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REVIEW:

Current Status of Extenders and Cryoprotectants on Fish Spermatozoa Cryopreservation

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ABSTRACT

An important component of many studies of cryopreservation of fish spermatozoa is the type of extenders and cryoprotectants. The suitability of extenders and cryoprotectants differs from one fish to another. There are many studies have been done in cryopreservation of fish spermatozoa. However, there are few review have been done. This review reveals some aspects of cryopreservation especially the role of extender and cryoprotectant in fish sperm cryopreservation. Fish produce high viscosity of sperm and in some cases only small volume is produced. Before cryopreserved in liquid nitrogen, sperm have to dilute with extenders and for long-term cryopreservation, cryoprotectants are needed to protect the sperm cell from cold and hot shock treatments and prevent cell dehydration during pre-freezing, freezing and post thawed. The suitability of extenders and cryoprotectants differs from one fish to another. Over the last decade, studies on the cryopreservation of mammalian sperm, animal husbandry sperm and human sperm have progressed significantly but studies on fish sperm is still confined to some aquatic.

Key words: fish, sperm, cryopreservation, extenders, cryoprotectants.

INTRODUCTION

Cryopreservation is the method to preserve the milt. This method has been applied in fish sperm. According to Chao and Liao (2001), cryopreservation of fish sperm has been well established for many years in many finfish species. This method offers several benefits such as stock protection from being totally eliminated due to sudden diseases outbreak, natural disaster, or accidents such as oil spills. Other advantages of cryopreservation include stable supply of sperm for optimal utilization in hatchery production and laboratory experiments, easy stock transportation among hatcheries, improvement in selective breeding whereby stock can be maintained more economically and effectively, experimental material for advanced studies, such as gene transfer.

The principle of cryopreservation is to cause cell dehydration and eventually concentrate the cytosol with minimum injury so that ice crystallization in the cytosol is minimized during quenching in liquid nitrogen. Major cryoinjuries can occur in relation to freezing and thawing process during conventional cryopreservation within the temperature ranges of generalized cryopreservation procedures due to the cold shock during freezing and hot shock when the samples thawed. The cryoinjuries occur during pre-freezing and post-thawing, at the temperature range between 0 and –40^{o}C. Other causes of cryoinjuries include pH fluctuation, ice crystal formation, osmotic pressure, and cryoprotectant toxicity (Chao and Liao, 2001).

In addition, Hammerstedt *et al*. (1990) reported that ice formation and changes in osmotic pressure are other major causes of spermatozoa damage during cryopreservation, and the ability of spermatozoa plasma membrane to resist structural damage during cryopreservation may be related to the type of fatty acids in the spermatozoa plasma membrane and the strength of the bonds between membrane components.

It was postulated that fatty acids protect the cell from osmotic pressure of extenders and cryoprotectants solution and hot or cold shock during freezing and thawing further prevent cell dehydration and damages.

EXTENDERS

In terms of cryopreservation, extenders have been very well studied because cryopreservation is difficult without them. An extender is a medium to dilute sperm and to get a larger amount of diluted sperm for artificial induce breeding purposes, while a cryoprotectant is a material which added into an extended sperm dilutions to protect the sperm from cold and hot shock and cryo toxicity during cryopreservation (Muchlisin, 2004b). Some of extenders that used for cryopreservation of sperm in various fish species was listed in Table 1. (Chao, 1996).

Fish produces high viscosity of sperm and in some cases only small volume is produced. Extenders are playing a vital role in cryopreservation, it needed for sperm dilution, and generally it induced initial motility and increased fertilization of cryopreserved sperm. It is known that spermatozoa can be preserved for a day to years and their motility could be retained under low temperatures.

Ringer and physiology solutions are common practical extenders to dilute the milt, since these solutions are easy to prepare. A physiology solution contains 7.98 g/L NaCl and 0.2 g/L $NaHCO_3$ (Alawi *et al.,* 1995) while Ringer solution has 7.5 g/L NaCl, 0.2 g/L KCl, 0.20 g/L $CaCl_2$, 0.20

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g/L and $NaHCO_3$. Ringer solution is commonly used in freshwater fish spermatozoa. Although, modified Ringer contains 13.5 g/l NaCl, 0.60 g/l KCl, 0.25 g/l $CaCl_2$, 0.35 g/l $MgCl_2$, and 0.2 g/l $NaHCO_3$ is used for marine fish spermatozoa. Ringer with milk and Ringer with honey are also reported to be suitable for tilapia (*Oreochromis niloticus*).and milkfish (*Chanos chanos*) or black porgy (*Acanthopagrus schlegeli*) spermatozoa respectively (Chao, 1991). In addition, Muchlisin *et al*. (2004a**)** find that Ringer solutions at dilution ratio of 1:20 resulted in higher sperm motility of bagrid catfish (*Mystus nemurus*).

