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A BRIEF OVERVIEW ON CRYOPRESERVATION METHOD OF STURGEON SPERM

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ABSTRACT

Cryopreservation and long-time storage of sperm has been studied in cultured fish species and in fish species with currently threatened stocks. Reasons of these studies on cryopreservation of fish sperm are to decrease cost associated with keeping live male brood stock, conservation of threatened species and benefiting from individuals having good gamete quality all year around for fertilization. Environmental pressures such as water pollution, dam construction and development of adjacent watersheds for irrigation purposes, which are responsible for the lost of spawning grounds, have caused declines in sturgeon populations and their diversity. As a result of these declines, all sturgeon species worldwide were included in Appendix II of the Convention on the International Trade of Endangered Species of Wild Fauna and Flora (CITES) regulations on April 1, 1998. Successful cryopreservation protocols of the 8 different sturgeon species have been reported today. In this review, we have provided overview of cryopreservation methods, commonly used of cryoprotectans and extenders for sturgeon, freezing-thawing procedure and fertilizing with cryopreserved sperm.

Key words: Cryopreservation, Sturgeon, Cryoprotectan, Extender

INTRODUCTION

The development of routine "artificial" fertilization in aquaculture has raised the requirement for storage of reproductive materials. The short period time for fish gametes remain in good condition after collection became a significant obstacle to hybridization between fish species inhabiting different geographical locations or having different spawning times. The asynchronous maturation of breeders invariably causes problems in aquaculture and has stimulated research to develop a suitable method for the prolonged storage of fish sperm (Stoss, 1983; Rana, 1995a;b).

The storage for short time or cryopreservation method has been applied widely and has become not only a routine tool in aquaculture for fish hybridization and selective breeding, but also an important for programs on biodiversity and the preservation of endangered species. Gamete banks are currently being created with the objective of protecting endangered, rare or almost extinct species (Chao and Liao, 2001; Cabrita *et al.*, 2008; Horvath *et al.*, 2009).

Sturgeons and paddlefish of the order Acipenseriformes are an ancient group of fishes. Several species are used in aquaculture programs and farmed for their roe sold as caviar and their high quality boneless meat. However, some species are critically threatened or endangered for reasons including destruction of habitat and spawning grounds, overexploitation of stocks and uncontrolled poaching (Dettlaff *et al.*, 1993; Wirgin *et al.*, 1997; Billard and Lecointre, 2001). To assist in aquaculture production and to make strong recovery/conservation programmes; cryopreservation of sturgeon sperm is considered a potentially strong method.

Burtsev and Serebryakova first attempted sperm cryopreservation of three sturgeon species; *Huso huso* (Beluga); *Huso dauricus* (Kaluga) and *Acipenser ruthenus* (Sterlet sturgeon) (Mims *et al.*, 2011). Since then, with these 3 speices, total 12 species's sperm, belongs to Acipenseriform are successfully cryopreserved in the world. And these species are; *Acipenser fulvescens* (Lake sturgeon); *Acipenser baer*i (Siberian sturgeon), *Acipenser sturio* (Atlantic sturgeon), *Acipenser sinensins* (Chinese sturgeon), *Acipenser persicus* (Persian sturgeon), *Acipenser stellatus* (Stellate sturgeon), *Acipenser gueldenstaedtii* (Russian sturgeon), *Acipenser transmontanus* (White sturgeon) and *Polyodon spathul*a (Paddlefish).

All these cryopreservation studies have focused that effective methods including the best cryoprotectant; extender; cryprotectant/extender combination; dilution rate, freezing and tawing methods, ect. (Dzyuba *et al.*, 1999; Billard *et al.*, 2004, Kopeika *et al.*, 2007). Sturgeon sperm cryopreservation studies success depend on the techniques and related method and are still need of refinement to avoid negative effects of species-specific cryo-resistance heterogenetiy.

The present review summarises of the techniques for cryopreservation sturgeons and paddlefish sperm.

