UNIVERSITI PUTRA MALAYSIA

EFFECTS OF CRYOPRESERVATION ON PERFORMANCE AND FERTILIZING ABILITY OF SPERM FROM *KERAI* (*Puntius daruphani)*

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By

MOHAMED M. M. EMHEMED

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malayisa, in Fulfilment of the Requirements for the Degree of Master Science

August 2008

DEDICATION

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To my parents

*****

To my beloved wife

*****

And

To my son and daughter

Thank you for your love, understanding and support

*****

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Master Science

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Chairman : Annie Christianus, PhD

Faculty : Agriculture

Puntius daruphani broodstocks bought from fishermen in Temerloh, Pahang, conditioned in the Hatchery Unit of Universiti Putra Malaysia were used for all the experiments. In this study fresh and cryopreserved sperm of *P. daruphani* were analyzed for their physical characteristics (motility, motile period, live sperm count and abnormality), morphology, fertilizing ability and hatching rate. Kurokura medium and dimethyl sulfoxide (DMSO) were used as extender and cryoprotectant, respectively in the cryopreservation of the sperm.

The percentage of motility, live sperm count and time of motile period after activation of frozen-thawed sperm were significantly lower than that of fresh sperm. Whereas the percentage of abnormality for frozen-thawed sperm was significantly higher than fresh sperm. ANOVA showed that there was no

significant different ($p>0.05$) between fresh samples for all the parameters, whereas results among frozen-thawed samples using different concentrations of DMSO (10 - 15%) were significantly different ($p<0.05$) for all the parameters. Results of fertilizing ability and hatching rate were significantly different ($p<0.05$) between fresh and cryopreserved samples, whereas there were no significant different ($p>0.05$) among frozen-thawed samples.

The entire testis with total length ranged from 9.8 to 11.3cm, were categorized as the anterior, middle and posterior parts. The anterior, middle and posterior parts were creamy in colour and having soft tissue. Anterior and middle parts involved in the production of spermatozoa. Posterior part as glandular testis is only displayed some ducts with smooth muscle layer and produce seminal fluids. *P. daruphani* sperm consisted from three parts head, midpice and flagellum, measuring 2.19±0.01, 0.955±0.02 and 17.08±0.05 µm respectively.

In the first experiment, the results on the physical characteristics (motile period, motility, abnormality and live sperm count) were significantly different ($p<0.05$) between the fresh and cryopreserved sperm (using 10% DMSO as cryoprotectant). However the results between 1 and 3-month cryopreserved sperm samples were significantly different ($p<0.05$) for all the above parameters. Liquid nitrogen affected the sperm membrane function and mitochondria, increased the percentage of abnormality by damaging the cells of the spermatozoa.

In the second experiment, the results on the physical characteristics (motile period, motility, abnormality and live sperm count), fertilizing ability and hatching rates were significantly different ($p<0.05$) between the fresh and cryopreserved samples of 1 and 3-month (using 15% DMSO). There were no significant different ($p>0.05$) between the cryopreserved sperm samples of 1 and 3-month for all the parameters above. However, there were significant different ($p<0.05$) for all the physical characteristics (motile period, motility, abnormality and live sperm count) between cryopreserved (1 and 3 month) sperm samples using 10 and 15% DMSO. These results are supported by the observation on the sperm morphology showing that by using 15% DMSO produced less damages on the sperm membrane, cytoplasmic mitochondria and flagellum as compared to 10% DMSO.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan ijazah Master Sains

Kesan kriopengawetan ke atas prestasi dan kebolehan mensenyawa sperma Puntius daruphani

Oleh

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Induk *Puntius daruphani* dibeli dari nelayan di Temerloh, Pahang, disesuaikan di Hatcheri Universiti Putra Malaysia telah digunakan untuk semua eksperimen. Dalam kajian ini, sperma *P. daruphani* segar dan yang telah dikrio-awet dianalisis untuk ciri-ciri fizikal (motiliti, jangka motil, jumlah sperma hidup dan abnormaliti), morfologi, kemampuan persenyawaan dan kadar penetasan. Medium Kurokura dan dimethyl sulfoxide (DMSO) masing-masing digunakan sebagai ekstender dan pelindung krio di dalam kryoawetan sperma.

