yielded a 20% survival of spermatozoa with no loss of motility after 60 days. Unsatisfactory results were,

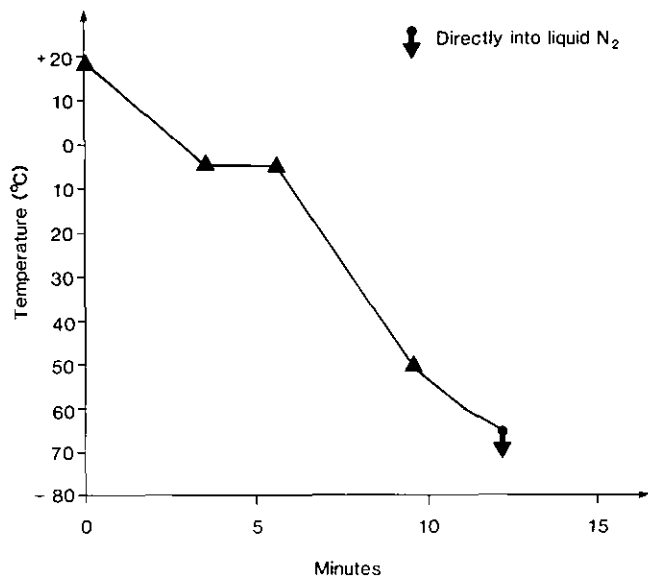

Figure 1: Freezing rate Z_1.

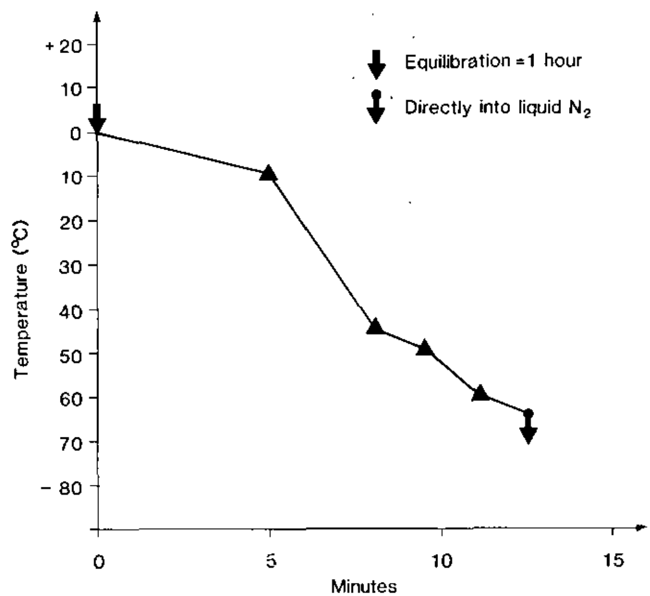

Figure 3: Freezing rate Z_3.

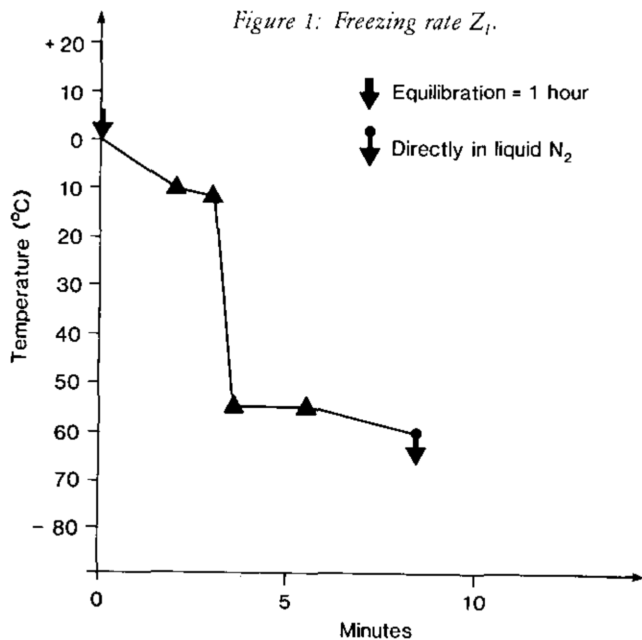

Figure 2: Freezing rate Z_2.

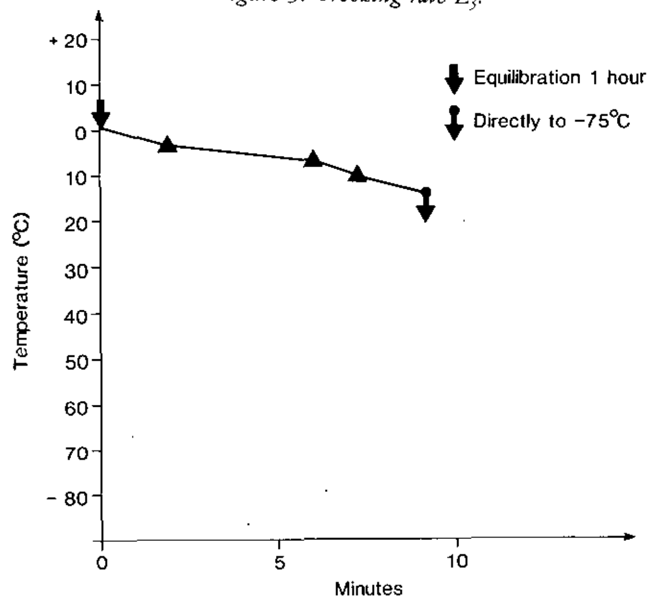

Figure 4: Freezing rate Z_4.