Another extender that can be used is saline solution consisting of 75 mmol/L NaCl, 70 mmol/L KCl, 2 mmol $CaCl_2$, 1 mmol/L $MgSO_4$, and 20 mmol/L tris (pH 8) which is suitable for cyprinid fish spermatozoa (Lahnsteiner *et al*., 2000). In addition, Kurokura-1 which contains of 128.4 mM NaCl. 2.7 mM KCl, 1.4 $mMCaCl_2$, 2.4 $mMNaHCO_3$ is suitable for common carp (*Cyprinus carpio*) spermatozoa (Linhart, *et al.*, 2000).

CRYOPROTECTANTS

Like extenders, cryoprotectants are also playing important role in cryopreservation particularly for long-term cryopreservation. Cryoprotectants are needed to protect the sperm cell from cold and hot shock treatments and prevent cell dehydration. Cryoprotectants provide cryoprotection to labile enzymes, for example catalase, and stabilize protein in unfrozen and aqueous solutions. They can also prevent ice formation during prefreezing, but the same levels of cryoprotectants can be lethal to unfrozen cell (Chao, 1991). However, cryoprotectants have some disadvantages in that it can induce protein denaturation at higher temperature and cause cryoprotectant toxicity in cellular systems. The toxicity of the diluents is a major limitation to successful spermatozoa cryopreservation of fishes. Some of cryoprotectants that used in cryopreservation studies was listed in Table 2. (Chao, 1996).

Since spermatozoa are very sensitive cells and are easily affected by diluents such as extenders and cryoprotectants, studies on the cryoprotectants and extenders, in terms of spermatozoa preservation are important to determine the most suitable extenders, cryoprotectants and their concentration for certain fish species. Chao (1991) also reported that very few spermatozoa survive at very low temperature without cryoprotectant due to the ice crystals that were formed.

In addition, cryoprotectants added to milt greatly extends the tolerance of spermatozoa to freezing particularly when it is done at slower rate. Here the optimal cooling rate depends on the nature and concentration of the cryoprotectant that is used. For instance, in grey mullet and black porgy, 5-15% glycerol showed satisfactory function, while in the tilapias, 15% milk and 5% methanol as diluents-cryoprotective agent mixture gave satisfactory motility and fertility.

DMSO (dimethyl-sulfoxide) at various concentrations is generally used as a cryoprotectant for animal cells, for example 10% DMSO resulted in a high percentage of motile milkfish spermatozoa (Chao, 1991), Artic charr (*Salvelinus alpinus*) (Richardson *et al*., 2000b), and spotted sea trout (*Cynoscion nebulosus*) (Wayman *et al*., 1996), whereas 12% DMSO was found suitable for Atlantic salmon (*Salmo salar* L) spermatozoa (Gallant *et al.*, 1993). However, 10% Dimethyl-acetamide (DMA) was more effective than 10% DMSO for cryopreservation of rainbow trout (*Ochorhynchus mykiss*) spermatozoa (McNiven *et al*., 1993; and Richardson, *et al*., 2000a) and African catfish (*Clarias gariepinus*) spermatozoa (Horvath and Urbanyi, 2000).

Also available as a cryoprotectant is glycerol which was found to be a more effective cryoprotectant than DMSO and ethylene glycol (EG) for European catfish (*Silarus glanis*) spermatozoa (Linhart *et al.*, 1993). In contrast, Marian and Krasznai (1987) found DMSO better than EG in European catfish, and propylene glycol (PG) in yellowtail founder (*Pleuronectes ferrugineus*) (Richardson *et al.,* 1995). Methanol also has a good protective effect for fish spermatozoa. For example, 10% methanol is suitable for cryopreservation of bitterling spermatozoa (Ohta *et al.,* 2001), spine foot fish (Chao, 1991), bagrid catfish (Muchlisin and Muhammadar, 2002), European catfish (Ogier de Baulny *et al*., 1999) and African catfish (Viveiros *et al.,* 2000), and 5% methanol is reported to be suitable for tilapia spermatozoa (Chao, 1991).

Table 2. Cryoprotectants used in cryopreservation studies (Chao, 1996).

Acetamide	Glycerol monoacetate	Proline
Aline (L)	Glycine	Propylene glycol
Albumin	Hydroxyethyl starch	Pyridine-N-Oxide
Ammonium acetate	Inositol	Ribose
Chloroform	Lactose	Serine
Choline	Magnesium chloride	Sodium bromide
Dextrans	Magnesium sulfate	Sodium chloride
Diethyl glycol	Maltose	Sodium iodide
Dimethyl acetamide	Mannitol	Sodium nitrate
Dimethyl formamide	Mannose	Sodium sulfate
Dimethyl sulphoxide	Methanol	Sorbitol
Erythritol	Methyl acetamide	Sucrose
Ethanol	Methyl formamide	Triethylene glycol
Ethylene glycol	Methyl urea	Trimethylamine acetate
Formamide	Phenol	Urea
Glucose	Pluronic polyols	Valine
Glycerol	Polyethylene glycol	Xylose
Glycerophosphate	Polyvinyl pyrrolidone	-

Table 1. The type of extenders used for cryopreservation of sperm in various fish species (Chao, 1996).