CRYOPRESERVATION METHODOLOGY

Extenders and Cryoprotectants

Extender (without cryoprotectant) is a solution containing organic and inorganic substances and its' pH is similar to seminal plasma. Extender solution is used as diluent aiming that; to increase the diluat ion of the sperm samples, protect the sperm cells from chemical and physical changes or contamination, and provide optimal conditions for fertilization. The extender solution allows to spermatozoa staying im-motile and an optimum sperm: eggs ratio for fertilization (Park and Chapman, 2005; Linhart *et al.*, 2006; Kopeika *et al.*, 2007).

The choice of appropriate extender depends on the sperm characteristics of species to be investigated. The extender which called modified Tsvetkova's (mT) has been used succesfully for the cryopreservation of sturgeon and paddlefish sperm. Its composition is 23.4 mM sucrose, 0.25 mM KCl, 30 mM Tris and pH adjusted to 8.0 by using hydrochloric acid (Glogowski *et al.*, 2002).

The osmolarity of the extender solution appears to be one of the most important factors in preparation of an appropriate extender. Because large inter-individual differences have been demonstrated in fish sperm about osmotic responses, in order to maximize the outcome of cryopreservation, the osmolarity of extender should be adjusted on the individual bases. Increased concentration of buffer or K^+ may be

used to in extenders inhibit sperm motility for some species such as sturgeon (Toth *et al.*, 1997; Alavi *et al.*, 2004a,b; Kopeika *et al.*, 2007).

Cryoprotectant are added to the sperm in order to increase the survival of spermatozoa during the freezing procedure. Their main feature is in water binding with reduced formation of crystals in the course of freezing or in structuring uniform crystals. Their another feature is in electrolyte binding, what limits the creation of concentrated residual solutions and thus decreases freezing point during the freezing procedure (Woods *et al.*, 2004; Kopeika *et al.*, 2007; Linhart *et al.*, 2009). Many substances were identified as a good quality cryoprotectants, e.g. dimethyl sulfoxide (DMSO), ethylene glycol, glycerol, methanol and dimethyl acetate (DMA) (Glogowski *et al.*, 2002; Billard *et al.*, 2004; Urbanyi *et al.*, 2004; Horvath *et al.*, 2005; Linhart *et al.*, 2009; Kopeika *et al.*, 2007). DMSO gave the best protective effect on Ponto-Caspian sturgeon sperm according to Cherepanov and Kopeika (1999). Horvath and Urbanyi (2000) reported better sperm fertilizing capacity with methanol (22% fertilization) compared to DMSO (2%) and dimethyl acetate (DMA) (0%). Glogowski *et al.* (2002) also used methanol as cryoprotectant for the Siberian sturgeon sperm. In addition, egg yolk as non-permeant cryoprotectant sometimes can be added into extender (Leung, 1991).

Both permeable (glycerol, DMSO, ethylene glycol, dimethylacetamide, methanol) and nonpermeable (glucose, sucrose, egg yolk) cryoprotectants may be used in protocols to cryopreserve fish sperm. The absence of an ideal cryoprotectant, as well as the lack of full understanding of their mechanisms in cell protection, make selection of a single cryoprotectant difficult for different species. However, the optimal cryoprotectant can be determined empirically (Leung, 1991; Horvath *et al.*, 2005; Kopeika *et al.*, 2007; Linhart *et al.*, 2009).

Concentration of cryoprotectants and dilution rates

The concentration of cryoprotectant is usually varies in the range between 5 and 12% (v/v) at fish (Kopeika *et al.*, 2007). Concentration of cryoprotectants in protective media or after final dilution of sperms differs from sturgeon species in usual range of 5 - 15% (Linhart *et al.*, 2009). Better cell protection can be achieved on employing higher concentrations, but this has to be balanced with toxicity effects of the cryoprotectant (Stoss, 1983).

The dilution rate of the medium and sperm is equally important and it has species sensitive. High-density sperm needs a greater rate of dilution. Usually one volume of sperm is mixed with one volume of medium (Tsvetkova *et al.*, 1996; Brown and Mims, 1999; Billard *et al.*, 2004; Kopeika *et al.*, 2007; Boryshpolets *et al.*, 2011).