Peratus motili, jumlah hidup dan jangka motil sperma diaktifkan selepas dinyahbekukan adalah sangat ketara lebih rendah daripada sperma segar. Manakala peratus abnormaliti sperma yang dinyahbekukan adalah ketara lebih tinggi berbanding dengan sperma segar. ANOVA menunjukkan bahawa tiada

perbezaan ketara ($p>0.05$) di antara sperma segar untuk semua parameter, manakala keputusan di antara sampel yang dinyahbekukan menggunakan kepekatan DMSO yang berbeza (10 - 15%) menunjukkan perbezaan yang ketara ($p<0.05$) untuk semua parameter. Keputusan kemampuan persenyawaan dan kadar penetasan adalah berbeza dengan ketara ($p<0.05$) di antara sampel segar dan yang dikrioawet, Manakala tiada perbezaan ketara ($p>0.05$) di antara sampel yang dinyahbekukan.

Keseluruhan testis dengan jumlah panjang dari 9.8 to 11.3cm, dibahagikan kepada bahagian anterior, tengah dan posterior. Bahagian anterior, tengah dan posterior berwarna krim dan mempunyai tisu lembut. Bahagian anterior dan tengah terlibat dalam penghasilan spermatozoa. Bahagian posterior hanya sebagai testis glandular degan kelihatan beberapa duktus dan lapisan otot licin, menghasilkan cecair seminal. Sperma *P. daruphani* terdiri daripada tiga bahagian, kepala, penengah dan flagelum, dengan masing-masingnya berukuran 2.19±0.01, 0.955±0.02 dan 17.08±0.05 µm.

Di dalam eksperimen pertama, terdapat perbezaan yang ketara ($p<0.05$) pada keputusan untuk ciri-ciri fizikal (motiliti, jangka motil, abnormaliti dan jumlah sperma hidup) di antara sperma segar dan yang dikrioawet (menggunakan 10% DMSO sebagai pelindung kryo). Walau bagaimanapun, terdapat perbezaan yang ketara ($p<0.05$) untuk semua parameter di atas di antara sperma yang dikrioawet untuk tempoh 1 dan 3-bulan. Cecair nitrogen memberi kesan ke atas

fungsi membran sperma dan mitokondria, meningkatkan peratus abnormaliti dengan merosakkan sel sperma.

Pada eksperimen kedua, keputusan ke atas ciri-ciri fizikal (jangka motil, motiliti, abnormaliti dan jumlah sperma hidup), kemampuan persenyawaan dan kadar penetasan menunjukkan perbezaan ketara ($p<0.05$) di antara sampel sperma segar dan yang dikrioawet selama 1 dan 3-bulan (menggunakan 15% DMSO). Tidak terdapat perbezaan ketara ($p>0.05$) di antara sampel sperma yang dikrioawet selama 1 dan 3-bulan untuk semua parameter di atas. Walau bagaimanapun, terdapat perbezaan yang ketara ($p<0.05$) untuk semua ciri-ciri fizikal (jangka motil, motiliti, abnormaliti dan jumlah sperma hidup) di antara sampel sperm yang dikrioawet (1 dan 3-bulan) menggunakan 10 dan 15% DMSO. Keputusan ini disokong oleh keputusan pemerhatian ke atas morfologi sperma, yang menunjukkan kurang kerosakan ke atas membran, sitoplasmik mitokondria dan flagelum sperma apabila menggunakan 15% DMSO berbanding dengan 10% DMSO.

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This thesis submitted to the Senate of Universiti Putra Malaysia and has been accepted was fulfillment of the requirement for the degree of Master of Sciences.
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DECLARATION

I declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or at any other institution.

MOHAMED EMHEMED

Date:

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LIST OF ABBREVIATIONS

cm	centimeter
CPE	Carp Pituitary Extract
CASA	Computer Assisted Sperm Motility Analysis
CPAs	Cryoprotective Agents
D	Duct
DMSO	Dimethyl Sulfoxide
gm	Gram
IBS	Institute of BioScience
Kg	Kilogram
L	Lumen
LN	Liquid Nitrogen
M	Mole
Min	Minute
mg/l	milligram per liter
µl	Micro liter
mM	mill Molar

mm^3	milli cubic meter
µm	Micro meter
n	Replicate number
N_2	Nitrogen
NS	No significant different
S	Significant different
SAS	Statistical analysis
S.E	Standard error
SEM	Scanning Electron Microscopy
SM	Smooth muscle
Spz	Spermatozoa
t	Ton
TEM	Transmission Electron Microscopy
TL	Total length
X	Magnification

CHAPTER 1

INTRODUCTION

Background of Study

Four native species belonging to the Family Cyprinidae are in demand and fetch a good market price. *Puntius daruphani* (Smith) locally known as Kerai (Eddy, 1980) is one of the most important and expensive fish in Malaysia. Fetch high market valued at RM 25 to 40/kg. However, supply of the fish is only from the wild catch and fry are collected from rivers and lakes.