Ingredient (g/L)	NaCl	KCl	$CaCl_2$	$MgCl_2$	$NaHCO_3$	Taps	Caps	Glucose	Yolk	Honey	Milk	Species Recommended
	(g/l)					(mmol)			(ml)			
Marine fish ringer	13.5	0.6	0.25	0.35	0.2	-	-	-	-	-	-	Marine fish
Freshwater fish ringer	7.5	0.2	0.20	-	0.2	-	-	-	-	-	-	Freshwat. fish
Taps	2.9	3.2	0.07	0.03	-	15	-	-	-	-	-	Tilapia
Caps	2.9	3.2	0.07	0.03	-	-	15	-	-	-	-	Tilapia
Milk in ringer	7.5	0.2	0.20	-	0.2	-	-	-	-	-	150	Tilapia
V_2e	7.5	0.38	-	-	2.0	-	-	1.0	0.2	-	-	Tilapia
V_2f	7.5	-	-	-	2.0	-	-	1.0	0.2	-	-	Tilapia
Honey in ringer	7.5	0.6	0.60	0.35	0.2	-	-	-	-	1	-	Black porgy, milkfish

"CO-CRYOPROTECTANTS"

Besides cryoprotectants and extenders, some researchers have used a combination of several materials known as "co-cryoprotectants" such as egg yolk, milk, honey, sucrose and glucose to the diluents (Table 3). For instance, Chao *et al*. (1987) reported that the simplest formulation such as 5% glucose has generally been the most successful for cryopreservation of tilapia sperm. Further simple or single component diluents such as diluted 0.5% honey or glucose in Ringer solution were found functional in both laboratory and field for milkfish and black porgy (Chao *et al*., 1987). Milt diluted with Ringer solution which has been previously combined in methanol is an ideal freezing medium in cryopreserved sperm of several tilapia species including *Oreochromis aureus, Oreochromis mossambicus, Oreochromis niloticus, Tilapia zilli, Oreochromis niloticus x Oroechromis aureus* hybrid and red tilapia (*Oreochromis* sp. hybrid) (Chao, 1991).

Egg yolk has been tested as an additive material to extenders and cryoprotectants in many cryopreservation studies. Ciereszko *et al*. (1993) compared two combined cryoprotectants and found that 8% DMSO with 10% egg yolk gave significantly higher fertilization rate than 20% glycerol with 0.3 M glucose in yellow perch. In addition, Lahnsteiner *et al*. (1996) pointed out that the addition of egg yolk (7% and 20%) and sucrose (0.5%) significantly increased the quality of rainbow trout semen in comparison without these additives.

Anuar and Chan (2000) used an extender containing of 0.16 g calcium chloride, 8 g sodium chloride, 0.4 g potassium chloride, 0.2 g magnesium sulfate, 0.12 g potassium dihydrogen phosphate, 0.06 g sodium hydrogen carbonate, 10 g glucose and 0.15 g amphicilin for *Clarias batrachus* spermatozoa, and reported a 68% of sperm motility at the beginning of storage with survival until day 12, but a combination of cryoprotectant with 15% DMSO and 15% egg yolk maintained sperm motility until day 28.

In marine fish, a combination of Erdahl-Graham`s solution (which contains 0.29 g of $CaCl_2.H_2O$, 0.4 g of $MgCl_2.6H_2O$, 0.5 g of Na_2HPO_4, 5.1 g of KCl, 11.7 g of NaCl., 0.2 g of citric acid, 20 g of glucose, 20 mL at 1.27 g $100mL^{-1}$ KOH, 20 mL at 5.3 g 100 mL^{-1} bicine, 2 dm^3 of distilled water) and 10% egg yolk showed positive effects on milt of northern pike (*Esox lucius*) with increasing fertility rate (Babiak *et al*., 1995; 1999). Moreover, Piironen (1993) found that the addition of egg yolk in extender for sperm cryopreservation increased the fertilization rates in brown trout (*Salmo trutta m. lacustris* L), although in Artic charr addition of egg yolk into the diluents decreased the fertilization rate. Similarly, in aspius (*Aspius aspius*), the presence of egg yolk in the extender significantly decreased cryopreservation success (Babiak *et al*., 1998).