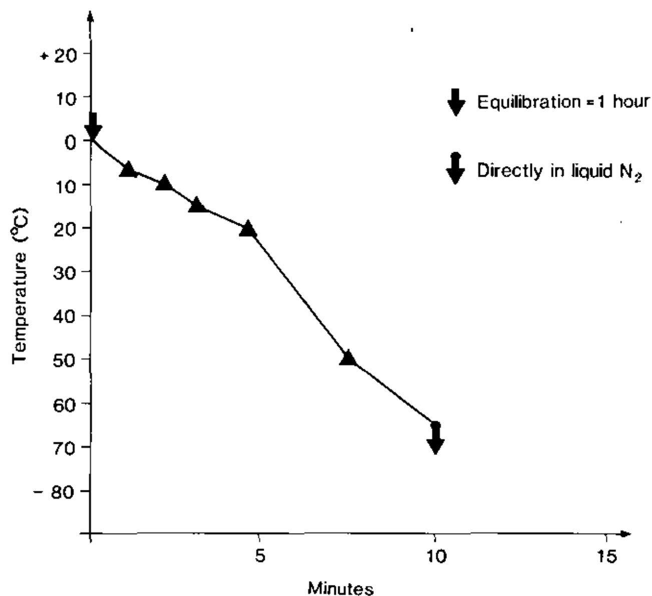

Figure 5: Freezing rate Z_5

however, obtained where the extender and cryoprotectant used by Mounib (1978) were employed (Tables 1 and 2). A rapid thawing procedure was found to be superior to a slow one (Experiments 14-23, Table 2) and a relatively slow freezing rate (Z_1, Fig. 1) was the most successful in this series of experiments. The percentage survival as well as duration of active movement of the sperm was reduced in the presence of a pressure release mechanism in the vial cap (Experiments 27 to 29, Table 2).

Discussion

According to Scott and Baynes (1980), most of the recent cryogenic preservation procedures do not provide time for equilibration. Harvey (1983) and Stoss and Holtz (1983), working on the equilibration of fish semen after mixing with cryoprotectants, concluded that the detrimental effects of any equilibration time were aggravated by increasing the concentration of the cryoprotective agent. In the present investigation equilibration times employed were determined by the period required to dilute and prepare the semen samples for cryopreserva-

tion. Thus the effects of equilibration time on the survival rate of the spermatozoa of this fish species still have to be evaluated experimentally.

Decreases in both motility and the percentage survival of *C. gariepinus* spermatozoa when pressure release mechanisms were employed, corresponded with results obtained by Tinnerberg *et al.* (1980) on rabbit spermatozoa. It was found that semen frozen at a high pressure (400 atmospheres) had higher spermatozoal survival rates than when frozen at 1 atmosphere.

Freezing rate is critical in cryogenics and appears to be species specific. It is known that the optimum freezing rate for salmonid sperm varies between 30 °C min^{-1} and 160 °C min^{-1} (Scott and Baynes, 1980). In contrast, the best freezing rate obtained for *C. gariepinus* sperm during the present study was 7 °C min^{-1}.

This rate is higher than that found for bull semen which varies between 0,8 °C min^{-1} and 5 °C min^{-1} (Salisbury *et al.*, 1978). From the results available in the literature and based on the author's own experience with *C. gariepinus* semen it is evident that the freezing rate is one of the most critical aspects in the cryopreservation of spermatozoa. This aspect requires further investigation.

From the results obtained in this study, the freezing rate Z_1 (Fig. 1) gave the most promising results on the survival and motility of *C. gariepinus* sperm. The present experiments further indicate that the motility grade of the surviving sperm remained virtually the same while a much lower percentage of live sperm was found after preservation in liquid nitrogen. This phenomenon should be investigated further. Motility, however, serves as a measure of flagellar movement and does not guarantee fertility (Salisbury *et al.*, 1978).

The results obtained so far suggest that it will be possible to store *C. gariepinus* sperm successfully over prolonged periods at sub-zero temperatures. To improve on the present results more research needs to be undertaken on the biochemistry of semen of this species. This will ensure that the most suitable extender and cryoprotective agent will be obtained with the osmolality of the diluent closely resembling that of the fluid in which the sperm are maintained *in vivo*.

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