P. daruphani are found along the whole Pahang river system throughout the year. This species tend to gather in deep pools with submerged tree trunks or branches during the day. At night they move to the shallower areas in search of food and subsequently return to the deep pools at dawn, when the water level rises and as the water becomes murky as a result of heavy rainfall they move along the river banks feeding on recently submerged vegetation (Woynarovich *et al*., 1981).

Spawning season of *P. daruphani* is still unclear; it is possible that the species may spawn more than once annually. For a long time, reproduction was not controlled and all methods were completely unsuccessful because of high dependency on the climate. The reproduction activity actually started in 1950s when research on reproduction and practical application of reliable technologies for artificial reproduction became available particularly information on

reproductive physiology in hatcheries (Woynarovich, 1962; Woynarovich and Horvath, 1981).

Cryopreservation of milt is a widely used technique for long term storage of fish milt because it can facilitate artificial reproduction practices and preserve desirable gene pools (Stoss, 1983; Rana, 1995). Procedures used for cryopreservation are similar in different species fish. They include dilution of fresh milt in extender containing cryoprotectant, frequent use of dimethyl sulfoxide, short equilibration period, freezing as pellets on dry ice or in vials or straws over liquid nitrogen, storage in liquid nitrogen, and thawing in appropriate solutions and at a specific temperature just before fertilization (Rana, 1995). However, the success using these procedures varies. Holtz (1993) used a simple extender with a good success in rainbow trout. However, consistently in high fertility of cryopreserved sperm rarely has been obtained. Many factors, such as thawing solutions, freezing and thawing speed likely affect the fertility of cryopreserved sperm (Stoss and Holtz, 1983).

A number of distinct protocols have been developed for particular fish due to species specific requirements for freezing and/ or thawing. Therefore, an optimal cryopreservation technique should be determined before the milt of an untested species is introduced for long term storage.

Successful storage of fish sperm in liquid nitrogen have been achieved in several fish species (Scott and Baynes, 1980; Stoss, 1983). However, since the

procedure for fish sperm cryopreservation varies from species to species, an applicable procedure for sperm is not yet available.

1.2 Importance of the Study

Cryopreservation of fish sperm is a new technology in aquaculture. Artificial insemination requires large quality semen; collection and storage of good quality semen for future use to improve the convenience of artificial insemination and reduce stress to male broodstock caused by repeated semen sampling which reduces semen quality (Yao *et al.,* 2000).

Cryopreservation is an effective method for long term storage of viable sperm. It has long been used in breeding many species of animals since Polge *et al*. (1949) found that the addition of glycerol allowed survival of human and fowl sperm after thawing.

Cryopreservation is considered as one component in an effective strategy to save endangered species by facilitating the storage of their gametes in a gene bank (Gausen, 1993). Cryopreservation offers several benefits such as stable supply of sperm for optimal utilization in hatchery production and laboratory experiments.

For biological, clinical and animal breeding research, banks of frozen tissues, culture cells, blood, sperm and embryos have been established in many countries (Ashwood-Smith, 1980; Tiersch and Mazik, 2000).

Use of the total volume of available milt is useful for sperm economy in species where milt is difficult to obtain, but also in species where only low volume of semen can be stripped in captivity, for instance in yellowtail flounder (Clearwater and Crim, 1998) or turbot (Suquet *et al.,* 1992).

In simplifying broodstock maintenance, off season spawning can be induced in most cultured fish species by the manipulation of photoperiod and temperature cycles (Bromage, 1995). However, the technique is very costly. When cryopreserved sperm is available all year around, the manipulation of the spawning season could be restricted by the females.

The cryopreservation of *P. daruphani* has a great scope due to reasons discussed as under:

- Fish is a native species.
- Supply of fish is coming only from the wild catch.
- Fry are collected only from rivers and lakes.
- Decreasing of wild stock population.
- Fresh milt is not easily available.

The present study has optimized methods of cryopreservation of fish sperm with the use of cryoprotectant and freezing rates, the sperm motility, motile period, live percentage, abnormality, density and fertilization ability of post-thawed sperm.