In addition to the cryoprotectant used, the acceptable dilution ratios of the extenders appear to vary in fish. For example 1 part milt: 1 part extender for the sperm of grey mullet, black porgy, and tilapia; 1:4 for milkfish; and 1:20 for grouper (Chao and Liao, 2001). Tables 1 and 2 show the types of, cryoprotectants, extenders and co-protectants are commonly used respectively.

EFFECT OF CRYOPRESERVATION ON THE SPERM MOTILITY AND FERTILITY

Motility and fertilization rate of cryopreservation spermatozoa is usually inferior to that of fresh milt. Thus, with frozen semen 15 times more spermatozoa are required for obtaining sufficient fertility in rainbow trout. The lower fertility observed could be due to several factors among them (i) low motility of post-thaw spermatozoa, (ii) the reduced percentage of motile spermatozoa after undergoing freezing and thawing and (iii) low fertility regardless of motility of cryopreserved sperm (Ohta *et al*., 1995).

In walking catfish (*Clarias batrachus*) for example, spermatozoa motility decreased from 80% to 59%, and 38% after 20, 40, and 120 minutes respectively at a temperature of 10^oC (Anuar and Chan, 2000). However, Oetome *et al.* (1996) studied the effect of different cryoprotectants on sperm motility of African catfish, and found that fresh and cryopreserved semen resulted in no significant difference in hatching rate of 82.25% and 78.9% for the fresh and cryopreserved sperm respectively.

Furthermore, Ohta *et al*. (2001) studied the effect of five cryoprotectants namely DMSO, glycerol, methanol, DMA and DMF in Japanese bitterling spermatozoa cryopreservation, they reported that the additional of 10 % DMSO or N, N-dimethylacetamide (DMA) to the diluents increased the percent motility significantly. The results also showed that 10% methanol plus 90% fetal bovine serum as suitable diluents for cryopreservation of Japanese bitterling spermatozoa and those samples should be cooled to $–40^oC$ at low freezing rate for effective storage.

Ohta *et al*. (1995) suggested a correlation between the percentage of fertility and motility of amago salmon (*Oncorhynchus masou*) after thawing, in that high percentage of motility can increase the fertility. Similar results were found in sea bass and turbot spermatozoa (Dreanno *et al.,* 1998).

Contrarily, Ciereszko *et al*. (1999) reported no relationship between the motility and sperm fertility in rainbow trout, since 0% motility had 67.9% fertilization success. They considered three possible explanations for the phenomenon of cryopreserved sperm for this observation: (i) sperm motility was suppressed by conditions of the sperm analysis chamber, (ii) sperm motility was activated by an egg factor, or (iii) eggs can be fertilized by immotile spermatozoa. The existence of a factor in egg and ovarian fluid that can stimulate sperm motility and could fertilize the egg support the theory that sperm motility was activated by an egg factor. Unfortunately, the mechanism for the stimulation of sperm motility by the egg factor remains unclear. It is suspected that some of the steroid hormones in egg and ovarian have played a role in this phenomenon.

Table 3. The types of co-cryoprotectants used in cryopreservation studies.

Co-protectants	Combined with	Fish species	References
Glucose 5%	Ringer	Milkfish	Chao *et al. (*1987); Muchlisin *et al*. (2004a).
Honey 0.5%	Ringer	Black porgy	Chao *et al*. (1987)
Milk	Ringer-methanol	Tilapias	Chao, (1991)
Egg yolk 10%	DMSO	Yellow perch	Ciereszko *et al*. (1993)
Egg yolk 7&20%+ sucrose (0.5%)	-	Rainbow trout	Lahnsteiner *et al. (*1996)
Egg yolk 15%	DMSO	Walking catfish	Annuar and Chan (2000)
Egg yolk 10%+ sucrose 0.6M	Erdahl-DMSO	Northern pike	Babiak *et al*. (1999)

However, in the most successful cryopreservation, sperm maybe viable and motile after cryopreservation, but their fertility was reduced. Roberts *et al*. (2000) reported many cryopreserved sperm were immotile, and that the motile sperm rapidly lost their motility and swimming velocity after dilution. They also found the highest fertilization rates obtained were only 20-40%.

CONCLUSIONS

Cryopreservation has several benefits such as stock protection, a stable supply of sperm for optimal utilization in hatchery production and laboratory experiments, easy stock transportation, improvement in selective breeding and gene transfer. In fact, fish produce a high viscosity of sperm and in some cases only small volume is produced. Extenders and cryoprotectants are important and play a vital role in cryopreservation. Extenders are needed for sperm dilution to produce large volume of diluted sperm while cryoprotectants are also needed to protect the sperm cell from cold and hot shock treatments and prevent cell dehydration during pre-freezing, freezing and post thawed, and its greatly extends the tolerance of spermatozoa to freezing particularly when it is done at slower rate. The optimal cooling rate depends on the nature and concentration of the cryoprotectant that is used. In addition, the suitability of extenders and cryoprotectants differs from one fish to another.

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