Equilibration time

In general, no equilibration time is needed and sperm was mixed with the medium and immediately frozen (Billard *et al.*, 2004). Equilibration has been reported often as deleterious for the sperm (Kopeika *et al.*, 2000; Alipour *et al.*, 2009) but Jähnichen *et al.* (1999) has been found no change in fertilizing capacity of sterlet that sperm frozen after 15-min equilibration in extender with 40% ethylene glycol by comparison with non-equilibrated samples.

However, this is not the case for all species. When the motility level of freeze–thawed sturgeon sperm was compared following different equilibrating durations, it was observed that the samples that had a 40-min exposure to the medium at 5°C had a significantly higher motility rate than the ones that were frozen almost immediately after placing them in cryoprotectants (Kopeika *et al.*, 2007).

Also using equilibration time, not only the sperm of sturgeon was sufficiently dehydrated, but also the toxicity of the cyroprotectant would be at negligible levels while the sperm motility can reach over 90% (Liu *et al.*, 2006).

Freezing Procedure

Sperms are frozen in 100 µL pellets which put directly on dry ice or in vials or straws which are placed in programmable freezers or simply in the vapours of liquid nitrogen. The use of larger straws for high sperm volume should desirable, but it requires longer diameter straws, at, in turn, increase the freezing time. Nowadays most used straws are made of polyethylene, with capacity ranging from 0.25 mL to 0.5

mL or 5 mL (micro and medium straws, respectively) (Rana, 1995a,b; Noveiri *et al*., 2002; Linhart *et al*., 2006; Kopeika *et al*., 2007).

The freezing rate was controlled by the height above the surface of liquid nitrogen (usually 3-5 cm), but it depends on the size of the straws. Starws after sealed and after 3 minutes (0.5 mL straws) and 10 minutes (5 mL straws) of freezing in liquid vapor, plunged into liquid nitrogen (-196°C) with in plastic goblets attached to canes (Billard *et al*., 2004; Urbanyi *et al*., 2004; Horvath *et al*., 2005; Horvath *et al*., 2009; Boryshpolets *et al*., 2011).

Slow freezing (from 4°C to -70°C) at the time of cryoprotectant penetration is the mostly used approach for spermatozoa and other cells. Ice crystals are formed in extracellular solution in the course of freezing, water is released from cells when balancing the osmotic level until they gain the osmotic equilibrium. When the low intracellular temperature -70°C is gained, cells can be kept safely at -196 °C in liquid nitrogen. The optimum freezing level is species specific and must be determined for each species seperately (Glogowski *et al*., 2002; Billard *et al*., 2004; Urbanyi *et al*., 2004; Horvath *et al*., 2005; Linhart *et al*., 2006; Horvath *et al*., 2009; Boryshpolets *et al*., 2011).

Another way of freezing is dry-ice. Small aliquots (100–250 µL) of sperm diluted with cryoprotectant are directly placed onto dry ice and allowed to cool for 4–5 min, then transfered to cryovials and stored in liquid nitrogen (Kopeika *et al*., 2007).

In a programmable freezer, a three step freezing regime was often used at sturgeon: 3.5 to 5°C/min from 2 to 14°C, 15–20°C /min down to 70°C and direct transfer to liquid nitrogen (Billard *et al*., 2004). Trukshin (2000) reported a deleterious effect of a 10°C /min freezing rate (0% fertilization) compared to 22% at 4°C /min.

Thawing Procedure

Studies on optimization of the thawing regime have demonstrated that the best thawing regime for 1–2 ml vials is using a 40°C water bath. For straws; in general, the sperm is be thawed in a water bath at 40°C for 8 s (0.5 ml straws) and 30 s (5 ml straws) (Rana, 1995b; Kopeika *et al*., 2007; Horvath *et al*., 2009).

Fertilizing

A semi-dry fertilization method is used for sturgeon and paddlefish sperm. Sperm is added in a ratio up to 1:200 (milt-water) to hatchery water and then poured onto the eggs. Egg batches up to 5 g have been fertilized with one 0.5 ml straw of thawed mixture (0.25 ml milt), while 40 g of eggs were fertilized with one 5 ml straw of thawed paddlefish mixture (2,5 ml milt) (Horvath *et al*., 2009).

In general, the fertilizing capacity was significantly decreased after thawing. The decrease in sperm motility was related to some loss of fertilizing capacity. Dettlaff *et al.* (1993) stated that when the motility of thawed sperm was as low as %20–40, there was almost no fertilization in some Ponto-Caspian sturgeons. This result related some factors such as alteration of the acrosome more than sperm motility. However, Jähnichen *et al.* (1999) reported that a high loss of motility but not fertilizing capacity in thawed sperm.

CONCLUSION

Storage of fish sperm for aquaculture purposes and preservation of biodiversity in fish steadily increases its' importance. In recent years' intensive new data and knowledge on this topic has accumulated.

Cryopreservation of fish spermatozoa is a complex but frequently used method, the success of which depends on a number of variables affecting the capacity of spermatozoa to fertilize eggs (Ciereszko *et al*., 1996; Babiak *et al*., 2000; Billard *et al*., 2004; Linhart *et al*., 2006; Kopeika *et al*., 2007; Horvath *et al*., 2009).

It has been estimated that sperm from 200 fish species are already successfully cryopreserved but optimization of this technology is still needed for sturgeons and paddlefish species due to limited hatching rate have been reported for sturgeons or paddlefish when cryopreserved sperm were used (Brown and Mims, 1999; Jähnichen *et al*., 1999; Glogowski *et al*., 2002).

Dimethyl-sulfoxide (DMSO), methanol and ethylene glycol were the most common cryoprotectants; DMSO gave the best protective effect to Ponto-Caspian sturgeon sperm according to Cherepanov and Kopeika (1999). Earlier works indicated that the cryopreservation of sturgeon spermatozoa using DMSO-sucrose extender resulted in recovery of motile spermatozoa with basic motility characteristics similar to those of fresh semen (Ciereszko *et al*., 1996). Unfortunately, despite good and sometimes excellent post-thaw motility performances, the fertilizing ability of such spermatozoa was poor and therefore DMSO-cryopreserved sturgeon sperm was unsuitable for practical applications. High post-thaw motility and low or non-existent fertilization rates of sturgeon and paddlefish spermatozoa cryopreserved with the use of DMSO has also been confirmed by Lahnsteiner *et al*. (2004), Horvath *et al*. (2005), Linhart *et al*. (2006) and Yamaner *et al*., (2015) Linhart *et al*. (2006) and Horvath *et al*. (2009) introduced methanol as a cryoprotectant for the cryopreservation of sturgeon and paddlefish spermatozoa.

Methanol as a cryoprotectant secures both high values of sperm motility and velocity, although sometimes lower motility and fertilization rates than DMSO was observed (Lahnsteiner *et al*., 2004; Glogowski *et al*., 2002). Usefulness of methanol for sperm cryopreservation has also been confirmed for sterlet (*A. ruthenus* L.). Lahnsteiner *et al*. (2004) have recorded a significant reduction in fertilizing ability of sterlet semen frozen in DMSO, while fertility rates with semen frozen in methanol was comparable to controls.

Various effects of different cryoprotectants and extenders depend on factors such as fish species (freshwater or marine), freezing method (straw or pellet), and freezing procedure (equilibration, cooling, thawing, etc.) (Dzyuba *et al*., 1999; Boryshpolets *et al*., 2011).

There are 27 Acipenserid species, and most of them are on to extinction. Sturgeons natural stocks are threatened by their long term adulthood period, vulnerable life cycle, anthropogenic effects, global warming, ect. Cryopreservation of the endangered species semen will be support hatchery activities, and accordingly studies on sustainability of the natural stocks. Besides the success of sturgeon sperm cryopreservation studies, the techniques and related methods are still in need of refinement to avoid negative effects of individual and species-specific cryo-resistance heterogeneity but also to achieve reproducible results. There is much work still to be done to improve this technology. Species-specific optimizations of technology are needed.

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